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No evidence for an open vessel effect in centrifuge-based vulnerability curves of a long-vesselled liana (*Vitis vinifera*)

Anna L. Jacobsen and R. Brandon Pratt

Department of Biology, California State University, Bakersfield, 9001 Stockdale Hwy, Bakersfield, CA 93311, USA

Author for correspondence:
Anna L. Jacobsen
Tel: +1 661 654 2572
Email: ajacobsen@csub.edu

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Summary

- Vulnerability to cavitation curves are used to estimate xylem cavitation resistance and can be constructed using multiple techniques. It was recently suggested that a technique that relies on centrifugal force to generate negative xylem pressures may be susceptible to an open vessel artifact in long-vesselled species.
- Here, we used custom centrifuge rotors to measure different sample lengths of 1-yr-old stems of grapevine to examine the influence of open vessels on vulnerability curves, thus testing the hypothesized open vessel artifact. These curves were compared with a dehydration-based vulnerability curve.
- Although samples differed significantly in the number of open vessels, there was no difference in the vulnerability to cavitation measured on 0.14- and 0.271-m-long samples of *Vitis vinifera*. Dehydration and centrifuge-based curves showed a similar pattern of declining xylem-specific hydraulic conductivity (K_s) with declining water potential. The percentage loss in hydraulic conductivity (PLC) differed between dehydration and centrifuge curves and it was determined that grapevine is susceptible to errors in estimating maximum K_s during dehydration because of the development of vessel blockages.
- Our results from a long-vesselled liana do not support the open vessel artifact hypothesis.

Introduction

The ability of plants to maintain water transport during periods of water stress is a key plant functional trait. This trait is often quantified through the generation of a vulnerability curve, which plots the percentage of decline in hydraulic conductivity with declining water potential. Vulnerability curves are also used to calculate the water potential at 50% loss in hydraulic conductivity (P_{50}) and this value is used as a proxy for xylem cavitation resistance. There are several different methods used to generate vulnerability to cavitation curves, including dehydration of large branches (Sperry & Tyree, 1988; Jarbeau *et al.*, 1995), air injection, acoustic emissions, and several different types of centrifuge-based methods (Pockman *et al.*, 1995; Alder *et al.*, 1997; Cochard *et al.*, 2005, 2010; Li *et al.*, 2008). These methods have generally been found to produce similar results (Jarbeau *et al.*, 1995; Pockman *et al.*, 1995; Hacke *et al.*, 2000, 2006; Jacobsen *et al.*, 2007a; Li *et al.*, 2008).

A recent study found that different vulnerability curve methods produced different results in a long-vesselled liana, grapevine (Choat *et al.*, 2010). In particular, dehydration-based vulnerability curves, which are often considered the 'gold standard' of vulnerability curve methods (Sperry *et al.*, 2012), were found to be more resistant than expected and these data disagreed with a centrifuge-based vulnerability curve that was less resistant. The presence of open vessels through centrifuged samples was hypothesized to be the cause for the disparity between these methods (Choat *et al.*, 2010; Cochard *et al.*, 2010). It was also suggested that air-injection methods may be susceptible to this open vessel artifact (Choat *et al.*, 2010).

Concerns about vessel length and some vulnerability curve methods have been expressed previously. This has included discussion of potential measurement artifact when long-vesselled species are measured using air-injection techniques (Martínez-Vilalta *et al.*, 2002; Limousin *et al.*, 2010; Ennajeh *et al.*, 2011) or centrifuge-based methods (McElrone *et al.*, 2004; Maherali *et al.*, 2006; Sperry *et al.*, 2007). Recent studies have shown that different sample lengths produce different vulnerability curves with air-injection techniques (Choat *et al.*, 2010; Ennajeh *et al.*, 2011). This may be the result of variation in the number of open vessels with length (Choat *et al.*, 2010; Ennajeh *et al.*, 2011) or of changes in the amount of the sample treated with increasing sample length (Cai *et al.*, 2010), suggesting that further research may be required to investigate the impact of vessel and sample length with air-injection techniques. In the present study, we focused specifically on the potential effect of open vessels on a centrifuge-based method.

A key prediction of the hypothesis that long vessels affect measurements of centrifuge-based vulnerability to cavitation curves is that vulnerability curves will vary with varying percentages of open vessels. To address this, in the present study, we used custom rotors that differed in their internal diameter to measure centrifuge-based vulnerability curves on different sample lengths of 1-yr-old shoots of a long-vesselled liana (grapevine, *Vitis vinifera* 'Glenora'). This allowed us to explicitly examine the influence of open vessels on centrifuge-based vulnerability to cavitation curves, because altering sample length also alters the percentage of open vessels. To our knowledge, this is the first time that the influence of open vessels on centrifuge-based vulnerability to cavitation curves has been examined in a long-vesselled liana, although different sample lengths have recently been used to examine the influence of open vessels in other non-liana woody species (Sperry *et al.*, 2012). Thus, this represents a crucial test of the hypothesis that long vessels affect vulnerability curves measured with a centrifuge. Centrifuge-generated data were also compared with data from a dehydration vulnerability curve to determine if centrifuge-based vulnerability curves were able to accurately predict declines in hydraulic efficiency during dehydration. This, too, is a crucial test because dehydration curves are generally thought to describe what occurs in an intact plant during dehydration. Finally, these data were compared with previous studies that have examined vulnerability to cavitation in grapevine.

