

RESEARCH PAPER

# Mind the bubbles: achieving stable measurements of maximum hydraulic conductivity through woody plant samples

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## Abstract

The maximum specific hydraulic conductivity ( $k_{\max}$ ) of a plant sample is a measure of the ability of a plants' vascular system to transport water and dissolved nutrients under optimum conditions. Precise measurements of  $k_{\max}$  are needed in comparative studies of hydraulic conductivity, as well as for measuring the formation and repair of xylem embolisms. Unstable measurements of  $k_{\max}$  are a common problem when measuring woody plant samples and it is commonly observed that  $k_{\max}$  declines from initially high values, especially when positive water pressure is used to flush out embolisms. This study was designed to test five hypotheses that could potentially explain declines in  $k_{\max}$  under positive pressure: (i) non-steady-state flow; (ii) swelling of pectin hydrogels in inter-vessel pit membranes; (iii) nucleation and coalescence of bubbles at constrictions in the xylem; (iv) physiological wounding responses; and (v) passive wounding responses, such as clogging of the xylem by debris. Prehydrated woody stems from *Laurus nobilis* (Lauraceae) and *Encelia farinosa* (Asteraceae) collected from plants grown in the Fullerton Arboretum in Southern California, were used to test these hypotheses using a xylem embolism meter (XYL'EM). Treatments included simultaneous measurements of stem inflow and outflow, enzyme inhibitors, stem-debarking, low water temperatures, different water degassing techniques, and varied concentrations of calcium, potassium, magnesium, and copper salts in aqueous measurement solutions. Stable measurements of  $k_{\max}$  were observed at concentrations of calcium, potassium, and magnesium salts high enough to suppress bubble coalescence, as well as with deionized water that was degassed using a membrane contactor under strong vacuum. Bubble formation and coalescence under positive pressure in the xylem therefore appear to be the main cause for declining  $k_{\max}$  values. Our findings suggest that degassing of water is essential for achieving stable and precise measurements of  $k_{\max}$  through woody plant samples. For complete rehydration of woody samples, incubation in water under vacuum for 24 h is suggested as a reliable technique that avoids bubble problems associated with flushing under high positive pressure.

**Key words:** Bubble coalescence, *Encelia farinosa*, *Laurus nobilis*, hydraulic conductivity, pectin hydrogel hypothesis, plant hydraulics, woody stems, xylem vulnerability curves.

## Introduction

The maximum hydraulic conductivity ( $k_{\max}$ ) is a measure of the plant vascular system's ability to transport water and solutes under optimum conditions. It is defined as the hydraulic conductivity in the absence of any reversible air embolisms in xylem conduits. Precise measurements of  $k_{\max}$  are needed for characterizing the resistance of plants to embolism formation (Sperry and Sullivan, 1992; Alder

*et al.*, 1997), in studies of embolism repair (Salleo *et al.*, 2004), and in comparative studies of hydraulic conductivity in different plant species (Maherali *et al.*, 2004). Xylem traits that affect  $k_{\max}$  include the abundance, diameters, lengths, and degree of connectedness of xylem conduits (Loepfe *et al.*, 2007). Plants can lower  $k_{\max}$  by filling xylem conduits with tyloses (Cochard and Tyree, 1990), gels

(Bonsen and Kučera, 1990; Crews *et al.*, 2003), or both (Sun *et al.*, 2008), usually in response to wounding or to pathogen attack or during heartwood formation. The ionic composition of xylem sap also affects  $k_{\max}$  (van Ieperen *et al.*, 2000; Zwieniecki *et al.*, 2001; Lopez-Portillo *et al.*, 2005; Gascó *et al.*, 2007). This process is hypothesized to involve the swelling of pectin hydrogels embedded into the pit membranes that connect xylem conduits (Zwieniecki *et al.*, 2001), which potentially could decrease  $k_{\max}$ .

When water or a solution with a constant ionic composition is fed through stems, roots, or petioles whose conduits are completely filled with water, one would not expect  $k_{\max}$  to change substantially in the short term (minutes to hours), because structural features do not change over such periods and because pit membranes are thought to retain a constant hydraulic conductivity for a given ionic composition in water (Zwieniecki *et al.*, 2001). Over longer periods (many hours to days), microbial growth and decomposition could clog or alter the structure of xylem conduits (Sperry *et al.*, 1988) and tyloses may plug conduits as well (Sun *et al.*, 2007). Despite the fact that short-term changes of  $k_{\max}$  are not expected in theory, they are frequently observed. When deionized water is used as the fluid medium,  $k_{\max}$  typically declines from initially high values, even in samples without embolized xylem conduits (Kelso *et al.*, 1963; Sperry *et al.*, 1988; Sperry and Tyree, 1990; van Ieperen and van Gelder, 2006; Canny *et al.*, 2007; Cochard *et al.*, 2010). Such short-term declines lead to unreliable measurements of  $k_{\max}$ , which can be a serious problem for studies in plant hydraulics. This study was designed to test the hypothesis that a short-term decline in  $k_{\max}$  was caused by one or more of the following mechanisms.

(i) Non-steady-state flow. Water forced into a cut plant sample under positive pressure may enter air-filled fibre cells, extracellular spaces, or parenchyma cells (van Ieperen *et al.*, 2000). Lateral water flow from conduits may cause initially high rates of inflow, declining over time as cells and extracellular spaces are filled (Tyree and Yang, 1992). Hydraulic conductance measurements based on inflow, for example, using the XYL'EM apparatus (Cochard *et al.*, 2000), could be affected by artefacts due to lateral flow.

(ii) Swelling of pectin hydrogels in pit membranes. Use of deionized water for flushing xylem could potentially deplete pit-membrane-residing pectins of cations, especially  $\text{Ca}^{2+}$ , thereby cause hydrogel swelling, and thus decrease hydraulic conductance (Zwieniecki *et al.*, 2001).  $\text{Ca}^{2+}$  ions are a structural component of some pectins, provide rigidity, and reduce pectin swelling potential (Ryden *et al.*, 2000).

(iii) Formation and coalescence of bubbles. Gassing-out can occur when air-saturated water flows through constrictions (Kelso *et al.*, 1963; Scardina and Edwards, 2004; Blatteau *et al.*, 2006; Canny *et al.*, 2007), such as xylem pit membranes or perforation plates. In a process termed 'air binding', small bubbles form through heterogeneous nucleation and then coalesce into larger ones that can block pit membranes or whole vessels.

(iv) Active wounding responses. Reduced water uptake by stems can be due to a physiological wounding response that may include mechanisms of damage control and repair or defences against pathogens that may take advantage of the wounded plant tissue (Cheong *et al.*, 2002; Ramonell and Somerville, 2002). Wounding responses that have been associated with reduced water uptake in cut stems appear to involve cell wall enzyme activity, possibly related to suberin, tylose, and gel formation (van Doorn and Cruz, 2000; van Doorn and Vaslier, 2002; Loubaud and van Doorn, 2004) or in defence against pathogenic cell-wall degrading enzymes (Cheong *et al.*, 2002; Li *et al.*, 2003).

(v) Passive wounding effects. A decline in  $k_{\max}$  could result if cutting a sample caused cell-wall debris to dislodge or cause gums and resins to distribute across the cut surface of the xylem (Schulte *et al.*, 1987; Sperry *et al.*, 1988). Flushing the samples under pressure could force such materials into vessels and clog them.

This study was designed to test for the five mechanisms described above for stem samples from two woody plant species, *Laurus nobilis* L. (Lauraceae), sweet bay, and *Encelia farinosa* Torrey & A. Gray (Asteraceae), brittlebush. *Laurus*, a small evergreen tree of Mediterranean origin, was chosen because it has been the subject of numerous studies in plant hydraulics (Tyree *et al.*, 1999; Zwieniecki *et al.*, 2001; Hacke and Sperry, 2003; Salleo *et al.*, 2004; Gascó *et al.*, 2006), while *Encelia*, a North American, drought-deciduous, desert shrub was chosen because we had previously experienced problems with unstable measurements of  $k_{\max}$  in this species.

