



**HAL**  
open science

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

Rosa Ana Lopez Rodriguez, Markus Nolf, Remko A. Duursma, Eric Badel, Richard J. Flavel, Hervé Cochard, Brendan Choat

► **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

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

## 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


 SCHOLARONE™  
Manuscripts

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

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

3

4

5 Rosana López<sup>1,2</sup>, Markus Nolf<sup>3</sup>, Remko A Duursma<sup>3</sup>, Eric Badel<sup>1</sup>, Richard J Flavel<sup>4</sup>, Hervé Cochard<sup>1</sup>,

6 Brendan Choat<sup>3</sup>

7

8 1- Université Clermont Auvergne, INRA, PIAF, Clermont-Ferrand, France.

9 2- Sistemas y Recursos Naturales, Universidad Politécnica de Madrid, Madrid, Spain.

10 3- Hawkesbury Institute for the Environment, Western Sydney University, Richmond, NSW, Australia.

11 4- School of Environmental and Rural Science, University of New England, Armidale, NSW, Australia.

12

13 **Corresponding author details:**

14 Dr. Rosana López

15 e-mail: rosana.lopez@upm.es

16 phone: +34 655868659

17 Address: ETSI Montes, Forestal y del Medio Natural. C/ José Antonio Novais, 10. 28040 Madrid

18

19

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**  
49

50  
51 41 Vulnerability to embolism, xylem embolism, drought, centrifuge technique, Cavitron, X-Ray microCT,  
52  
53 42 CAVITOPEN.  
54

55  
56 43  
57  
58  
59  
60

## 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).

1  
2  
3 69 The most straightforward technique for inducing embolism is bench dehydration, wherein whole  
4  
5 70 plants or long branches are gradually dehydrated to various xylem tensions and hydraulic  
6  
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  
12  
13 74 with which to validate other techniques (Cochard et al. 2013; Ennajeh et al. 2011; Sperry et al. 2012).  
14  
15 75 This method is not completely free of artefacts and issues associated with disequilibrium in water  
16  
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  
52  
53 94 and allowed for flow measurements to be made under tension.  
54  
55  
56  
57  
58  
59  
60

1  
2  
3 95 Although centrifuge based techniques induce embolism by increasing tension in sample xylem, the  
4  
5 96 patterns of embolism spread through the sample may differ from a naturally dehydrated sample (Cai  
6  
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).

24  
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  
31  
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  
37  
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  
45  
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).

49  
50  
51  
52  
53 118 In this study we evaluated the performance of both centrifuge techniques against bench  
54  
55 119 dehydration in order to examine possible discrepancies associated with each technique. First, we

1  
2  
3 120 tested two methods for inducing embolism: bench dehydration and centrifugation. We then tested  
4  
5 121 three ways of measuring the resulting loss of conductivity: gravimetric flow measured in bench-  
6  
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.*  
14  
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  
18  
19 128 measured hydraulic flow both in the whole, spun segments and excised middle sections. Spatial  
20  
21 129 patterns of embolism within samples were visualized with X-ray microCT after centrifugation in  
22  
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  
26  
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  
34  
35 136 vulnerability curves.  
36  
37  
38

## 39 137 **Material and methods**

### 40 138 *Plant Material*

41  
42  
43  
44  
45 139 Experiments were carried out on branch material of two diffuse-porous species of the same genus  
46  
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  
54  
55 144 exposed branches of 1.5-2.0 m length were collected in the field in the early morning and  
56  
57  
58  
59  
60



1  
2  
3 145 immediately placed in black plastic bags with moistened paper towels to prevent transpiration with  
4  
5 146 their cut ends covered with Parafilm. In the laboratory they were kept at 4 °C until measured.  
6  
7

8 147 *Midday xylem water potential in the field and Native embolism*  
9

10  
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.  
23

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  
27

28 156 because branches were cut at different times during late autumn-winter 2016 to avoid long storage.  
29

30 157 Branch proximal end was cut underwater to release tension for 30 min (Torres-Ruiz et al. 2015;  
31

32 158 Wheeler et al. 2013) and then the branch was progressively recut under water to segments 50 mm  
33

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  
37

38 161 conductivity ( $K_h$ ) was measured in 50 mm long segments with filtered, degassed 2 mmol KCl solution  
39

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  
43

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  
47

48 166 each segment as:  
49  
50

51  
52 167  $PLC = 100 \times (1 - K_h / K_{max})$  (equation 1)  
53  
54  
55  
56  
57  
58  
59  
60

1  
2  
3 168 Specific hydraulic conductivity ( $K_S$ ) was calculated dividing  $K_{max}$  by the xylem cross-sectional area  
4  
5 169 (average distal and proximal xylem area measured with a calliper).  
6

7  
8 170 *Maximum vessel length and vessel length distribution*  
9

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  
18  
19 175 0.05 MPa at its distal end with an aquarium air pump while the basal end was repeatedly shortened  
20  
21 176 by 2 cm under water until air bubbles emerged. The remaining sample length was assumed as  
22  
23 177 maximum vessel length.  
24

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  
49  
50 190 equal diameter, this percentage would correspond to the number of vessels longer than the distance  
51  
52 191 from the injection point. In this case of the two *Hakea* species used are diffuse porous and vessel  
53  
54 192 diameters within the same sample did not vary greatly. Thus the curves in Fig. 1 represent a proxy of  
55  
56  
57  
58  
59  
60

1  
2  
3 193 vessel distribution of the two species, although not as accurate as anatomy, and allow to estimate  
4  
5 194 the amount of open vessels from a certain cut point.

6  
7  
8 195 *Bench dehydration technique*

9  
10 196 Branches were dehydrated gradually in the laboratory at ca. 23 °C. Xylem water potential ( $\Psi_x$ ) was  
11  
12 197 measured with a pressure chamber (PMS Instrument Co., Albany, OR, USA) in bagged leaves  
13  
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  
35  
36 209 collection the cutting surface was covered with parafilm to avoid air entry in the rest of the sample.  
37  
38  
39  
40

41 210 *Centrifuge techniques*

42  
43  
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).

1  
2  
3 217 Static centrifuge measurements were carried out on 20 branches per species. Branches were  
4  
5 218 trimmed under water and both ends were shaved to a final length of 14 or 27 cm. The initial  
6  
7 219 hydraulic conductivity was measured as described above (see Native embolism section) with a  
8  
9 220 pressure head of 7.5 kPa. Subsequently, 14-cm long branches were spun in the centrifuge (Sorvall RC  
10  
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  
26  
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.

30  
31  
32  
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  
42  
43 236  $K_h$  was again determined while the sample was spinning. The PLC at each pressure step was  
44  
45 237 quantified as

46  
47  
48 238 
$$\text{PLC} = 100 \times (1 - K_h / K_i).$$
 (equation 2)  
49

50  
51 239 *X-ray microCT imaging*  
52  
53  
54  
55  
56  
57  
58  
59  
60

1  
2  
3 240 A subset of branches of *H. leucoptera* was transported to the University of New England in Armidale  
4  
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.  
38  
39  
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  
56  
57  
58  
59  
60

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

$$275 \quad K_{sth} = \frac{\sum \left( \frac{D^4 \pi}{128 \eta} \cdot \frac{\Delta p}{\Delta x} \right)}{A} \quad \text{(equation 3)}$$

276

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

279

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

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

286 Vulnerability curves were fitted using a Weibull function (Ogle et al. 2009) in R 3.2.0 (R Core Team,  
 287 2015) using the fitplc package (Duursma and Choat 2017). Confidence intervals of  $P_{12}$ ,  $P_{50}$  and  $P_{88}$  ( $\Psi_x$   
 288 at 12, 50 and 88 % loss of conductivity, respectively) and the slope of the curve at 50% loss of  
 289 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

1  
2  
3 313 sample and null at the extremities (eq. 5). Since the loss of conductivity is measured on the whole  
4  
5 314 sample, an underestimation of the degree of embolism in the middle of the sample is predicted. This  
6  
7 315 effect of vessel length was further tested with the CAVITOPEN model and found marginal, i.e. the  
8  
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  
12  
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.

26  
27  
28 324 The second assumption of the model is that open vessels drain when the minimum pressure in the  
29  
30 325 vessel exceeds a threshold value  $P_{open}$ . Because of the quadratic distribution of the pressure in the  
31  
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.

35  
36  
37 328 The branch segment was discretised in 0.1 mm thick sections arranged in serial. The xylem pressure  
38  
39 329 in the middle of the segment was set to a pressure varying from 0 to -12 MPa in 1 MPa steps. The  
40  
41 330 model then computes the pressure at steady state in each 0.1 mm section and determines the PLC  
42  
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  
46  
47 333 the model for different theoretical  $L_{max}$  values and the 4 rotors sizes used in our experiments. To  
48  
49 334 validate the model we used the values of PLC obtained for *H. leucoptera* in the static centrifuge with  
50  
51 335 the 27 cm rotor. The CAVITOPEN model was fit to the measurements using constrained numerical  
52  
53  
54  
55  
56  
57  
58  
59  
60



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

1  
2  
3 359 MPa) (Fig. 2). VCs obtained with bench dehydration had the most negative  $P_{12}$  and the steepest  
4  
5 360 slopes of all methods (Table S2), meaning that embolism formation started at more negative  $\Psi_x$  and  
6  
7 361 conductivity was lost across a narrower range of  $\Psi_x$  compared with VCs generated by centrifugation.  
8  
9  
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,  
13  
14 364 Cavitron and microCT (average  $P_{50}$  with the 27 cm rotor in the static centrifuge and the Cavitron -4.8  
15  
16 365 MPa), and the CI at 95% overlapped with bench dehydration ( $P_{50} = -5.0$  MPa). The VC generated with  
17  
18 366 the 14 cm rotor for *H. dactyloides* yielded slightly less negative values ( $P_{50} = -4.3$  MPa; Table S2; Fig.  
19  
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  
25  
26 370 samples spun in the static centrifuge, we found a significant effect both of the rotor size and the  
27  
28 371 segment used to measure flow (whole, spun segment or excised middle section in the 27 cm rotor)  
29  
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 -  
33  
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).  
37  
38 376 Both VCs were almost linear when flow was measured across the whole segment with a shift  
39  
40 377 towards more vulnerable values with the 14 cm rotor, but became s-shaped when only the middle  
41  
42 378 section of the 27 cm segment was measured (Fig. 2). Removing the segment ends resulted in a  
43  
44 379 steeper slope and significantly more negative values of  $P_{12}$  and  $P_{50}$ . The Cavitron and the middle  
45  
46 380 segment techniques yielded similar results and agreed well with the dehydration technique in  $P_{50}$   
47  
48 381 and  $P_{88}$  and with microCT image analysis (red triangles in Fig. 2).  
49  
50  
51  
52

53 382 *Patterns of embolism across a centrifuged sample*

1  
2  
3 383 Within 27-cm-length centrifuged samples of *H. leucoptera*, microCT scans revealed that embolism  
4  
5 384 levels were consistently at their highest near the sample ends (at 12 cm from the axis of rotation)  
6  
7 385 when spun at equivalents of -5, -7 and -9 MPa in the static centrifuge (Fig. 3). At -5 and -7 MPa loss  
8  
9 386 of conductivity decreased from the basal end to the centre, contradicting theoretical expectations.  
10  
11 387 This trend was observed even at  $\Psi_x$  inducing less than 40% PLC based on the bench dehydration VC  
12  
13 388 (Fig. 3). Only at -9 MPa, that is, below  $P_{88}$  on bench dehydration, did levels of embolism converge  
14  
15 389 along the length of the sample at 80-90%.

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

19  
20  
21 391 The simulations produced by the CAVITOPEN model confirmed that the shape of the VCs generated  
22  
23 392 by the centrifugation was largely dependent on vessel and sample lengths. As maximum vessel  
24  
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  
28  
29 395 increased (Fig. 4b). For instance, with 14-cm-length centrifuged samples,  $P_{50}$  ranged from -0.6 MPa  
30  
31 396 to -7.7 MPa varying the maximum vessel length of the sample from 50 cm to 5 cm. Likewise, the  $P_{50}$   
32  
33 397 of a centrifuged sample with maximum vessel length of 15 cm ranged from -2.1 MPa in the 14-cm  
34  
35 398 rotor to -7.7 MPa using a 40-cm rotor. Embolism of vessels open from the cut surface (Fig. S1)  
36  
37 399 influenced values of PLC at high negative pressures, even in short-veaseled samples, resulting in  
38  
39 400 rapid loss of conductivity followed by a plateau. The more open vessels and the less negative the  
40  
41 401 threshold of embolism of open vessels (Fig. 4c), the higher is this plateau and stronger the impact on  
42  
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  
46  
47 404 measurements of PLC obtained in 27-cm centrifuged samples of *H. leucoptera*. When the  
48  
49 405 CAVITOPEN model was fit (black circles and grey solid line, respectively) and we used the inflection  
50  
51 406 point as starting point for  $K_i$ , the corrected curve matched the reference VC obtained with bench  
52  
53 407 dehydration (Fig. 4d black solid line and orange dashed line, respectively). Alternatively, by fitting  
54  
55  
56  
57  
58  
59  
60

1  
2  
3 408 the model using numerical optimization we estimated values of  $P_{50} = -6.9$  MPa,  $S_{50} = 49.7$ ,  $L_{max} =$   
4  
5 409 15.21 and  $P_{open} = -0.75$ .