Materials and Methods

The vessel length distribution of 1-yr-old shoots of *Vitis vinifera* L. 'Glenora' was measured on six stem segments using a silicon injection method (Sperry *et al.*, 2005; Wheeler *et al.*, 2005). Shoot segments 5–7 mm in diameter and longer than 250 mm were harvested from a privately owned field site in Bakersfield, CA, USA. Before injection, stems were cut to length under water and stem ends were shaved with fresh razor blades. Samples were then inserted into a tubing apparatus and flushed for 1 h at 100 kPa using a degassed 20 mM KCl solution filtered to 0.1 μm (inline filter; GE Water and Process Technologies, Trevose, PA, USA). The basal end of the segments were then injected with a 10 : 1 silicone-hardener mixture (Rhodorsil RTV 141; Bluestar Silicones, East Brunswick, NJ, USA) mixed with a soluble fluorescent dye dissolved in chloroform (1% w/w, Uvitex OB; Ciba Specialty Chemicals, Tarrytown, NY, USA) at 50 kPa for 24 h. Stem segments were cured at room temperature for 72 h before sectioning. Stems were sectioned serially and the percentage of vessels filled at each distance was used to calculate the vessel length distribution, using the equations reported in Wheeler *et al.* (2005). The vessel length distribution was then used to calculate the mean vessel length and the percentage of open vessels through 0.14 and 0.271 m segments.

Vulnerability to cavitation curves were constructed using 1-yr-old stems of *V. vinifera* 'Glenora' from the same field site. Very long stems (> 2 m) were harvested and transported immediately to a laboratory (< 2 km distant) with their cut ends maintained under water during transport. In the laboratory, stems were cut under water to either 0.14 or 0.271 m in length and sample ends were trimmed with fresh razor blades. Each segment contained a central internode and at least two nodes. For one experiment, 0.14- and 0.271-m-long samples were paired, with samples of each length taken from immediately adjacent one another from the same shoot segment. Additionally, all shoots for this experiment were harvested from the same individual. This was to carefully control for intershoot and interplant variability so that the only experimental variable was the number of open vessels as varied by sample length. A second experiment measured 0.14 and 0.271 m samples (n = 12 for each length), all harvested from the same site, but samples were not paired in the same manner as described earlier.

Samples for determination of vulnerability curves were inserted into a tubing apparatus and flushed for 1 h at 100 kPa using the solution described earlier. Stem conductivity (K_h) was then measured as described in Jacobsen *et al.* (2007a) with flow through stems recorded gravimetrically on a balance (CP124S; Sartorius, Goettingen, Germany) connected to a computer (Inspiron 5000e; Dell Computer Corporation, Round Rock, TX, USA). The xylem area of stems was measured at the distal internode end of each stem segment and this area was then used to calculate xylem area specific conductivity (K_s). Because stem segments were all 1 yr old and were composed of only the current year's growth, and because plants had also been kept well watered, all of the xylem was assumed to be functional. During all conductivity measurements, the pressure head was kept below 2 kPa to avoid refilling any open vessels with large diameters. Depending on sample length, stems were then mounted in a custom rotor of either 0.14 or 0.271 m inner diameter and spun in a centrifuge (Sorvall RC-5C; Thermo Fisher Scientific, Waltham, MA, USA) that contained reservoirs at stem ends so that the cut ends of stems were submerged during centrifugation (Alder *et al.*, 1997). Foam pads were also added to reservoirs to maintain solution contact with stem ends at all times even when the rotor was not spinning. The percentage loss in hydraulic conductivity (PLC) was calculated following each successive centrifuge spin using the measured K_h relative to the initial measurement of maximum K_h. Vulnerability curves were fit using a Weibull model (Excel 2010; Microsoft Corporation, Redmond, WA, USA) and this model was used to determine the water potential at 50% loss in hydraulic conductivity (P_{50}). The initial flushed K_s (K_{smax}) and P_{50} measured on different sample lengths from the first experiment using paired samples were analysed using a paired t-test and K_{smax} and P_{50} from the second, unpaired, experiment were examined using a t-test. All data were analysed using Minitab (Minitab 16.1.1.0., College Station, PA, USA).

For dehydration-based vulnerability curves, large shoots > 3 m were cut under water from the same plants as used for centrifuge-based vulnerability curves. The ends of the cut shoots were sealed with high vacuum grease and the shoots were sealed in a large triple-layered bag to equilibrate. After at least 12 h of equilibration, leaves were harvested from shoot segments and their water potential determined using a pressure chamber (PMS Instrument Company, Albany, OR, USA). Stem segments, 0.14 m in length, were then cut under water from the large shoot from

segments immediately adjacent to leaves that were sampled for water potentials and all segments were taken from a location > 1 m from the cut end of the large shoot, which is greater than the longest vessel length as determined using air injection (*c.* 0.8–0.9 m). An entire single large shoot was measured for each sampling time, with three to seven samples gathered from each large shoot. The remaining portions of the large shoots were discarded following sampling to prevent air entry into samples from previously sampled points. All shoots were measured within 5 d of their original collection from the field.

Psychrometers (Model C-30; Wescor, Logan, UT, USA) controlled by a datalogger (Model CR7; Campbell Scientific, Logan, UT, USA) were used to evaluate whether leaf water potentials measured with a pressure chamber were indicative of xylem water potentials. All psychrometers were calibrated with a range of NaCl solutions (five different concentrations, ranging from −0.25 to −6.0 MPa). Psychrometers were held at 20°C inside an insulated box lined with copper coil connected to a circulating water bath to control for any temperature effects (Neslab RTE; Thermo Scientific, Newington, NH, USA). Samples were run at the same temperature that was used for calibration of the psychrometers. Samples were placed in psychrometer chambers and allowed to equilibrate for at least 6 h. This duration was determined by monitoring values over time and the point that they stopped changing was taken as the equilibrium water potential. We found agreement between water potentials measured with psychrometers and those measured with a pressure chamber as previously reported for grapevine in Choat *et al.* (2010).

