Xylem Wall Collapse in Water-Stressed Pine Needles

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Wall reinforcement in xylem conduits is thought to prevent wall implosion by negative pressures, but direct observations of xylem geometry during water stress are still largely lacking. In this study, we have analyzed the changes in xylem geometry during water stress in needles of four pine species (*Pinus* spp.). Dehydrated needles were frozen with liquid nitrogen, and xylem cross sections were observed, still frozen, with a cryo-scanning electron microscope and an epifluorescent microscope. Decrease in xylem pressure during drought provoked a progressive collapse of tracheids below a specific threshold pressure (P_{collapse}) that correlates with the onset of cavitation in the stems. P_{collapse} was more negative for species with smaller tracheid diameter and thicker walls, suggesting a tradeoff between xylem efficiency, xylem vulnerability to collapse, and the cost of wall stiffening. Upon severe dehydration, tracheid walls were completely collapsed, but lumens still appeared filled with sap. When dehydration proceeded further, tracheids embolized and walls relaxed. Wall collapse in dehydrated needles was rapidly reversed upon rehydration. We discuss the implications of this novel hydraulic trait on the xylem function and on the understanding of pine water relations.

In vascular plants, xylem sap is transported under negative pressure in lignified conduits (Pockman et al., 1995). From a physical point of view, such a transport is constrained by two major limitations: (a) a risk of collapse of the water columns; and (b) a risk of collapse of the conduit walls. The first constraint originates from the metastable state of water under negative pressures. Under such conditions, vapor nucleation can occur, disrupting the water columns in the xylem and therefore the sap flow. This phenomenon is referred to the risk of cavitation. The second physical limitation, wall collapse, is due to the centripetal forces exerted on conduit walls that may implode if wall mechanical reinforcement is deficient.

Over the past decades, the risk of xylem cavitation in upper plants has received considerable attention. Significant advances were obtained after techniques for detecting cavitation events acoustically (Milburn, 1966; Tyree and Dixon, 1983) or hydraulically (Sperry et al., 1988) were introduced. These techniques confirmed that xylem transport can remarkably operate under large negative pressures for prolonged periods. Cavitation occurs when plants are exposed to severe drought and pressures exceed species-specific thresholds. These thresholds are determined by the structure of pit membranes in the xylem wall because cavitation is thought to be caused by the aspiration of an air bubble through these membranes (Zimmermann, 1983; Sperry and Tyree, 1988). In the recent literature, results obtained with new techniques have suggested that the risk of xylem cavitation was much more pronounced than previously thought (Zimmermann et al., 1994; Canny, 1995). However, more recent studies (Wei et al., 1999; Cochard et al., 2000) have demonstrated that cavitation was overestimated in these studies.

The risk of xylem wall collapse has yet received very little attention. It has long been recognized that lignification of xylem conduit walls was a mechanical adaptation to prevent vessel implosion and support plant body (Raven, 1987). However, experimental evidences and theoretical considerations for the risk of xylem wall collapse are largely lacking. Johnson (1977) has reported xylem wall bending between the helicoidal thickening of a water lily, but xylem pressures were not recorded in this study. *Vigna* sp. plants grown in presence of a lignin inhibitor exhibited collapsed xylem conduits (Smart and Amrhein, 1985). More recently, xylem wall collapse in Arabidopsis mutants deficient in cellulose deposition in their secondary cell wall has been reported (Turner and Somerville, 1997; Taylor et al., 1999). A similar pattern was shown in transformed *Nicotiana* sp. plants (Hepworth and Vincent, 1998; Piquemal et al., 1998). Furthermore, a striking correlation between xylem vulnerability to cavitation and wall resistance to bending stress has been established (Hacke et al., 2001). All of these results suggest that the risk of xylem collapse was an evolutionary constraint for vascular plants. However, to the best of our knowledge, there is no direct evidence of xylem collapse in planta to substantiate this hypothesis.

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The objective of this study was to analyze, in planta, the concurrent patterns of xylem cavitation and xylem wall collapse during water stress in the needles of four *Pinus* spp. Because the xylem structure in conifers is fairly homogeneous and regular, this simplified the observations and the calculations. We have developed different techniques to observe both tracheid collapse and cavitation. These patterns were compared with the patterns of stomatal closure and water loss regulation during drought.

RESULTS

Xylem Anatomy

Tracheid dimensions differed significantly between species (Table I). The two alpine species exhibited smaller tracheid lumen (*b*) and thicker wall (*t*) and, as a result, much larger $(t/b)^2$ values (an estimate of wall resistance to bending stress).

