ORIGINAL ARTICLE

Neither xylem collapse, cavitation, or changing leaf conductance drive stomatal closure in wheat

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Abstract

Identifying the drivers of stomatal closure and leaf damage during stress in grasses is a critical prerequisite for understanding crop resilience. Here, we investigated whether changes in stomatal conductance (g_s) during dehydration were associated with changes in leaf hydraulic conductance (K_{leaf}), xylem cavitation, xylem collapse, and leaf cell turgor in wheat (*Triticum aestivum*). During soil dehydration, the decline of g_s was concomitant with declining K_{leaf} under mild water stress. This early decline of leaf hydraulic conductance was not driven by cavitation, as the first cavitation events in leaf and stem were detected well after K_{leaf} had declined. Xylem vessel deformation could only account for <5% of the observed decline in leaf hydraulic conductance during dehydration. Thus, we concluded that changes in the hydraulic conductance of tissues outside the xylem were responsible for the majority of K_{leaf} decline during leaf dehydration in wheat. However, the contribution of leaf resistance to whole plant resistance was less than other tissues (<35% of whole plant resistance), and this proportion remained constant as plants dehydrated, indicating that K_{leaf} decline during water stress was not a major driver of stomatal closure.

KEYWORDS

crops, drought stress, hydraulic conductance

1 | INTRODUCTION

Decreased crop production after dry conditions has been reported worldwide over the last decades (The Food and Agriculture Organization Corporate Statistical Database, n.d.; Lesk, Rowhani, & Ramankutty, 2016). These substantial drops in production result from the negative effect of dry conditions (Farooq, Wahid, Kobayashi, Fujita, & Basra, 2009; Flexas, Bota, Loreto, Cornic, & Sharkey, 2004) and abnormally warm temperatures (Ben-Ari et al., 2018) on photosynthesis and seedling growth. A predicted increase of drought events in the future (Dai, 2013) adds urgency to the challenge of understanding crop responses to water stress. The water transport system is of particular interest due to its vulnerability to damage under water stress, making it a potential target for breeding that may produce enhanced performance under drier conditions. Water movement through the vascular system of plants is driven by leaf transpiration that generates tension in the xylem. As the environment becomes drier, the tension increases and may lead to a disruption of water transport in the vascular system (Dixon & Joly, 1895; Tyree & Sperry, 1989). Maximum rates of transpiration decline during water stress as stomatal conductance (g_s) is reduced thereby delaying the onset of damage to the xylem.

Dynamic changes in g_s and leaf hydraulic conductance (K_{leaf}) during water stress have been largely studied; however, interactions between them are complex and remain unresolved. A primary role for stomatal closure is restricting the drop of xylem pressure under drier atmospheric conditions (Cardoso, Brodribb, Lucani, DaMatta, & McAdam, 2018), thus avoiding the decline of leaf hydraulic conductance due to cavitation and embolism of xylem vessels (Brodribb & McAdam, 2017). The difference between the xylem pressure at which stomatal closure is complete (P_{gs90}) and the xylem pressure inducing 12% or 50% loss of hydraulic conductance in leaf or stem (P_{12} or P_{50}) is called the stomatal safety margin (SSM), whereas the hydraulic safety margin corresponds to the difference between the minimum water potential experienced under natural conditions (Pmin) and the xylem pressure inducing 12% or 50% loss of hydraulic conductivity (P₁₂ or P₅₀). The latter one (hydraulic safety margin) is based on the minimum water potential assessed in a comparable and ecologically meaningful way by including seasonal minima through repeated measurements across years. However, the two physiological traits used to estimate SSM can be assessed on a batch of plants by carrying out dry-down experiments. Variations in SSM among species are mainly due to species-specific differences in xylem vulnerability to embolism (Martin-StPaul, Delzon, & Cochard, 2017). Species that are highly resistant to xylem embolism have a greater SSM than species with more "risky" behaviour, which close their stomata right before the onset of xylem cavitation.

Strong evidence links cavitation in leaf veins and a decline of K_{leaf} during acute water stress (Brodribb et al., 2016; Nardini, Salleo, & Raimondo, 2003). Cavitation has been shown to reduce the functionality of the water transport system, leading to a reduced capacity of the plant to recover from drought after being exposed to water stress (Brodribb & Cochard, 2009; Urli et al., 2013) and ultimately the death of the plant under prolonged water stress (McDowell et al., 2008). Xylem vulnerability to cavitation has been widely studied and provides a predictive indication of plant tolerance and mortality during drought (Anderegg et al., 2015). However, some studies report declines in K_{leaf} at water potentials insufficient to cause cavitation but before or concomitant with stomatal closure (Brodribb & Holbrook, 2006: Holloway-Phillips & Brodribb, 2011: Scoffoni et al., 2018: Torres-Ruiz, Diaz-Espejo, Perez-Martin, & Hernandez-Santana, 2015; Wang, Du, Huang, Peng, & Xiong, 2018). One possible explanation for this behaviour is xylem wall implosion (i.e., xylem collapse) under negative pressure (Cochard, Froux, Mayr, & Coutand, 2004). Even though this process has not been extensively studied, it has been shown to correlate with a decrease of the hydraulic conductance of leaves (Brodribb & Holbrook, 2005) and is thought to be reversible after rehydration. In trees, thickening of the xylem cell wall appears to prevent collapse from occurring before cavitation in stems. However, leaf xylem is less reinforced, and recent observations of xylem collapse in leaves of Quercus rubra (Zhang, Rockwell, Graham, Alexander, & Holbrook, 2016) suggest the possibility that collapse in the terminal parts of the xylem network may cause decreases in leaf hydraulic conductance that could accelerate stomatal closure in response to changes in evaporative demand. An alternative explanation for declines in K_{leaf} prior to cavitation is that the outside-xylary conductance (K_{ox}) could decline under mild water deficit conditions (Scoffoni et al., 2017). This has then been discussed as a possible driver of stomatal closure that could delay further decline of xylem conductance (K_x) by cavitation (Flexas, Carriquí, & Nadal, 2018; Wang et al., 2018).