## 6 7 8 410 **Discussion**

9  
10 411 We evaluated the reliability of two centrifuge based techniques commonly used to measure  
11  
12 412 vulnerability to embolism in angiosperm species and present a protocol that mitigates experimental  
13  
14 413 artefacts associated with open xylem vessels. Both the static centrifuge method and the *in-situ* flow  
15  
16 414 centrifuge method (Cavitron) were prone to artefactual embolism caused by open vessels, although  
17  
18 415 the errors were significantly greater in the static centrifuge method. In a species with maximum  
19  
20 416 vessel length longer or similar to the centrifuge rotor diameter, the static centrifuge significantly  
21  
22 417 overestimated xylem vulnerability to embolism if the whole spun segment was used to measure  
23  
24 418 flow. Observations with microCT indicated that artefactual embolism caused by centrifugation of  
25  
26 419 samples occurred in the outer most portions of samples. However, we demonstrated that  
27  
28 420 artefactual embolism was largely eliminated from static centrifuge if flow was measured in an  
29  
30 421 excised central part of the segment. This altered protocol yielded VCs similar to those obtained on  
31  
32 422 the same species with bench dehydration thus allowing these centrifuge techniques to accurately  
33  
34 423 measure vulnerability to embolism in longer vesseled species. We also present a new model  
35  
36 424 (CAVITOPEN) that simulates the impact of vessel draining at the cut end on the whole VC curve and  
37  
38 425 showed that errors were largely dependent on vessel length and rotor diameter. This model allows  
39  
40 426 researchers to quantitative test and avoid errors associated with the artefactual embolism. The  
41  
42 427 bench dehydration technique indicated that significant embolism was only initiated in both species  
43  
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  
47  
48 430 a span of 1 MPa (Fig. 2). These vulnerability curves have been classified as sigmoidal or s-shaped as  
49  
50 431 opposed to exponential or r-shaped curves, characterized by rapid conductivity losses as soon as the  
51  
52 432 water potential declines below zero (Cochard et al. 2013; Sperry et al. 2012). A third type of VC,

1  
2  
3 433 intermediate between these two, exhibits a linear response, and is mainly found in diffuse porous  
4  
5 434 species when using centrifugation to induce embolism (Cochard et al. 2013).

6  
7  
8 435 Our results showed that VCs obtained with the static centrifuge technique and the Cavitron are  
9  
10 436 similar to bench dehydration in a short-vesseled species, i.e. a species with no through vessels (open  
11  
12 437 at both ends) in the segment and with few vessels open from the cut surface to the middle of the  
13  
14 438 segment. All centrifuge generated VCs for *H. dactyloides* were sigmoidal and similar to bench  
15  
16 439 dehydration VCs, with a slight shift towards more vulnerable values when using the 14 cm rotor (Fig.  
17  
18 440 2, Table S2) as recently found by Pengxian et al. (2018) in *Acer mono* when comparing in the static  
19  
20 441 centrifuge the 14 cm and 27 cm rotors. VCs of other short vesseled angiosperms such as *Betula*  
21  
22 442 *pendula* (Cochard et al. 2010), *Fagus sylvatica* (Aranda et al. 2014), *Populus tremuloides* (Schreiber et  
23  
24 443 al. 2011) or *Acer negundo* (Christman et al. 2009) were also sigmoidal when the static centrifuge or  
25  
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  
29  
30 446 population (Fig. 2, Table S2). This dramatic change was observed previously in peach (*Prunus persica*)  
31  
32 447 when the length of the centrifuged samples was varied in a Cavitron; shorter samples were more  
33  
34 448 vulnerable to embolism ( $P_{50}$  shifted from -4.5 to -1 MPa) and VCs became r-shaped (Cochard et al.  
35  
36 449 2010). However, when using the static centrifuge to measure the same population, Sperry et al.  
37  
38 450 (Sperry et al. 2012) found that VCs were linear and relatively insensitive to the number of open  
39  
40 451 vessels with  $P_{50}$  less negative than -2 MPa using 14 cm and 27 cm samples. This difference in  
41  
42 452 sensitivity to the proportion of open vessels in the centrifuged samples has led some to conclude  
43  
44 453 that the original centrifuge method and rotor design are not subject to the open vessel artefact  
45  
46 454 (Hacke et al. 2015; Sperry et al. 2012). However, Torres-Ruiz et al. (2017) demonstrated that if the  
47  
48 455 amount of open vessels is relatively high in both rotors, 14 and 27 cm, VCs could be equally biased  
49  
50 456 and would appear statistically indistinguishable.  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

1  
2  
3 457 Recent publications have addressed this controversy, showing that long-vesseled species such as  
4  
5 458 grape vine, oaks, robinia or olive, with a high proportion of open vessels, produce similarly biased  
6  
7 459 results with both the static centrifuge and the Cavitron when compared with reference curves  
8  
9 460 generated by dehydration or non-invasive imaging (Choat et al. 2016; Choat et al. 2010; Pengxian et  
10  
11 461 al. ; Torres-Ruiz et al. 2014). Li et al. (2008) and Pengxian et al. (2018) tested the two centrifuge  
12  
13 462 methods head to head and found close correspondence in VCs across species with different xylem  
14  
15 463 anatomy. An extended literature survey of methods to measure vulnerability to embolism showed  
16  
17 464 that when using the centrifuge, VCs were sigmoidal in conifers and in long vessel species  
18  
19 465 exponential, whereas in diffuse porous species VCs varied from sigmoidal to linear or exponential  
20  
21 466 (Cochard et al. 2013). Our measurements and simulations made with the CAVITOPEN model explain  
22  
23 467 the different shapes of VCs and some disagreements between the static centrifuge and the Cavitron.  
24  
25 468 In short-vesseled angiosperms, we have shown that VCs by centrifugation agreed with each other  
26  
27 469 and closely matched the curves based on bench dehydration and microCT (Choat et al. 2016;  
28  
29 470 Cochard et al. 2010). In angiosperms with a proportion of vessels open to the middle but not the  
30  
31 471 whole way through, the standard protocol in the static centrifuge produces linear VCs (Sperry et al.  
32  
33 472 2012). Here the initial conductivity is measured before spinning, thus if the native embolism is low,  
34  
35 473 all the vessels are conductive, regardless of their length. As soon as the sample is spun, the  
36  
37 474 conductivity would be artificially reduced relative to the native state in proportion to the amount of  
38  
39 475 vessels open to centre. Sample with open vessels thus become artificially vulnerable to embolism at  
40  
41 476 the beginning of the VC (i.e. at less negative water potentials). For *H. leucoptera*, this translated into  
42  
43 477 less negative values of  $P_{12}$  in all centrifuged samples compared with those measured with the bench  
44  
45 478 dehydration technique creating a linear response or a plateau at high water potentials. Higher  
46  
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  
50  
51 481 measurement was made while spinning at low tension and many open to centre vessels would  
52  
53 482 already be embolised in the initial measurement of conductivity, resulting in a lower artefactual loss  
54  
55  
56  
57  
58  
59  
60

1  
2  
3 483 of conductivity in the subsequent water potentials of the VC. This may bias the curves slightly  
4  
5 484 pushing them to more negative values but it did not appear to be significant effect here as the  
6  
7 485 Cavitron curves for *H. leucompta* were similar to bench dehydration curves.  
8  
9

10 486 The simulations of PLC with the CAVITOPEN model confirmed that the impact of open vessels on the  
11  
12 487 VC was higher when vessels were long, samples short and when open vessels cavitated at less  
13  
14 488 negative pressures (Fig. 4). If the samples were much shorter than the maximum vessel length of the  
15  
16 489 branch (see the results in Fig. 4 for the 14 cm rotor with  $L_{max}$  50cm), the resulting VC was exponential  
17  
18 490 (r-shaped), as observed in long-vessel angiosperms, and shifted to more linear or s-shaped when  
19  
20 491  $L_{max}$  was decreased or the sample length increased. One of the assumptions in the model is that  
21  
22 492 vessels open at the cut surface cavitate when they reach a threshold value; that is far less negative  
23  
24 493 than intact vessels whose two ends are included within the spun segment. This influences the shape  
25  
26 494 the VC at higher pressures creating a “bump” in the VC followed by a plateau. This effect can be  
27  
28 495 corrected to some extent if the first inflexion point of the VC is considered to be the 0% point for loss  
29  
30 496 of conductivity. In this case the initial conductivity ( $K_i$ ) value is shifted to a lower value corresponding  
31  
32 497 to the hydraulic conductivity of the plateau (Fig. 4d). The estimated values of  $P_{50}$  and  $S_{50}$  when the  
33  
34 498 CAVITOPEN model was fit to actual measurements agreed quite well with those obtained with  
35  
36 499 reference techniques and confirmed that this model can be used to correct open vessel artefacts for  
37  
38 500 centrifuge based VCs. The estimated  $L_{max}$  was however significantly shorter than  $L_{max}$  measured with  
39  
40 501 the air injection technique. The air injection technique has shown to produce higher  $L_{max}$  than the  
41  
42 502 rubber injection method (Pan et al. 2015), thus our values could be overestimated. On the other  
43  
44 503 hand, the model assumed that vessel lengths in a sample follow the density function proposed by  
45  
46 504 Cohen et al. (2003) which can be sensitive to the clustering of vessel lengths (Cai and Tyree 2014). It  
47  
48 505 is clear that the actual distribution of vessel lengths, network topology and connectivity are crucial  
49  
50 506 for the sensitivity to an open vessel artefact.  
51  
52  
53  
54

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

56  
57  
58  
59  
60

1  
2  
3 508 The physical mechanisms underlying this open-vessel artefact are yet to be fully elucidated. Some  
4  
5 509 studies suggest that microbubbles and particles can act as nucleation sites when they flow through  
6  
7 510 the sample as it spins in the Cavitron, causing premature embolism (Cochard et al. 2010; Sperry et al.  
8  
9 511 2012; Wang et al. 2014). In the static centrifuge, bubbles might be drawn into vessels while starting  
10  
11 512 the spin or while mounting or dismounting the stems to measure flow (Wang et al. 2014). In both  
12  
13 513 centrifuge techniques bubbles in open vessels can move by buoyancy while spinning toward the  
14  
15 514 region of lowest pressure at the center of rotation (Rockwell et al., 2014). Draining from open  
16  
17 515 vessels as a consequence of artefactual embolism when the centrifuge starts spinning appears to be  
18  
19 516 a common phenomenon in both rotors. Our microCT images showed that after spinning in the  
20  
21 517 centrifuge, most of the vessels were empty near the ends even though tension ought to be zero  
22  
23 518 (Cochard et al. 2005). The use of water saturated foam pads to avoid desiccation did not prevent this  
24  
25 519 (Hacke et al. 2015; Tobin et al. 2013). We discarded the possibility that sample manipulation before  
26  
27 520 spinning or during wax embedding had triggered vessel draining because we scanned control  
28  
29 521 samples that were not spun. These samples showed no embolism (Fig. 3). Furthermore, patterns of  
30  
31 522 embolism did not follow theoretical expectations based on the distribution of tension within the  
32  
33 523 spun sample. The embolism levels decreased from the ends to the center in a fashion consistent  
34  
35 524 with the amount of vessels open to center, opposite to that expected from profile in tension and in  
36  
37 525 agreement with the assumption of the CAVITOPEN model than open vessels artificially cavitate  
38  
39 526 when they reach a threshold pressure that is much less negative than in intact vessels. This pattern  
40  
41 527 was observed at water potentials inducing less than 40% loss of conductivity based on the VC  
42  
43 528 obtained using the middle segment of the centrifuged sample (Fig. 3), even though the centre of the  
44  
45 529 sample experienced the highest tensions. Embolism levels converged within the sample at -9 MPa at  
46  
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  
50  
51 532 made previously by Cochard et al. (2010) using the Cavitron; they reported that embolism was  
52  
53 533 higher in the basal and upstream ends relative to the centre of samples from species with vessels  
54  
55  
56  
57  
58  
59  
60



1  
2  
3 534 that are predominately at least half as long as the spun segment. Cai et al. (2010) and Pengxian et al.  
4  
5 535 (2018) also reported higher PLC values than predicted by theory at both ends after spinning samples  
6  
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  
10  
11 538 technique.