Xylem Collapse in Excised Shoots

When pine shoots where dehydrated on a bench, we observed a progressive collapse of tracheid wall in the needle only when a threshold water potential was reached. The collapse patterns are shown qualitatively on the pictures in Figure 1 for *P. cembra* and quantified for all of the four species on the graphs in Figure 2. Figure 1, a to f, shows vascular bundles of *P. cembra* needles at different xylem pressures observed with the cryo-scanning electron microscopy (cryo-SEM) technique. In well-hydrated (control) needles (Fig. 1a), tracheids were all sap filled and almost circular. When P_{xylem} was decreased to −3.5 MPa (Fig. 1b), virtually no change was noticed. At −4 MPa (Fig. 1c), tracheids walls started to bend especially where in contact with living parenchyma cells (see arrows in Fig. 1c). At −4.6 MPa (Fig. 1d), all of the tracheids were severely distorted, but their lumen were still filled with sap. At −5 MPa (Fig. 1e), walls were so distorted that it was difficult to distinguish tracheid lumens. Finally for leaf water potential (Ψ_{leaf}) below −5.1 MPa (Fig. 1f), tracheids were distinctly filled with gas (embolized), and their walls were relaxed.

Observations of frozen *Pinus nigra* needle cross sections with the epifluorescent technique yielded similar results: Tracheids in control samples appeared circular (Fig. 1g) and were completely collapsed at −3.5 MPa (Fig. 1h). We noticed that wall collapse rapidly disappeared when samples were returned to ambient temperatures.

The patterns of wall deformation in pine needle tracheids were quantified by their isoperimetric quotient (*Q*). For the all of the species, we found that tracheid perimeter and xylem pressure were not statistically correlated ($P = 0.05$; needles with embolized tracheids were excluded from the analysis), suggesting that tracheids remained isoperimetric during dehydration. Figure 2 gives the changes in tracheid *Q* versus xylem pressure for the four pine species. From these graphs, it is clear that wall collapse occurred only when P_{xylem} dropped below a threshold value P_{collapse}. Above P_{collapse}, *Q* was constant and not significantly different from *Q* for controls. P_{collapse} varied greatly between species (Table I) with *P. nigra* being the most vulnerable species (−1.8 MPa) and *P. cembra* the least (−3.5 MPa). When P_{xylem} decreased below P_{collapse}, *Q* dropped progressively until tracheids embolized ($P_{\text{cavitation}}$). *Q* values of embolized tracheids were not different from that of controls for *P. cembra* and *Pinus mugo* (Fig. 2, white symbols). $P_{\text{cavitation}}$ values were very low, below −4 MPa, and not correlated with P_{collapse} ($r^2 = 0.03$). Scots pine (*Pinus sylvestris*) samples were not dehydrated down to the point of leaf tracheid cavitation.

When *P. nigra* needles at −3.5 MPa were rehydrated before freezing, the collapsed wall recovered within minutes (Fig. 3). After 30 min of rehydration, *Q* values were not significantly different from those of well-watered leaves. Cryo-SEM observations of leafy stems suggested that tracheids cavitated before exhibiting detectable wall deformation.

Figure 4 shows the predicted changes in needle xylem hydraulic conductance during dehydration. These graphs represent "vulnerability curves" for water stressed-induced wall collapse. For the reason that percent loss of xylem conductance in the needles (PLC) values scale to Q^3, it can be predicted that wall collapse had a considerable effect on xylem conduc-

Table I. *Xylem anatomical and functional properties for four pine species*

Axis cavitation pressures are from published data for *P. mugo* (Mayr et al., 2003a), *P. cembra* (Mayr et al., 2003b) *P. nigra* (Froux et al., 2002), and *P. sylvestris* (Cochard, 1992). Means ± SE. Across species, data having a letter in common are not significantly different at $P = 0.01$ (*t* test).

	P. mugo	*P. cembra*	*P. sylvestris*	*P. nigra*
Maximum diameter (*b*, μm, $n = 30$)	7.78 ± 0.27a	8.1 ± 0.19a	11.6 ± 0.41b	11.8 ± 0.35b
Minimum diameter (μm, $n = 30$)	5.94 ± 0.25a	6.31 ± 0.24a	8.49 ± 0.5b	8.26 ± 0.29b
Wall thickness (*t*, μm, $n = 60$)	1.9 ± 0.03a	1.44 ± 0.03b	1.46 ± 0.03b	1.31 ± 0.03c
$(t/b)^2$ ($n = 30$)	$6.73e^{-2} \pm 5.9e^{-3}$a	$3.43e^{-2} \pm 2.4e^{-3}$b	$1.75e^{-2} \pm 1.4e^{-3}$c	$1.4e^{-2} \pm 1.2e^{-3}$c
Needle collapse pressure (MPa)	−3.4	−3.5	−2.6	−1.8
Needle cavitation pressure (MPa)	−3.7	−5.0	–	−4.2
Stem cavitation pressure (MPa)	−3.3	−3.0	−2.6	−1.8