The most important crop species are nonwoody monocots and due to their leaf structure may be especially prone to both xylem collapse and cavitation. Traditional hydraulic methods are not well suited to measuring hydraulic processes in grasses. However, the recent development of methods such as X-ray microtomography that allow a direct observation of the hydraulic system (Cochard, Delzon, & Badel, 2014), and optical visualization of cavitation (Brodribb et al., 2016) provides new insights into the hydraulic vulnerability of major crop species to drought. A recent study demonstrated the nonreversibility of cavitation in wheat leaves (Johnson, Jordan, & Brodribb, 2018), showing that cavitation occurred only under extreme drought, at the same time as damage to photosynthetic tissue. The proximity of cavitation and leaf death in wheat refocuses attention on the function of the hydraulic system prior to cavitation and whether K_{leaf} is fixed or dynamic prior to cavitation.

Here, we focus on the common wheat, Triticum aestivum L., the most widely grown crop worldwide in terms of cultivated area. Until now, studies have investigated drought resistance of this species in terms of root architecture (Manschadi, Hammer, Christopher, & Devoil, 2008), chemical response (Bartoli et al., 1999) or genetic selection to improve yields under drier conditions (Bennett et al., 2012; Eid, 2009); but very little is known about the failure of the vascular system in this crop. The aims of our study are (a) to investigate the sensitivity of stomatal conductance and leaf hydraulic conductance to declining water potential and (b) to determine whether cavitation, mechanical deformation, or changes in leaf hydraulic conductance are associated with stomatal behaviour. Direct observational methods are used to visualize the spread of embolism and xvlem collapse during plant dehydration.

MATERIALS AND METHODS 2

2.1 Plant material

The X-ray micro computed tomography (microCT) experiments (i.e., leaf and stem vulnerability to cavitation and xylem collapse in leaf) were performed at the synchrotron facility SOLEIL (Saint-Aubin, France) on plants of T. aestivum var. SY Mattis (seed producer: Syngenta) in April 2015, March 2017, and May 2018. For the 2015 and 2017 campaigns, plants were grown in the field (Lot-et-Garonne, France) to the stem extension stage (≈2.5-months-old) and individually transplanted into 15×15 cm pots containing potting mix and kept well hydrated in a growth chamber (mean temperature of 22°C, light exposure of 16 hr/day; INRA Bordeaux, France). For the 2018 synchrotron campaign, 2-month-old plants were grown in a growth chamber (see above) under well-watered conditions. For each of these three synchrotron campaigns, several subsets of three plants were exposed to drought by withholding irrigation from 3 days to 2 weeks before starting the campaign at the synchrotron to induce a range of water potentials in the plants from 0 to -5 MPa.

Stomatal conductance, leaf hydraulic conductance, pressurevolume relations, and leaf vulnerability to cavitation curves using the optical technique were performed on plants of T. aestivum var. SY Mattis that reached the end of tillering (~2-months-old). They were grown in 10×10 -cm pots containing potting mix and perlite in a

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glasshouse between November 2017 and March 2018 (University of Tasmania, Australia). They were watered daily and exposed to natural light (\approx 16 hr/day) and mean temperatures of 25°C and 15°C during day and night, respectively.

2.2 | Sensitivity of stomatal conductance to water stress

Seven individuals were used to measure the relationship between leaf water potential (Ψ_{leaf}) and stomatal conductance during water stress. Stomatal conductance was measured using a LI-6400XT gas analyser (LiCor, Inc., Lincoln, NE). Plants were maintained under glasshouse conditions (described above) and measured daily between 10 and 11 a.m. Several leaves were enclosed in the cuvette (3 × 2 cm) at once, and leaf water potential (Ψ_{leaf}) was determined on these leaves immediately after measurement using a pressure chamber. Stomatal conductance values were adjusted by the total leaf area (i.e., sum of each leaf area, cm²) enclosed in the cuvette.

Two days after starting the measurements, irrigation was withheld and stomatal conductance monitored until reaching values close to zero (typically 2–4 days after suspending irrigation). Conditions in the cuvette were set as follows: cuvette temperature at 25°C, CO₂ concentration at 400 ppm, photosynthetically active radiation (PAR) at 1500 μ mol m⁻² s⁻¹ and vapour pressure deficit (VPD) between 1 and 1.7 for all measurements. Five equations were considered as most likely to fit the data: one linear, one logistic, two sigmoids, and one exponential (see Section 2.8). The following equation provided the best fit to the mean stomatal conductance curve:

$$y = a / (1 + e^{b^*[x - x50]}),$$
 (1)

where *a* is the maximum value of *y*, *b* is the slope, *x* is the water potential (MPa), and x_{50} is the water potential causing a 50% decrease in *y* (MPa).