## Materials and methods

### Collection of plant samples

Branch samples were collected between July 2008 and September 2010 from a single *Laurus nobilis* tree and a group of *Encelia farinosa* shrubs growing in the Fullerton Arboretum in Fullerton, California, USA. At least 50-cm-long branches with leaves were cut off from the plants under water and, with their cut ends submerged under water, were transported to the laboratory, where they were placed in a vase filled with tap water or with an aqueous-solution, depending on treatment (see below), for 24 h to allow the stems to rehydrate. In one experiment, stems were cut under water from very well-watered plants at predawn and used as a control treatment to test for the effectiveness of the vase rehydration treatment. The stems were then submerged in a water-filled tray, where a 15-cm-long central portion was cut out with sharp anvil clippers. Stem segments were 2–6 mm in diameter (mean 3.5 mm for both species), 1–2-years-old, and did not contain any heartwood. Maximum vessel lengths in both species exceed 15 cm (*Laurus* 40 cm; Gascó *et al.*, 2006; *Encelia* 50 cm; S Espino, unpublished data), thus, due to a few open vessels, measured  $k_{\max}$  values may slightly overestimate true values of  $k_{\max}$  in these species. For each treatment, 10 replicates were used for each species, but in some cases replication was reduced to 6–9 replicates, usually because leaky or damaged stems had to be discarded.

### Measurements of hydraulic conductivity

A XYL'EM embolism meter (Bronkhorst, Montigny les Cormeilles, France; Cochard *et al.*, 2000) was used for all

measurements of hydraulic conductivity. Keeping stem segments submerged under water at all times, they were connected at their basal inflow end to the tubing of the apparatus. Except for treatments measured in ice water (5–11 °C), all measurements were conducted at room temperature (18.1–22.3 °C). Aqueous solutions used for conductance measurements and for flushing stems under high pressure varied between treatments. Treatments and their predicted effects are summarized in Table 1. Except where noted in Table 1, all solutions were degassed under laboratory vacuum for at least 3 h in a side-arm flask on a magnetic stirrer at approximately 30 kPa absolute pressure (=71 kPa vacuum) and passed through a 0.2 µm filter (model Polycap AS 75, Whatman Inc., Piscataway, NJ). All flow rate measurements were preceded by a measurement without a pressure differential ( $\Delta p=0$ ) between inflow and outflow to determine baseline flow-rates ( $F_0$ ) into the samples. Water pressure for flushing of stems was generated with the high pressure vessel in the XYL'EM apparatus, which prevents contact between air and water during pressurization. All measurements were recorded after flow rates stabilized, typically within 1–5 min. The first measurement was conducted under a  $\Delta p$  of 3 kPa ( $F_{\Delta p}$ ). Hydraulic conductance,  $k$ , was determined as  $k=(F_{\Delta p}-F_0)/\Delta p$  and specific hydraulic conductivity,  $k_s$ , for a given length,  $L$ , and wood-cross-sectional area,  $A$ , as:

$$K_s = \frac{(F_{\Delta p} - F_0)L}{\Delta p A} \quad (1)$$

The reported conductivities include a temperature correction to 20 °C to allow for changes of water viscosity with temperature, except for ice water, because it was impossible to control the ice-water temperature carefully during measurements and the exact water temperature inside the stems was therefore unknown. Stems

were then flushed under a pressure differential of 150 kPa for 3 min, after which  $F_{\Delta p}$  was determined again. In one treatment, 3 kPa ‘flushes’ were used to test for effects of high-pressure versus low-pressure flushing. This was followed by another high-pressure flush for 3 min, followed by measurement of  $F_{\Delta p}$ . In a few cases indicated in the results, this sequence was repeated five times to determine  $k_{\max}$  stability. High-pressure flushing was used in this study because it is the most commonly used method for measuring  $k_{\max}$  in stems (Table 2).

The following experimental protocols were used to test the five alternative hypotheses. Some treatments potentially affect more than one of the five mechanisms. See Table 1 for a comprehensive listing of treatments and their predicted effects on the five mechanisms.

**Control treatments:** The two control treatments included non-degassed, deionized water, and deionized water degassed as described above.

**Non-steady-state flow:** To test for lateral flow from vessels into adjacent cells and extracellular spaces, the outflow of the measurement solution was measured simultaneously with the inflow by combining the XYL'EM apparatus with a Sperry apparatus (Sperry *et al.*, 1988). In this set-up, the XYL'EM apparatus provided the water supply under gravitational pressure for measurements and high pressure for flushing. The distal outflow end of the stem segments were connected with tubing to a reservoir placed on an electronic analytical balance (model Explorer Pro EP214DC, Ohaus, Pine Brook, NJ, USA), which was connected to a computer and recorded the outflow rate. Inflow and outflow rates were measured simultaneously at 5 s intervals. To

**Table 1.** Predicted outcomes of Experimental treatments on the decline of  $k_{\max}$  measurements in woody stems, assuming that the mechanism listed in the corresponding column header is operative (see Introduction for further explanations)

Symbols: o=no alleviation predicted; + =some alleviation predicted; ++ =strong alleviation predicted.

Experimental treatment	Non-steady-state flow	Pectin swelling	Bubbles	Active wounding response	Passive wounding effects
Not degassed	o	o	o	o	o
Flask-degassed at 30 kPa	o	o	+	o	o
Degasser at 30 kPa	o	o	+	o	o
Degasser at 3 kPa	o	o	++	o	o
Hydrated at 3 kPa, degasser at 3 kPa	o	o	++	o	o
Ice water	o	+	+	+	o
Ice water <sup>b</sup>	o	o	+	+	o
Bark removed before cutting	o	o	o	+	+
KCl, 10 mM	o	++	o	o	o
KCl, 100 mM	o	++	+	o	o
KCl, 300 mM	o	++	++	o	o
CaCl <sub>2</sub> , 10 mM	o	++	o	o	o
CaCl <sub>2</sub> , 40 mM	o	++	+	o	o
CaCl <sub>2</sub> , 100 mM	o	++	++	o	o
MgSO <sub>4</sub> , 0.5 mM	o	+	o	o	o
MgSO <sub>4</sub> , 20 mM	o	++	+	o	o
MgSO <sub>4</sub> , 50 mM	o	++	++	o	o
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , 100 mM	o	o	++	o	o
Cu(II)SO <sub>4</sub> , 0.1 mM <sup>b</sup>	o	o	o	+	o
Cu(II)SO <sub>4</sub> , 0.25 mM	o	o	o	+	o
4-HR, 10 mM <sup>b</sup>	o	o	o	+	o
Hydroquinone, 10 mM <sup>b</sup>	o	o	o	+	o
Degasser, in- and outflow measured	+ <sup>a</sup>	o	+	o	o

<sup>a</sup> Experimental treatment accounts for, but does not alleviate, declines in  $k_{\max}$ .

<sup>b</sup> Treatment applied during vase rehydration and during measurement.

**Table 2.** Methods used in representative studies of maximum hydraulic conductivity through stem and root samples

All studies were conducted at room temperature, except for Tyree and Yang (1992), which was conducted at 1–3 °C.