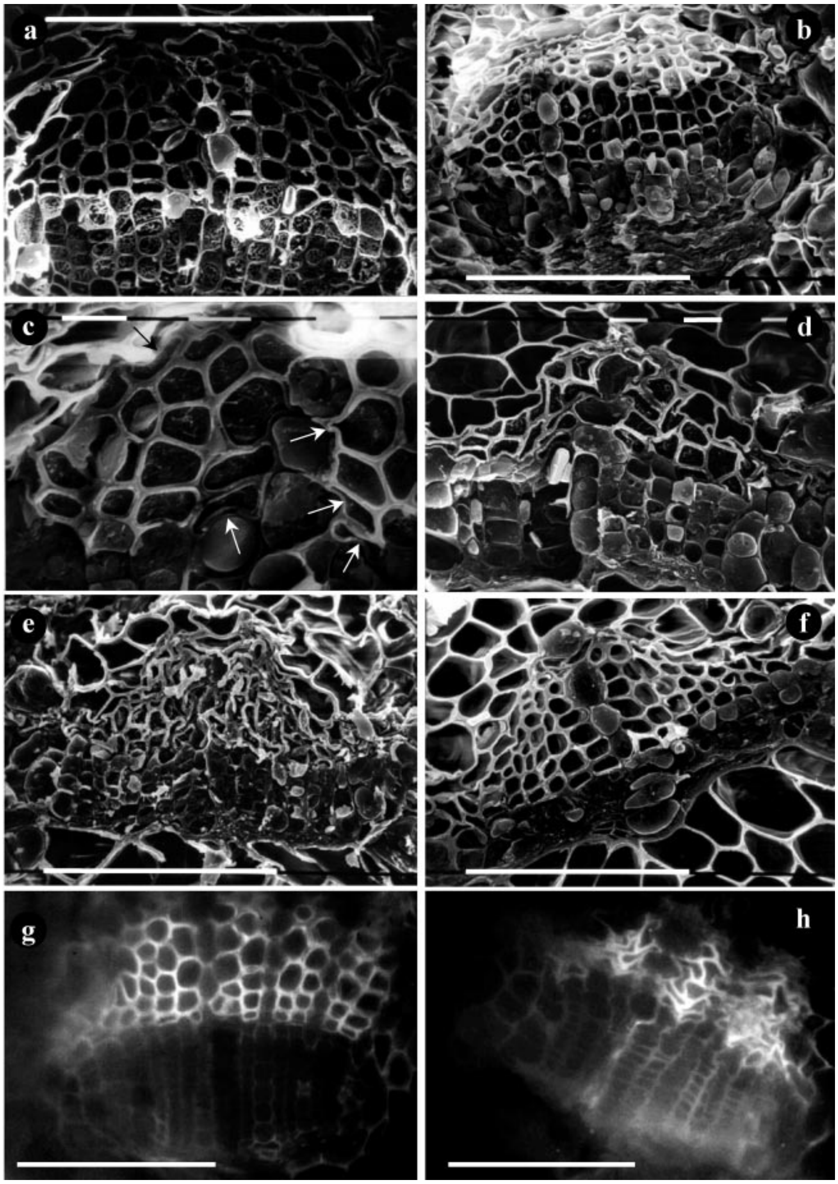

Figure 1. Representative pictures of vascular bundles in pine needles exposed to different xylem pressures. a to f, Vascular bundles in *P. cembra* observed with the Cryo-SEM technique on frozen samples coated with gold. g and h, Bundles in *P. nigra* observed with the epifluorescent technique on frozen samples. The xylem is oriented upwards and the phloem downwards. Xylem pressures were: a, 0 MPa; b, −3.5 MPa; c, −4 MPa; d, −4.6 MPa; e, −5 MPa; f, −5.1 MPa; g, 0 MPa; h, −3.5 MPa. The arrows in c indicate early collapses of tracheid walls in contact with living cells. The white bars = 100 μm except for c and d (10 μm).

tance, with more than 80 PLC noticed for the most severely collapsed samples.

Xylem Collapse in a Potted *P. nigra* Sapling

Figure 5 shows the time courses of water relations and xylem collapse of a *P. nigra* sapling exposed to a soil drought cycle. Drought effects on water relations were typical of a drought-avoiding species: Transpiration was drastically reduced a soon as drought was induced and Ψ_{leaf} started to decrease. Ten days after drought induction, stomata were completely closed, and Ψ_{leaf} values were close to −2 MPa. At that time, the differences between Ψ_{leaf} values measured in the dark or in the light were not distinguishable by our technique. This justified the hypothesis that Ψ_{leaf} equaled P_{xylem} in dehydrating shoots. After rewatering, Ψ_{leaf} recovered within 2 d but plant transpiration was reduced to one-half of its value before treatment. Significant changes in tracheid *Q* were noticed on the early stage of drought. Tracheids on the sides of the vascular bundles tended to collapse first. At the end of the drought treatment, most of the tracheids were collapsed, but their lumen was still filled with sap. After rehydration, *Q* values rapidly returned to control values.