2.3 | Leaf cell turgor loss

Changes in cell turgor were measured following the pressure-volume method (PV curves). Seedlings of *T. aestivum* were kept well hydrated during the experiment. Leaves were detached early in the morning (when fully hydrated) from the plant with a razor blade and progressively dehydrated on a bench. During dehydration, the leaf water potential and weight were measured 6 to 10 times with a pressure chamber and a balance, respectively. The leaf dry mass was determined after leaves were dried at 70°C for 3 days. The relative water content (RWC) was then calculated as follows to construct the PV curves:

$$RWC = (WW_i - DW) / (WW_{max} - DW), \qquad (2)$$

where WW_i is the mass of leaf water (g), WW_{max} is the mass of leaf water at 100% RWC (g), and DW is the leaf dry weight (g). The turgor

loss point was visually estimated to split the data into two groups, assumed to correspond to pre- and post-turgor loss. The data were then fitted by group using a linear function (y = ax + b). The turgor loss point (Ψ_{TLP}) was finally determined at the intercept of the two linear regressions.

Leaf capacitance pre- and post-turgor loss ($C_{\rm ft}$ and $C_{\rm tlp}$, respectively) were determined as

$$C = (\Delta RWC / \Delta \Psi_{leaf}) \times (DW / LA) \times (WW / DW) / M,$$
 (3)

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where LA is leaf area (m^2) and *M* is molar mass of water $(g \mod^{-1})$. A mean PV curve was calculated based on the parameters of all individual curves (*n* = 6).

2.4 | Dynamic of leaf hydraulic conductance under mild-water stress

The leaf hydraulic conductance was measured using the rehydration method in 12 plants of *T. aestivum*. Plants were kept well-watered during the experiment. One to three shoots per individual were cut with a razor blade below the junction with roots and dehydrated on a bench. For each shoot, the two youngest leaves were cut with a razor blade at the junction with the shoot and individually wrapped in wet paper to stop leaf transpiration. The initial water potential (Ψ_0) was measured for each leaf with a pressure chamber. Leaves were then rehydrated from 10 to 30 s while cutting under water two to three times above the original cut with a sharp razor blade; then, the final water potential (Ψ_f) was measured. Both initial and final leaf water potential were measured on the same leaf as Ψ_{leaf} happened to vary between leaves within the same shoot or individual. The leaf hydraulic conductance, K_{leaf} , was calculated as follows:

$$K_{\text{leaf}} = C \times \ln \left(\Psi_0 / \Psi_f \right) / t \tag{4}$$

where *C* is leaf capacitance determined from the mean PV curve (mmol m^{-2} MPa⁻¹) and *t* the rehydration duration (s). Data were fitted using the same method as for stomatal conductance data and the best equation to fit the mean hydraulic conductance is given in Equation 1.

2.5 | Vulnerability to embolism in leaves and stems of *T. aestivum*

2.5.1 | The optical technique

Visual assessment of cavitation was performed on leaves of four individuals of wheat using the "optical technique." Plants were kept wellhydrated under glasshouse conditions during their maturation and removed from their pot at the beginning of the experiment. Water potential measurements and imaging were done on the main shoot for each intact plant. The leaf water potential (Ψ_{leaf}) was recorded on the midrib of the youngest leaf every 10 min using a psychrometer (ICT International, Armidale, NSW, Australia). The leaf cuticle and epidermis were carefully removed with a razor blade on 5 mm along the midrib to expose xylem to the psychrometer chamber. Polystyrene was attached to the sample holder to avoid leaf damage during psychrometer installation. The psychrometer (except for the thermocouples chamber) was covered with Parafilm to maximize contact between the sample and the psychrometer chamber. Sealing of the chamber was optimized by adding a thin layer of grease between the psychrometer and the leaf. The clamp was tightened periodically as the leaf dehydrated to ensure sealing of the psychrometer chamber. As psychrometers can yield inaccurate measurements when water potential is high (from 0 to -0.5 MPa), Ψ_{leaf} was measured in neighbouring leaves with a pressure chamber in the first days of dehydration to check the proper installation and functioning of the psychrometer. Both methods resulted in similar values once the water potential started to drop. Embolism events were assessed in the nearest leaf to the psychrometer by capturing images with a custombuilt OpenSourceOV clamp (http://www.opensourceov.org/) every 2 min during plant dehydration. The leaf was fixed to the clamp with adhesive tape all around the scanned area to prevent movements during the experiment. A microscope slide was added on top of the leaf and fixed with adhesive tape to minimize leaf curling and ensure that the leaf would remain flat during dehydration. Measurements were stopped once no further embolism events were detected in the last hours of recording, assuming that all xylem vessels were embolized by that time.

The resulting stacks of images were analysed using ImageJ software (National Institute of Health, New York, NY, USA). Embolisms were visualised following the instructions given in OpenSourceOV. Aligning water potential and cavitation accumulation through time yielded a xylem vulnerability curve for each leaf plotted as the cumulative percentage of embolized pixels (PEP) against the leaf water potential. For each plant, an individual curve was fitted using the Pammenter and Van der Willigen's (1998) equation:

$$PEP = 100 / \left(1 + e^{(S/25*[P-P50])}\right),$$
(5)

where *S* is slope at the inflexion point, *P* is xylem pressure (MPa), and P_{50} is xylem pressure inducing 50% of embolized pixels. A mean vulnerability curve was finally obtained by averaging the P_{50} and the slope from the four replicate curves.