Reference	Sample length (cm)	Measured inflow or outflow? <sup>a</sup>	Filtered and degassed? <sup>b</sup>	Measuring pressure (kPa) <sup>b</sup>	Flushing pressure and flushing time <sup>b</sup>	Ionic composition
Sperry <i>et al.</i> (1987)	Various	Out	0.22 µm filter, degassed <sup>5</sup>	≤10 <sup>5</sup>	170 kPa, repeated flushes to $k_{max}$	10 mM NaCl, 0.05% formaldehyde
Sperry <i>et al.</i> (1988)	10–15	Out*	0.22 µm filter, degassed <sup>5</sup>	≤10	175 kPa, 20–200 min	10 mM NaCl, (1 mM NaCl+0.5 mM CaCl <sub>2</sub> +0.2 mM KCl), 'maple sap', 10 mM citric acid (pH 4), 10 mM citric acid (pH <3), formaldehyde (0.05%, 0.5%), gluteraldehyde (0.05%), 10 mM oxalic acid (pH 1.3–2.4)
Tyree and Yang (1992)	25–75	In and out	0.2 and 0.1 µm filter	2–14	150 kPa, both ends, up to 200 h	10 mM oxalic acid
Sperry and Sullivan (1992)	4–15	Out*	0.22 µm filter, degassed <sup>5</sup>	40 or 70	175 kPa, repeated flushes to $k_{max}$	10 mM oxalic acid
Sperry and Saliendra (1994)	15–25	Out*	0.2 µm filter, not degassed <sup>5</sup>	10	175 kPa, no time info	HCl, pH 2
Jarbeau <i>et al.</i> (1995)	10	Out*	0.1 µm filter, degassed	3	175 kPa, 1 h	10 mM citric acid
Pockman and Sperry (2000)	10	Out*	0.22 µm filter, not degassed <sup>5</sup>	≤10	100 kPa, repeated flushes to $k_{max}$	HCl, pH 2
Hacke <i>et al.</i> (2000)	14	Out*	0.2 µm filter, not degassed <sup>4</sup>	–7 to –10, –350–70 kPa, 30 min, $k_{max}$ for roots	–350–70 kPa, 30 min, $k_{max}$ at –0.5 MPa	Deionized water
Cochard <i>et al.</i> (2000)	3–4	In**	0.2 µm filter, degassed	3	100 kPa, no time info	Water
van Ieperen <i>et al.</i> (2000)	20	In <sup>†</sup>	?	–40	N/A	Deionized water, 0.01–200 mM KCl, 6.7 mM K <sub>2</sub> SO <sub>4</sub> , 10 mM NaCl, 67 mM CaCl <sub>2</sub> , 20 mM mannitol, 20 mM melizitose
Zwieniecki <i>et al.</i> (2001)	3–6	Out	0.2 µm filter, not degassed <sup>6</sup>	40	200 kPa, 10 min	Deionized water, 0.1–100 mM KCl, 10 mM sucrose, 10 mM ethanol, 10 mM NaCl, 10 mM KNO <sub>2</sub> , 10 mM CaCl <sub>2</sub> , 10 to 95% ethanol
Bucci <i>et al.</i> (2003)	10–15	Out <sup>8</sup>	0.22 µm filter, degassed	4.9	200 kPa, repeated 15 min flushes to $k_{max}$	Distilled water
Hukin <i>et al.</i> (2005)	2	In**	0.2 µm filter, degassed <sup>2</sup>	1.5	150 kPa, no time info	10 mM KCl
Jacobsen <i>et al.</i> (2005)	10–27	Out*	0.1 µm filter, degassed <sup>1</sup>	1.5–3.5 <sup>1</sup>	100 kPa, 1 h, $k_{max}$ at –0.5 MPa	HCl, pH 2
Gascò <i>et al.</i> (2006)	1–36	In**	0.1 µmm filter	9	190 kPa, 10 min	Deionized water, 5–150 mM KCl, 200 mM sucrose, 100 mM NaCl
Maherali <i>et al.</i> (2006)	Stems 14, roots: 27–59	Out*	0.2 µm filter, not degassed <sup>3</sup>	1.5–2	100 kPa, 15–20 min	Distilled water
van Meeteren <i>et al.</i> (2006)	22	In <sup>†</sup>	Degassed and not degassed	–40	–40 kPa 1.5 h, then 3.4–3 kPa 30 min	0.7 mM CaCl <sub>2</sub> , 1.5 mM NaHCO <sub>3</sub> , 50 mM CuSO <sub>4</sub>
van Ieperen and van Gelder (2006)	7–13	In <sup>†</sup>	?	–20	N/A	Ultrapure deionized water, 0.1 mM CaCl <sub>2</sub> , 1 mM CaCl <sub>2</sub> , 0.1 mM CaCl <sub>2</sub> +10 mM KCl, 1 mM CaCl <sub>2</sub> +10 mM KCl, 0.1 mM CaCl <sub>2</sub> +100 mM KCl, 1 mM CaCl <sub>2</sub> +100 mM KCl, 0.1–100 mM KCl, 0.1–10 mM CaCl <sub>2</sub>
Nardini <i>et al.</i> (2007)	6–16	In**	0.1 µm filter	9	190 kPa, 10 min	Deionized water, 25 mM KCl, 0.5 mM CaCl <sub>2</sub> , 1 mM CaCl <sub>2</sub> , 25 mM KCl+0.5 mM CaCl <sub>2</sub> , 25 mM KCl+1 mM CaCl <sub>2</sub> , mineral water

Table 2. Continued

Reference	Sample length (cm)	Measured inflow or outflow? <sup>a</sup>	Filtered and degassed? <sup>b</sup>	Measuring pressure (kPa) <sup>b</sup>	Flushing pressure and flushing time <sup>b</sup>	Ionic composition
Lovisollo <i>et al.</i> (2008)	Petioles 1, shoots 40, roots ?	In <sup>††</sup>	0.1 µm filter, degassed	Petioles 40; shoots 20; roots 10	Petioles 600 kPa <sup>7</sup> ; shoots 300 kPa <sup>7</sup> ; roots 300 kPa <sup>7</sup>	15 mM KCl

<sup>a</sup> Apparatus used for hydraulic conductivity measurements: Sperry apparatus (Sperry *et al.*, 1988), \*\*XYL'EM apparatus (Instrutec, Montigny les Cormeilles, France; Cochard *et al.*, 2000), <sup>†</sup>van Ieperen *et al.* (2000). <sup>††</sup>Hydraulic conductance flow meter (model HCFM-XP, Dynamax, Houston, TX; Tyree *et al.*, 1995).

<sup>b</sup> <sup>1</sup>Anna Jacobsen, personal communication; <sup>2</sup>Hervé Cochard, personal communication; <sup>3</sup>Hafiz Maherali, personal communication; <sup>4</sup>Uwe Hacke personal communication; <sup>5</sup>John Sperry, personal communication; <sup>6</sup>Maciej Zwieniecki, personal communication; <sup>7</sup>Pressure gradually increased to the maximum listed, Claudio Lovisollo personal communication; <sup>8</sup>Sandra Bucci, personal communication.

calibrate measurements using the balance against measurements with the high-precision flowmeter (model LIQUI-FLOW L1, Bronkhorst, Montigny les Cormeilles, France) that is at the core of the XYL'EM apparatus, stem samples were replaced with a 0.45 µm syringe filter and 15 cm of luer PVC tubing, which was compressed with a clamp to generate flow rates similar to those observed in *Encelia* stems. Flow rates were measured the same way as in all other experiments, first at  $\Delta p=0$  kPa, then at  $\Delta p=3$  kPa, then at  $\Delta p=3$  kPa after a first 3-min flush under  $\Delta p=150$  kPa, and again at  $\Delta p=3$  kPa after a second 3-min flush under  $\Delta p=150$  kPa.