Samples from large shoots were trimmed under water to 0.14 m in length using fresh razor blades and their K_s was determined as described earlier. Samples were then flushed for 1 h using the procedure described earlier and their flushed K_s determined. This flushed value compared with the native value was then used to determine PLC for each sample. Vulnerability to cavitation curves were constructed by plotting the PLC for each sample at each sample's measured water potential.

The flushed K_s values of dehydration samples were found to decline in branches that were dehydrated for increasing lengths of time, suggesting irreversible clogging of vessels. Stem segments from dehydrated shoots were microscopically examined to determine the cause of this decline (Zeiss Stereo Discover V.12 with Axiocam HRc digital camera and/or Zeiss Imager D.12 with Axiocam MRc digital camera). Samples that had been dehydrated to *c.* −2 MPa were also flushed as described earlier and their active xylem vessels stained using a 0.1% (mass/volume) dye solution of crystal violet following the methods of Jacobsen *et al.* (2007a). Stained samples were observed to determine which vessels were able to be flushed and therefore conduct dye solution and which vessels were not conductive following flushing.

To correct for the observed decline in flushed K_s, which influenced the calculation of PLC and made it impossible to accurately use PLC as an estimate of the level of embolism formed in dehydrated shoots, we also calculated the percentage decline in K_s relative to the mean flushed K_s of freshly collected and very hydrated (> −0.25 MPa) samples from the dehydration curves (n = 4, K_s = 8.86 kg m^{-1} MPa^{-1} s^{-1}). Calculating the percentage loss in decline in K_s of dehydrated stems relative to hydrated stem flushed K_s also allowed us to account for the difference in maximum conductivity between dehydration and centrifuge curves so that these data were comparable. The initial K_s and PLC values differ slightly between these curves when they are not corrected in this manner because large branches were not flushed before initiation of the dehydration curves and so measured K_s values included native-state embolism that may have been present in the field before large shoot collection. Data were plotted as the K_s or percentage decline in K_s for each sample at its sampled water potential. PLC and percentage decline in K_s curves were fitted using a cumulative Weibull model and this model was used to calculate P_{50}.

To further investigate the observed decline in flushed K_s values over the course of the construction of dehydration-based vulnerability curves, we examined the influence of embolism and the timing of flushing on K_s decline. We collected eight stems from a single shoot. These samples were cut from the larger shoot under water and then trimmed under water to 0.14 m using fresh razor blades. These samples were flushed and their K_s determined. Stem segments were spun to −2.0 MPa in the centrifuge using the same protocol as described earlier for spinning of stems in order to induce embolism formation within the central portion of these stems (*c.* 86 PLC). Four of these samples were then immediately flushed again for 1 h to determine if stems could be returned to the same K_s (i.e. if spinning and subsequent embolism formation had triggered clogging of stems). The remaining four stems were not flushed immediately and were floated in water for 48 h at room temperature. After 48 h, the ends of these segments were carefully trimmed with fresh razor blades under water and stems were flushed for 1 h. The final flushed K_s values of these stems were then determined and compared with their initial flushed K_s.

Previously published studies were examined to determine dehydration and whole-plant vulnerability curves for grapevine. In addition to Choat *et al.* (2010), three previous studies were found that had reported conductivity or PLC values in tandem with water potential measures for grapevine (Schultz, 2003; Lovisolo *et al.*, 2008; Zufferey *et al.*, 2011). An additional study reported only PLC-based curves and P_{50} from dehydration curves, but did not show the data from which these values were derived (Alsina *et al.*, 2007). For three of the studies (Schultz, 2003; Lovisolo *et al.*, 2008; Zufferey *et al.*, 2011), data were not originally plotted as vulnerability curves and data were therefore extracted and plotted as vulnerability curves for comparison with data from the present study. For two of these studies, vulnerability curves were generated using leaf-specific hydraulic conductance (k_l) and these data therefore represent an estimate of whole-plant vulnerability and the response of the whole plant to declines in water potential rather than just stem xylem vulnerability to cavitation (Schultz, 2003; Zufferey *et al.*, 2011). Presumably, stems are hydraulically coordinated with whole-plant conductance (Pratt *et al.*, 2010). Data from these curves were fitted using a Weibull model and this model was used to calculate P_{50}.

Results

One-year-old shoots had a mean vessel length of 0.116 ± 0.017 m, with 9.4 ± 3.0% of vessels open through 0.14 m samples compared with 1.5 ± 0.7% vessels open through 0.271 m samples. Although samples differed significantly in the number of open vessels ($P < 0.001$), there was no difference in the vulnerability to cavitation curves measured on paired samples of *V. vinifera* that were 0.14 and 0.271 m long (Fig. 1). Varying the sample length did not alter the stem sample K_{smax} (Fig. 1; paired *t*-test, $T_3 = -0.16$, $P = 0.883$, $n = 4$; $K_s = 13.5 \pm 3.43$ kg m^{-1} MPa^{-1} s^{-1} for 0.14 m samples and 14.3 ± 4.4 kg m^{-1} MPa^{-1} s^{-1} for 0.271 m samples) or water potential at 50% loss in hydraulic conductivity (Fig. 1, Table 1; P_{50}: paired *t*-test, $T_3 = 1.59$, $P = 0.211$, $n = 4$).

When a larger sample size of 0.14 and 0.271 m samples was examined, but the samples were not paired in the same way as the earlier data, there was also no difference between the vulnerability to cavitation curves of samples of different lengths and different percentages of open vessels. Differing percentages of open vessels did not alter P_{50} (Table 1; *t*-test, $T_{16} = -1.49$, $P = 0.156$, $n = 12$) or K_{smax} (*t*-test; $T_3 = 0.45$, $P = 0.659$, $n = 12$; $K_s = 14.1 \pm 2.2$ kg m^{-1} MPa^{-1} s^{-1} for 0.14 m samples and 12.9 ± 5.1 kg m^{-1} MPa^{-1} s^{-1} for 0.271 m samples). These samples were not paired as in the earlier experiment and ranged in sampled stem diameter from 5.36 to 9.50 mm. The cavitation resistance of samples (P_{50}) was significantly correlated with the xylem area of samples, such that segments with greater cross-sectional xylem area were also more vulnerable to cavitation (Fig. 2; $P < 0.001$, $r^2 = 0.475$). This relationship was consistent for both 0.14- and 0.271-m-long samples (Fig. 2).