12  
13  
14 539 The hydraulic continuity between vessels cut open at each end of the sample and vessels with their  
15  
16 540 terminal ends in this portion of the sample is probably re-established by refilling of vessels immersed  
17  
18 541 under water at both ends (Fig. 2 in Cochard et al. (2010)). This refilling would occur by capillarity  
19  
20 542 either while spinning in the Cavitron or while flow is measured gravimetrically (Fig. S2). Since the  
21  
22 543 middle of the centrifuged sample contains the majority of intact vessels, VCs constructed with the  
23  
24 544 static centrifuge technique of angiosperm species using only the central segment are more reliable  
25  
26 545 and in closer agreement with PLC generated by natural dehydration (Fig. 2). This modification is  
27  
28 546 technically easy to achieve and mitigates the open vessel artefact; however, it carries the  
29  
30 547 disadvantage that samples cannot be spun repeatedly to construct replicate curves for each sample  
31  
32 548 and thus more plant material is needed to construct each curve.

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

37  
38  
39 550 We confirmed the validity of vulnerability curves constructed with both centrifuge methods for short  
40  
41 551 conduit angiosperm species, those with most conduits shorter than half the length of the centrifuge  
42  
43 552 rotor. A new model, CAVITOPEN was developed to simulate the effect of vessel length, rotor size and  
44  
45 553 vulnerability of open vessels in loss of conductivity of centrifuged samples. In species with maximum  
46  
47 554 vessel length similar to the centrifuge rotor, we recommend constructing vulnerability curves with  
48  
49 555 the Cavitron or measuring flow exclusively in the central part of the spun segment when using the  
50  
51 556 static centrifuge. Alternatively, artefactual embolism at low xylem tensions can be corrected if the  
52  
53 557 first inflexion point of the VC is considered to be the starting point for  $K_{max}$  (0 % loss of conductivity)  
54  
55 558 or by fitting the CAVITOPEN model to the measurements to estimate  $P_{50}$  and  $S_{50}$ . When samples

1  
2  
3 559 contained a high proportion of open to centre vessels, the centrifuge technique is prone to error and  
4  
5 560 overestimates vulnerability to embolism. Determining the proportion of open to centre vessels or  
6  
7 561 performing the simple test recently proposed by Torres-Ruiz et al. (2017), which compares changes  
8  
9 562 in  $K_s$  before and after spinning in the centrifuge at low tensions, are highly advisable before using  
10  
11 563 any of the centrifuge techniques.

12  
13  
14 564 The shape of the vulnerability curves obtained with bench dehydration were always sigmoidal while  
15  
16 565 in centrifuged samples the shape was determined by the presence of open vessels. While previous  
17  
18 566 studies have demonstrated that species with the longest vessel classes (eg. lianas, ring porous trees)  
19  
20 567 open vessels tend to exhibit exponential curves when measured in the centrifuge. Here we showed  
21  
22 568 that VCs with a linear shape are symptomatic of species with intermediate vessel lengths in which a  
23  
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  
27  
28 571 segment.

### 31 572 **Acknowledgments**

32  
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  
39  
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.

43  
44  
45  
46 578

579 **References**

- 580 Alder N, Pockman W, Sperry J, Nuismer S (1997) Use of centrifugal force in the study of xylem  
581 cavitation. *Journal of Experimental Botany*. 48:665-674.
- 582 Aranda I, Cano FJ, Gascó A, Cochard H, Nardini A, Mancha JA, López R, Sánchez-Gómez D (2014)  
583 Variation in photosynthetic performance and hydraulic architecture across European beech  
584 (*Fagus sylvatica* L.) populations supports the case for local adaptation to water stress. *Tree*  
585 *physiology*. 35:34-46.
- 586 Brodersen CR, McElrone AJ, Choat B, Lee EF, Shackel KA, Matthews MA (2013) In vivo visualizations  
587 of drought-induced embolism spread in *Vitis vinifera*. *Plant Physiology*. 161:1820-9.
- 588 Brodrribb TJ (2017) Progressing from 'functional' to mechanistic traits. *New Phytologist*. 215:9-11.
- 589 Brodrribb TJ, Cochard H (2009) Hydraulic failure defines the recovery and point of death in water-  
590 stressed conifers. *Plant Physiology*. 149:575-84.
- 591 Cai J, Hacke U, Zhang S, Tyree MT (2010) What happens when stems are embolized in a centrifuge?  
592 Testing the cavitron theory. *Physiologia plantarum*. 140:311-320.
- 593 Cai J, Tyree MT (2014) Measuring vessel length in vascular plants: can we divine the truth? History,  
594 theory, methods, and contrasting models. *Trees*. 28:643-655.
- 595 Choat B, Badel E, Burrett R, Delzon S, Cochard H, Jansen S (2016) Noninvasive Measurement of  
596 Vulnerability to Drought-Induced Embolism by X-Ray Microtomography. *Plant Physiology*.  
597 170:273-82.
- 598 Choat B, Drayton WM, Brodersen C, Matthews MA, Shackel KA, Wada H, McElrone AJ (2010)  
599 Measurement of vulnerability to water stress-induced cavitation in grapevine: a comparison  
600 of four techniques applied to a long-vesseled species. *Plant, Cell & Environment*. 33:1502-12.
- 601 Choat B, Jansen S, Brodrribb TJ, Cochard H, Delzon S, Bhaskar R, Bucci SJ, Feild TS, Gleason SM, Hacke  
602 UG, Jacobsen AL, Lens F, Maherali H, Martinez-Vilalta J, Mayr S, Mencuccini M, Mitchell PJ,  
603 Nardini A, Pittermann J, Pratt RB, Sperry JS, Westoby M, Wright IJ, Zanne AE (2012) Global  
604 convergence in the vulnerability of forests to drought. *Nature*. 491:752-5.
- 605 Christman MA, Sperry JS, Adler FR (2009) Testing the 'rare pit' hypothesis for xylem cavitation  
606 resistance in three species of *Acer*. *New Phytologist*. 182:664-674.
- 607 Cobb AR, Choat B, Holbrook NM (2007) Dynamics of freeze-thaw embolism in *Smilax rotundifolia*  
608 (*Smilacaceae*). *American Journal of Botany*. 94:640-9.
- 609 Cochard H (2002) A technique for measuring xylem hydraulic conductance under high negative  
610 pressures. *Plant, Cell & Environment*. 25:815-819.
- 611 Cochard H, Badel E, Herbette S, Delzon S, Choat B, Jansen S (2013) Methods for measuring plant  
612 vulnerability to cavitation: a critical review. *Journal of Experimental Botany*. 64:4779-91.
- 613 Cochard H, Damour G, Bodet C, Tharwat I, Poirier M, Améglio T (2005) Evaluation of a new  
614 centrifuge technique for rapid generation of xylem vulnerability curves. *Physiologia*  
615 *Plantarum*. 124:410-418.
- 616 Cochard H, Delzon S, Badel E (2015) X-ray microtomography (micro-CT): a reference technology for  
617 high-resolution quantification of xylem embolism in trees. *Plant, Cell & Environment*.  
618 38:201-206.
- 619 Cochard H, Ewers F, Tyree M (1994) Water relations of a tropical vine-like bamboo (*Rhipidocladum*  
620 *racemiflorum*): root pressures, vulnerability to cavitation and seasonal changes in embolism.  
621 *Journal of Experimental Botany*. 45:1085-1089.
- 622 Cochard H, Herbette S, Barigah T, Badel E, Ennajeh M, Vilagrosa A (2010) Does sample length  
623 influence the shape of xylem embolism vulnerability curves? A test with the Cavitron  
624 spinning technique. *Plant, Cell & Environment*. 33:1543-52.
- 625 Cohen S, Bennink J, Tyree M (2003) Air method measurements of apple vessel length distributions  
626 with improved apparatus and theory\*. *Journal of Experimental Botany*. 54:1889-1897.

- 1  
2  
3 627 Dalla-Salda G, Fernández ME, Sergent A-S, Rozenberg P, Badel E, Martinez-Meier A (2014) Dynamics  
4 628 of cavitation in a Douglas-fir tree-ring: transition-wood, the lord of the ring? *Journal of Plant*  
5 629 *Hydraulics*. 1:005.
- 6 630 Duursma R (2017) An R implementation of the CAVITOPEN model  
7 631 Duursma R, Choat B (2017) fitplc - an R package to fit hydraulic vulnerability curves. 2017. 4  
8 632 Ennajeh M, Simoes F, Khemira H, Cochard H (2011) How reliable is the double-ended pressure sleeve  
9 633 technique for assessing xylem vulnerability to cavitation in woody angiosperms? *Physiologia*  
10 634 *Plantarum*. 142:205-10.
- 11 635 Ewers FW, Fisher JB (1989) Variation in vessel length and diameter in stems of six tropical and  
12 636 subtropical lianas. *American Journal of Botany*:1452-1459.
- 13 637 Hacke UG, Venturas MD, MacKinnon ED, Jacobsen AL, Sperry JS, Pratt RB (2015) The standard  
14 638 centrifuge method accurately measures vulnerability curves of long-vesselled olive stems.  
15 639 *New Phytologist*. 205:116-27.
- 16 640 Holbrook NM, Burns MJ, Field CB (1995) Negative xylem pressures in plants: a test of the balancing  
17 641 pressure technique. *Science*. 270:1193-1195.
- 18 642 Jacobsen AL, Pratt RB (2012) No evidence for an open vessel effect in centrifuge-based vulnerability  
19 643 curves of a long-vesselled liana (*Vitis vinifera*). *New Phytologist*. 194:982-990.
- 20 644 Jansen S, Schuldt B, Choat B (2015) Current controversies and challenges in applying plant hydraulic  
21 645 techniques. *New Phytologist*. 205:961-964.
- 22 646 Li Y, Sperry JS, Taneda H, Bush SE, Hacke UG (2008) Evaluation of centrifugal methods for measuring  
23 647 xylem cavitation in conifers, diffuse- and ring-porous angiosperms. *New Phytologist*.  
24 648 177:558-68.
- 25 649 Nardini A, Battistuzzo M, Savi T (2013) Shoot desiccation and hydraulic failure in temperate woody  
26 650 angiosperms during an extreme summer drought. *New Phytologist*. 200:322-329.
- 27 651 Nolf M, Lopez R, Peters JM, Flavel RJ, Koloadin LS, Young IM, Choat B (2017) Visualization of xylem  
28 652 embolism by X-ray microtomography: a direct test against hydraulic measurements. *New*  
29 653 *Phytologist*. 214:890-898.
- 30 654 Ogle K, Barber JJ, Willson C, Thompson B (2009) Hierarchical statistical modeling of xylem  
31 655 vulnerability to cavitation. *New Phytologist*. 182:541-554.
- 32 656 Pan R, Geng J, Cai J, Tyree MT (2015) A comparison of two methods for measuring vessel length in  
33 657 woody plants. *Plant, Cell & Environment*. 38:2519-2526.
- 34 658 Pengxian Y, Feng M, Qing L, Rui A, Jing C, Guangyuan D A comparison of two centrifuge techniques  
35 659 for constructing vulnerability curves: insight into the 'open-vessel' artifact. *Physiologia*  
36 660 *Plantarum*. 0
- 37 661 Pockman WT, Sperry JS, Leary JW (1995) Sustained and significant negative water pressure in xylem.  
38 662 *Nature*. 378:715.
- 39 663 Rodríguez-Calcerrada J, Li M, López R, Cano FJ, Oleksyn J, Atkin OK, Pita P, Aranda I, Gil L (2017)  
40 664 Drought-induced shoot dieback starts with massive root xylem embolism and variable  
41 665 depletion of nonstructural carbohydrates in seedlings of two tree species. *New Phytologist*.  
42 666 213:597-610.
- 43 667 Sala A, Piper F, Hoch G (2010) Physiological mechanisms of drought-induced tree mortality are far  
44 668 from being resolved. *New Phytologist*. 186:274-81.
- 45 669 Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis.  
46 670 *Nature methods*. 9:671-675.
- 47 671 Schreiber SG, Hacke UG, Hamann A, Thomas BR (2011) Genetic variation of hydraulic and wood  
48 672 anatomical traits in hybrid poplar and trembling aspen. *New Phytologist*. 190:150-160.
- 49 673 Sperry JS (1985) Xylem embolism in the palm *Rhapis excelsa*. *IAWA Journal*. 6:283-292.
- 50 674 Sperry JS, Christman MA, Torres-Ruiz JM, Taneda H, Smith DD (2012) Vulnerability curves by  
51 675 centrifugation: is there an open vessel artefact, and are 'r' shaped curves necessarily invalid?  
52 676 *Plant, Cell & Environment*. 35:601-10.