DISCUSSION

In this study, cryo-SEM and epifluorescent observations revealed the existence of collapsed tracheids in dehydrated pine needles. To our knowledge, this is the first direct evidence of a water stress-induced xylem wall collapse in planta. We will analyze our results in terms of xylem anatomy and discuss the

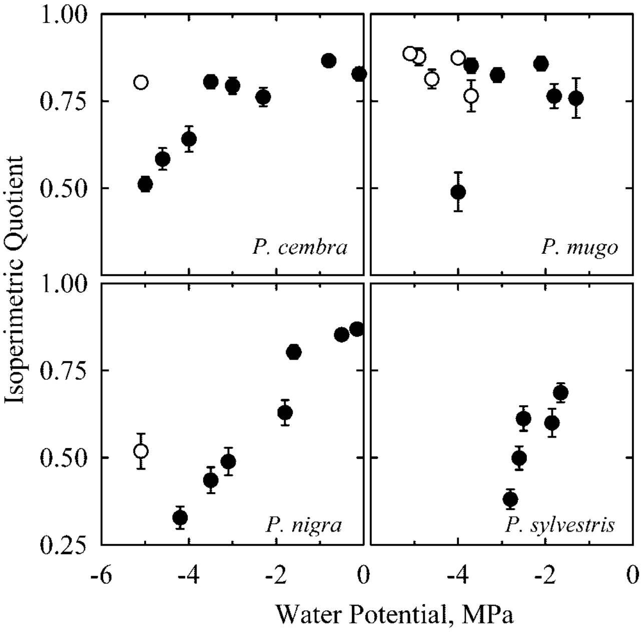

Figure 2. Tracheid Q versus Ψ_{leaf} in bench dehydrated shoots of four pine species. Q equals 1 for a circle and decreases with tracheid eccentricity. White symbols are for embolized (air-filled) tracheids. Errors bars are SE ($n = 39$ on average).

significance of this novel hydraulic trait for the understanding of pine water relations during drought.

Pine needles in this work were dehydrated and frozen in liquid nitrogen (LN_2) for cryo-SEM observations. The possibility that xylem wall collapse was an artifact of the freezing procedure seems low if we consider the following points. First, sap volume expand by 10% upon freezing, therefore tracheids lumen area might have been overestimated (6.5% increase). Second, tracheid wall geometry in moderately dehydrated needles (Fig. 1, a and b) and air filled tracheids (Fig. 1f) were similar. Finally, wall deformation was restricted to the vascular bundles and affected only xylem tracheids. When pine needles were dehydrating, we noticed an overall shrinkage of the needle so one may wonder if the xylem collapse was caused by negative sap pressures or compressive forces exerted by the surrounding tissues. We are confident with the former explanation for three reasons: First, the vascular bundles are surrounded by a pericycle composed of large thin-walled cells. If compressive forces were the cause of xylem collapse, then cells in the pericyle would have collapsed well before tracheids in the xylem. This was not the case. Second, when xylem cavitation occurred in severely dehydrated needles and thus xylem pressure was released to atmospheric, the patterns of collapsed walls disappeared (Fig. 1f). Finally, wall collapse was not observed in the phloem or parenchyma cells because the turgor pressures are much higher in living cells than in xylem conduits (Tyree, 1976). We are thus confident with the interpretation that highly negative sap pressures caused xylem wall collapses in this study.

From a mechanical point of view, large pipes with thin walls are more vulnerable to wall collapse (Niklas, 1992). Qualitatively, tracheid collapse in planta obeyed this law. Thin-walled tracheids on the sides of a vascular bundle tended to collapse first (Fig. 1c). Furthermore, walls in contact with living cells where more prone to collapse. This fact might be explained by two nonexclusive reasons. First, living cells have thinner and thus weaker walls. Second, there is a greater pressure difference across the wall because pressure is usually positive in living cells and negative in xylem conduits.

Between species, vulnerability to collapse was also related to tracheid anatomy, the most vulnerable species having tracheids with large lumens (b) and thin walls (t). Hacke et al. (2001) have proposed that, at the conduit level, wall resistance to bending stress scales with $(t/b)^2$. The relation between P_{collapse} and $(t/b)^2$, although negative as expected, was rather weak ($r^2 = 0.57$) for tracheids in pine needles. Furthermore, predictions of wall collapse pressures using their equation yield values two to six times more negative than the actual P_{collapse} values. This suggests that one or more assumptions in their study did not apply to our data. It is probably necessary to take into account explicitly the multicellular and heterogeneous structure of the xylem tissue and the actual tracheid wall shape. It is worth noting that vulnerabilities to cavitation and wall collapse for tracheids in the needles were not correlated ($r^2 = 0.03$). Apparently, wall properties determined the risk of collapse but had no impact on cavitation thresholds. Interestingly, the difference between $P_{\text{cavitation}}$ and P_{collapse} was highly correlated with wall mechanical properties $(t/b)^2$: The weaker the wall, the higher the dif-