We did not find any evidence for an open vessel effect in centrifuge vulnerability curves. Different sample lengths did not differ in any of the parameters examined. Because of this, data from 0.14- and 0.271-m-long samples were pooled for comparisons to dehydration curves. When pooled, centrifuge curves had a K_{smax} of 13.5 ± 1.3 kg m^{-1} MPa^{-1} s^{-1} and a P_{50} of −0.44 ± 0.07 MPa ($n = 24$).

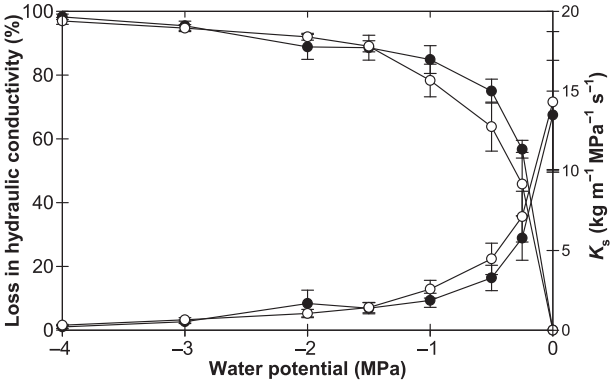

Fig. 1 Vulnerability to cavitation as determined using a centrifuge-based method for paired 1-yr-old shoots of grapevine (*Vitis vinifera*) of differing sample lengths (closed circles, 0.14 m samples; open circles, 0.271 m samples; $n = 4$). Sample lengths varied the number of open vessels through samples with 9.4 ± 3.0% of vessels open through 0.14 m samples compared to 1.5 ± 0.7% vessels open through 0.271 m samples. Samples of differing length did not differ in K_{smax} ($P = 0.883$) or water potential at 50% loss in hydraulic conductivity (P_{50}; $P = 0.211$).

Centrifuge- and dehydration-based vulnerability curves both described a similar decline in K_s with declining water potential (Fig. 3a; Table 1); however, this result was not consistent with the PLC as determined from the difference between native and flushed values of K_s (Fig. 3b; Table 1). This discrepancy was related to variability in flushed K_s over the course of vulnerability curve construction. In particular, flushed K_s values declined as samples became more dehydrated. To account for this decline, we calculated the PLC of samples as the decline in K_s of native samples relative to the flushed K_{smax} of hydrated samples (i.e. dehydration samples were all compared with the same initial flushed K_{smax} values from samples that had not been dehydrated rather than compared with individual flushed values for each stem; Fig. 3c). The percentage loss in K_s determined in this way described the loss in xylem conductivity as measured in samples (compare Fig. 3a with Fig. 3c) and was not susceptible to the same K_s flushing error as PLC estimates based on flushed values from each sample (Fig. 3b). Indeed, flushed K_s values as measured from each sample predicted the difference between PLC- and K_s-based curves, suggesting that the difference between the curves depicted in Fig. 3(b) and (c) is driven by this K_s error ($P < 0.001$, $r^2 = 0.558$; data not shown).

Instability in K_{smax} over the course of dehydration measures impacted estimates of the water potential at 50% loss in hydraulic conductivity (P_{50}). When PLC values based on the comparison of flushed and native samples were used to calculate the P_{50} of the dehydration curve, this value (Fig. 3b, Table 1; $P_{50} = -1.58$ MPa) was much more negative than the point of 50% decline in K_s (Fig. 3c and Table 1; $P_{50} = -0.51$ MPa). Thus, the PLC of the dehydration curve as calculated using standard methods of comparing flushed with native samples was more negative (i.e. appeared more cavitation-resistant) than suggested by measured declines in K_s.

Declines in K_s appeared to be caused by the formation of gels within the vessels of benchtop dehydrated shoots (Fig. 4a–d). These gels did not appear to be caused by wounding, because they occurred well away from the cut end of shoots (> 1 m). However, the continued formation of large amounts of gel following sectioning (Fig. 4e) suggests that gels may also be involved in responding to wounding. Vessels that contained gels were not conductive (Fig. 4b).

Declines in K_s, presumably as a result of blockage by gels, appeared to take time to develop, which is why they were likely not apparent in centrifuge vulnerability curves. Stem segments that were spun to −2.0 MPa and *c.* 86 PLC (Fig. 5a) could be immediately flushed back to their pre-spin K_s (Fig. 5a; *c.* 5.9 PLC relative to the initial K_s value). By contrast, stem segments that were spun to the same pressure and allowed to remain embolized for 48 h were not able to be flushed back to their pre-spin K_s and exhibited a decline of 47.5% in their conductivity (Fig. 5b). The timing of flushing significantly affected the PLC of stems ($P < 0.001$), with stems that were allowed to sit for

Table 1 Water potential at 50% loss in hydraulic conductivity (P_{50}) calculated from centrifuge- and dehydration-based vulnerability to cavitation curves and arranged in order from the least cavitation-resistant to the most cavitation-resistant within each method

