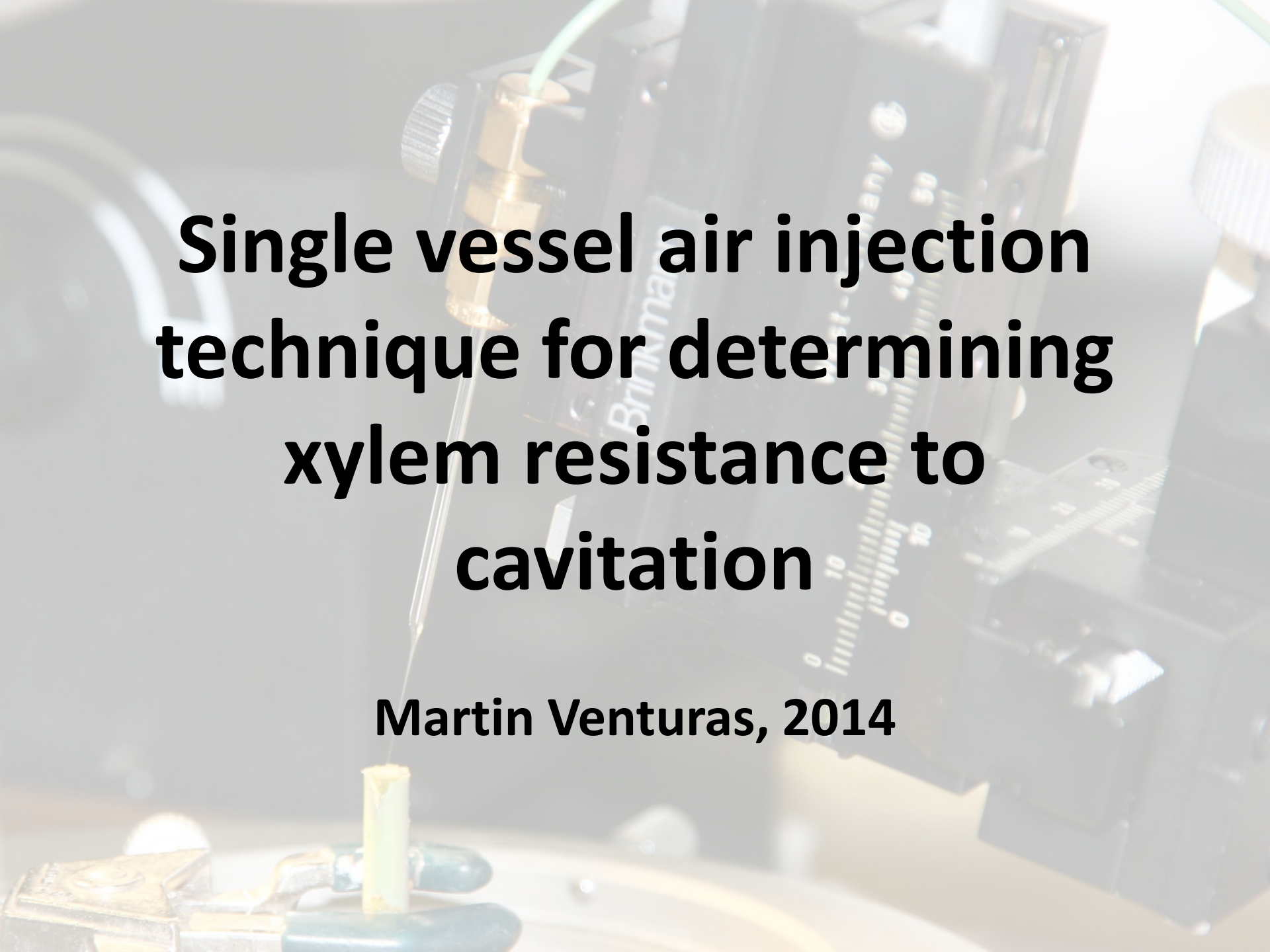


Single vessel air injection technique for determining xylem resistance to cavitation

Martin Venturas, 2014

Important!