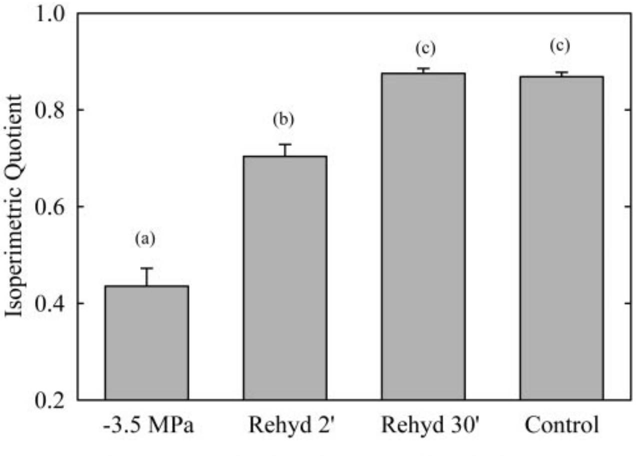

Figure 3. Change in tracheid Q during needle rehydration. Q was measured for needles of *P. nigra* dehydrated to −3.5 MPa and frozen intact for 2 or 30 min after rehydration. Q values for needles at −0.15 MPa are given as control values for well-hydrated needles. Treatments having a letter in common are not statistically different at $P = 0.05$. Error bars represent one SE.

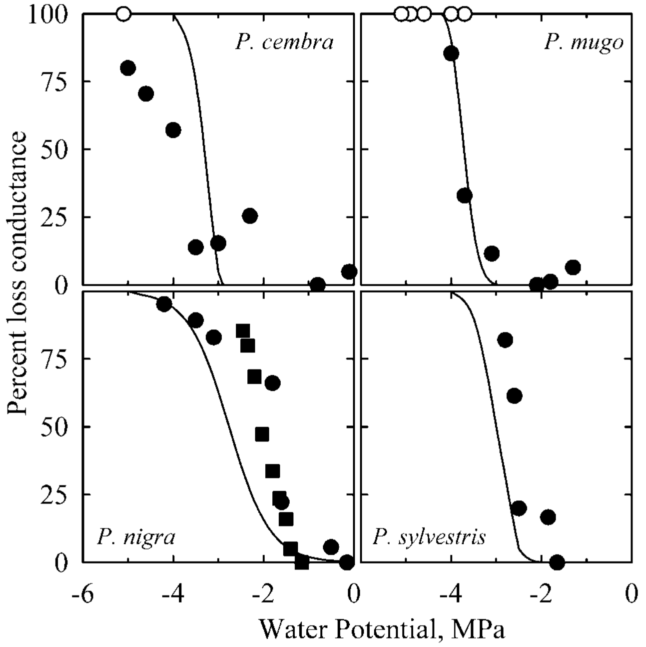

Figure 4. Predicted loss of needle xylem conductance due to wall collapse versus Ψ_{leaf} for bench dehydrated and pot dehydrated (*P. nigra* squares) pine shoots. White symbols are for embolized tracheids. Lines represent stems vulnerability to cavitation (data from previous works; see refs. in the text).

ference ($r^2 = 0.99$). In other words, it seems that when walls collapse during water stress, the risk of cavitation is avoided or delayed. In conifers, cavitation occurs when pit membranes are disrupted (Sperry and Tyree, 1990), but the mechanism by which wall collapse could lower tracheid vulnerability remains unclear.

What is the physiological significance of xylem wall collapse during water stress in pine? A thorough answer to this question will be possible only when the phenomenon has been documented for a larger number of environmental conditions. However some reasonable speculations can be formulated from the results in this study. Pines respond to soil drought by closing their stomata to reduce water losses. The mechanisms triggering stomatal closure during water stress are still poorly understood and probably very complex (Cochard et al., 2002). It has been suggested that stomata may close in response to hydraulic signals and that early cavitation events in the leaf veins might trigger stomatal closure (Comstock and Mencuccini, 1998; Sperry, 2000; Salleo et al., 2001). This was clearly not the case for the pine species in this study, because tracheid cavitation occurred at a very high degree of dehydration. However, stomata may have responded to earlier cavitation events in the stems. Wall collapse reduced lumen area and accordingly considerably increased tracheid hydraulic resistance to water flow. Therefore, wall collapse might also cause hydraulic signals and trigger stomatal closure. To better unravel the mechanism, it is important to consider the timing of events during a drought period. For the four species we have documented, there is a significant statistical correlation ($r^2 = 0.93$) between the onset of wall collapse in the needles and cavitation in the twigs (see Table I; lines on Fig. 4). Alpine *Pinus* spp. are resistant to cavitation because they are exposed to severe dehydration during the winter period when soil is frozen (Mayr et al., 2003b, 2003a). The more detailed information we have for *P. nigra* suggests that wall collapse is an earlier phenomenon than stem cavitation. This can be seen on Figure 6 where plant transpiration, stem embolism, and wall collapse in the needles are expressed as a function of Ψ_{leaf}. When plant transpiration was reduced to zero, stem embolism was still low, but tracheids conductance was already severely