Comparisons of inflow and outflow rates for ten replicates of *Encelia* stems, *Laurus* stems, and tubing were conducted using separate paired, two-sided *t* tests for the four flow rates measured. Observed differences between the four measured inflow and outflow rates ( $\Delta F=F_{in}-F_{out}$ ) were also compared between *Encelia* stems, *Laurus* stems, and tubing using two-sided *t* tests. Test results were corrected for false discovery rate in multiple comparisons (Benjamini and Hochberg, 1995).

*Swelling of pectin hydrogels in pit membranes:* To reduce the chance that swelling pectin hydrogels cause a decline in  $k_s$  in response to flushing, aqueous solutions of 10 mM KCl and 10 mM CaCl<sub>2</sub> were used, which are hypothesized to prevent hydrogel swelling at these low concentrations and have been shown to increase flow rates through stems compared to deionized water (Zwieniecki *et al.*, 2001; Gascó *et al.*, 2006; van Ieperen and van Gelder, 2006). A solution of 0.5 mM MgSO<sub>4</sub> was also tested, because Mg<sup>2+</sup> ions are also known to prevent pectin swelling (Zsivánovits *et al.*, 2005). Ice water was also used as a treatment, because it can cause pectin hydrogels to shrink and become more rigid by forming hydrogen bonds (Lootens *et al.*, 2003; Kjøniksen *et al.*, 2004).

*Formation and coalescence of bubbles:* To prevent bubble formation in the stem completely, deionized water was degassed using a membrane contactor (Liqui-Cel mini-module 1.7×5.5, Membrana, Charlotte, NC, USA) in line between the external water supply and the XYL'EM apparatus. The mini-module was used in vacuum mode under 3 kPa absolute pressure, generated using a vacuum diaphragm pump (model DAA-V715A-EB, Gast, Benton Harbor, MI, USA). In addition, solutions were created that contained ionic solutes at concentrations that have been found to prevent the coalescence of small bubbles into larger ones (Craig *et al.*, 1993). Solutions of 50 mM MgSO<sub>4</sub>, 100 mM CaCl<sub>2</sub>, 300 mM KCl, and 100 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> were used because they completely inhibit bubble coalescence, while solutions of 20 mM MgSO<sub>4</sub>, 40 mM CaCl<sub>2</sub>, and 100 mM KCl were used because they prevent about 50% of bubble coalescence (Craig *et al.*, 1993). Solutions of 0.5 mM MgSO<sub>4</sub>, 10 mM CaCl<sub>2</sub>, and 10 mM KCl used to test for pectin swelling are not expected to inhibit bubble coalescence (Craig *et al.*, 1993). Di-ammonium sulphate

(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> at 100 mM is expected to inhibit bubble coalescence completely (Craig *et al.*, 1993), while only having minor osmotic effects on pectin swelling. Ice water was also used as a treatment, because it can reduce bubble coalescence in deionized water (Ribeiro and Mewes, 2006).

*Active wounding responses:* To slow enzyme activities at low water temperatures, branches were both rehydrated and measured in ice water for one treatment and rehydrated at room temperature, but measured in ice water for another treatment. The latter treatment was expected only to affect immediate wounding responses to cutting stems to 15 cm in length just before taking the measurements. In addition, three chemical treatments were applied that previously had been found to reduce xylem blockage and increase the water uptake into cut plant stems, including treatment with 0.25 mM Cu(II)SO<sub>4</sub> (Loubaud and van Doorn, 2004) during measurement or 0.10 mM Cu(II)SO<sub>4</sub> (Vaslier and van Doorn, 2003) during prehydration and measurement, 10 mM hydroquinone (Loubaud and van Doorn, 2004) during prehydration and measurement, and 10 mM 4-hexylresorcinol (4-HR) (Vaslier and van Doorn, 2003; He *et al.*, 2006) during prehydration and measurement. Ice water as a measuring solution also was expected to slow enzyme activity and thereby inhibit active wound responses.

*Passive wounding effects:* The only treatment directly to address passive wounding responses was to remove the bark of stems before cutting them in order to avoid the transfer of resins from the bark to the cut surface, especially in the resin-rich *Encelia*.

#### Rehydration of partially embolized stem sections

Because most woody plant stems under natural conditions will at least be partially embolized and because flushing of stems under high pressure is time-consuming (Tyree and Yang, 1992; Yang and Tyree, 1992) and can cause a decline in  $k_s$ , a prehydration treatment under vacuum (Sellin, 1991; Hietz *et al.*, 2008) was tested to determine whether it could result in stable measurements of  $k_{max}$ . Ten stem segments (15 cm long) each of *Encelia* and *Laurus* were placed on a laboratory bench for 24 h, as previous studies (data not shown) had found that this treatment would induce a high degree of embolism. They were then infiltrated with deionized water under vacuum for 24 h and hydraulic conductance measured as described above.

#### Data analysis

The effect of flushing time and flushing pressure on specific hydraulic conductance,  $k_s$ , through stems of *Encelia farinosa* and *Laurus nobilis* were tested using repeated measures ANOVA using SYSTAT (version 12.02, SYSTAT Inc., San Jose, CA, USA).

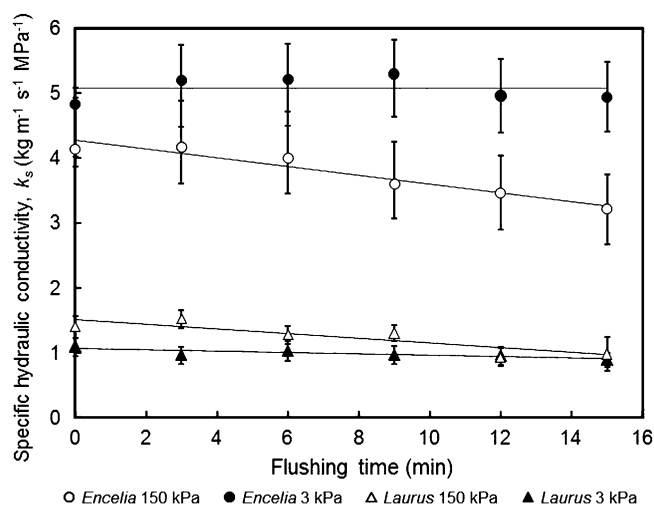
Because the experiments included measurements of  $k_{\max}$  spread out over 24 dates during a period of 8 months, for temporal effects on  $k_{\max}$  were tested by linearly regressing the treatment means of  $k_{\max}$  against date separately for each species using SYSTAT. To test for serial correlation between measurement dates, Durbin-Watson D statistics, calculated by SYSTAT for these regressions, were compared to critical values given in Savin and White (1977). ANOVA was used to analyse  $k_{\max}$  as a function of species, treatments, and species  $\times$  treatment interactions using SYSTAT. To identify  $k_{\max}$  values that were significantly higher or lower than the respective overall mean  $k_{\max}$  value for the species calculated across 22 separate experiments, post hoc tests were conducted for each species  $\times$  treatment combination to test the null hypothesis that the treatment mean was equal to the overall species mean. Resulting  $p$ -values were corrected for false discovery rate in multiple comparisons (Benjamini and Hochberg, 1995). Responses of  $k_s$  to repeated high-pressure flushing were characterized by calculating the mean percent change in  $k_s$  after each 3 min flush for each treatment. These rates of change were analysed by ANOVA in SYSTAT with species, treatments, and species  $\times$  treatment interactions as effects. To test the null hypothesis of no change in  $k_s$ , post hoc tests were conducted for each species  $\times$  treatment combination. Resulting  $p$ -values were corrected for false discovery rate in multiple comparisons (Benjamini and Hochberg, 1995).