- 1  
2  
3 677 Sperry JS, Tyree MT (1988) Mechanism of water stress-induced xylem embolism. *Plant Physiology*.  
4 678 88:581-7.  
5 679 Tobin MF, Pratt RB, Jacobsen AL, De Guzman ME (2013) Xylem vulnerability to cavitation can be  
6 680 accurately characterised in species with long vessels using a centrifuge method. *Plant*  
7 681 *Biology*. 15:496-504.  
8 682 Torres-Ruiz JM, Cochard H, Mayr S, Beikircher B, Diaz-Espejo A, Rodriguez-Dominguez CM, Badel E,  
9 683 Fernandez JE (2014) Vulnerability to cavitation in *Olea europaea* current-year shoots: further  
10 684 evidence of an open-vessel artifact associated with centrifuge and air-injection techniques.  
11 685 *Physiologia Plantarum*. 152:465-74.  
12 686 Torres-Ruiz JM, Cochard H, Mencuccini M, Delzon S, Badel E (2016) Direct observation and modelling  
13 687 of embolism spread between xylem conduits: a case study in Scots pine. *Plant, Cell &*  
14 688 *Environment*. 39:2774-2785.  
15 689 Torres-Ruiz JM, Jansen S, Choat B, McElrone AJ, Cochard H, Brodribb TJ, Badel E, Burrett R, Bouche  
16 690 PS, Brodersen CR (2015) Direct X-ray microtomography observation confirms the induction  
17 691 of embolism upon xylem cutting under tension. *Plant Physiology*. 167:40-43.  
18 692 Torres-Ruiz JM, Cochard H, Choat B, Jansen S, López R, Tomášková I, Padilla-Díaz CM, Badel E, Burrett  
19 693 R, King A (2017) Xylem resistance to embolism: presenting a simple diagnostic test for the  
20 694 open vessel artefact. *New Phytologist*. 215:489-499.  
21 695 Tyree MT, Dixon MA (1986) Water stress induced cavitation and embolism in some woody plants.  
22 696 *Physiologia Plantarum*. 66:397-405.  
23 697 Tyree MT, Sperry JS (1988) Do woody plants operate near the point of catastrophic xylem  
24 698 dysfunction caused by dynamic water stress? : answers from a model. *Plant Physiology*.  
25 699 88:574-80.  
26 700 Tyree MT, Zimmermann MH (2002) Hydraulic architecture of whole plants and plant performance  
27 701 Xylem structure and the ascent of sap. Springer, pp 175-214.  
28 702 Urli M, Porte AJ, Cochard H, Guengant Y, Burrett R, Delzon S (2013) Xylem embolism threshold for  
29 703 catastrophic hydraulic failure in angiosperm trees. *Tree Physiology*. 33:672-83.  
30 704 Venturas MD, MacKinnon ED, Dario HL, Jacobsen AL, Pratt RB, Davis SD (2016) Chaparral shrub  
31 705 hydraulic traits, size, and life history types relate to species mortality during California's  
32 706 historic drought of 2014. *PloS one*. 11:e0159145.  
33 707 Wang R, Zhang L, Zhang S, Cai J, Tyree MT (2014) Water relations of *Robinia pseudoacacia* L.: do  
34 708 vessels cavitate and refill diurnally or are R-shaped curves invalid in *Robinia*? *Plant, cell &*  
35 709 *environment*. 37:2667-2678.  
36 710 Wheeler JK, Huggett BA, Tofte AN, Rockwell FE, Holbrook NM (2013) Cutting xylem under tension or  
37 711 supersaturated with gas can generate PLC and the appearance of rapid recovery from  
38 712 embolism. *Plant, Cell & Environment*. 36:1938-1949.  
39  
40  
41 713  
42  
43  
44 714  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

1  
2  
3 715 **Figure legends**  
4

5  
6 716 **Figure 1.** Distribution of PLC in air-injected branches of *H. dactyloides* (black circles) and *H.*  
7  
8 717 *leucoptera* (open circles) at different positions from the injected end. Vertical bars represent the  
9  
10 718 standard error. Dashed lines indicate the two sample lengths used for the centrifuge methods, 14 cm  
11  
12 719 and 27 cm and dot lines indicate their respective half sample length.

13  
14  
15 720 **Figure 2.** Xylem vulnerability to embolism curves and 95% confidence intervals (grey shaded areas)  
16  
17 721 of *Hakea dactyloides* (left panels) and *Hakea leucoptera* (right panels) obtained with two methods to  
18  
19 722 induce cavitation in the xylem, bench dehydration and centrifuge force and three methods to  
20  
21 723 measure the loss of conductivity, flowmeter (close circles), in situ flow method (open circles) and X-  
22  
23 724 ray microCT visualisation (red triangles). Vertical solid lines indicate  $P_{50}$  and vertical dashed lines  
24  
25 725 indicate the 95% confidence interval for  $P_{50}$ . Horizontal dashed lines indicated native xylem  
26  
27 726 embolism measured in the field. Two rotor sizes, 14 cm and 27 cm, were used in the static  
28  
29 727 centrifuge, and water flow in the whole segment or only in the central part was measured (see  
30  
31 728 methods for details).

32  
33  
34 729 **Figure 3.** Transverse slices from X-ray microtomography (X-ray micro-CT) scans of branches of *Hakea*  
35  
36 730 *leucoptera* (maximum vessel length =  $25 \pm 5$  cm) scanned at three positions before spinning (left  
37  
38 731 column) and after spinning in the centrifuge at 5, 7 and 9 MPa. Embolized vessels appear as black  
39  
40 732 and water-filled conduits appear as grey. The estimated percent loss of conductivity (PLC) is shown  
41  
42 733 in each picture. Scale bar, 1 mm.

43  
44  
45 734 **Figure 4.** Simulations with the CAVITOPEN model of the effect of threshold of embolism formation  
46  
47 735 (MPa) of cut open vessels (A), maximum vessel length (cm) (B), and rotor size (cm) (C) on xylem  
48  
49 736 vulnerability to embolism curves generated with centrifugation. In red, vulnerability curve of close  
50  
51 737 vessels at both ends. (D) The CAVITOPEN model was fit to measurements in *H. leucoptera* using  
52  
53 738 numerical optimization to estimate all four parameters: water potential at 50% loss of conductivity  
54  
55  
56  
57  
58  
59  
60

1  
2  
3 739 ( $P_{50}$ ), slope of the vulnerability curve ( $S_{50}$ ), maximum vessel length ( $L_{max}$ ) and threshold of embolism  
4  
5 740 formation of cut open vessels ( $P_{open}$ ). Circles represent the values obtained in our study with the  
6  
7 741 static centrifuge, 27 cm rotor in *H. leucoptera* when flow was measured in the whole segment (see  
8  
9 742 Methods for details); grey solid line is the fitted curve with the CAVITOPEN model; black solid line  
10  
11 743 represent the curve after correction and orange dashed line is the reference curve obtained with  
12  
13 744 bench dehydration for the species.

14  
15  
16 745  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

For Peer Review

1  
2  
3  
4  
5  
6  
7  
8 1 **Title:** Mitigating the open vessel artefact in centrifuge based measurement of embolism resistance

9  
10 2 **Running head:** Mitigating artefacts in vulnerability curves

11  
12  
13 3

14  
15 4

16  
17 5 Rosana López<sup>1,2</sup>, Markus Nolf<sup>3</sup>, Remko A Duursma<sup>3</sup>, Eric Badel<sup>1</sup>, Richard J Flavel<sup>4</sup>, Hervé Cochard<sup>1</sup>,

18  
19 6 Brendan Choat<sup>3</sup>

20  
21 7

22  
23 8 1- Université Clermont Auvergne, INRA, PIAF, Clermont-Ferrand, France.

24  
25 9 2- Sistemas y Recursos Naturales, Universidad Politécnica de Madrid, Madrid, Spain.

26  
27 10 3- Hawkesbury Institute for the Environment, Western Sydney University, Richmond, NSW, Australia.

28  
29 11 4- School of Environmental and Rural Science, University of New England, Armidale, NSW, Australia.

30  
31  
32 12

33  
34  
35 13 **Corresponding author details:**

36  
37 14 Dr. Rosana López

38  
39 15 e-mail: rosana.lopez@upm.es

40  
41 16 phone: +34 655868659

42  
43 17 Address: ETSI Montes, Forestal y del Medio Natural. C/ José Antonio Novais, 10. 28040 Madrid

44  
45  
46  
47 18

48  
49 19

50  
51  
52  
53 1



**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).

1  
2  
3  
4  
5  
6  
7  
8 68 The most straightforward technique for inducing embolism is bench dehydration, wherein whole  
9  
10 69 plants or long branches are gradually dehydrated to various xylem tensions and hydraulic conductivity  
11  
12 70 of excised segments is measured gravimetrically before and after removing air from embolised  
13  
14 71 conduits (Sperry and Tyree 1988; Tyree and Zimmermann 2002). Bench dehydration relies on natural  
15  
16 72 desiccation of plant tissues and is therefore considered as the best reference method with which to  
17  
18 73 validate other techniques (Cochard et al. 2013; Ennajeh et al. 2011; Sperry et al. 2012). This method  
19  
20 74 is not completely free of artefacts and issues associated with disequilibrium in water potential within  
21  
22 75 a stem, blockage of flow by resin/mucilage (Cobb et al. 2007), and excision of samples under tension  
23  
24 76 can all alter the vulnerability curve significantly (Wheeler et al., 2013). Although most of these issues  
25  
26 77 can be minimised by adoption of suitable protocols (eg. Torres-Ruiz et al., 2015), the bench  
27  
28 78 dehydration technique requires several days and a substantial amount of plant material to obtain a  
29  
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  
39  
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  
53  
54 91 allowed measuring the conductivity of the segment while it is spinning and under tension. This further  
55  
56 92 increased the efficiency of measurement and allowed for flow measurements to be made under  
57  
58 93 tension.

1  
2  
3  
4  
5  
6  
7  
8 94 Although centrifuge based techniques induce embolism by increasing tension in sample xylem, the  
9  
10 95 patterns of embolism spread through the sample may differ from a naturally dehydrated sample (Cai  
11  
12 96 et al. 2010). The tension profile in the centrifuged segment is highest in the axis of rotation (i.e. in the  
13  
14 97 middle section of the segment) and declines towards the segment ends (Cochard et al. 2005), while  
15  
16 98 during natural dehydration the tension profile across the segment is expected to remain  
17  
18 99 approximately constant (Cai et al. 2010). Nevertheless, the vulnerability curves generated by  
19  
20 100 centrifugation agree well with the bench-top method in conifers and short-vesseled angiosperm  
21  
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  
25  
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  
34  
35 108 spinning (static centrifuge method), or while centrifuging (Cavitron method). However, we are yet to  
36  
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  
48  
49 115 temporal patterns of embolism formation (Brodersen et al. 2013; Choat et al. 2016; Dalla-Salda et al.  
50  
51 116 2014; Torres-Ruiz et al. 2016).

52  
53 117 In this study we evaluated the performance of both centrifuge techniques against bench dehydration  
54  
55 118 in order to examine possible discrepancies associated with each technique. First, we tested two

1  
2  
3  
4  
5  
6  
7  
8 119 methods for inducing embolism: bench dehydration and centrifugation. We then tested three ways of  
9  
10 120 measuring the resulting loss of conductivity: gravimetric flow measured in bench-dehydrated and  
11  
12 121 centrifuged samples (static centrifuge), *in situ* flow measured under tension during spinning in the  
13  
14 122 centrifuge (Cavitron), and direct imaging using X-ray microCT observation. All experiments were  
15  
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  
19  
20 125 vessels and maximum vessel length is ca. 25 cm. Additionally, we compared results obtained using  
21  
22 126 two rotor diameters (14 and 27 cm) to assess the effect of sample length, and measured hydraulic  
23  
24 127 flow both in the whole, spun segments and excised middle sections. Spatial patterns of embolism  
25  
26 128 within samples were visualized with X-ray microCT after centrifugation in order to provide further  
27  
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

6

1  
2  
3  
4  
5  
6  
7  
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.

12  
13  
14 146 *Midday xylem water potential in the field and Native embolism*

15  
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  
49  
50 165 as:

51  
52  
53  
54  
55  
56  
57  
58  
59  
60 166 
$$PLC = 100 \times (1 - K_i / K_{max})$$
 (equation 1)

1  
2  
3  
4  
5  
6  
7  
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).

10  
11  
12 169 *Maximum vessel length and vessel length distribution*

13  
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. leucoptera* 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  
48  
49 188 contribution to flow from vessels longer than this distance. If all the vessels were of equal diameter,  
50  
51 189 this percentage would correspond to the number of vessels longer than the distance from the injection  
52  
53 190 point. In this case of the two *Hakea* species used are diffuse porous and vessel diameters within the  
54  
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

1  
2  
3  
4  
5  
6  
7  
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).



1  
2  
3  
4  
5  
6  
7  
8 216 Static centrifuge measurements were carried out on 20 branches per species. Branches were trimmed  
9  
10 217 under water and both ends were shaved to a final length of 14 or 27 cm. The initial hydraulic  
11  
12 218 conductivity was measured as described above (see Native embolism section) with a pressure head of  
13  
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.