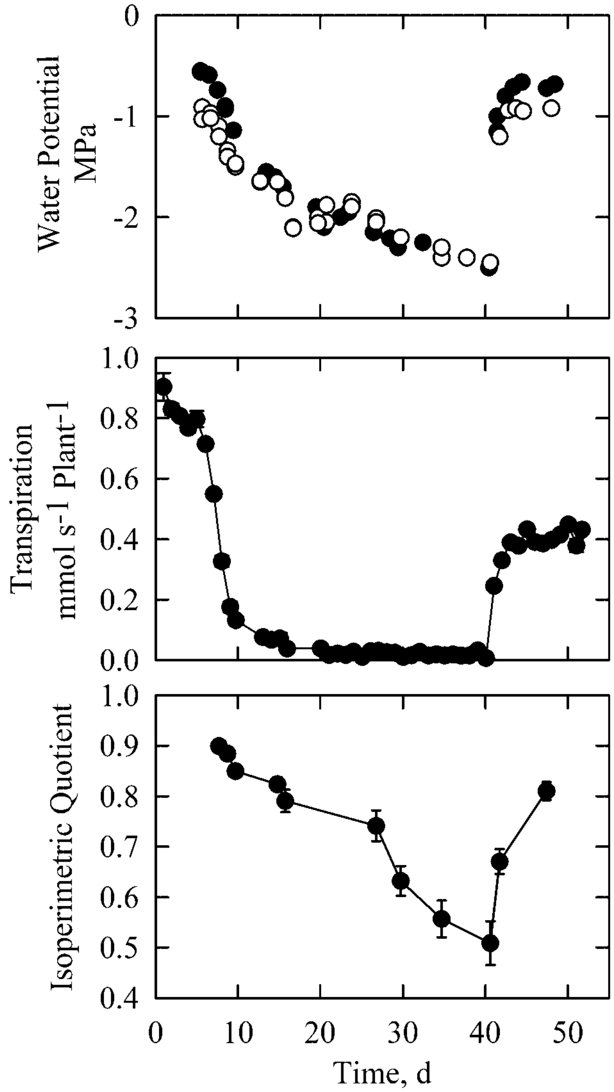

Figure 5. Time courses of Ψ_{leaf} (top), transpiration (middle), and tracheid *Q* (bottom) during a drought cycle for a potted *P. nigra* sapling. Irrigation was stopped between d 5 and 40. Black and white circles in the top panel represent Ψ_{leaf} measured in dark and full-light conditions, respectively. Means ± SE.

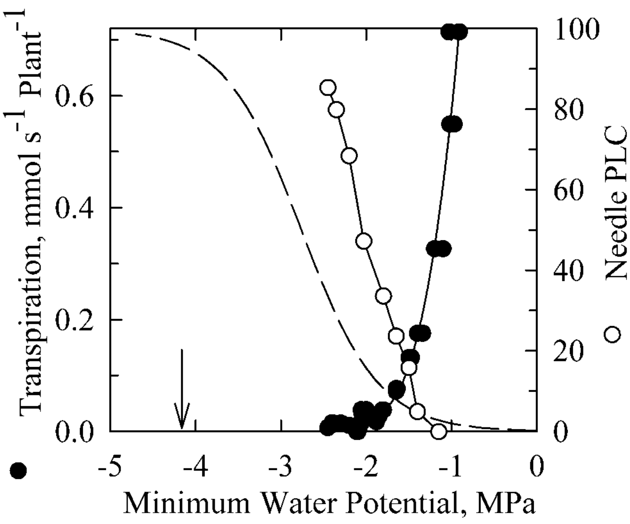

Figure 6. Dependence of transpiration (black circles), percentage of loss conductance in the needles (white circles) and the stems (dashed line) on Ψ_{leaf} during a drought cycle (data similar to Fig. 4). The arrow on the *x* axis indicates the onset of tracheid cavitation in the needles. Stem vulnerability is replotted from Froux et al. (2003).

reduced in the needles. Therefore, stomata closed at the expanse of no embolism but substantial wall collapse. The curve on Figure 6 represents the mean collapse of all of the tracheids. This hides the fact that some tracheids were already collapsed at the onset of stomatal closure. Therefore, xylem wall collapse in leaves seems a potential candidate for hydraulic signals during water stress in pine. It causes variable and reversible hydraulic conductances in the very terminal part of plant vascular system therefore minimizing the risk of xylem dysfunction in more downstream parts. Recovery from wall collapse can occur while pressures are still largely negative, contrary to cavitation-induced dysfunctions (Tyree and Yang, 1992).