## Results

The overnight vase rehydration treatment used to refill gas-filled vessels worked very well in *Encelia*, resulting in a specific hydraulic conductivity,  $k_s$  [ $3.29 \pm 0.55$  (standard error)  $\text{kg m}^{-1} \text{s}^{-1} \text{MPa}^{-1}$ ] that was not statistically different ( $P=0.18$ ) from that measured in stems harvested at predawn from the same plants after they had been well-watered ( $4.13 \pm 0.25 \text{ kg m}^{-1} \text{s}^{-1} \text{MPa}^{-1}$ ). In *Laurus*, vase rehydration was less successful, as rehydrated stems had a lower  $k_s$  ( $1.28 \pm 0.15 \text{ kg m}^{-1} \text{s}^{-1} \text{MPa}^{-1}$ ) than stems from the same tree after thorough watering ( $1.83 \pm 0.26 \text{ kg m}^{-1} \text{s}^{-1} \text{MPa}^{-1}$ ). The difference, which was not statistically significant ( $p=0.08$ ), was alleviated by rehydrating the stems under vacuum for 20 h, which resulted in a  $k_s$  of  $2.40 \pm 0.44$  and  $1.83 \pm 0.26 \text{ kg m}^{-1} \text{s}^{-1} \text{MPa}^{-1}$ , which was similar ( $p=0.27$ ) to that observed after thorough watering.

Consecutive 150 kPa flushes with deionized, non-degassed water resulted in significant declines in  $k_s$  over the 15 min flushing period in both species, while 15 min of low pressure flushing at 3 kPa did not cause such a decline (Fig. 1). No significant differences were observed between inflow rates measured with the XYL'EM apparatus and outflow rates measured with a Sperry apparatus for the control (PVC tubing) and *Laurus nobilis* (Fig. 2a, c). Inflow rates for *Encelia farinosa* before flushing under high pressure were slightly higher than outflow rates ( $p < 0.05$ ; Fig. 2b), but the difference disappeared after flushing, and none of the differences between inflow and outflow for *Laurus* or *Encelia* were significantly different from those observed for the tubing control.

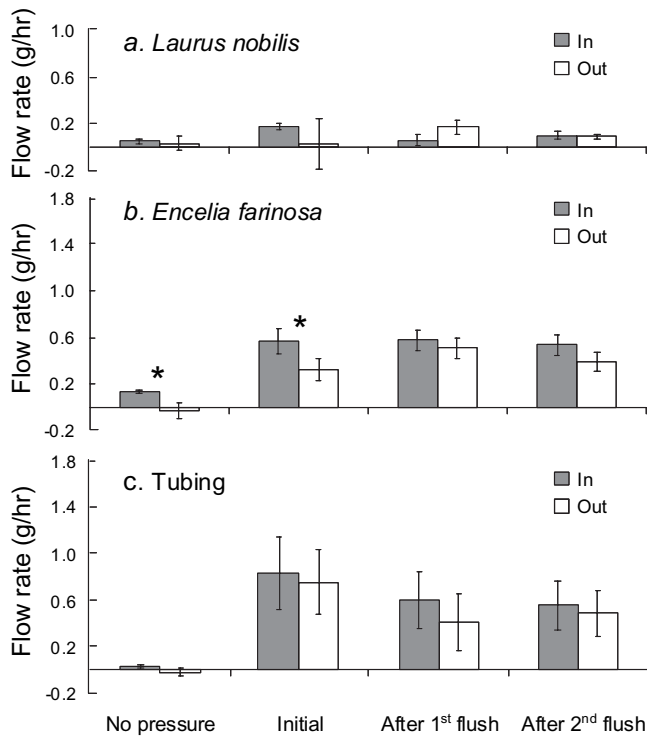
The overall mean  $k_{\max}$  across 20 experiments (excluding the two ice-water treatments) for *Encelia* was  $2.75 \pm 0.20 \text{ kg m}^{-1} \text{s}^{-1} \text{MPa}^{-1}$  and for *Laurus* was  $1.24 \pm 0.10 \text{ kg m}^{-1} \text{s}^{-1} \text{MPa}^{-1}$ . Means for individual experiments varied from 1.40 to  $4.70 \text{ kg m}^{-1} \text{s}^{-1} \text{MPa}^{-1}$  for *Encelia* and from 0.52 to 2.61



**Fig. 1.** Effect of flushing time and flushing pressure on specific hydraulic conductance,  $k_s$ , through stems of *Encelia farinosa* and *Laurus nobilis* when flushing with deionized, non-degassed water. Effects of species, pressure, time, and all their interactions on  $k_s$  were tested with repeated measures ANOVA (Species: Sum of Squares (SS) 639.19, degrees of freedom (df) 1,  $F$ -ratio ( $F$ ) 58.982,  $p$ -value ( $p$ )  $< 0.001$ ; pressure: SS 16.17, df 1,  $F$  1.492,  $p$  0.230; species  $\times$  pressure: SS 35.68, df 1,  $F$  3.292,  $p$  0.078; time: SS 6.43, df 5,  $F$  7.832,  $p < 0.0001$ ; time  $\times$  species: SS 0.74, df 5,  $F$  0.899,  $p$  0.48313; time  $\times$  pressure SS 3.486, df 5,  $F$  4.247,  $p$  0.001; time  $\times$  species  $\times$  pressure: SS 1.542, df 5,  $F$  1.878,  $p$  0.100). Slopes of linear trend-lines shown in the graph were significantly different from zero only in the 150 kPa treatments for both species.

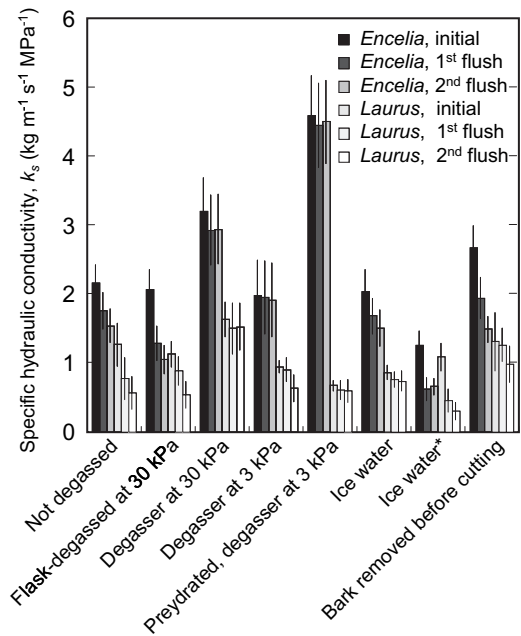
$\text{kg m}^{-1} \text{s}^{-1} \text{MPa}^{-1}$  for *Laurus*. Small rates of inflow into well-hydrated stems without any driving pressure differential were observed for all treatments. No positive serial correlations among  $k_{\max}$  measurements conducted over an 8-month period were found for either species (data not shown), suggesting that treatment effects on  $k_{\max}$  masked any seasonal variation that may have existed. This was also evident by significant differences between treatments conducted within just a few days of each other (data not shown). For *Encelia*, four treatment means were significantly ( $p < 0.05$ ) higher than the overall species mean, including 10 mM KCl, 10 mM  $\text{CaCl}_2$ , 50 mM  $\text{MgSO}_4$ , and prehydration under vacuum and using a degasser at 3 kPa. Hydration and measurement in ice water, 100 mM KCl, 100 mM  $(\text{NH}_4)_2\text{SO}_4$ , and 0.25 mM  $\text{Cu(II)SO}_4$  in the measurement solution resulted in  $k_{\max}$  values that were significantly lower ( $p < 0.05$ ) than the overall mean. For *Laurus*, the only treatment that differed significantly from the overall mean  $k_{\max}$  was an exceptionally high  $k_{\max}$  measured with 0.25 mM  $\text{Cu(II)SO}_4$  solution.