Please read the following papers were single vessel measurements and single-vessel air injection technique are first described:

- Zwieniecki MA, Melcher PJ, Holbrook NM (2001) Hydraulic properties of individual xylem vessels of *Fraxinus americana*. Journal of Experimental Botany 52, 257-264
- Melcher PJ, Zwieniecki MA, Holbrook NM (2003) Vulnerability of xylem vessels to cavitation in sugar maple. Scaling from individual vessels to whole branches. Plant Physiology 131, 1775-1780

Another article I suggest you read if you are planning to use this technique is:

- Christman MA, Sperry JS, Smith DD (2012) Rare pits, large vessels and extreme vulnerability to cavitation in a ring-porous tree species. New Phytologist 193, 713-720

The technique described in the present document is a modification of prior papers. Manuscripts describing our technique include:

- Pratt RB, MacKinnon ED, Venturas MD, Crous CJ, Jacobsen AL. 2015. Root resistance to cavitation is accurately measured using a centrifuge technique. Tree Physiology 35: 185-196.
- Venturas MD, Rodriguez D, Percolla M, Crous C, Jacobsen AL, Pratt RB. Single vessel air injection estimates of xylem resistance to cavitation are impacted by vessel network characteristics and sample length. (in review)

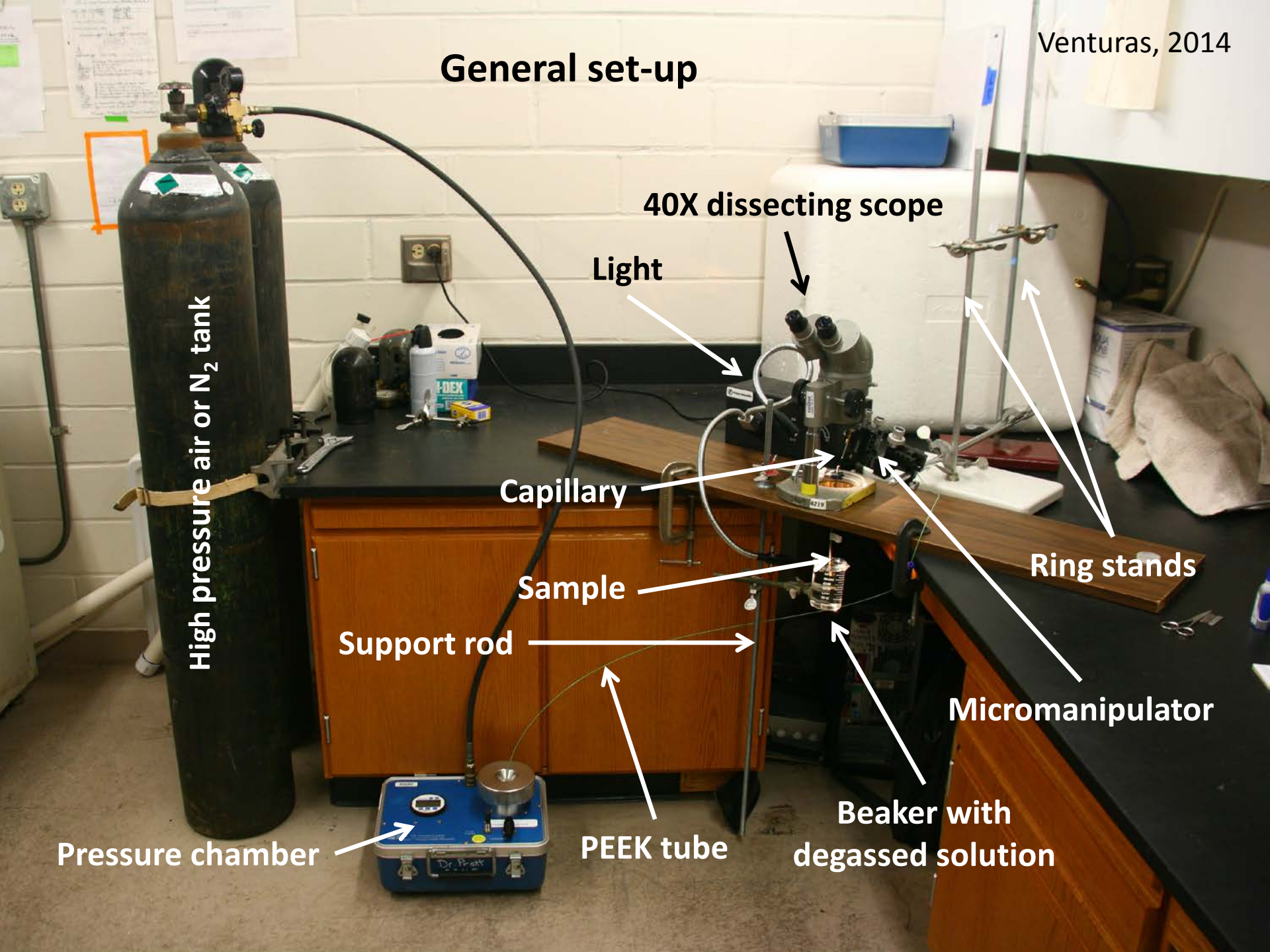
Materials

These are the materials and supplies we used to set up our system as described in this document:

- 1 high pressure air or nitrogen tank
- 1 Scholander pressure chamber (Model 2000, PMS Instruments, Albany, Oregon, USA)
- 1 dissecting microscope (Oympus 40X)
- 1 beaker (150 mL)
- 1 micromanipulator (Brinkmann)
- 2 rod stands, 4 right-angle clamp holders, and 2 extra metal rods (to use as cross-bars) for supporting the micromanipulator