Zimmermann (1983) speculated that increases in xylem efficiency were balanced by decreases in xylem safety. Xylem efficiency scales to the fourth power of conduit diameter. Safer xylem conduits have the capacity to preserve xylem efficiency at lower xylem pressures. Safety has so far been quantified by the xylem vulnerability to cavitation. Zimmermann (1983) hypothesized that wider conduits were more prone to cavitation events. However, when different species are compared, a rather weak correlation between the above parameters (Tyree et al., 1994) is found, suggesting a lack of such a tradeoff. Within a given xylem tissue, a correlation may exist (Sperry and Tyree, 1990). Our results illustrate another aspect of the tradeoff between xylem efficiency and safety in pine needles. Xylem safety shall not be thought in terms of vulnerability to cavitation but rather in terms of vulnerability to wall collapse. Tracheids less prone to wall collapse were safer because they maintained xylem efficiency at lower xylem pressures. Larger tracheid diameters conferred higher hydraulic efficiency at the expanse of a higher risk of collapse. The situation was different for woody axis because wall collapse was not observed and cavitation occurred first. This is consistent with other cryo-SEM observations on lignified plant parts in other species (Canny, 1997; Cochard et al., 2000). Contrary to needles, conifer axes have both mechanical and hydraulic functions. In the stems, xylem walls have to be thick enough to avoid collapse upon negative pressures and prevent stem rupture under dynamic or static loads (Wagner et al., 1998). Walls in the stems might thus be over-reinforced and less prone to collapse. In needles, mechanical stability is not such an important function of the xylem, and wall reinforcement is dimensioned only to avoid collapse due to negative pressures. Thicker walls and higher wood densities could compensate the risk of collapse associated with larger conduits (Hacke et al., 2001), but the cost of carbon investment might become the limiting factor.

CONCLUSIONS

Our study has revealed a novel aspect of hydraulic functioning in pine. Xylem conduits in pine needles are subject to the collapse of their walls upon dehydration. Vulnerability to collapse seems related to tracheid anatomy, although a mechanical model taking explicitly into account the xylem structure and geometry remains to be elaborated. Wall collapse induces a drastic change in xylem conductance but is rapidly reversible upon rehydration. Our data on pine species suggest that wall collapse might have significant implication for the understanding of plant water relations and xylem function. We do not know yet whether these findings are specific to pine or whether they apply to other plants.

MATERIALS AND METHODS

Plant Material

Experiments were conducted on four different pine species (*Pinus cembra* L., *Pinus mugo* Turra s. str., *Pinus nigra* subsp. *nigra* Arnold, and Scots pine [*Pinus sylvestris*]). The former two species typically grow at high elevation in the alpine mountains. Mayr et al. (2003b, 2003c) have shown that these species were exposed to very low xylem pressures during the winter period when the soil was frozen. *P. nigra* and Scots pine are more temperate species. These different pine species are known to greatly differ in terms of shoot vulnerability to cavitation (Cochard, 1992; Froux et al., 2003; Mayr et al., 2003b, 2003c). Two types of plant material were used in this study. First, shoots were collected in situ on mature trees of *P. cembra*, *P. mugo*, and Scots pine. The first two species were sampled in the Botanical Garden, University Innsbruck, Tyrol (elevation, 600 m). *P. cembra* is the tree species reaching highest altitudes in the European Central Alps, *P. mugo* is a coniferous shrub naturally growing above tree line in the alpine "Krummholzzone". Shoots of Scots pine originated from trees growing in grasslands at Berzet near Clermont-Ferrand, France (elevation, 800 m). The more thorough experiments on *P. nigra* were conducted on 2-year-old saplings grown in 10-L pots filled with a mixture of sand/peat and forest soil. The plants were well watered and fertilized regularly.

Leaf Dehydration

Two protocols were used to provoke shoot dehydration. First, terminal shoots of the four species were cut from the trees and allowed dehydrating on the bench of a laboratory. Periodically, one shoot was selected, and its Ψ_{leaf} was determined with a Scholander pressure chamber. Because samples dehydrated very slowly, we assumed that Ψ_{leaf} equaled the xylem pressure in the needles (P_{xylem}) in our study. The shoot was then enclosed in a plastic bag (to stop dehydration) until leaves were sampled for microscopic analyses (see below). Sample leaves were from the current and previous-year growth units. Shoots of *P. cembra* and *P. mugo* were prepared in Innsbruck (Austria) and sent by rapid mail to Clermont-Ferrand in their bags for analysis. On a sub-sample, we confirmed that Ψ_{leaf} did not change significantly during transport.

For the second procedure, we provoked a more progressive leaf dehydration by exposing a potted *P. nigra* sapling to a soil drought. A 2-year-old sapling was installed in a growth cabinet with 16 h, 28°C, 50% relative humidity/8 h, 20°C, 70% relative humidity day/night conditions. During the first 5 d, the soil was maintained at field capacity by repetitive watering. Then, water was withheld, and the plant was allowed to dehydrate for 35 d. The pot was finally rehydrated and maintained at field capacity for 12 more d. The sapling was placed on a PC interfaced digital balance (1 g resolution) to measure water losses continuously. Periodically, we determined Ψ_{leaf} by detaching a pair of needles. We measured Ψ_{leaf} in morning before lights were turned on and on transpiring leaves near the middle of the light period.