Most treatments, including the two control treatments, resulted in declines of  $k_s$  after flushing at high pressure (Figs 3, 4), with slopes of decline varying from 0% to  $-41.4\% \text{ min}^{-1}$  (Fig. 5). The only significant increase in  $k_s$  was observed for treatment with 300 mM KCl for *Laurus* (Fig. 5). Overall treatment effects on rates of decline in  $k_s$  were significant, but differences between the two species were



**Fig. 2.** Comparison of inflow rates into plant stems of *Encelia farinosa*, *Laurus nobilis*, and a tubing control, measured with a XYL'EM apparatus (Cochard *et al.*, 2000) and simultaneous outflow rates from these samples measured with a Sperry apparatus (Sperry *et al.* 1988). Significant differences ( $p < 0.05$ ) between inflow and outflow rates are designated by an asterisk.

not, and the species responded similarly to the 22 experimental treatments (Fig. 5), as there was no significant species  $\times$  treatment interaction. All treatments that were expected to alleviate declines of  $k_s$  due to prevention of bubble formation or coalescence (Table 1) resulted in rates of change in  $k_s$  that were not significantly different from zero, with the exception of 100 mM  $\text{CaCl}_2$  in *Laurus*. Most treatments predicted to cause at least some alleviation of decline in  $k_s$  (Table 1), through the prevention of bubble formation or coalescence, also had the predicted effect, except, notably, for measurement with flask-degassed, deionized water and for treatment with ice water during prehydration and measurement for both species. Those treatments predicted to prevent a decline in  $k_s$  by reducing the swelling of pectins in pit membranes (Table 1), but not by reducing bubble coalescence, such as 10 mM KCl, 10 mM  $\text{CaCl}_2$ , or 0.5 mM  $\text{MgSO}_4$ , did not prevent a decline in  $k_s$ , but a treatment that strongly affected bubble coalescence but not pectin swelling, 100 mM  $(\text{NH}_4)_2\text{SO}_4$ , did (Fig. 5). Treatments applied to reduce active wounding responses did not prevent a decline of  $k_s$ , except for 0.25 mM  $\text{Cu(II)SO}_4$  in the measurement solution. Removing the bark before cutting the stems under water did not prevent a decline in  $k_s$ , in *Encelia*, but produced what appeared to be an experimental artefact in *Laurus*, in which bark removal was difficult and required substantial handling of the stems. This resulted in low initial flow rates that increased after the first

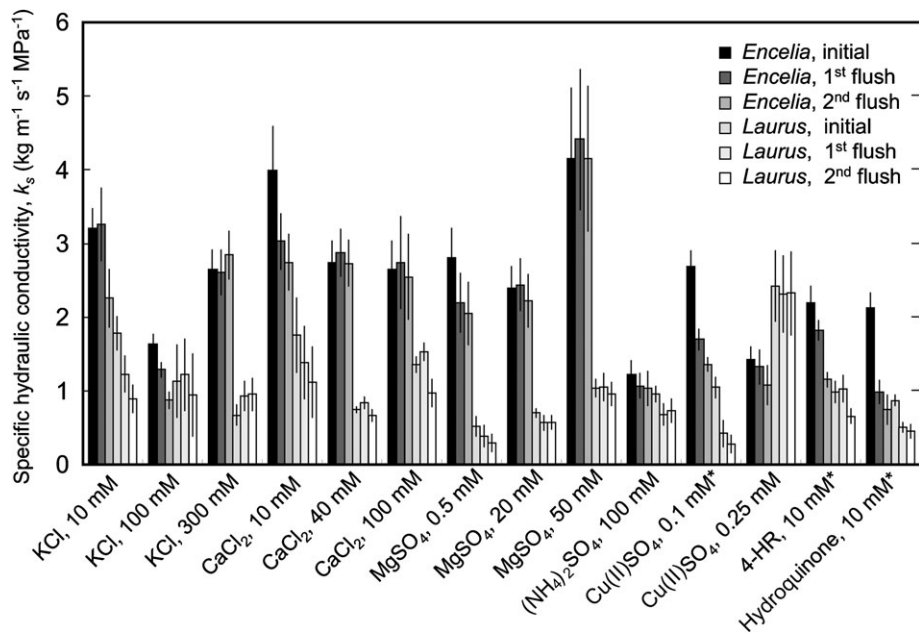


**Fig. 3.** Effects of physical treatments on the specific hydraulic conductivity,  $k_s$ , of fully hydrated *Encelia* and *Laurus* stems before high-pressure flushing (initial), after the first 3-min high-pressure flush, and after a second 3-min high-pressure flush. \*Treatment applied during vase rehydration and during measurement.

flush and then decreased after the second flush, causing an overall change in  $k_s$  that was not significantly different from zero. Prehydration for 24 h under 3 kPa vacuum resulted in stable measurements of  $k_s$ , whether the stems were well hydrated or embolized (Fig. 6).

## Discussion

Stems of *Laurus nobilis* proved to be very difficult to rehydrate. Most flushing treatments did not result in specific hydraulic conductivities,  $k_s$ , as high as those measured in stems taken at predawn after thorough watering over two nights. The failure of vase rehydration was most probably due to stomatal closure, observed for leaves of cut branches (data not shown), and rehydrating the stems under vacuum for 20 h restored  $k_s$  to the levels seen in well-watered plants. This suggests that vase storage overnight did not induce rapid gel or tylose formation in vessels. *Encelia farinosa* branches rehydrated well in the vase, and their stomata did not close. Thus, for *Laurus*, measurements of  $k_s$  probably remained below  $k_{\text{max}}$  in this study, as *Laurus* stems remained partially embolized. This does not affect the conclusions to be drawn from this study, which addressed the problem of declines in  $k_s$  rather than the question of how to achieve true measurements of  $k_{\text{max}}$ , but it raises the question of how reliable measurements of  $k_{\text{max}}$  can be achieved in different species. Our results show that flushing stems under high pressure can be problematic. Rehydration under vacuum (Hietz *et al.*, 2008) may affect xylem structure and function, so it may not be suitable for all studies.



**Fig. 4.** Effects of chemical treatments on the specific hydraulic conductivity,  $k_s$ , of fully hydrated *Encelia* and *Laurus* stems before high-pressure flushing (initial), after the first 3-min high-pressure flush, and after a second 3-min high-pressure flush. \*Treatment applied during vase rehydration and during measurement.