35  
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

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

49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60 238 *X-ray microCT imaging*

1  
2  
3  
4  
5  
6  
7  
8 239 A subset of branches of *H. leucomelaena* was transported to the University of New England in Armidale  
9  
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).  
52  
53 264 A median Z projection of c. 100  $\mu\text{m}$  along the sample axis was extracted from the middle of the scan

1  
2  
3  
4  
5  
6  
7  
8 265 volumes following the protocol in Nolf et al. (2017). PLC of each sample was estimated calculating the  
9  
10 266 theoretical hydraulic conductance based on the conduit dimensions of embolized and functional  
11  
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

1  
2  
3  
4  
5  
6  
7  
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  
41  
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

Con formato: Fuente: Cursiva

1  
2  
3  
4  
5  
6  
7  
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.

11  
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)

1  
2  
3  
4  
5  
6  
7  
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  
9  
10 384 conductivity decreased from the basal end to the centre, contradicting theoretical expectations. This  
11  
12 385 trend was observed even at  $\Psi_x$  inducing less than 40% PLC based on the bench dehydration VC (Fig.  
13  
14 386 3). Only at -9 MPa, that is, below  $P_{88}$  on bench dehydration, did levels of embolism converge along the  
15  
16 387 length of the sample at 80-90%.

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

18  
19 389 The simulations produced by the CAVITOPEN model confirmed that the shape of the VCs generated  
20  
21 390 by the centrifugation was largely dependent on vessel and sample lengths. As maximum vessel length  
22  
23 391 decreased, PLC of the whole sample decreased at a given  $\Psi_x$ , and the shape of the VC shifted from  
24  
25 392 exponential to sigmoidal (Fig. 4a). The same pattern was observed when the sample length increased  
26  
27 393 (Fig. 4b). For instance, with 14-cm-length centrifuged samples,  $P_{50}$  ranged from -0.6 MPa to -7.7 MPa  
28  
29 394 varying the maximum vessel length of the sample from 50 cm to 5 cm. Likewise, the  $P_{50}$  of a centrifuged  
30  
31 395 sample with maximum vessel length of 15 cm ranged from -2.1 MPa in the 14-cm rotor to -7.7 MPa  
32  
33 396 using a 40-cm rotor. Embolism of vessels open from the cut surface (Fig. S1) influenced values of PLC  
34  
35 397 at high negative pressures, even in short-vesseled samples, resulting in rapid loss of conductivity  
36  
37 398 followed by a plateau. The more open vessels and the less negative the threshold of embolism of open  
38  
39 399 vessels (Fig. 4c), the higher is this plateau and stronger the impact on the VC (Fig. 4). VCs can be  
40  
41 400 corrected if the first inflection point of the curve is considered the starting point for initial conductivity  
42  
43 401 ( $K_i$ ), i.e. 0% loss of conductivity. This is shown in Fig. 4d with actual measurements of PLC obtained in  
44  
45 402 27-cm centrifuged samples of *H. leucoptera*. When the CAVITOPEN model was fit (black circles and  
46  
47 403 grey solid line, respectively) and we used the inflection point as starting point for  $K_i$ , the corrected  
48  
49 404 curve matched the reference VC obtained with bench dehydration (Fig. 4d black solid line and orange  
50  
51 405 dashed line, respectively). Alternatively, by fitting the model using numerical optimization we  
52  
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**



1  
2  
3  
4  
5  
6  
7  
8 408 We evaluated the reliability of two centrifuge based techniques commonly used to measure  
9  
10 409 vulnerability to embolism in angiosperm species and present a protocol that mitigates experimental  
11  
12 410 artefacts associated with open xylem vessels. Both the static centrifuge method and the *in-situ* flow  
13  
14 411 centrifuge method (Cavitron) were prone to artefactual embolism caused by open vessels, although  
15  
16 412 the errors were significantly greater in the static centrifuge method. In a species with maximum vessel  
17  
18 413 length longer or similar to the centrifuge rotor diameter, the static centrifuge significantly  
19  
20 414 overestimated xylem vulnerability to embolism if the whole spun segment was used to measure flow.  
21  
22 415 Observations with microCT indicated that artefactual embolism caused by centrifugation of samples  
23  
24 416 occurred in the outer most portions of samples. However, we demonstrated that artefactual  
25  
26 417 embolism was largely eliminated from static centrifuge if flow was measured in an excised central part  
27  
28 418 of the segment. This altered protocol yielded VCs similar to those obtained on the same species with  
29  
30 419 bench dehydration thus allowing these centrifuge techniques to accurately measure vulnerability to  
31  
32 420 embolism in longer vesseled species. We also present a new model (CAVITOPEN) that simulates the  
33  
34 421 impact of vessel draining at the cut end on the whole VC curve and showed that errors were largely  
35  
36 422 dependent on vessel length and rotor diameter. This model allows researchers to quantitative test  
37  
38 423 and avoid errors associated with the artefactual embolism. The bench dehydration technique  
39  
40 424 indicated that significant embolism was only initiated in both species after water potential dropped  
41  
42 425 below a threshold value, -3.8 MPa in *H. dactyloides* and -6.3 MPa in *H. leucoptera*. PLC then increased  
43  
44 426 rapidly and hydraulic conductivity was lost almost completely within a span of 1 MPa (Fig. 2). These  
45  
46 427 vulnerability curves have been classified as sigmoidal or s-shaped as opposed to exponential or r-  
47  
48 428 shaped curves, characterized by rapid conductivity losses as soon as the water potential declines  
49  
50 429 below zero (Cochard et al. 2013; Sperry et al. 2012). A third type of VC, intermediate between these  
51  
52 430 two, exhibits a linear response, and is mainly found in diffuse porous species when using  
53  
54 431 centrifugation to induce embolism (Cochard et al. 2013).

55  
56  
57  
58  
59  
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

1  
2  
3  
4  
5  
6  
7  
8 460 that when using the centrifuge, VCs were sigmoidal in conifers and in long vessel species exponential,  
9  
10 461 whereas in diffuse porous species VCs varied from sigmoidal to linear or exponential (Cochard et al.  
11  
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-  
14  
15 464 vesseled angiosperms, we have shown that VCs by centrifugation agreed with each other and closely  
16  
17 465 matched the curves based on bench dehydration and microCT (Choat et al. 2016; Cochard et al. 2010).  
18  
19 466 In angiosperms with a proportion of vessels open to the middle but not the whole way through, the  
20  
21 467 standard protocol in the static centrifuge produces linear VCs (Sperry et al. 2012). Here the initial  
22  
23 468 conductivity is measured before spinning, thus if the native embolism is low, all the vessels are  
24  
25 469 conductive, regardless of their length. As soon as the sample is spun, the conductivity would be  
26  
27 470 artificially reduced relative to the native state in proportion to the amount of vessels open to centre.  
28  
29 471 Sample with open vessels thus become artificially vulnerable to embolism at the beginning of the VC  
30  
31 472 (i.e. at less negative water potentials). For *H. leucomptera*, this translated into less negative values of  
32  
33 473  $P_{12}$  in all centrifuged samples compared with those measured with the bench dehydration technique  
34  
35 474 creating a linear response or a plateau at high water potentials. Higher differences in  $P_{12}$  were  
36  
37 475 observed in *H. leucomptera* than in *H. dactyloides* according with a higher proportion of vessels open to  
38  
39 476 centre in the former species (Fig. 1). In the Cavitron, the initial measurement was made while spinning  
40  
41 477 at low tension and many open to centre vessels would already be embolised in the initial  
42  
43 478 measurement of conductivity, resulting in a lower artefactual loss of conductivity in the subsequent  
44  
45 479 water potentials of the VC. This may bias the curves slightly pushing them to more negative values but  
46  
47 480 it did not appear to be significant effect here as the Cavitron curves for *H. leucomptera* were similar to  
48  
49 481 bench dehydration curves.

46 482 The simulations of PLC with the CAVITOPEN model confirmed that the impact of open vessels on the  
47  
48 483 VC was higher when vessels were long, samples short and when open vessels cavitated at less negative  
49  
50 484 pressures (Fig. 4). If the samples were much shorter than the maximum vessel length of the branch  
51  
52 485 (see the results in Fig. 4 for the 14 cm rotor with  $L_{max}$  50cm), the resulting VC was exponential (r-

20

1  
2  
3  
4  
5  
6  
7  
8 486 shaped), as observed in long-vesseled angiosperms, and shifted to more linear or s-shaped when  $L_{max}$   
9  
10 487 was decreased or the sample length increased. One of the assumptions in the model is that vessels  
11  
12 488 open at the cut surface cavitate when they reach a threshold value; that is far less negative than intact  
13  
14 489 vessels whose two ends are included within the spun segment. This influences the shape the VC at  
15  
16 490 higher pressures creating a “bump” in the VC followed by a plateau. This effect can be corrected to  
17  
18 491 some extent if the first inflexion point of the VC is considered to be the 0% point for loss of  
19  
20 492 conductivity. In this case the initial conductivity ( $K_i$ ) value is shifted to a lower value corresponding to  
21  
22 493 the hydraulic conductivity of the plateau (Fig. 4d). The estimated values of  $P_{50}$  and  $S_{50}$  when the  
23  
24 494 CAVITOPEN model was fit to actual measurements agreed quite well with those obtained with  
25  
26 495 reference techniques and confirmed that this model can be used to correct open vessel artefacts for  
27  
28 496 centrifuge based VCs. The estimated  $L_{max}$  was however significantly shorter than  $L_{max}$  measured with  
29  
30 497 the air injection technique. The air injection technique has shown to produce higher  $L_{max}$  than the  
31  
32 498 rubber injection method (Pan et al. 2015), thus our values could be overestimated. On the other hand,  
33  
34 499 the model assumed that vessel lengths in a sample follow the density function proposed by Cohen et  
35  
36 500 al. (2003) which can be sensitive to the clustering of vessel lengths (Cai and Tyree 2014). It is clear that  
37  
38 501 the actual distribution of vessel lengths, network topology and connectivity are crucial for the  
39  
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  
41  
42 505 studies suggest that microbubbles and particles can act as nucleation sites when they flow through  
43  
44 506 the sample as it spins in the Cavitron, causing premature embolism (Cochard et al. 2010; Sperry et al.  
45  
46 507 2012; Wang et al. 2014). In the static centrifuge, bubbles might be drawn into vessels while starting  
47  
48 508 the spin or while mounting or dismounting the stems to measure flow (Wang et al. 2014). In both  
49  
50 509 centrifuge techniques bubbles in open vessels can move by buoyancy while spinning toward the region  
51  
52 510 of lowest pressure at the center of rotation (Rockwell et al., 2014). Draining from open vessels as a

1  
2  
3  
4  
5  
6  
7  
8 511 consequence of artefactual embolism when the centrifuge starts spinning appears to be a common  
9  
10 512 phenomenon in both rotors. Our microCT images showed that after spinning in the centrifuge, most  
11  
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;  
14  
15 515 Tobin et al. 2013). We discarded the possibility that sample manipulation before spinning or during  
16  
17 516 wax embedding had triggered vessel draining because we scanned control samples that were not  
18  
19 517 spun. These samples showed no embolism (Fig. 3). Furthermore, patterns of embolism did not follow  
20  
21 518 theoretical expectations based on the distribution of tension within the spun sample. The embolism  
22  
23 519 levels decreased from the ends to the center in a fashion consistent with the amount of vessels open  
24  
25 520 to center, opposite to that expected from profile in tension and in agreement with the assumption of  
26  
27 521 the CAVITOPEN model than open vessels artificially cavitate when they reach a threshold pressure  
28  
29 522 that is much less negative than in intact vessels. This pattern was observed at water potentials  
30  
31 523 inducing less than 40% loss of conductivity based on the VC obtained using the middle segment of the  
32  
33 524 centrifuged sample (Fig. 3), even though the centre of the sample experienced the highest tensions.  
34  
35 525 Embolism levels converged within the sample at -9 MPa at 80-90%. These results confirm that  
36  
37 526 centrifugation drains open vessels and only reliably measure the vulnerability of intact xylem vessels  
38  
39 527 within the sample (Fig. S1). This is consistent with observations made previously by Cochard et al.  
40  
41 528 (2010) using the Cavitron; they reported that embolism was higher in the basal and upstream ends  
42  
43 529 relative to the centre of samples from species with vessels that are predominately at least half as long  
44  
45 530 as the spun segment. Cai et al. (2010) and Pengxian et al. (2018) also reported higher PLC values than  
46  
47 531 predicted by theory at both ends after spinning samples in a Cavitron. Given that our results were  
48  
49 532 obtained with the static centrifuge it is clear that the overestimation of vulnerability for open to centre  
50  
51 533 vessels occurs in both versions of the centrifuge technique.