Xylem Anatomical Observations

Needle xylem characteristics were determined on samples snap-frozen by immersion in LN_2. Needles were detached from the dehydrated shoots and immediately soaked in a bath of LN_2. About 2-cm-long segments were taken from the central part of each needle and stored at −80°C until observation. The samples were thus frozen while the xylem pressure in the needles was close to the xylem pressure before excision. A few observations were also made on stem segments prepared similarly. On *P. nigra* needles dehydrated to −3.5 MPa, xylem pressure was first relaxed before freezing. Needles were sampled as before but first immersed and recut under tap water. After 2 and 30 min, needles segments were immerged in LN_2 and treated as above.

Most of the observations were done with a SEM (model SEM 505, Philips, Eindhoven, The Netherlands) equipped with a cryogenic stage (model CT 1000, Hexland, Oxford Instruments Ltd, Oxford, UK) in the Laboratory of Electron Microscopy at the Institut National de la Recherche Agronomique-Theix center near Clermont-Ferrand. This equipment enables observations of xylem content on intact frozen samples (Cochard et al., 2000; Mayr and Cochard, 2003; Tyree et al., 2003). Samples stored at −80°C were first immersed in LN_2 and installed in 1-cm-deep holes in a sample holder. The samples were then rapidly transferred to a cryo-chamber on a stage at −180°C. Once vacuum was made in the chamber, the samples were cryo-fractured inside the chamber to expose the vascular bundles on a cross section. This procedure limited considerably the deposit of frost on the cross sections. The samples were then transferred to the SEM chamber and installed on a stage maintained at −160°C. Samples were first observed at a low voltage (<5 kV), and the presence of ice or air in the tracheids was noted. To better delimitate cell walls, the samples were then etched by raising the SEM stage temperature to −80°C. Finally, the samples were coated with a gold deposit in the cryo chamber (−180°C). Coating was necessary for obtaining high resolution and contrasted pictures, but this often had the effect of sublimating most of the ice in the tracheids. Nevertheless, we verified that ice sublimation had no effect on wall shape because the samples always remained below −80°C. Pictures were scanned and analyzed with standard image analysis software. Measurements were restricted to tracheids with bordered pits in the metaxylem. For each tracheid, we measured the wall perimeter (P) and the lumen area (A). Tracheid shape was estimated by their Q defined as (Weisstein, 1999):

$$Q=4\,\Pi.A.P^{-2} \tag{1}$$

Q, a dimensionless shape factor, equals 1 if the tracheid is a perfect circle, equals 0.79 for a square, and decreases below these values with wall collapse. On average, each Q value on the graphs represents the mean value of 39 different tracheids.

We determined the effect of wall collapse on loss of hydraulic conductance (K) as follows. We assumed (a) that tracheids remained elliptical as they collapse, (b) that wall perimeter was constant, and (c) that Poiseuille's equation remained valid for collapsed tracheids. Our data show that assumption b was correct (see "Results"), but it is clear that assumptions a and c were not entirely satisfied. Our PLC estimates are thus rather imprecise and probably underestimate the actual PLC values. If a and b are the axes of the ellipse, we have:

$$a = r\,[1 + (1-Q^2)^{1/2}]^{1/2} \tag{2}$$

$$b = r\,[1 - (1-Q^2)^{1/2}]^{1/2} \tag{3}$$

with r as the radius of the circle having the same perimeter. For an ellipse, K scales to $a^3b^3/(a^2+b^2)$ (Lewis and Boose, 1995). Therefore K scales to $r^4\,Q^3$. The PLC due to wall collapse was then estimated by:

$$\mathrm{PLC} = 100[1-(Q/Q_0)^3] \tag{4}$$

with Q_0 being the Q of tracheids in well-hydrated samples.

For well-hydrated samples of each species, we also measured the mean wall thickness (t; n = 60) and maximum tracheid beam (b; n = 30). Following Hacke et al. (2001), we determined the relative wall resistance to bending stress as $(t/b)^2$.

On some *P. nigra* samples, we used a second technique to observe wall collapse. Although the results of this technique were not precisely quantified in this study, it is worth mentioning because people without access to a cryo-SEM could employ it. The principle of the technique is to observe tracheid walls on frozen cross sections with an epifluorescent microscope. A 2.2- × 2.2-cm, 0.8-cm-thick piece of copper placed under the microscope objective was used as a cooling stage. The free end of a copper wire (0.16 cm in diameter) attached to the stage was immersed in a bath of LN_2. We adjusted the length of the wire to obtain a stage temperature around −80°C (as measured by a thermocouple inserted into the stage). Samples stored at −80°C were rapidly placed on the stage where a few drops of absolute ethanol were added. This was necessary to prevent frost deposition on the samples during observation. About 1-mm-thick cross sections were obtained with a fresh razor blade near the center of the sample and observed at a 400× magnification.

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