How to measure xylem embolism with the XYL'EM apparatus

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Foreword

This chapter was written with the intention of helping new users with their first determination of xylem embolism values. Although the principle of the technique is rather simple, several conditions must be respected in order to obtain reliable data. Our objective is to share our experience with the technique and to describe the major difficulties we have encountered. These are only hints and recommendations that you may follow or just ignore. We can guarantee that the XYL'EM apparatus is measuring pressures, flow and temperature correctly but we can absolutely not guarantee that your embolism measurements will be accurate if the recommendations below are not followed.

Cavitation and embolism in plants

Functional xylem conduits (vessels and tracheids) normally contain water and are hydraulically connected to upstream and downstream conduits. Under some circumstances (drought or frost stresses for instance), xylem conduits can cavitate and consequently become embolised. Xylem cavitation corresponds to the rapid breakdown of the water column in the conduits and to the formation of a cavity filled with vapor water (near vacuum pressure). Rapidly air degasses into this cavity which increases pressure to atmospheric. At this stage the whole conduit lumen contains a large air bubble that block the water flow through the conduit. An air embolism has formed.

Principle of the technique

The principle of the XYL'EM apparatus is to measure the relative decrease in xylem hydraulic conductance caused by the presence of air in the conduits. Contrary to other techniques (dye coloration, acoustic emissions, cryo-SEM observations) this technique is quantitative, i.e., it quantifies the amount of loss of xylem functionality at any given time. John Sperry first proposed the technique in the 80's (Sperry et al 1988).

Let us assume a xylem segment (a petiole, a shoot internode, a root segment etc...) fully functional (no embolism) having an hydraulic conductance equal to K (mmol s-1 MPa-1 or kg s-1 MPa-1). Following a drought or a frost stress, embolism forms which reduce K to K'. The percentage of loss of xylem conductance (PLC) can then be computed as:

$$PLC=100*(1-K'/K)$$

If PLC=0%, K'=K, i.e., none of the conduits were embolised.
If PLC=100%, K'=0, i.e., all the conduits were embolised.
Therefore, to compute PLC, we need to measure K and K'. However, it is usually not possible to measure K for the trivial reason that the technique is destructive. When a sample is collected on an experimental plant, it is likely to contain embolism. Hence, K' is measured first. To estimate K, the idea is to resaturate the sample in order to dissolve any air bubble that may have formed during the treatment. To remove

embolism, the segment is perfused ("flushed") with water at a relatively high pressure (0.1 to 0.2 MPa or 1 to 2 bars). Degassing the water facilitate the dissolution. This is the reason why the XYL'EM contains a captive air tank. After the flush, the conductance of the xylem segment is K". PLC is then computed as:

$$PLC=100*(1-K'/K")$$

It is easy to see that the technique will meet our expectation only if K"=K, i.e., if the xylem conductance of the resaturated segment equals the conductance of the same segment before treatment.

Our experience with many woody species and several herbaceous ones shows that this condition is generally satisfied. We will discuss below the situation when PLC values might be misleading for a proper usage of the XYL'EM apparatus.

1- Possible problems with K'

K' is suppose to correspond to the hydraulic conductance of the xylem segment *in planta*, i.e., before it was excised. By the time the sample was collected and measured no embolism must have formed or dissolved, unless the value will be artifactual.

Problems during sample collection

Xylem vessels *in planta* are usually exposed to large negative pressures. Therefore, when a cut is made in the xylem, water in the open vessels is exposed to atmospheric pressure and will thus be sucked upward and downward into the xylem in a few seconds. The capillary pressure that develops at the cut end of the conduits is far too small to maintain intact the air-water meniscus (see figure 1). Sap will be sucked back the entire length of the conduits. This length represents a few millimeters for tracheids but several meters in some large vessel bearing plants (ringporous trees for instance). Therefore, it is essential to know the maximum vessel length if a xylem segment is to be cut in air. The PLC value of a segment containing cut open vessels will be an artifact of the sampling procedure. Experimental procedures exist to measure vessel length (air injection, paint perfusion, vessel microcasting, etc). We will describe another technique that uses the XYL'EM apparatus below. It is sometimes possible to excise the samples on the plant under water, which greatly reduces this problem.