2.5.2 | X-ray microtomography

X-ray microCT was performed at the PSICHE beamline of the synchrotron facility SOLEIL (France; King et al., 2016) during three different campaigns in 2015, 2017, and 2018. In total, 22 intact plants of wheat were used to assess vulnerability to cavitation in leaf and stem. Plants were progressively dried down by stopping irrigation before or during the experiment (see Section 2.1). Stems were scanned at mid-height from the base at a 3.09 μm^3 resolution. whereas midrib and neighbouring leaf veins were scanned at a higher resolution of 0.87 μ m³ as vessel diameter is smaller in leaves. Samples were scanned using a high flux (3.10¹¹ photons mm⁻¹) 25-keV monochromatic X-ray beam and a continuous rotation from 0° to 180°. X-ray projections were collected with a 50 ms exposure time during rotation and recorded with an Orca-flash sCMOS camera (Hamamatsu Photonics K.K., Naka-ku, Japan) equipped with a 250-µm thick LuAG scintillator. Scan times were extremely rapid, ranging from 75 s (standard resolution) to 3 min (high resolution) and resulted in a stack of 752 to 1,024 TIFF images slices over 0.9 (high resolution) to 3 mm (standard resolution) along the plant organ. Tomographic reconstructions were performed following the Paganin method (Paganin et al., 2002) in PyHST2 software (Mirone et al., 2014) and resulted in 32-bit volumic images. Water potential measurements were made with a pressure chamber (DGMeca, Gradignan, France) right after the scan and values ranged from -0.3 MPa to –5 MPa. Ψ_{leaf} was measured on leaves and Ψ_{stem} on leaves that were bagged 1–2 hr before the scan.

For each sample (i.e., an individual at a given water potential), one projection was analysed using ImageJ to follow the spread of embolism as samples were increasingly dehydrated. The same individual was scanned two to four times from being well hydrated to being highly stressed. A final scan was performed after the sample was cut in the air below the scanned area, inducing air entry in the remaining functional vessels and therefore 100% of embolized vessels in the area. This scan was used to estimate the maximum theoretical hydraulic conductivity (k_{max}). Stem and leaf measurements were done on different individuals. The images were cleaned by applying a threshold, thus leading to a mask of the image containing embolized vessels only. For each scan, the number of embolized vessels and their area (A, μ m²) were measured with the *Analyze Particles* function. Vessels diameter (*D*, m) was calculated as

$$D = 2 (A/\pi)^{1/2} \times 10^{-6}.$$
 (6)

The theoretical hydraulic conductivity (k_{th} , m⁴ s⁻¹ MPa⁻¹) of embolized vessels, corresponding to the loss of hydraulic conductivity at a given water potential, was calculated based on Poiseuille's law (Tyree and Ewers, 1991):

$$k_{\rm th} = \sum \left(\left[\pi \times D^4 \right] / 128 \eta \right), \tag{7}$$

where η is the water viscosity (1.002 10^{-6} MPa s at 20°C).

The theoretical hydraulic conductivity of the entire section (k_{max} , m⁴ MPa⁻¹ s⁻¹) was calculated from the final scan for each individual and each organ. The percentage loss of hydraulic conductivity (%) was calculated as

Percentage loss of hydraulic conductivity =
$$(k_{th}/k_{max}) \times 100.$$
 (8)

A mean vulnerability curve was finally fitted for both stem and leaf using Equation 6.

2.6 | Xylem collapse

The variation of vessel shape with increasing water stress in stems of *T. aestivum* was measured in the same 12 individuals used to determine vulnerability to cavitation using the X-ray microtomography (see above). The full projection (volume of images) of each sample was kept for the image analysis on ImageJ. At least 10 vessels per hydraulic status (i.e., functional or embolized) were randomly selected throughout the sample. Each individual was scanned one to three times, such that some vessels were functional on the first scan(s) at high water potential and embolized on the last scan(s) under water stress. The image analysis was done on one vessel at a time. Brightness and contrast were adjusted when needed, and a threshold was applied to remove the noise and to obtain a mask of the vessel only. Images that were close to a junction between two vessels in the volume were removed as vessel shape varies at this point.

Feret parameters, that are minimum and maximum diameters, were measured for each vessel along the entire volume of images. A Feret ratio was calculated as the ratio of vessel minimum diameter to the maximum diameter and used to describe vessel shape. Its value ranges from 0 (flat vessel) to 1 (circular vessel). For each vessel, the mean Feret ratio was used to determine whether xylem collapse occurs in stems of common wheat exposed to water stress.

A mean relative diameter was calculated using parameters of Feret ratio where the maximum diameter for each sample was 1. Thus, the variation in vessel area between samples was not considered to determine changes in xylem conductivity that could be attributed to vessel deformation. The theoretical xylem conductivity of functional vessels was calculated for each sample (i.e., scan at a given water potential) using Equation 8. A mean xylem conductivity was then calculated at -0.5 MPa (n = 5) and -1.6 MPa (n = 4). These water potentials represent the range over which the mean K_{leaf} declined from 77% to 15%.