Vulnerability curve method	Plant material	P_{50} (MPa)	*n*	Sample length (m)	Source*
Centrifuge	***Vitis vinifera* L. 'Glenora'**	**−0.16 ± 0.03**	**4**	**0.14**	
	***V. vinifera* L. 'Glenora'**	**−0.31 ± 0.11**	**4**	**0.27**	
	***V. vinifera* L. 'Glenora'**	**−0.34 ± 0.06**	**12**	**0.14**	
	***V. vinifera* L. 'Glenora'**	**−0.55 ± 0.12**	**12**	**0.27**	
	V. vinifera L. 'Chardonnay'	−0.7	6	0.145	Drayton (2009); Choat *et al.* (2010)
	V. vinifera L.	−0.76	≥ 6	0.142	Wheeler *et al.* (2005)
Standard dehydration (PLC)	*V. vinifera* L.	−0.90 to −2.63		Not available	Alsina *et al.* (2007) (data from 8 cultivars)
	V. vinifera L. 'Grenache'	−0.91	17	*c.* 0.4; apical shoot	Lovisolo *et al.* (2008) (pooled data)
	***V. vinifera* L. 'Glenora'**	**−1.58**	**31**	**0.14**	
	V. vinifera L. 'Chardonnay'	−2.17	15	≥ 0.145	Drayton (2009); Choat *et al.* (2010)
	V. vinifera L. 'Chardonnay'	−2.97	14	≥ 0.145	Drayton (2009); Choat *et al.* (2010)
Dehydration decline relative to hydrated plant K_s	***V. vinifera* L. 'Glenora'**	**−0.51**	**31**	**0.14; K_s**	
Dehydration decline in k_l	*V. vinifera* L. 'Grenache'	−0.32	19	Whole shoot	Schultz (2003)
	V. vinifera L. 'Chasselas'	−0.41	12	Whole shoot	Zufferey *et al.* (2011)
	V. vinifera L. 'Syrah'	−0.51	15	Whole shoot	Schultz (2003)

k_l, leaf-specific hydraulic conductance; K_s, xylem-specific hydraulic conductivity; PLC, percentage loss in hydraulic conductivity.
*If a source is not listed, data are derived from the current study. These data are shown in bold.

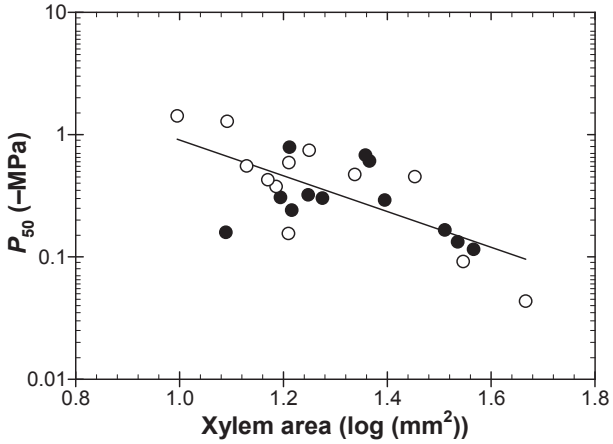

Fig. 2 Vulnerability to cavitation (P_{50}) changes with stem sample xylem area such that larger-diameter 1-yr-old shoots of grapevine (*Vitis vinifera*) are less resistant to cavitation than smaller diameter shoots ($P < 0.001$, $r^2 = 0.475$, $n = 24$; $P_{50} = 1.417 - (1.462\times$ xylem area)). Data are shown for P_{50} calculated from both short (0.14 m, closed circles) and long (0.271 m, open circles) samples; however, data were pooled for the regression analysis because sample length did not affect stem cavitation resistance (see Fig. 1).

48 h after centrifugation exhibiting significantly greater losses in conductivity than stems that were flushed immediately.

Centrifuge-based vulnerability curves generally agreed with previously published data for grapevine (Table 1). This includes agreement with previously published centrifuge-based vulnerability curves (Wheeler *et al.*, 2005; Choat *et al.*, 2010). Reported P_{50} from centrifuge vulnerability curves ranged from −0.16 to −0.76 MPa (Table 1). These data agree with P_{50} from the dehydration vulnerability curve from the present study when this value was calculated as the percentage decline in K_s (−0.51 MPa; Table 1). Additionally, these data agree with data from whole-plant dehydration experiments that measured declines in k_l (Schultz, 2003; Zufferey *et al.*, 2011), suggesting that these values are accurately describing the response of grapevine to water stress (P_{50} of k_l ranged from −0.32 to −0.51 MPa; Table 1). All P_{50} values reported from standard dehydration curves (PLC-based curves using native vs flushed values) have more negative P_{50} than those generated using these other methods (−0.9 to −2.97 MPa; Table 1).

Discussion

A test of the open vessel artifact hypothesis for centrifuge-based vulnerability curves

We found that experimentally altering the number of open vessels, from 9.4 to 1.5%, did not alter the vulnerability to cavitation of 1-yr-old grapevine shoots. This finding does not support one of the key predictions of the open vessel artifact hypothesis suggested by Choat *et al.* (2010) and Cochard *et al.* (2010), namely that the proportion of open vessels affects centrifuge-based vulnerability curves. Apparently, the disagreement between the centrifuge method and other methods found by Choat *et al.* (2010) is not on account of long and open vessels affecting centrifuge-based measures. Indeed, another recent study also failed to find support for the open vessel artifact (Sperry *et al.*, 2012). Sperry *et al.* (2012) found that centrifuge-based vulnerability curves generated using short and long stem segments, and thus more or less open vessels, for a long-vesselled oak species as well as other species were not different.