Problems due to accidental embolism dissolution

Some authors have argued that the hydraulic determination of xylem embolism greatly underestimates the actual levels *in planta* for the reason that xylem pressure has to be released to atmospheric or supra-atmospheric values during measurements. The problem could be potentially acute if xylem conduits remained saturated with vapor pressure (not air) for a long period. Using a spinning experiment (see Cochard et al 2000 and Cochard 2002 for details), we have established that this underestimation was very unlikely in walnut petiole. However, for very tiny samples (leaf petioles, or leaf veins for instance) having small diameter lumens (and thus high capillary pressures) one may observe a rapid embolism dissolution under near zero xylem pressure. This will translate into a progressive increase in sample conductance during the initial K' measurement. One way around this problem is to place the sample in an hydraulic circuit in order to expose it to a negative pressure during measurement.

The major risk of embolism dissolution is not this passive refilling during measurement. Rather, the critical phase is when samples are inserted into the

manifold. Indeed, it is very easy to displace the air bubbles trapped in an open vessel during this phase. Figure 1 gives the theoretical threshold water head (in cm of H2O) that will displace an air/water meniscus in a conduit of a given diameter. One can see that a water head of 60 cm is enough to refill an embolised open vessel having a 50 microns lumen diameter. For larger conduits, the threshold pressure can be as low as 10 cm. Therefore, one must be sure that the water head used for the initial K' estimate is lower than this threshold value. This threshold pressure can be determined experimentally as follows: prepare a representative xylem segment as described above (excised under water). Connect the sample to an air filled tubing and pressurize for a minute at ca 0.1 MPa. This will cause air to enter the vessels and induce 100PLC. Connect carefully the sample to the XYL'EM manifold with a pressure head as small as possible (a few centimeter as indicated by the low-pressure transducer). Measure K', which should, in theory, be close to zero. Move up the low-pressure reservoir and note when K' rapidly increases. You should note air bubbles coming out of the sample free end at this moment. This is the threshold pressure you do not want to exceed. Working with longer segments decreases the number of open vessels and thus the risk of refilling described above. However, longer samples are more difficult to resaturate! We describe below a procedure to test if your PLC data are trustworthy.

2- Possible problems with K''

K'' is the xylem conductance of the resaturated segment. K'' is supposed to be an estimate of K, the conductance of the same segment before treatment. However, under certain circumstances, or for several species, K'' can be very different of K.

Situations where K''may overestimate K

We have encountered two situations where K'' was greatly overestimating K. The first situation was found in Festuca lamina (see Martre et al 2001). In this species, intercellular air spaces are well developed in the leaves and form continuous pipe in the axial direction. When leaves are flushed, the air spaces become water filled and conduct water! Therefore, very high PLC values were noted in the leaf lamina of this species, even for control plants, but this was because K'' was overestimating K. There is not a practical solution to this problem, and K must be estimated statistically (see below).

A more common overestimation of K is found in species having a high native state of embolism. Temperate ring porous species are illustrative of this problem. We know that large vessels embolise during winter are never refilled (see for instance Cochard and Tyree 1990 or Cochard et al 1997.). Therefore, large vessels conduct water only in the current year ring in such species. Older rings have non-functional air filled vessels. When such a sample is flushed, these >1 yr old vessels are refilled and conduct water. The PLC value can then be quite high, especially for old samples. But this does not necessary mean that the sample has experienced any drought stress! To overcome this problem, work on current-year shoots, or follow the trends in embolism during a whole year.

Situations where K'' underestimate K

These situations are of common occurrence. They are found when xylem conduits get plugged for one or more reasons.

First, segment conductance may decline continuously during k measurements or after each flush. This may indicate a change in xylem conductance caused by the perfusion solution itself. This might be due to the presence of tiny particles in the solution that can pass through the pores in the filters (usually > 0.2 microns) but not through the pores in the pit membrane (usually < 0.2 microns). It is critical to use distilled or dionised water, to change periodically the filters and to clean the tubing frequently to avoid such problems. Change in K values might also be caused by the presence or the absence of certain ions in the solution. Usually when a small concentration of KCl is added to the solution (10 mmol per liter) a constant K is achieved. But different species may require different concentrations or different ions. We used to add HCl or oxalic acid to lower the pH and prevent bacterial growth. We now prefer to use neutral water and change the solution more often. It is easy to understand that these plugging problems will be exacerbated if a lot of solution has to pass through the sample (long flushes for instance). This is why we prefer to work on relatively short samples (about 2cm) and used very short flushes (a few seconds). Short samples will have many open vessels that will be easy to refill. However, they are more difficult to install for the same reason! Long samples will contain many bubbles trapped into close vessels and will require prolonged flushes to refill (and hence will be more likely to plug).