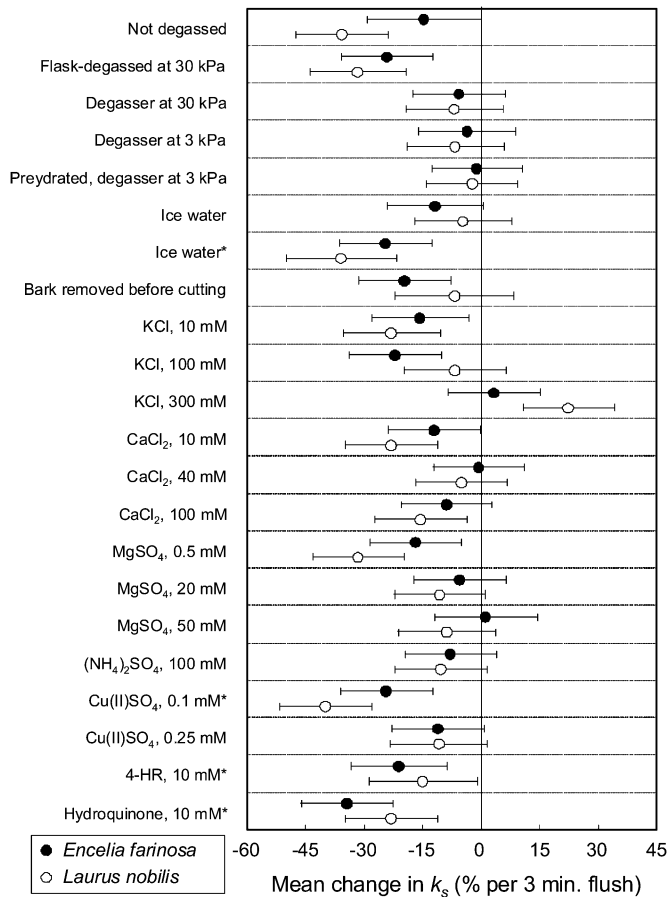
Our comparison of methods between a XYL'EM apparatus (Cochard *et al.*, 2000) that measures inflow into a stem and a Sperry apparatus (Sperry *et al.*, 1988) that measures outflow from a stem showed no significant differences between flow rates, suggesting that both methods lead to similar measurements of maximum hydraulic conductivity ( $k_{max}$ ). Initial inflow into *Encelia* stems slightly exceeded outflow, but did not significantly exceed inflow and outflow comparisons for a tubing control. Observations of higher inflow than outflow had been previously reported by Tyree and Yang (1992). As the differences observed in this study were minor, it appears that there is little concern about potential unpredictable secondary responses to lateral water flow, such as the movement of ions into vessels, that may affect hydraulic conductance (van Ieperen, 2007). Interestingly, inflow into well-hydrated stems was consistently observed, even without any driving pressure differential. Interestingly, this no-pressure flow declined to zero in *Laurus* after repeated flushes, but not in *Encelia* (data not shown). Initial flow through stems without a driving pressure differential is commonly observed in plant hydraulics studies (John Sperry, personal communication) and is routinely deduced from measured flow rates under pressure. The mechanisms responsible for such no-pressure flow would certainly seem to warrant some attention.

Declines in hydraulic conductance have been attributed to the swelling of pectin hydrogels in pit membranes as  $Ca^{2+}$  ions are flushed from the pectins by deionized water (Zwieniecki *et al.*, 2001). Cations, such as  $K^+$ ,  $Na^+$ , and  $Ca^{2+}$ , can reduce hydration of pectin gels by associating with negatively-charged galacturonic acid groups (Zwieniecki *et al.*, 2001; van Ieperen, 2007). In addition,  $Ca^{2+}$  ions crosslink demthylesterized pectin molecules (Jarvis,

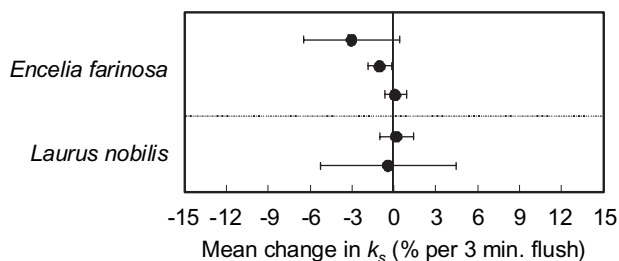
1984; Willats *et al.*, 2001) and thereby increase gel rigidity and decrease hydration.  $Mg^{2+}$  ions appear to have a similar effect (Zsivánovits *et al.*, 2005). In our experiments, low concentrations of  $K^+$ ,  $Mg^{2+}$ , and  $Ca^{2+}$  salts caused high values of  $k_{max}$  in *Encelia*, but did not prevent decline in  $k_s$  in either species. Observed alleviations of declines at high salt concentrations were probably due to the property of these salts to inhibit bubble coalescence almost completely at these higher concentrations (Marrucci and Nicodemo, 1967; Lessard and Zieminski, 1971; Craig *et al.*, 1993; Zahradnik *et al.*, 1995). This interpretation is strongly supported by the finding that  $(NH_4)_2SO_4$ , a very effective bubble coalescence inhibitor (Craig *et al.*, 1993), alleviated the decline in  $k_s$ , even though ammonium ions are unlikely to affect pectin hydration. The stem segments used in this study were shorter than the maximum vessel length, which means that at least some water could pass through open vessels without crossing pit membranes. Other studies conducted to date to test the pectin hydrogel hypotheses have used stem lengths ranging from 3 cm to 16 cm (Zwieniecki *et al.*, 2001; Boyce *et al.*, 2004; Nardini *et al.*, 2007), with only one study examining stem lengths of up to 36 cm (Gascó *et al.*, 2006). Flow through open vessels did not obscure membrane effects in any of these studies.

Four chemical and two physical treatments that were predicted to alleviate declines in  $k_s$  by preventing either the formation or coalescence of gas bubbles in the xylem all had the predicted effect, providing very strong support for the conclusion that declines in  $k_s$  are caused by the blockage of vessels by bubbles. Similarly, cut flowers have been found to take up degassed water more rapidly than non-degassed water (van Ieperen *et al.*, 2002; van Meeteren *et al.*, 2006). Our findings confirm the conclusions of Kelso *et al.* (1963)





**Fig. 5.** Effects of treatments on the change in specific hydraulic conductivity  $k_s$  ( $\pm 95\%$  confidence intervals) of fully hydrated *Encelia* and *Laurus* stems in response to high-pressure flushing. Values with 95% confidence intervals overlapping the zero-line are not significantly different from zero. Overall effects of treatments on change in  $k_s$  were highly significant (sum of squares,  $SS=5,638.4$ , degrees of freedom,  $df=21$ ,  $F$ -ratio= $6.765$ ,  $p < 0.001$ ), but differences in rates of change in  $k_s$  between species were not ( $SS=59.457$ ,  $df=1$ ,  $F$ -ratio= $1.498$ ,  $p=0.222$ ), nor were species  $\times$  treatment interactions ( $SS=1,215.037$ ,  $df=21$ ,  $F$ -ratio= $1.458$ ,  $p=0.089$ ).



**Fig. 6.** Effects of 24 h prehydration of submerged woody plant stems under 3 kPa (absolute pressure) vacuum on the change in specific hydraulic conductivity  $k_s$  ( $\pm 95\%$  confidence intervals) in response to repeated high pressure flushing. Values with 95% confidence intervals overlapping the zero-line are not significantly different from zero. Multiple points for *Laurus* and *Encelia* stand for separate experiments.

and Canny *et al.* (2007), who proposed that a thorough degassing of the measurement solution is essential for stable and precise measurements of hydraulic conductivity. Our findings show that thorough degassing, as provided by the membrane contactors used in this study, is needed to obtain stable  $k_{max}$ . It is important to note that the common degassing method of stirring water under laboratory vacuum was not sufficient to prevent a decline of  $k_{max}$  (Fig. 5).

Our findings of bubbles as the main cause for declining hydraulic conductivity through woody stems while under positive pressure raise the question whether similar effects occur in the transpiration stream under natural conditions, as proposed by Canny *et al.* (2007). This could indeed be the case if xylem sap is air-saturated, as normally assumed (Lybeck, 1959; Hammel, 1967; Sperry and Sullivan, 1992; Yang and Tyree, 1992; McCully *et al.*, 2000; Cobb *et al.*, 2007). Passage of air-saturated water through pit membranes could cause the formation of bubbles through heterogeneous nucleation, followed by bubble coalescence, and the air blockage of vessels. It should be noted, however, that contrary to the suggestion by Canny *et al.* (2007), this would not occur due to the water pressure drop that occurs as water flows through constrictions, because decreasing water pressure actually increases air solubility (Mercury and Tardy, 2001; Mercury, 2006). The familiar gassing-out of water under vacuum only occurs when water is in contact with a gas phase under partial vacuum (Mercury *et al.*, 2003), which is not the case in xylem because it is in contact with a gas phase under atmospheric pressure. Gassing-out of xylem sap could still occur due to heterogeneous nucleation or in response to lower air solubility with rising temperatures as the xylem sap emerges from cool roots into warmer stems and branches.