Grapevine is a very wide and long-vesselled species. Indeed, a recent survey of all published vessel length data, which included data for 120 woody species, suggests that 1-yr-old grapevine shoots have longer vessels than 83.3% of measured species and wider and longer vessels than 94.4% of measured species (A. L. Jacobsen, unpublished). Choat *et al.* (2010) also reported that

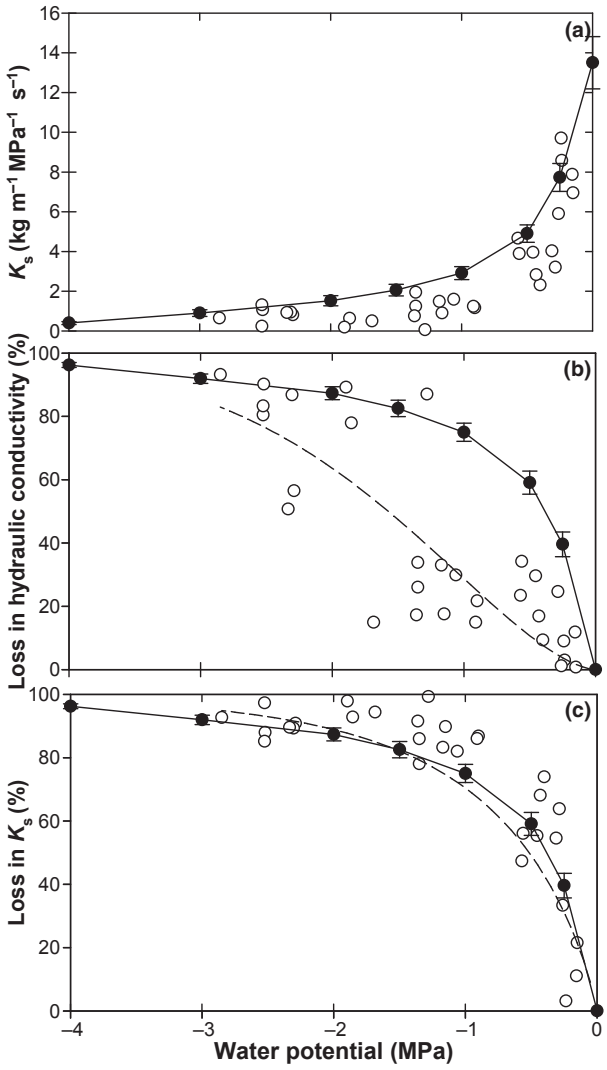

Fig. 3 Vulnerability to cavitation as determined using a centrifuge-based method (closed circles, solid line) and a dehydration-based method (open circles, dashed line, Weibull curve fit) for 1-yr-old shoots of grapevine (*Vitis vinifera*). Xylem-specific conductivity (K_s) declined with declining water potential (a). The percentage loss in hydraulic conductivity (PLC) with declining water potential differed between centrifuge-based and dehydration-based vulnerability curves when dehydration PLC values were calculated as the percentage loss in K_s native compared with K_s flushed (b). This difference was the result of errors in the estimate of PLC in the dehydration curve owing to declines in flushed K_s values during the dehydration. When PLC values were calculated as the loss in K_s relative to the K_s of hydrated samples, centrifuge- and dehydration-based vulnerability to cavitation curves were similar (c). See Table 1 for a summary of water potentials at 50% loss in hydraulic conductivity (P_{50}) for these curves.

grapevine was extremely long-vesselled. Although their reported estimate for the number of open vessels differs from those present here, perhaps as a result of methodological differences between these studies including the use of a much higher silicon injection pressure and the sampling of vessel clusters instead of individual vessels by Choat *et al.* (2010), these data are consistent with long vessels in grapevine. Finally, the mean vessel length of 0.116 m found in the present study is consistent with two previous studies that have measured the vessel length distribution of similarly sized grapevine shoots and reported a mean vessel length of 0.128 m (Sperry *et al.*, 2005; Wheeler *et al.*, 2005). If long and perhaps even wide vessel dimensions affect centrifuge vulnerability curves, one would certainly expect to see an effect in grapevine. Yet, even in this extremely long-vesselled species, we did not find evidence that suggested that open vessels impacted centrifuge-based vulnerability curve measures.

While we did not find evidence of an open vessel artifact in the present study when using a centrifuge technique based on the technique originally described in Alder *et al.* (1997), other centrifuge-based vulnerability curve techniques may be susceptible to this artifact. The centrifuge based technique described in Cochard *et al.* (2005) and referred to as the 'cavitron' technique has been shown to consistently produce anomalous results when used to measure long-vesselled species (Cochard *et al.*, 2005, 2010). The susceptibility of this particular technique to vessel length may be the result of differences between it and other centrifuge techniques, especially in the design of the rotor used to spin samples (Sperry *et al.*, 2012).

PLC-based dehydration curves may not accurately estimate declines in xylem conductivity

Similar to Choat *et al.* (2010), we found a discrepancy between centrifuge and dehydration-based vulnerability curves in grapevine. However, while Choat *et al.* (2010) concluded that this may be the result of open vessels through centrifuge samples, we found that this discrepancy may be the result of errors in dehydration measurements of PLC. These estimates are affected both by changes in conductivity as a result of cavitation and by flushed conductivity values. For many species, flushed K_s is stable over time (A. L. Jacobsen & R. B. Pratt, unpublished; Jacobsen *et al.*, 2007b). However, in the present study, flushed K_s was not stable over the several days required for the generation of a dehydration vulnerability to cavitation curve. Instead, flushed K_s values declined over time, which was probably a result of blockages that developed in the vessels of samples as they dehydrated that could not be reversed by flushing. These blockages reduced flushed K_s and made it appear that PLC was artificially low, that is, that there were low levels of emboli. Thus, a more negative P_{50} calculated from dehydration curves in grapevines did not indicate greater than expected cavitation resistance because they were not accurately representing the decline in previously functional vessels as a result of cavitation.