Species with resin or latex or gums in their xylem will also cause plugging problems. Removing the bark can help, but not always.

Another source of plugging is the formation of tyloses or gums in embolised vessels. Tyloses are invaginations of contact cells into vessel lumens (usually only in large vessels). We know that vessels have to be embolised for tyloses to form. Tyloses will obviously lower the hydraulic conductance of these vessels once refilled. The extreme situation is when all embolised vessels are entirely filled with tyloses. Then the water flow through these vessels will remain null even after many flushes. The PLC values for such samples will always be close to zero whatever the actual degree of embolism *in planta*. A similar situation is found in conifers where pit membranes become permanently aspirated against the cell walls of embolised tracheids. These membranes cannot return to their initial position and flow through these tracheids is permanently disrupted. To identify and then overcome these problems is rather difficult. First, it is always wise to do simple dye coloration (with safranine or phloxine B for instance) to find out if flushed samples are indeed entirely resaturated. Second, you may compute the sample xylem-area-specific conductivity of the resaturated samples and compare it to values obtained for control segments. If the saturated specific conductivity decreases with the intensity of the treatment or with time then you may have identified a refilling problem. This may lead to incorrect interpretation of what is going on in your plant. You may think that as the PLC values are decreasing over time the species is recovering or is reversing its embolism but it may just be that tyloses are being formed, or that new functional vessels are being added! This is where the statistical technique can be very useful. The principle is to establish a reference relationship between segment xylem area and segment conductivity for control plants and use this relationship to predict saturated K values for experimental samples. This technique is obviously less precise but you may have no other options.

In most instances you will rapidly learn how to avoid the problems listed above and you will rapidly learn to avoid troublesome species!

Test experiments

We describe below "test" experiments that we normally perform when we start a study on a new species or when we are training people to this technique. The idea is to do PLC measurements on samples having known or predictable PLC values. They are applicable to vessel bearing species (not for conifers). The first two experiments are just for practice, the third one is always useful.

1- Collect an intact branch (leafy or leafless) and connect its cut end to the XYL'EM outlet. Immerge the branch under water and flush it with a 0.2 MPa pressure for one hour. This should be enough to remove any embolism in the branch. Collect samples as you would normally do and determine their PLC values. Repeat until you find something very close to zero!

2- Cut in the air a small segment from a plant. Blow 0.1 MPa air on both sides and connect carefully the sample to the XYL'EM manifold to determine its PLC value. Repeat until you find something very close to 100PLC!

3- First determine the maximum vessel length. For this, the easiest way is to use the air perfusion technique: cut a branch on a plant and blow air at 0.1MPa through the cut end. Then successively remove branch parts starting at the most distal position (leaf petiole for instance). The remaining branch segment will thus become smaller and smaller. Put the terminal cut end in a beaker with tap water and notice if air is bubbling out of a vessel. If not, recut the branch a few more centimeters and repeat the operation. When you see a continuous flow of air bubbles emerging from the terminal end this means that at least one vessel was cut open on its both sides. The branch length will then be an estimate of the maximum vessel length for this sample. You will need to repeat the operation with different branches because maximum vessel length can vary substantially between samples. You may need to perfuse the branch with water first in order to reestablish all the air / water meniscus in the pit membranes. You can also use the paint perfusion technique but this is more laborious (see Tyree and Zimmerman for instructions). Second, cut a similar intact leafy branch in the air from a plant and let it transpire for a couple of minutes. This will be enough to empty all the lumens of the vessels cut open. Immerge the plant under water, excise different samples and note their location relative to the cut end. You want to collect sample from the cut end up to a distance longer than the maximum vessel length measured above. Measure the PLC values for these samples. You expect to find PLC values as follow: The proximal segment (that includes the cut end) will have a PLC value close to 100%. The PLC values will then progressively decrease in the apical direction. Then the PLC values become constant. This value corresponds to the native state of embolism. It can be close to zero or relatively high if the branch has experienced a drought or a frost stress. The length at which the values become constant should correspond to the maximum vessel length determined above. If your results look different, then the PLC values were not properly determined!