2.7 | Effect of the leaf hydraulic conductance decline on stomatal conductance decline under mild water stress

The influence of K_{leaf} decline on stomatal behaviour was assessed by measuring the percentage contribution of leaf resistance (R_{leaf}) to whole plant resistance (R_{plant}), assuming that the importance of R_{leaf} would increase during stomatal closure if K_{leaf} decline was driving stomatal closure.

Nine plants grown at the stem extension stage were exposed to a range of evaporative conditions to produce a range of water potentials where resistance partitioning could be calculated. Plants were transferred to high irradiance growth cabinets (1,000 μ mol quanta m² s⁻¹ photosynthetic photon flux density) set at 25°C or 30°C and 13.5 hr/day of light exposure. They were kept well hydrated during the experiment, to ensure that the soil water potential Ψ_{soil} remained close to zero. The relative humidity (Rh, %) was set at 25% to reach an airvapour pressure deficit close to 2 kPa at 25°C and 3 kPa at 30°C, whereas a VPD = 1 kPa was reached with Rh = 70% at 25°C.

conductance was measured using a LI-6400XT gas analyser with the cuvette temperature at 25°C or 30°C depending on the targeted VPD, CO₂ concentration at 430 ppm, and PAR at 1500 µmol m⁻² s⁻¹. K_{I_plant} , the leaf specific whole-plant conductance, was then calculated as

$$K_{l_plant} = (g_s * VPD) / ([\Psi_{soil} - \Psi_{leaf}] * P_{atm}).$$
(9)

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With g_s measured over a range of leaf water potential from -0.3 to -0.8 MPa (i.e., before complete stomatal closure), VPD the vapour pressure deficit in the air (in kPa), $\Psi_{soil} = 0$ MPa, and the atmospheric pressure $P_{atm} = 101.33$ kPa.

As K_{I plant} was measured with an evaporative flux method and K_{leaf} with the rehydration kinetics, additional measurements of leaf hydraulic conductance using an evaporative flux method ($K_{\text{leaf EFM}}$) were done to ensure the estimation of K_{leaf} was the same between both methods. Twelve individuals grown at the stem extension stage were measured using the LI-6400XT gas analyser. The plants were kept well hydrated in the glasshouse, and measurements were performed on sunny days to ensure plant functioning. On each plant, a leaf was wrapped in wet paper and bagged the day before the measurement. Conditions in the cuvette were set as follows: cuvette temperature at 20°C and PAR at 1,000 µmol m⁻² s⁻¹ for all measurements. The average VPD was 1.6 kPa and the CO₂ concentration was the ambient value. The bagged leaf was cut at the junction with the stem and immediately place in a plastic bag with wet paper. The adjacent leaf was placed in the cuvette and cut with a razor blade after the measurement. This leaf was wrapped in wet paper and put into a plastic bag. The water potential of both leaves was then measured with a pressure chamber after 30 min of equilibration. $K_{\text{leaf EFM}}$ was finally calculated as $K_{\text{leaf}_{EFM}} = E / |\Psi_{\text{measured leaf}} - \Psi_{\text{bagged leaf}}|$. The values of $K_{\text{leaf}_{EFM}}$ ranged from 15 to 45 mmol m⁻² s⁻¹ MPa⁻¹ over a range of water potential going from -0.29 to -0.60 MPa, that is, similar to K_{leaf} values obtained with the rehydration kinetics method.

Thus, the plant resistance, R_{plant} , was determined as the inverse of $K_{\text{l_plant}}$, and leaf resistance R_{leaf} was calculated using the mean curve of K_{leaf} from the rehydration method (see above). The contribution of leaf resistance to the whole plant resistance ($R_{\text{leaf}}/R_{\text{plant}}$, in %) was calculated at binned water potentials (classes of 0.2 MPa). Changes in R_{leaf} contribution to R_{plant} with increasing water stress were tested using a Wilcoxon test.

2.8 | Statistics

The nonlinear least squares function (i.e., *nls* function) was used to find the best fit for stomatal conductance and leaf hydraulic conductance data. Five equations were tested (including the ones proposed by Scoffoni, McKown, Rawls, & Sack, 2012): a linear function ($y = ax + y_0$), two sigmoid functions { $y = a / [1 + e^{-(x-xO)/b}]$ } and $y = a / (1 + e^{b*[x-x50]})$, a logistic function { $y = a / [1 + (x/x_0)^b]$ } and an exponential function ($y = y_0 + a*exp^{-bx}$), where *x* always corresponds to the leaf water potential.

A sigmoidal regression was fitted on vulnerability to embolism data from both the optical technique and the X-ray microCT (Equation 5). An analysis of variance and Tukey post-hoc tests were performed to compare vulnerability to embolism between plant organs (microCT data) and within the same organ measured with two different methods, the optical vulnerability (OV) method and and microCT). As we obtained only one mean curve per organ with the X-ray microtomography, the mean and standard error of the mean were used to perform the analysis. The relation of xylem deformation with water potential was tested with the Pearson correlation test, and changes in k_{leaf} caused by vessel deformation was tested using t test.



All statistical analyses were performed on R version 3.4.4 (R Core Team, 2018).