Vessels can become blocked in a relatively short period of time in grapevine (< 48 h) and techniques that rely on measures taken over several days or longer may not be able to accurately measure the PLC by comparing flushed with native values in this species. In the present study, sectioned samples did not show evidence of tyloses, but instead showed the formation of gels that blocked vessels. The formation of gels in grapevine xylem has been described previously and may be related to refilling of embolized vessels in grapevine (Sun *et al.*, 2008). Grapevine can also rapidly form tyloses, especially in response to wounding, but formation of tyloses has not been found to be

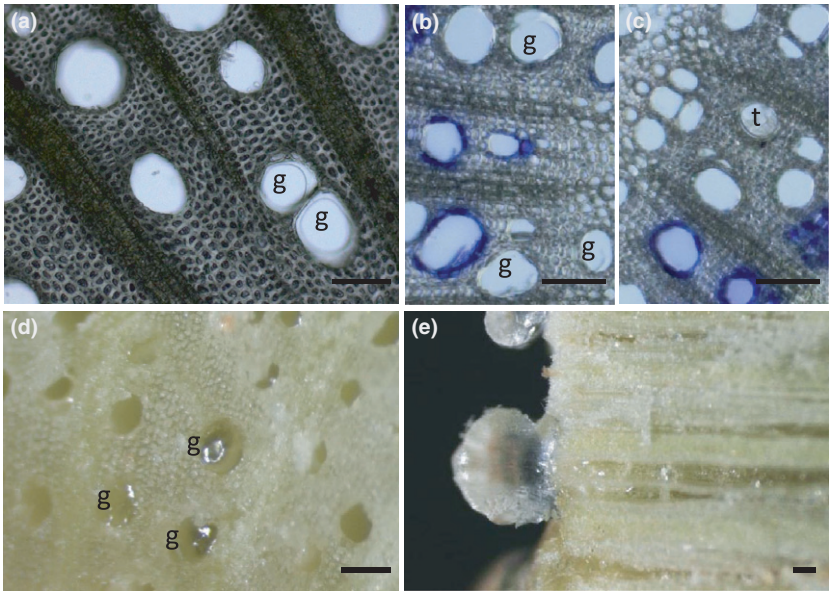

Fig. 4 (a) Vessels from stems of grapevine (*Vitis vinifera*) that were dehydrated during benchtop dehydration showed evidence of the formation of gels (g). (b) When dehydrated stems were flushed and then stained for conductive vessels using crystal violet, gel-filled vessels did not stain as being conductive (i.e. they did not have the purple colouring that indicates that solution was able to move through vessels). (c) Although rare, we saw some evidence of tylose formation (t) and these vessels also did not stain as conductive following flushing. (d) When segments from dehydrated branches were sectioned in the middle of segments used to measure hydraulic conductivity and the cut end was observed, gels could be seen filling some vessels, whereas other vessels, which could have been previously water- or air-filled, were visible as being air-filled following sectioning. (e) When these samples were sectioned longitudinally, gels could be seen filling vessels. When cut samples were allowed to sit in air, gel production appeared to continue, with gels exuding from cut vessels, indicating that gel formation may also be part of a plant wound response (e). Bars, 100 μm (all panels).

linked to embolism (Sun *et al.*, 2007). Because of this, grapevine may represent a species in which centrifuge curves are more informative than dehydration curves because they are not susceptible (over the course of the generation of a vulnerability to cavitation curve) to vessel blockages on account of the speed that a full response can be characterized with the centrifuge method (6–8 h). Indeed, centrifuged stems could be flushed back to their initial K_s and did not exhibit clogging of vessels over the time period in which centrifuge-based vulnerability curves were constructed.

When dehydration curves were analysed as the percentage decline in K_s over the course of the dehydration, dehydration vulnerability curves were not different from centrifuge-based data. This suggests that decline in K_s rather than PLC may be preferred in the evaluation of grapevine response to dehydration, such as has been reported previously for some other species (Hacke *et al.*, 2006; Pratt *et al.*, 2008; Sperry *et al.*, 2012). Additionally, this may explain why previous studies that have examined vulnerability to cavitation in grapevine using shoot dehydration over many days have suggested greater resistance to cavitation (Alsina *et al.*, 2007; Choat *et al.*, 2010) compared with dehydration data from over the course of a single day (Lovisolo *et al.*, 2008) and from centrifuge-based curves (Wheeler *et al.*, 2005; Choat *et al.*, 2010). Analyzing K_s values instead of PLC may be preferable in grapevine and other species that have unstable K_s maximum values when dehydrated (cf. Sperry *et al.*, 2012).

Error in determination of K_s as a result of vessel blockages may also affect native measures. Native measures may be particularly difficult to interpret because of the combination of vessel blockages with the formation of new xylem, since previous studies have reported large seasonal changes in stem conductivity in grapevine over the course of the growing season (Sperry *et al.*, 1987; Tibbetts & Ewers, 2000; Choat *et al.*, 2010). Additionally, the formation of gels may vary seasonally (Sun *et al.*, 2008), which may make seasonal measures and native embolism measures particularly difficult to interpret in grapevine. In the present study, these issues were minimized by completion of dehydration and centrifuge curves concurrently and the completion of all hydraulic measures over a period of several weeks to minimize seasonal effects.