Physiological wounding responses are a concern in hydraulic studies of cut plant samples. Responses to wounding could be induced by a rapid increase in water potential, which sets off hydraulic and/or electrical signals (Stahlberg and Cosgrove, 1995; Stahlberg *et al.*, 2005; Fromm and Lautner, 2007). Cell-wall proteins, some of which reside in pit membranes (Harrak *et al.*, 1999), can react within minutes to wounding (Bradley *et al.*, 1992). Experiments with cut flowers have shown that physiological wounding responses can be reduced by keeping stems at low temperatures (van Meeteren, 1992; van Doorn and Cruz, 2000). In this study, measurement in deionized water at 4–6 °C reduced the decline in  $k_s$ , but this effect may have been due to several causes, including increasing air solubility that reduced bubble formation, reduced bubble coalescence (Ribeiro and Mewes, 2006), shrinking of pectin hydrogels (Kjønksen *et al.*, 2004; Lootens *et al.*, 2003), or reduced physiological activity.

A more specific inhibition of active wounding responses was expected from chemical treatments that had been found previously to reduce active wounding responses, reduce xylem blockage, and increase water uptake into cut plant stems (van Meeteren *et al.*, 2000; van Doorn and Vaslier, 2002; Vaslier and van Doorn, 2003; Loubaud and van Doorn, 2004; He *et al.*, 2006). In this study,  $Cu^{2+}$  ions,

applied at a concentration of 0.25 mM for measurement and flushing, reduced the decline in hydraulic conductance, as found in previous studies (van Doorn and Vaslier, 2002; Vaslier and van Doorn, 2003; Loubaud and van Doorn, 2004). Copper is an essential micronutrient, is toxic at super-optimal levels, and plays a wide variety of roles in plant biochemistry (Maksymiec, 1997). Its functions in xylem may include effects on peroxidases, which are among the most common enzymes in xylem sap (Buhtz *et al.*, 2004; Kehr *et al.*, 2005), and whose activities  $\text{Cu}^{2+}$  ions have been found to increase (Chen *et al.*, 2002) or inhibit (Zancani *et al.*, 1995), probably depending on the nature of specific peroxidases.  $\text{Cu}^{2+}$  ions also bind strongly to cell wall pectins (Dronnet *et al.*, 1996; Wehr *et al.*, 2004) and may thereby inhibit enzymatic attack (Wehr *et al.*, 2004) and/or contribute to cell wall loosening by causing non-enzymatic scission of other wall polysaccharides (Fry *et al.*, 2002). In our study, 0.25 mM  $\text{Cu(II)SO}_4$  caused exceptionally high values of  $k_{\text{max}}$  in *Laurus* and exceptionally low values of  $k_{\text{max}}$  in *Encelia*, which means that this is probably not a treatment to be recommended for a standardized protocol of  $k_{\text{max}}$  measurements.

The other two chemicals applied to reduce active wounding responses, hydroquinone and 4-hexylresorcinol (4-HR), had no effect on preventing the decline in  $k_{\text{max}}$ . These chemicals were chosen because they have been found to reduce xylem blockage and delay wilting in cut flowers (van Doorn and Vaslier, 2002; Vaslier and van Doorn, 2003; Loubaud and van Doorn, 2004). Hydroquinone, a peroxidase substrate (Bagirova *et al.*, 2001) and inhibitor (Martinez *et al.*, 2001) is a powerful phytotoxin at the concentration used in this study (10 mM) that strongly affects cell membrane integrity (Pandey *et al.*, 2005). The polyphenol oxidase inhibitor 4-hexylresorcinol (Dawley and Flurkey, 1993) had no effect in our study. Polyphenol oxidases play roles in lignifying xylem and in wounding responses (Richardson *et al.*, 2000; Mayer, 2006). They do not appear to occur normally in mature xylem (Buhtz *et al.*, 2004; Kehr *et al.*, 2005), so it is not clear how they could affect water uptake through cut stems.

Cutting of plant stems could decrease hydraulic conductivity by clogging of vessels with gums, resins, or debris from the xylem and the bark. To reduce the transfer of substances from the bark to the xylem, bark was removed from submerged stems before cutting them. This treatment had no effect on alleviating the decline of  $k_s$  in *Encelia*, and produced experimental artefacts in *Laurus* that lead to inconclusive results. Examination of stems for evidence of debris in the xylem was initially planned for this study, but the plans were dropped as too labour-intensive and unnecessary after it was found that the declines in  $k_s$  could be alleviated with methods that affect bubble formation and coalescence.

Arguing that a lack of method standardization in plant hydraulics could negatively affect progress in the field, Pratt *et al.* (2008) recently called for a common toolbox of methods. A comparison of methods used by different plant hydraulics research laboratories to measure  $k_{\text{max}}$  through

stems (Table 2) shows that methods vary widely in the length of samples, pressure differentials and flushing times, chemical composition of measurement solutions, and in whether or not solutions are degassed. As a result, measurements of  $k_{\text{max}}$  and the percentage loss of conductance (PLC) in xylem vulnerability curves from different published studies are strictly comparable only within laboratories and among collaborating researchers. Two conclusions from this study would seem to merit incorporation into future protocols to measure  $k_{\text{max}}$ : (i) Thorough degassing of measurement solutions using a degassing unit, such as the Liqui-Cel mini-module used in this study, and complete air removal from the measuring apparatus. (ii) Hydration of submerged samples under vacuum instead of flushing at high pressure. The latter is recommended based on the findings of this study for angiosperm stems, based on a previous study of conifer stems (Hietz *et al.*, 2008), and, most importantly, based on theoretical considerations and measurements which showed that complete air removal from a severely embolized stems by high-pressure flushing takes many hours (Yang and Tyree, 1992). Eliminating high-pressure flushing from protocols to measure native embolisms and  $k_{\text{max}}$  has the added advantage of allowing increased sample sizes by shifting measurements of  $k_{\text{max}}$  to the next day after the samples have been incubated for 20–24 h under vacuum. Subsequent to the experiments described in this paper, it was found that vacuum hydration lead to stable measurements of  $k_{\text{max}}$  for eight out of ten species tested, with relatively small declines for the remaining two species (1–3%  $\text{min}^{-1}$ ; data not shown). A detailed protocol for measurements of native embolisms and  $k_{\text{max}}$ , especially for XYL'EM users, is available from the authors upon request.

Vacuum hydration was found to lead to stable measurements of  $k_{\text{max}}$  in this study, but further comparative research on the effects of vacuum versus high pressure infiltration of angiosperm wood seems warranted. Both techniques could cause artefacts involving non-conducting xylem cells and could potentially damage xylem structures. Several hours of flushing with thoroughly degassed water under very low pressure may be the optimal solution. Another technique that reduces the chance of bubble formation during flushing is to measure and flush very short stem segments (2–4 cm). This technique used by Hervé Cochard and colleagues (e.g. Hukin *et al.*, 2005) greatly reduces the number of vessel endings at which bubbles could form or get entrapped within a stem segment. By allowing water to flow through a stem without crossing vessel ends, this technique would tend to overestimate  $k_{\text{max}}$ , but this may be of little concern for measurements of PLC within a species.

Obviously, the choice of methods used for measuring  $k_{\text{max}}$  will depend on the study system and the questions asked. Adding chemicals to the measurement solution provided no clear advantage in this study over the use of thoroughly degassed, deionized water, which is why the latter is suggested as the easiest choice for a standard solution. Responses of  $k_{\text{max}}$  to salts vary widely between

species and seasonally (Gascó *et al.*, 2007; Trifilò *et al.*, 2008), which means that there is no recipe for a chemical composition of the measuring solution that could be a standard choice for all studies. Deionized and thoroughly degassed water therefore appears to be the most parsimonious choice for a standard measurement solution.

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