52  
53  
54  
55  
56  
57  
58  
59  
60 534 The hydraulic continuity between vessels cut open at each end of the sample and vessels with their  
61  
62 535 terminal ends in this portion of the sample is probably re-established by refilling of vessels immersed  
63  
64 536 under water at both ends (Fig. 2 in Cochard et al. (2010)). This refilling would occur by capillarity either

1  
2  
3  
4  
5  
6  
7  
8 537 while spinning in the Cavitron or while flow is measured gravimetrically (Fig. S2). Since the middle of  
9  
10 538 the centrifuged sample contains the majority of intact vessels, VCs constructed with the static  
11  
12 539 centrifuge technique of angiosperm species using only the central segment are more reliable and in  
13  
14 540 closer agreement with PLC generated by natural dehydration (Fig. 2). This modification is technically  
15  
16 541 easy to achieve and mitigates the open vessel artefact; however, it carries the disadvantage that  
17  
18 542 samples cannot be spun repeatedly to construct replicate curves for each sample and thus more plant  
19  
20 543 material is needed to construct each curve.

#### 20 544 **Conclusion**

21  
22  
23 545 We confirmed the validity of vulnerability curves constructed with both centrifuge methods for short  
24  
25 546 conduit angiosperm species, those with most conduits shorter than half the length of the centrifuge  
26  
27 547 rotor. A new model, CAVITOPEN was developed to simulate the effect of vessel length, rotor size and  
28  
29 548 vulnerability of open vessels in loss of conductivity of centrifuged samples. In species with maximum  
30  
31 549 vessel length similar to the centrifuge rotor, we recommend constructing vulnerability curves with the  
32  
33 550 Cavitron or measuring flow exclusively in the central part of the spun segment when using the static  
34  
35 551 centrifuge. Alternatively, artefactual embolism at low xylem tensions can be corrected if the first  
36  
37 552 inflexion point of the VC is considered to be the starting point for  $K_{max}$  (0 % loss of conductivity) or by  
38  
39 553 fitting the CAVITOPEN model to the measurements to estimate  $P_{50}$  and  $S_{50}$ . When samples contained  
40  
41 554 a high proportion of open to centre vessels, the centrifuge technique is prone to error and  
42  
43 555 overestimates vulnerability to embolism. Determining the proportion of open to centre vessels or  
44  
45 556 performing the simple test recently proposed by Torres-Ruiz et al. (2017), which compares changes in  
46  
47 557  $K_s$  before and after spinning in the centrifuge at low tensions, are highly advisable before using any of  
48  
49 558 the centrifuge techniques.

50  
51 559 The shape of the vulnerability curves obtained with bench dehydration were always sigmoidal while  
52  
53 560 in centrifuged samples the shape was determined by the presence of open vessels. While previous  
54  
55 561 studies have demonstrated that species with the longest vessel classes (eg. lianas, ring porous trees)

1  
2  
3  
4  
5  
6  
7  
8 562 open vessels tend to exhibit exponential curves when measured in the centrifuge. Here we showed  
9  
10 563 that VCs with a linear shape are symptomatic of species with intermediate vessel lengths in which a  
11  
12 564 higher proportion of vessels open to centre of the test segment. The occurrence of this incipient open  
13  
14 565 vessel artefact can be mitigated by measurement of the excised central portion of the segment.

15 566 **Acknowledgments**

16  
17  
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

573 **References**

- 574 Alder N, Pockman W, Sperry J, Nuismer S (1997) Use of centrifugal force in the study of xylem  
575 cavitation. *Journal of Experimental Botany*. 48:665-674.
- 576 Aranda I, Cano FJ, Gascó A, Cochard H, Nardini A, Mancha JA, López R, Sánchez-Gómez D (2014)  
577 Variation in photosynthetic performance and hydraulic architecture across European beech  
578 (*Fagus sylvatica* L.) populations supports the case for local adaptation to water stress. *Tree*  
579 *physiology*. 35:34-46.
- 580 Brodersen CR, McElrone AJ, Choat B, Lee EF, Shackel KA, Matthews MA (2013) In vivo visualizations  
581 of drought-induced embolism spread in *Vitis vinifera*. *Plant Physiology*. 161:1820-9.
- 582 Brodribb TJ (2017) Progressing from 'functional' to mechanistic traits. *New Phytologist*. 215:9-11.
- 583 Brodribb TJ, Cochard H (2009) Hydraulic failure defines the recovery and point of death in water-  
584 stressed conifers. *Plant Physiology*. 149:575-84.
- 585 Cai J, Hacke U, Zhang S, Tyree MT (2010) What happens when stems are embolized in a centrifuge?  
586 Testing the cavitron theory. *Physiologia plantarum*. 140:311-320.
- 587 Cai J, Tyree MT (2014) Measuring vessel length in vascular plants: can we divine the truth? History,  
588 theory, methods, and contrasting models. *Trees*. 28:643-655.
- 589 Choat B, Badel E, Burlett R, Delzon S, Cochard H, Jansen S (2016) Noninvasive Measurement of  
590 Vulnerability to Drought-Induced Embolism by X-Ray Microtomography. *Plant Physiology*.  
591 170:273-82.
- 592 Choat B, Drayton WM, Brodersen C, Matthews MA, Shackel KA, Wada H, McElrone AJ (2010)  
593 Measurement of vulnerability to water stress-induced cavitation in grapevine: a comparison  
594 of four techniques applied to a long-vesseled species. *Plant, Cell & Environment*. 33:1502-12.
- 595 Choat B, Jansen S, Brodribb TJ, Cochard H, Delzon S, Bhaskar R, Bucci SJ, Feild TS, Gleason SM, Hacke  
596 UG, Jacobsen AL, Lens F, Maherali H, Martinez-Vilalta J, Mayr S, Mencuccini M, Mitchell PJ,  
597 Nardini A, Pittermann J, Pratt RB, Sperry JS, Westoby M, Wright IJ, Zanne AE (2012) Global  
598 convergence in the vulnerability of forests to drought. *Nature*. 491:752-5.
- 599 Christman MA, Sperry JS, Adler FR (2009) Testing the 'rare pit' hypothesis for xylem cavitation  
600 resistance in three species of *Acer*. *New Phytologist*. 182:664-674.
- 601 Cobb AR, Choat B, Holbrook NM (2007) Dynamics of freeze-thaw embolism in *Smilax rotundifolia*  
602 (*Smilacaceae*). *American Journal of Botany*. 94:640-9.
- 603 Cochard H (2002) A technique for measuring xylem hydraulic conductance under high negative  
604 pressures. *Plant, Cell & Environment*. 25:815-819.
- 605 Cochard H, Badel E, Herbette S, Delzon S, Choat B, Jansen S (2013) Methods for measuring plant  
606 vulnerability to cavitation: a critical review. *Journal of Experimental Botany*. 64:4779-91.
- 607 Cochard H, Damour G, Bodet C, Tharwat I, Poirier M, Améglio T (2005) Evaluation of a new  
608 centrifuge technique for rapid generation of xylem vulnerability curves. *Physiologia*  
609 *Plantarum*. 124:410-418.
- 610 [Cochard H, Delzon S, Badel E \(2015\) X-ray microtomography \(micro-CT\): a reference technology for](http://mc.manuscriptcentral.com/tp)  
611 [high-resolution quantification of xylem embolism in trees. \*Plant, Cell & Environment\*.](http://mc.manuscriptcentral.com/tp)  
612 [38:201-206.](http://mc.manuscriptcentral.com/tp)
- 613 Cochard H, Ewers F, Tyree M (1994) Water relations of a tropical vine-like bamboo (*Rhipidocladum*  
614 *racemiflorum*): root pressures, vulnerability to cavitation and seasonal changes in embolism.  
615 *Journal of Experimental Botany*. 45:1085-1089.
- 616 Cochard H, Herbette S, Barigah T, Badel E, Ennajeh M, Vilagrosa A (2010) Does sample length  
617 influence the shape of xylem embolism vulnerability curves? A test with the Cavitron  
618 spinning technique. *Plant, Cell & Environment*. 33:1543-52.
- 619 Cohen S, Bennink J, Tyree M (2003) Air method measurements of apple vessel length distributions  
620 with improved apparatus and theory\*. *Journal of Experimental Botany*. 54:1889-1897.



- 1  
2  
3  
4  
5  
6  
7
- 8 621 Dalla-Salda G, Fernández ME, Sergent A-S, Rozenberg P, Badel E, Martinez-Meier A (2014) Dynamics  
9 622 of cavitation in a Douglas-fir tree-ring: transition-wood, the lord of the ring? *Journal of Plant*  
10 623 *Hydraulics*. 1:005.
- 11 624 Duursma R (2017) An R implementation of the CAVITOPEN model  
12 625 Duursma R, Choat B (2017) fitplc - an R package to fit hydraulic vulnerability curves. 2017. 4
- 13 626 Ennajeh M, Simoes F, Khemira H, Cochard H (2011) How reliable is the double-ended pressure sleeve  
14 627 technique for assessing xylem vulnerability to cavitation in woody angiosperms? *Physiologia*  
15 628 *Plantarum*. 142:205-10.
- 16 629 Ewers FW, Fisher JB (1989) Variation in vessel length and diameter in stems of six tropical and  
17 630 subtropical lianas. *American Journal of Botany*:1452-1459.
- 18 631 Hacke UG, Venturas MD, MacKinnon ED, Jacobsen AL, Sperry JS, Pratt RB (2015) The standard  
19 632 centrifuge method accurately measures vulnerability curves of long-vesselled olive stems.  
20 633 *New Phytologist*. 205:116-27.
- 21 634 Holbrook NM, Burns MJ, Field CB (1995) Negative xylem pressures in plants: a test of the balancing  
22 635 pressure technique. *Science*. 270:1193-1195.
- 23 636 Jacobsen AL, Pratt RB (2012) No evidence for an open vessel effect in centrifuge-based vulnerability  
24 637 curves of a long-vesselled liana (*Vitis vinifera*). *New Phytologist*. 194:982-990.
- 25 638 Jansen S, Schuldt B, Choat B (2015) Current controversies and challenges in applying plant hydraulic  
26 639 techniques. *New Phytologist*. 205:961-964.
- 27 640 Li Y, Sperry JS, Taneda H, Bush SE, Hacke UG (2008) Evaluation of centrifugal methods for measuring  
28 641 xylem cavitation in conifers, diffuse- and ring-porous angiosperms. *New Phytologist*.  
29 642 177:558-68.
- 30 643 Nardini A, Battistuzzo M, Savi T (2013) Shoot desiccation and hydraulic failure in temperate woody  
31 644 angiosperms during an extreme summer drought. *New Phytologist*. 200:322-329.
- 32 645 Nolf M, Lopez R, Peters JM, Flavel RJ, Koloadin LS, Young IM, Choat B (2017) Visualization of xylem  
33 646 embolism by X-ray microtomography: a direct test against hydraulic measurements. *New*  
34 647 *Phytologist*. 214:890-898.
- 35 648 Ogle K, Barber JJ, Willson C, Thompson B (2009) Hierarchical statistical modeling of xylem  
36 649 vulnerability to cavitation. *New Phytologist*. 182:541-554.
- 37 650 Pan R, Geng J, Cai J, Tyree MT (2015) A comparison of two methods for measuring vessel length in  
38 651 woody plants. *Plant, Cell & Environment*. 38:2519-2526.
- 39 652 Pengxian Y, Feng M, Qing L, Rui A, Jing C, Guangyuan D A comparison of two centrifuge techniques  
40 653 for constructing vulnerability curves: insight into the 'open-vessel' artifact. *Physiologia*  
41 654 *Plantarum*. 0
- 42 655 Pockman WT, Sperry JS, Leary JW (1995) Sustained and significant negative water pressure in xylem.  
43 656 *Nature*. 378:715.
- 44 657 Rodríguez-Calcerrada J, Li M, López R, Cano FJ, Oleksyn J, Atkin OK, Pita P, Aranda I, Gil L (2017)  
45 658 Drought-induced shoot dieback starts with massive root xylem embolism and variable  
46 659 depletion of nonstructural carbohydrates in seedlings of two tree species. *New Phytologist*.  
47 660 213:597-610.
- 48 661 Sala A, Piper F, Hoch G (2010) Physiological mechanisms of drought-induced tree mortality are far  
49 662 from being resolved. *New Phytologist*. 186:274-81.
- 50 663 Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis.  
51 664 *Nature methods*. 9:671-675.
- 52 665 Schreiber SG, Hacke UG, Hamann A, Thomas BR (2011) Genetic variation of hydraulic and wood  
53 666 anatomical traits in hybrid poplar and trembling aspen. *New Phytologist*. 190:150-160.
- 54 667 Sperry JS (1985) Xylem embolism in the palm *Rhapis excelsa*. *IAWA Journal*. 6:283-292.
- 55 668 Sperry JS, Christman MA, Torres-Ruiz JM, Taneda H, Smith DD (2012) Vulnerability curves by  
56 669 centrifugation: is there an open vessel artefact, and are 'r' shaped curves necessarily invalid?  
57 670 *Plant, Cell & Environment*. 35:601-10.