Grapevine is highly susceptible to water-stress induced cavitation

Grapevine appears to be highly susceptible to water stress-induced cavitation as indicated by rapid declines in xylem-specific hydraulic conductivity (K_s) with small declines in water potential as measured by both centrifuge and dehydration techniques. The vulnerability to cavitation curves presented in the current study agree with previously published centrifuge curves (Wheeler *et al.*, 2005; Choat *et al.*, 2010). These curves are also consistent with measured acoustic emissions (AEs). AEs have been commonly shown to accurately describe stem cavitation resistance and to produce curves consistent with those derived from other techniques (Hacke *et al.*, 2000); however, the technique has well-documented limitations (Tyree & Sperry, 1989). A previous study reported that dehydrated grapevine shoots from two different grapevine varieties both displayed a majority

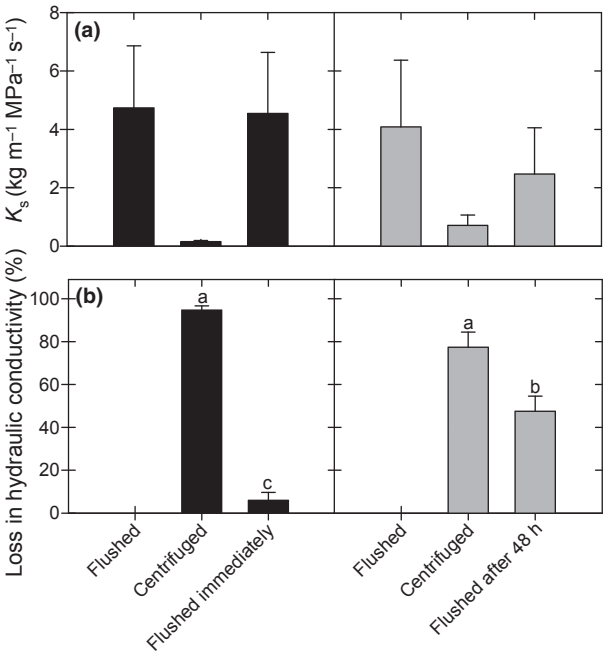

Fig. 5 Stem segments of grapevine (*Vitis vinifera*) that were flushed and then spun in a centrifuge to −2 MPa using a custom rotor exhibited large declines in K_s (a) and large losses in conductivity (b) (n = 4 for each treatment). Stems that were flushed immediately after centrifugation (black bars) exhibited a return to their pre-centrifugation K_s and very low losses in hydraulic conductivity relative to their initial K_s. By contrast, stems that were centrifuged and then allowed to float for 48 h on water before being flushed again (grey bars) showed a decline in K_s and exhibited a greater loss in hydraulic conductivity relative to their initial K_s. The timing of flushing significantly affected the percentage loss in hydraulic conductivity of stems (b) ($P < 0.001$, different letters indicate significant difference), with stems that were allowed to sit for 48 h after centrifugation exhibiting significantly greater losses in conductivity than stems that were flushed immediately.

of AEs between −0.2 and −1.5 MPa, with early peaks in AEs at *c.* −0.5 MPa, and these data were consistent with measured declines in whole-plant k_l with declining water potential from the same grapevine varieties (Schultz, 2003). This is consistent with centrifuge- and K_s-based data, which all suggest that grapevine is highly vulnerable to cavitation. This appears to be generally consistent among different grapevine varieties (Schultz, 2003), although some varieties may be more resistant to cavitation than others (Alsina *et al.*, 2007).

High vulnerability to cavitation in 1-yr-old shoots of grapevine is consistent with grapevine physiology. Grapevines are affected by even small declines in water potential. Whole-plant k_l has been shown to rapidly decline with minor declines in water potential (Schultz, 2003; Zufferey *et al.*, 2011) and shoot resistance was shown to increase rapidly when water potentials declined from −0.6 to −1.2 MPa (Schultz & Matthews, 1988). Even when experimentally dehydrated, intact grapevine plants rarely experience water potentials < −2.0 MPa. Indeed, the water potentials of 'stressed' dehydrated plants have been reported to range from −0.8 to −2.0 MPa in many studies (Loveys & Kriedemann, 1973; Matthews & Anderson, 1988; Schultz & Matthews, 1988; Lovisolo & Schubert, 1998; Schultz, 2003; Lovisolo *et al.*, 2008).

While many studies have suggested that grapevine is highly susceptible to water stress and highly vulnerable cavitation, other data have been cited as being inconsistent with this interpretation. For example, Choat *et al.* (2010) used NMR observations to suggest that grapevine was more resistant to water stress than was previously thought; however, these imaging data may be particularly difficult to interpret without concomitant functional measures and staining to identify potentially functional conduits. Choat *et al.* (2010) observed that it was 'the larger vessels toward the outer edge of the growth ring [that] remained conductive even at very low water potentials (−3.0 MPa)' and this was used as confirmation of grapevine being more cavitation-resistant than previously reported. However, a recent study suggests that these outer vessels may not be conductive because the outer ring of vessels are still living in 1-yr-old grapevine shoots as sampled by Choat *et al.* (2010). These developing vessel elements, which contain large water-filled vacuoles, may only be distinguishable from conductive vessels by staining for active xylem (Halis *et al.*, 2011), which was not done in the Choat *et al.* (2010) study. Thus, while NMR data should be interpreted with caution, it appears that these data indicate that by −3 MPa the mature conduits of the inner rings were apparently embolized, consistent with the many studies that suggest that grapevine is highly sensitive to declines in water potential.

Our results do not support the hypothesis that the centrifuge method for the determination of vulnerability to cavitation curves is susceptible to open vessel artifacts in a long-vesselled liana. The chief evidence for this is that vulnerability curves from short segments with many open vessels agree with vulnerability curves from longer segments with few open vessels. These data are consistent with a recent study that found that open vessels do not impact vulnerability curves in long-vesselled tree species (Sperry *et al.*, 2012). Additionally, centrifuge curves are able to accurately describe losses in hydraulic conductivity with declining water potential in grapevine when these declines are expressed as K_s, but not when expressed as PLC as a result of blockages that affect K_s measures. High vulnerability to cavitation in grapevine is consistent with the bulk of existing hydraulic and physiological data for grapevine. Contrary to the conclusions of Choat *et al.* (2010) and Cochard *et al.* (2010), our data suggest that centrifuge-based vulnerability curves are able to estimate vulnerability to cavitation accurately, even when used to measure a long-vesselled liana.

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