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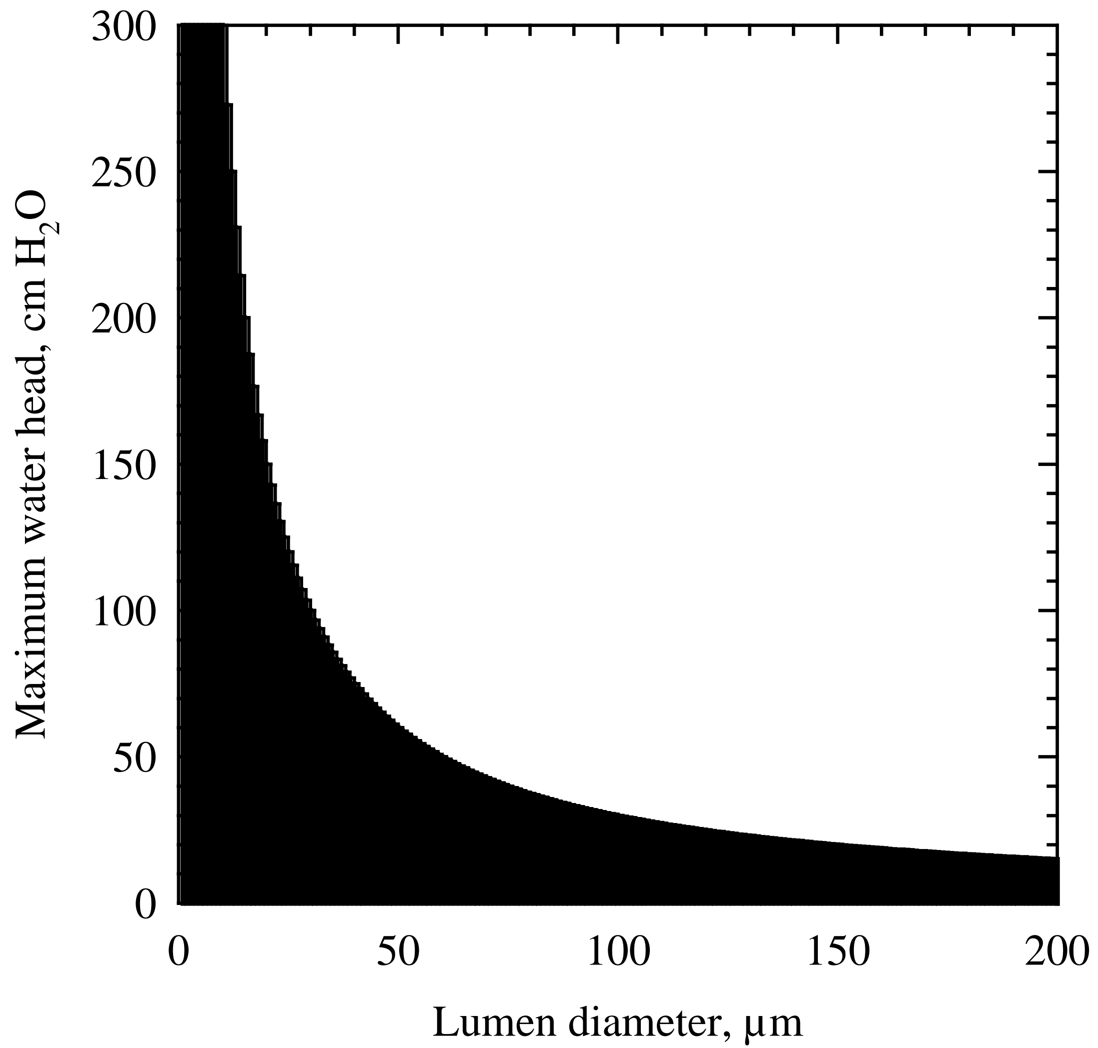

Figure 1: Maximum water head for measuring the conductance of an embolised vessel according to its internal (lumen) diameter. The computation assumes that vessels are behaving like perfect pipes and follow the capillary equation. For a proper estimate of xylem embolism, the hydrostatic pressure difference between the two ends of the sample must be in the black area.