3 | RESULTS

3.1 | Stomatal, turgor, and hydraulic response of *T. aestivum* to water stress

The mean (± standard error [SE]) maximum stomatal conductance (g_s) at $\Psi_{\text{leaf}} > -0.5$ MPa was 340.88 ± 31.27 mmol m⁻² s⁻¹. Stomatal conductance started to decrease at -0.64 MPa (i.e., 10% loss of g_s), and stomatal closure was 90% complete at -1.38 MPa (Figure 1a). The mean xylem pressure inducing loss of cell turgor (Ψ_{tip}) was determined as -0.97 ± 0.06 MPa (Figure 1b). In the turgor loss region, leaf capacitance increased from 752.07 to 2,631.37 mmol MPa⁻¹ m⁻². Measurements of K_{leaf} showed a mean maximum of 38.41 ± 2.63 mmol m⁻² s⁻¹ MPa⁻¹ at water potentials > -0.6 MPa and 90% loss of leaf hydraulic conductance at -1.70 MPa (Figure 1c). In the region of cell turgor loss, a 52% and 57% decline in leaf hydraulic conductance and stomatal conductance was recorded, respectively.

3.2 | Vulnerability to embolism

Seedlings of *T. aestivum* L. were dehydrated for 5 to 9 days while recording cavitation using the optical technique. Initial Ψ_{leaf} was > – 0.5 MPa, whereas final Ψ_{leaf} ranged from –3.72 to –5.07 MPa. Cavitation started between –1.57 and – 1.8 MPa (Figure 2a) in leaf veins, and all xylem vessels were embolized by the time the water potential reached –3.7 MPa. The mean Ψ_{leaf} at which 50% of maximum cavitation occurred (P_{50}) was –2.21 ± 0.17 MPa (Figure 3a).

Similar patterns of cavitation were observed with the X-ray microCT in both leaf (Figure 2b) and stem (Figure 2c). At high water potential (i.e., $\Psi_{\text{leaf}} > -1$ MPa), native embolism in metaxylem vessels did not exceed 0.13% of the maximum theoretical hydraulic conductivity (k_{th}) in the stem, whereas leaf vessels did not show any embolism. The first embolized vessels were detected at -1.5 MPa in both

FIGURE 1 Leaf response to dehydration in winter wheat (*Triticum aestivum L*.). (a). The decline of stomatal conductance (g_s) during dehydration in seven individuals. The curve shows the best fit calculated using a sigmoid equation, and the grey area corresponds to the 95% confidence interval. (b). Changes in leaf relative water content were evaluated in six individuals. The solid line represents the mean pressure–volume curve, and the dashed line corresponds to the xylem pressure inducing cell turgor loss (Ψ_{TLP}) in leaves. (c). The decline of leaf hydraulic conductance (K_{leaf}) under mild water stress observed in 12 seedlings. The mean (± standard error) values of K_{leaf} at binned water potentials (classes of 0.2 MPa) are represented by black dots (and error bars). The solid line shows the best fit calculated using a sigmoid equation, and the grey area corresponds to the 95% confidence interval



FIGURE 2 Embolism spread in Triticum aestivum leaves and stems under water stress. (a) Series of images taken using the optical technique during the dehydration of a wheat leaf. Coloured pixels show cumulative embolisms over time, with a colour scale used to identify the water potential at which events occurred (see colour scale at the top left corner). (b and c). Cavitation patterns observed in the leaf (b) and stem (c) using the X-ray micro-computed tomography. The organ of interest is delimited by the yellow area. On bottom right corner images, cavitated vessels (in black) are easily distinguishable from the water-filled vessels (in light grey). For both methods, the leaf (Ψ_{leaf}) or stem water potential (Ψ_{stem}) is indicated below each image, as well as the corresponding percentage of embolized pixels (PEP, optical technique) or percentage loss of hydraulic conductivity (PLC, X-ray micro computed tomography)

organs, and their P_{50} values were estimated as -2.22 ± 0.12 and -2.44 ± 0.16 MPa in the leaf and stem, respectively (Figure 3b). Thus, the X-ray microCT suggested a slightly higher vulnerability to embolism in leaves than stems.

3.3 Xylem collapse

Changes in vessel shape of T. aestivum were evaluated in 384 vessels covering a water stress gradient going from -0.3 to -3.3 MPa. For each vessel, the analysis resulted in a minimum of 80 measurements of Feret ratio along the scanned organ, with values ranging from 0.18 (flat vessel) to 0.93 (circular vessel). The image analysis revealed an important variation of shape within the same vessel for both fully hydrated and air-filled vessels (Figure 4). The Feret ratio was also found to vary between vessels at a sample level (i.e., scan of a plant at a given water potential). However this variation was negligible as $\Delta_{\text{Feret ratio}}$ equals 0.21 ± 0.05 (Figure 5a) for all samples.

A significant correlation ($R^2 = 0.270$, p value = .018) was found between leaf water potential and average Feret ratio (Figure 5a),

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meaning that xylem vessels, regardless if they were functional or cavitated, tended to be slightly flatter with increasing water stress. This result was supported by the significant difference in vessel shape