- 1  
2  
3  
4  
5  
6  
7  
8 671 Sperry JS, Tyree MT (1988) Mechanism of water stress-induced xylem embolism. *Plant Physiology*.  
9 672 88:581-7.
- 10 673 Tobin MF, Pratt RB, Jacobsen AL, De Guzman ME (2013) Xylem vulnerability to cavitation can be  
11 674 accurately characterised in species with long vessels using a centrifuge method. *Plant*  
12 675 *Biology*. 15:496-504.
- 13 676 Torres-Ruiz JM, Cochard H, Mayr S, Beikircher B, Diaz-Espejo A, Rodriguez-Dominguez CM, Badel E,  
14 677 Fernandez JE (2014) Vulnerability to cavitation in *Olea europaea* current-year shoots: further  
15 678 evidence of an open-vessel artifact associated with centrifuge and air-injection techniques.  
16 679 *Physiologia Plantarum*. 152:465-74.
- 17 680 Torres-Ruiz JM, Cochard H, Mencuccini M, Delzon S, Badel E (2016) Direct observation and modelling  
18 681 of embolism spread between xylem conduits: a case study in Scots pine. *Plant, Cell &*  
19 682 *Environment*. 39:2774-2785.
- 20 683 Torres-Ruiz JM, Jansen S, Choat B, McElrone AJ, Cochard H, Brodribb TJ, Badel E, Burrell R, Bouche  
21 684 PS, Brodersen CR (2015) Direct X-ray microtomography observation confirms the induction  
22 685 of embolism upon xylem cutting under tension. *Plant Physiology*. 167:40-43.
- 23 686 Torres-Ruiz JM, Cochard H, Choat B, Jansen S, López R, Tomášková I, Padilla-Díaz CM, Badel E, Burrell  
24 687 R, King A (2017) Xylem resistance to embolism: presenting a simple diagnostic test for the  
25 688 open vessel artefact. *New Phytologist*. 215:489-499.
- 26 689 Tyree MT, Dixon MA (1986) Water stress induced cavitation and embolism in some woody plants.  
27 690 *Physiologia Plantarum*. 66:397-405.
- 28 691 Tyree MT, Sperry JS (1988) Do woody plants operate near the point of catastrophic xylem  
29 692 dysfunction caused by dynamic water stress? : answers from a model. *Plant Physiology*.  
30 693 88:574-80.
- 31 694 Tyree MT, Zimmermann MH (2002) Hydraulic architecture of whole plants and plant performance  
32 695 Xylem structure and the ascent of sap. Springer, pp 175-214.
- 33 696 Urli M, Porte AJ, Cochard H, Guengant Y, Burrell R, Delzon S (2013) Xylem embolism threshold for  
34 697 catastrophic hydraulic failure in angiosperm trees. *Tree Physiology*. 33:672-83.
- 35 698 Venturas MD, MacKinnon ED, Dario HL, Jacobsen AL, Pratt RB, Davis SD (2016) Chaparral shrub  
36 699 hydraulic traits, size, and life history types relate to species mortality during California's  
37 700 historic drought of 2014. *PLoS one*. 11:e0159145.
- 38 701 Wang R, Zhang L, Zhang S, Cai J, Tyree MT (2014) Water relations of *Robinia pseudoacacia* L.: do  
39 702 vessels cavitate and refill diurnally or are R-shaped curves invalid in *Robinia*? *Plant, cell &*  
40 703 *environment*. 37:2667-2678.
- 41 704 Wheeler JK, Huggett BA, Tofte AN, Rockwell FE, Holbrook NM (2013) Cutting xylem under tension or  
42 705 supersaturated with gas can generate PLC and the appearance of rapid recovery from  
43 706 embolism. *Plant, Cell & Environment*. 36:1938-1949.

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).

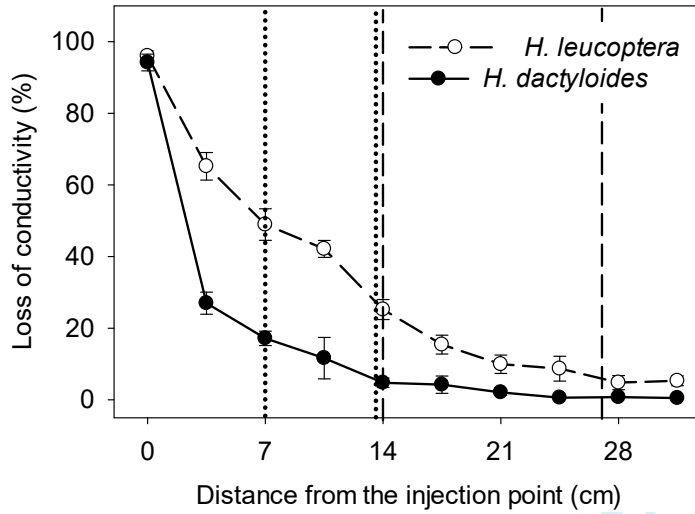
723 **Figure 3.** Transverse slices from X-ray microtomography (X-ray micro-CT) scans of branches of *Hakea*  
 724 *leucoptera* (maximum vessel length =  $25 \pm 5$  cm) scanned at three positions before spinning (left  
 725 column) and after spinning in the centrifuge at 5, 7 and 9 MPa. Embolized 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

1  
2  
3  
4  
5  
6  
7  
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.  
14  
15  
16  
17  
18  
19 739

For Peer Review

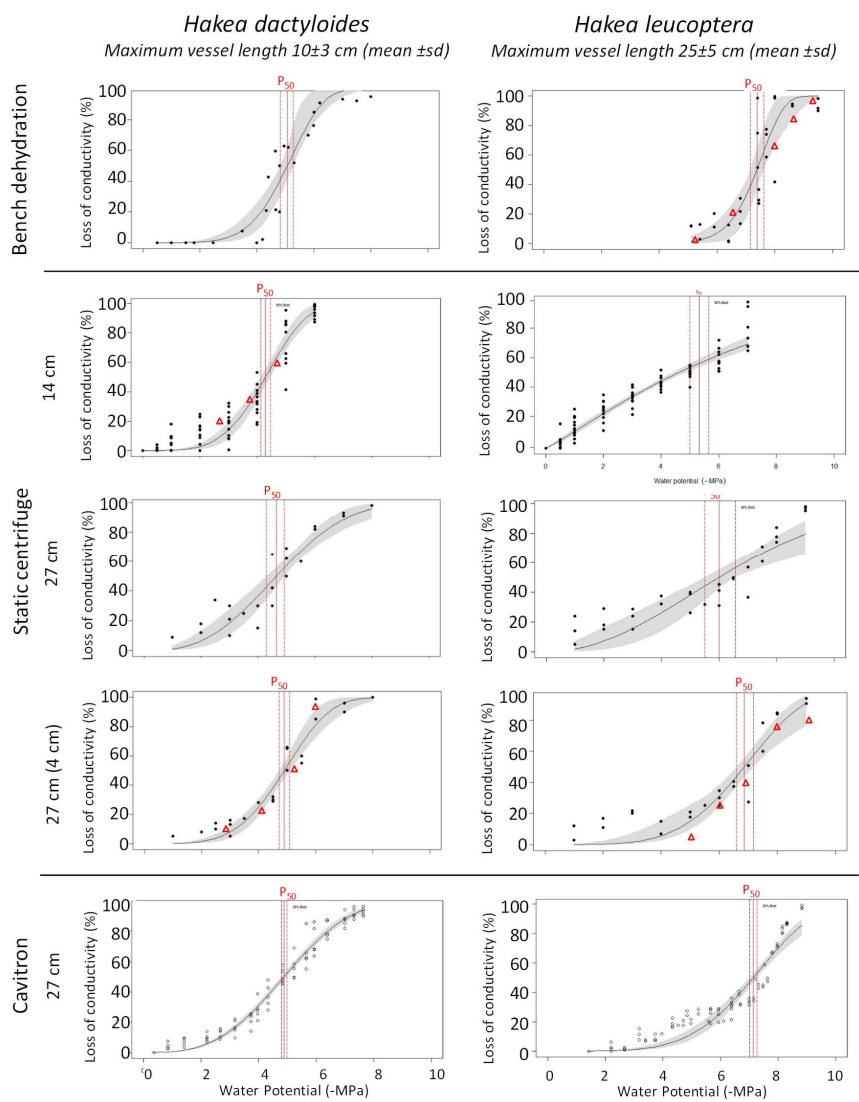
740 Figures



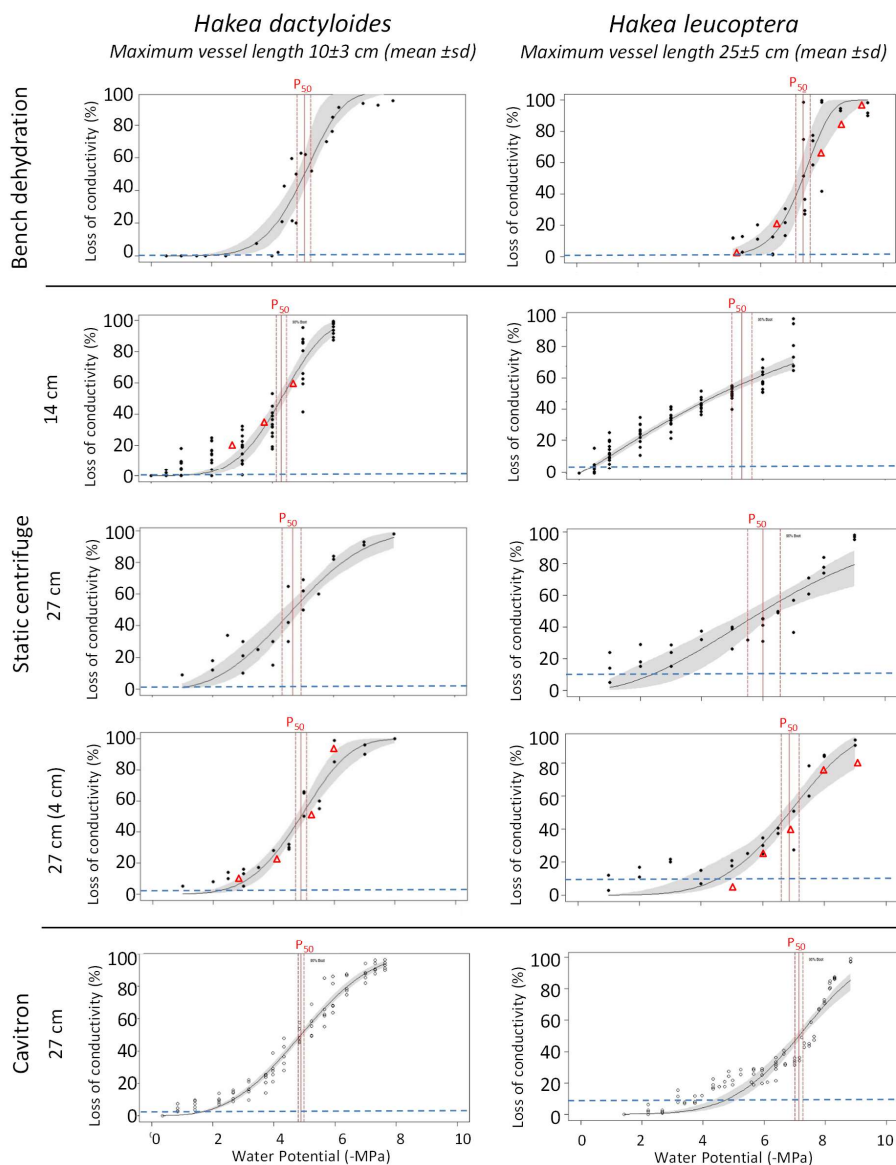
741

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  
 744 standard error. Dashed lines indicate the two sample lengths used for the centrifuge methods, 14 cm  
 745 and 27 cm and dot lines indicate their respective half sample length.