FIGURE 3 Vulnerability to cavitation in leaf and stem of *Triticum aestivum*. (a) Direct visualization of embolism spread in leaves of four individuals (grey points). The loss of hydraulic functioning is represented as a cumulative percentage of embolized pixels (PEP). (b) Percentage loss of hydraulic conductivity (PLC_{th}) in leaf (red points) and stem (blue points) measured in 12 and 10 individuals, respectively, using X-ray microCT. For both panels, mean vulnerability curves in leaf (red curves) and stem (blue curve) are fitted following the Pammenter equation; the standard error of estimated parameters is represented by light red and light blue areas for leaf and stem respectively. Dotted lines correspond to P_{50} (the xylem pressure inducing 50% of embolized pixels or loss of hydraulic conductivity) determined in leaf (red) and stem (blue)

found between fully watered and air-filled vessels (Figure S1, mean *p* value = .03). Nonetheless, vessel deformation seemed to be a gradual process, and completely collapsed vessels were rarely observed (the average Feret ratio ranged from 0.79 to 0.48). According to calculation of theoretical vessel conductivity using Poiseuille's equation, this change in Feret ratio with increasing water stress would cause a maximum of 9% decline of the mean hydraulic diameter when comparing individuals at -0.3 MPa to individuals at -3.3 MPa, thus resulting in a maximum ca. 30% decrease in xylem conductivity. Considering that measured K_{leaf} declined by 60% over the water potential range of -0.5 to -1.6 MPa, less than 5% could be attributed to xylem deformation (Figure 5b).

Some vessels were scanned two to three times (see M&M), being initially water-filled then subsequently cavitated. The image analysis revealed that when a vessel was flat before cavitation, it did not recover a circular shape when cavitating. Even when the sample was cut to get the fully embolized scan, vessels remained deformed (Figure S2).

3.4 | Effect of the leaf hydraulic conductance decline on stomatal conductance decline under mild water stress

The contribution of leaf resistance (R_{leaf}) to plant resistance (R_{plant}) was determined in well-watered plants, using different evaporative conditions to impose a range of leaf water potentials. The aim was to determine whether stomatal closure under these conditions occurred as a result of declining K_{leaf} becoming a major limiting resistor on hydraulic supply. The mean $K_{l_{\text{plant}}} \pm \text{SE}$ calculated at binned water potentials was 8.2 ± 2.5 mmol m⁻² s⁻¹ MPa at $\Psi_{\text{leaf}} = -0.4$ MPa and declined slightly to 6.7 ± 2.2 mmol m⁻² s⁻¹ MPa at $\Psi_{\text{leaf}} = -0.8$ MPa.

The proportion of R_{leaf} in R_{plant} at binned water potentials ranged between 26.07 ± 2.32% and 32.73 ± 2.66% (Figure S3), and no significant change in the $R_{\text{leaf}}/R_{\text{plant}}$ proportion with increasing water stress was evidenced (*F* = 0.869, *p* value = .03). Additionally, we calculated the *F* index from Scoffoni et al. (2018), which describes the contribution of K_{leaf} decline to g_s decline (Figure S4). We observed that declining K_{leaf} was contributing at most to 9.1% of g_s decline, at a water potential of -0.87 MPa corresponding to a 55% decline in stomatal conductance.



FIGURE 4 Variation of vessel shape in a wheat stem. Images illustrate the change in shape of a fully hydrated vessel (a–b) and an air-filled vessel (c–d), in yellow. For each hydraulic status, the two images correspond to the same vessel scanned at a different height in the stem (<3-mm difference). Values of Feret ratio (i.e., minimum diameter/maximum diameter) are indicated above each image and exhibit the great variation of vessel shape along the stem. The scale bar (100 µm) is indicated in the top right corner of each image



FIGURE 5 Xylem collapse in vessels of *Triticum aestivum*. (a) Variation of vessel shape along a water stress gradient. Points represent the average Feret ratio measured in at least six vessels (functional or cavitated) from the same individual, used as an indicator of vessel shape. Error bars correspond to the variation of Feret ratio within a sample. The linear regression (black line) represents the positive relation between water potential and vessel shape ($R^2 = 0.27$, *p* value < .05). Light and dark grey areas correspond to cavitation spread and stomatal closure respectively. (b) Changes in the theoretical mean hydraulic conductivity attributed to xylem vessels deformation (t = 0.78, *p* value = .46). The box plots represent the relative theoretical hydraulic conductivity calculated from vessel shape at the beginning (-0.5 MPa) and after (-1.6 MPa) the decline of leaf hydraulic conductance. PLC, percentage loss of hydraulic conductivity

4 | DISCUSSION

This study used imaging, hydraulic, and gas exchange data to investigate to what extent xylem collapse and cavitation were associated with changing hydraulic conductance and stomatal closure in wheat leaves. Wheat was chosen for study not only for its commercial importance, but also because it has large metaxylem vessels that should be particularly vulnerable to collapse and cavitation. Although some evidence of xylem deformation was found, there was no evidence that either xylem collapse or cavitation caused the steep decline in K_{leaf} observed in the turgor loss region.

It was previously reported that stomatal closure occurred at -1.60 MPa in the adaxial surface of winter wheat (Biscoe, Cohen, & Wallace, 1976). Here, stomatal conductance measured as the sum of both surfaces approached zero at -1.42 MPa. Studies on corn (Cochard, 2002), rice (Wang et al., 2018), and tomato (Skelton, Brodribb, & Choat, 2017) reported similar patterns of stomatal closure during dehydration suggesting that crops are sensitive to a changing environment and limit water losses early during water stress.

As previously reported in grasses and crops (Holloway-Phillips & Brodribb, 2011), a decrease of leaf hydraulic conductance under mild leaf water deficit was also detected in *T. aestivum*. Declining K_{leaf} was found to occur concomitantly with declining g_s in wheat leaves exposed to water stress, questioning the generality of stomatal protection towards cavitation, one cause of declining K_{leaf} , in leaves.

To investigate the cause of K_{leaf} decline during water stress in wheat, xylem cavitation was visualized using microCT and the optical technique. One advantage of these recently developed methods is that they allow direct identification of cavitation in xylem tissue and differentiation of xylem versus nonxylem effects on hydraulic conductivity (Klepsch et al., 2018). The first cavitation events in leaf veins of wheat plants were detected at water potentials that were very close to that measured for 98% stomatal closure. Thus, the reported SSM in wheat leaves was positive (SSM = 0.42 MPa) and in the same range as

the SSMs recently reported in tree species where cavitation was recorded with the optical technique (SSM between 0.2 and 1 MPa for most studied species; Creek et al., 2019). This is consistent with the general dynamics of stomatal closure observed in dicot and tree species whereby stomatal conductance is strongly reduced before the onset of significant xylem cavitation, thereby delaying damage to the water transport network (Brodribb & McAdam, 2017; Martin-StPaul et al., 2017). The temporal sequence of stomatal closure followed by cavitation suggests that there is no causal association between these phenomena.

Close agreement was found between the xylem pressure inducing 50% loss of hydraulic functioning (i.e., P_{50}) in wheat leaves as determined by the optical technique and microCT as the two estimates differed by only 0.01 MPa and the confidence intervals of these estimates overlapped. These methods have been used to compare vulnerability to cavitation in different organs of the same species (Lamarque et al., 2018), but here, we provide a cross validation of the two techniques in the quantification of cavitation spread in the xylem of wheat leaves.

The decline of leaf hydraulic conductance observed here in wheat leaves exposed to mild water deficit (i.e., $\Psi_{\text{leaf}} > -1$ MPa) cannot be explained by xylem cavitation, as this occurred under much greater water stress. Xylem collapse was investigated in stems and leaves of wheat as it was previously demonstrated that cell wall implosion is associated with a decline of the hydraulic conductivity (Brodribb & Holbrook, 2005; Cochard et al., 2004). Vessels were found to flatten slightly with an increasing water stress. However, the relation between vessel shape and water potential must be considered with caution as vessels were not found to be completely collapsed and vessel shape varied considerably within the same sample and along the same vessel. In addition, analysis of flow properties in deformed vessels indicated that less than 5% of the observed decline in K_{leaf} could be attributed to xylem vessel collapse.

The analysis highlighted different behaviour of vessel shape under increasing water stress. Indeed, some vessels were non-

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circular under well-watered conditions, whereas others remained circular even under great water stress. Although a partial recovery of vessel shape after rehydration was observed in oak trees (Zhang et al., 2016), vessel deformation was found to be irreversible in wheat as neither xylem cavitation nor sample cutting caused a change in vessels shape, suggesting that flattening of hydraulic conduits might be caused by a force other than xylem pressure in wheat. Furthermore, the expected dichotomy in vessel shape characterizing vessel implosion (Brodribb & Holbrook, 2005) was not observed in wheat as vessels flattened slowly along the water stress gradient. Cavitated vessels were less circular than functional ones, which is opposite to the expectation if internal hoop stress was responsible for xylem collapse (Cochard et al., 2004). Further studies are needed to determine the mechanism responsible for the change in vessel shape of wheat.

The decline of K_{leaf} under high water potential could not be explained by xylem cavitation or xylem deformation, suggesting that some changes in the hydraulic conductance of tissue outside the xylem were responsible. Recent studies proposed that dynamic changes in the outside-xylem conductance (K_{ox}) can affect K_{leaf} early during dehydration (Scoffoni et al., 2017; Trifiló, Raimondo, Savi, Lo Gullo, & Nardini, 2016) and that K_{ox} can decline before stomatal closure (Scoffoni et al., 2018). In this context, changing xylem conductance (K_x) would only change K_{leaf} under severe water stress. Our results agreed with the hypothesis that unknown processes outside the xylem drive the decline of leaf hydraulic conductance at high water potential.

Both decline in leaf hydraulic conductance and stomatal conductance occurred in the same range of water potential. However, the leaf resistance makes a small contribution to plant resistance ($R_{\text{leaf}} < 1/3 R_{\text{plant}}$), and this proportion did not increase during the process of stomatal closure as would be expected if declining stomatal conductance was driven by changing K_{leaf} . Reduced whole plant hydraulic conductance during dehydration may have hastened stomatal closure during mild water stress, but this is more likely to be associated with reduced root hydraulic conductance as demonstrated recently in olive (Rodriguez-Dominguez & Brodribb, 2019).

5 | CONCLUSIONS

Parallel declines in stomatal conductance and leaf hydraulic conductance during dehydration show a loss of functionality in the turgor loss region. Declining K_{leaf} was not associated with either cavitation or vessel deformation, but rather with an apparent loss of conductance between vessels and evaporative sites. Despite its sensitivity to dehydration, K_{leaf} remained too high to be directly responsible for stomatal closure.

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CONFLICT OF INTEREST

There is no conflict of interest.

AUTHOR CONTRIBUTIONS

DC and TJB conceived the research plans. DC conducted the experiments at University of Tasmania. DC, LJL, HC, JMTR, and SD conducted the experiments at the synchrotron SOLEIL. AK provided technical assistance. DC wrote the article with contributions of all the authors. TJB completed the writing. TJB agrees to serve as the author responsible for contact and ensures communication.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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