746



747

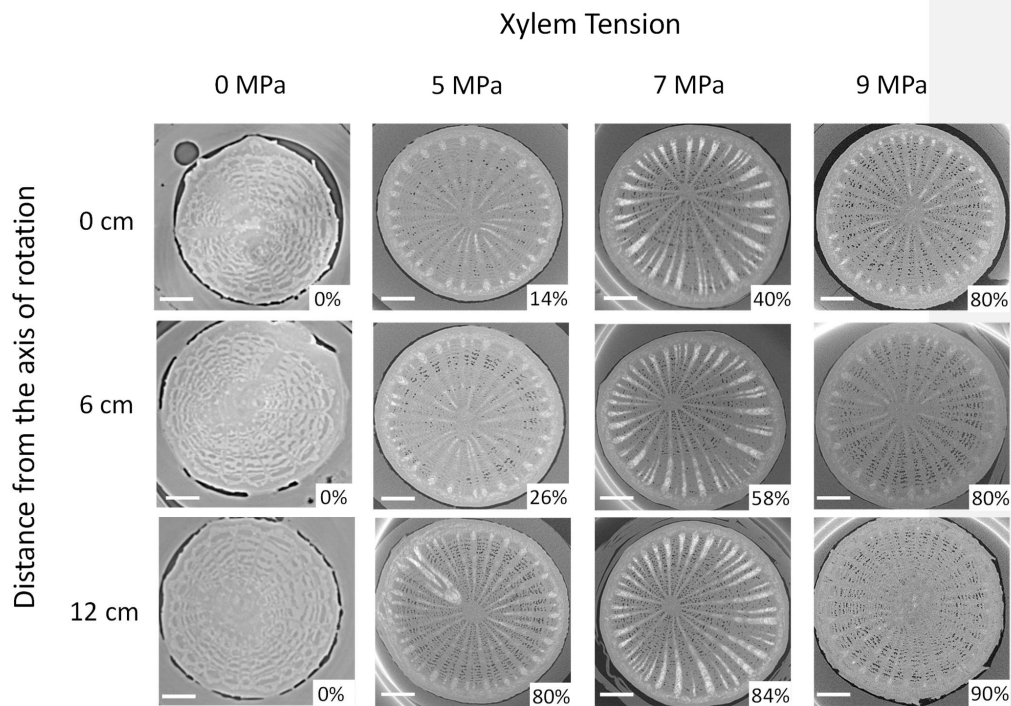


748

749 **Figure 2.** Xylem vulnerability to embolism curves and 95% confidence intervals (grey shaded areas)  
 750 of *Hakea dactyloides* (left panels) and *Hakea leucoptera* (right panels) obtained with two methods to  
 751 induce cavitation in the xylem, bench dehydration and centrifuge force and three methods to  
 752 measure the loss of conductivity, flowmeter (close circles), in situ flow method (open circles) and  
 753 X-ray microCT visualisation (red triangles). Vertical solid lines indicate  $P_{50}$  and vertical dashed lines  
 754 indicate the 95% confidence interval for  $P_{50}$ . Two rotor sizes, 14 cm and 27 cm, were used in the

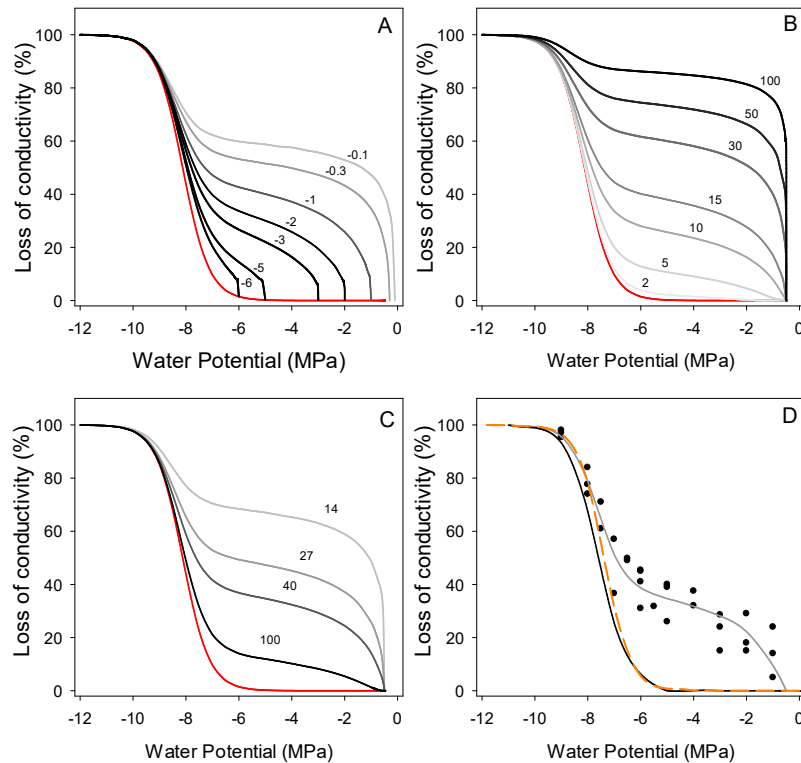
32

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



757  
 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.

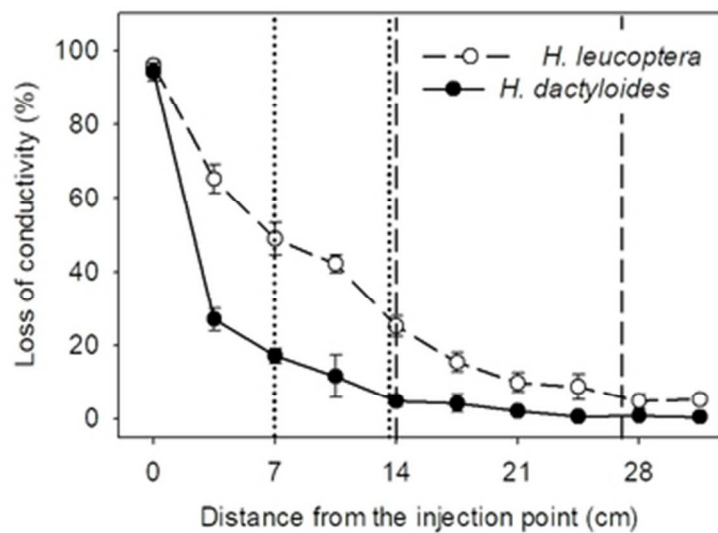


Figure 1. Distribution of PLC in air-injected branches of *H. dactyloides* (black circles) and *H. leucoptera* (open circles) at different positions from the injected end. Vertical bars represent the standard error. Dashed lines indicate the two sample lengths used for the centrifuge methods, 14 cm and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

42x32mm (300 x 300 DPI)

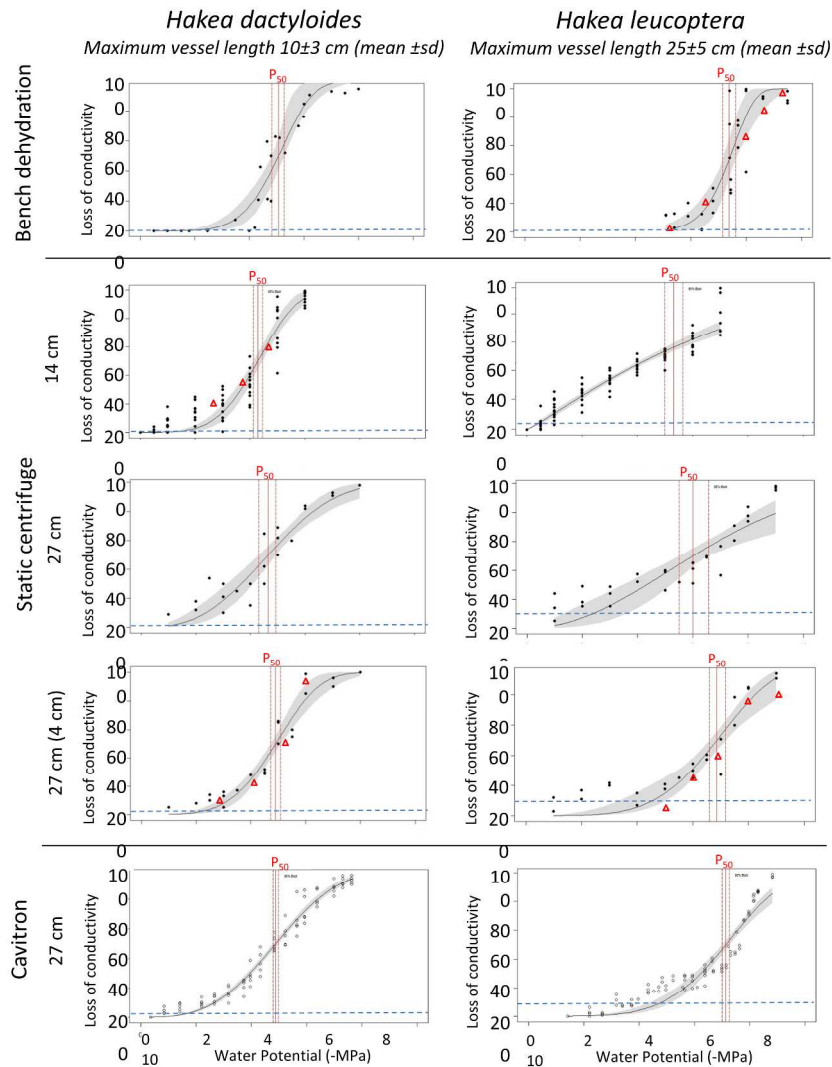


Figure 2. Xylem vulnerability to embolism curves and 95% confidence intervals (grey shaded areas) of *Hakea dactyloides* (left panels) and *Hakea leucoptera* (right panels) obtained with two methods to induce cavitation in the xylem, bench dehydration and centrifuge force and three methods to measure 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).

254x338mm (300 x 300 DPI)

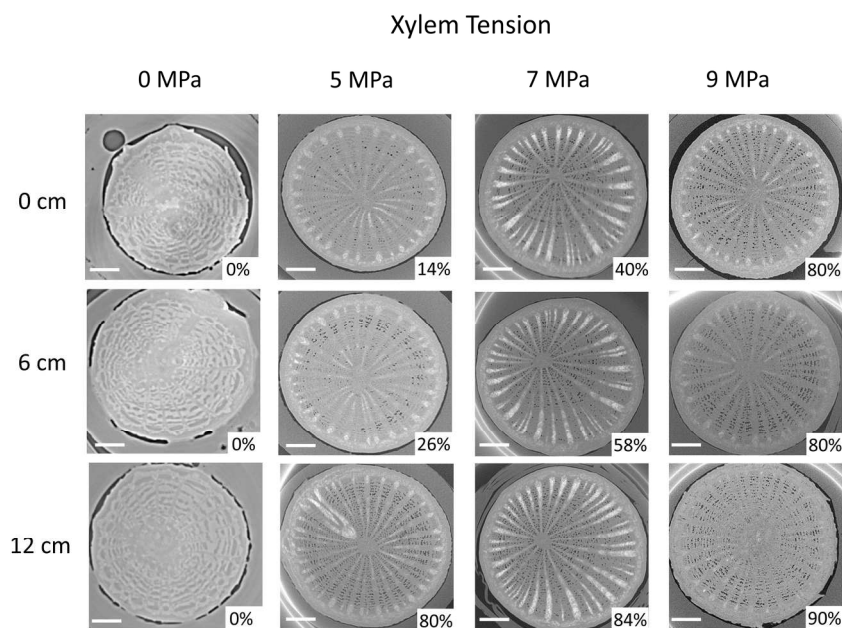


Figure 3. Transverse slices from X-ray microtomography (X-ray micro-CT) scans of branches of *Hakea leucoptera* (maximum vessel length =  $25 \pm 5$  cm) scanned at three positions before spinning (left column) and after spinning in the centrifuge at 5, 7 and 9 MPa. Embolized vessels appear as black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

190x142mm (300 x 300 DPI)

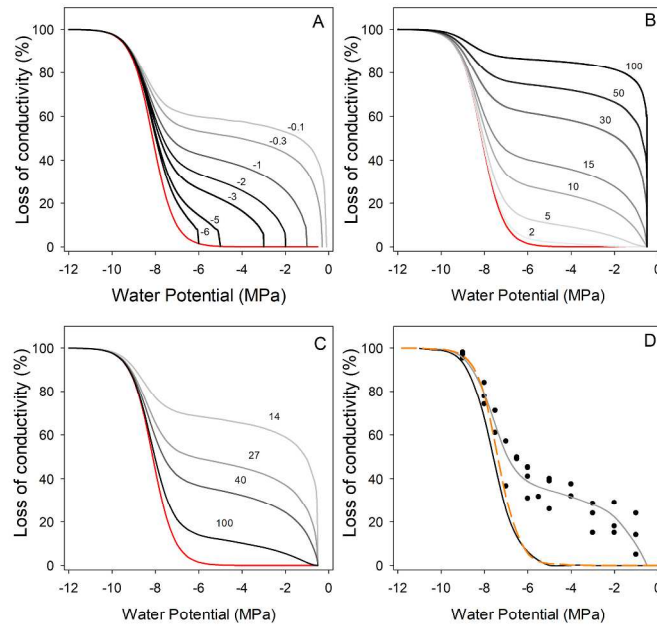


Figure 4. Simulations with the CAVITOPEN model of the effect of threshold of embolism formation (MPa) of cut open vessels (A), maximum vessel length (cm) (B), and rotor size (cm) (C) on xylem vulnerability to embolism curves generated with centrifugation. In red, vulnerability curve of close vessels 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.

254x338mm (300 x 300 DPI)

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

For Peer Review