- 2 ring stand clamps with three rubber covered prongs (a large one to hold the 150 mL beaker and a small extension clamp to hold samples)
- 1 threaded rod (50 cm long, 10.8 mm diameter), 2 washers and 2 bolts to attach the rod to the table or plank, and 2 to right-angle clamp holders for supporting the ring stand clamps that hold the sample and beaker
- 1 piece of green PEEK (polyetheretherketone) tubing (1/16'' outer diameter, 0.016'' wall thickness, 1.4 m length, item[1] # 51085K48)
- 1 quick-assembly brass tube fitting straight connector for 1/16" tube outer diameter (item [1] # 7473T113)
- Borosilicate glass capillaries (1.5 mm outer diameter, 0.84 mm internal diameter, item [2] # 1B150-4)
- 1 plank or table with a 80 mm diameter perforation for being able to place the sample through it
- 1 vacuum pump for preparing a degassed solution (membrane contactor Liqui-Cel mini-module 1.735.5, Membrana, Charlotte, NC, USA)
- A system for flushing or vacuum infiltrating samples (see http://www.csub.edu/~ajacobsen/Flushing%20Methods.pdf)
- 1 vertical capillary puller Model P-30, Sutter Instrument Co.
- Fast drying glue (Loctite, Super Glue Gel Control, Henkel Corp., Rocky Hill, Connecticut, USA)
- Fisher Scientific™ Stereomaster™ Microscope Fiber-Optic Light Sources (catalogue # 12-562-36)

[1] Item # corresponds to McMaster-Carr (http://www.mcmaster.com)

[2] Item # corresponds to World Prescision Instruments (www.wpiinc.com)


General set-up
Venturas, 2014
40X dissecting scope
Light
High pressure air or N_2 tank
Capillary
Ring stands
Sample
Support rod
Micromanipulator
Beaker with degassed solution
PEEK tube
Pressure chamber

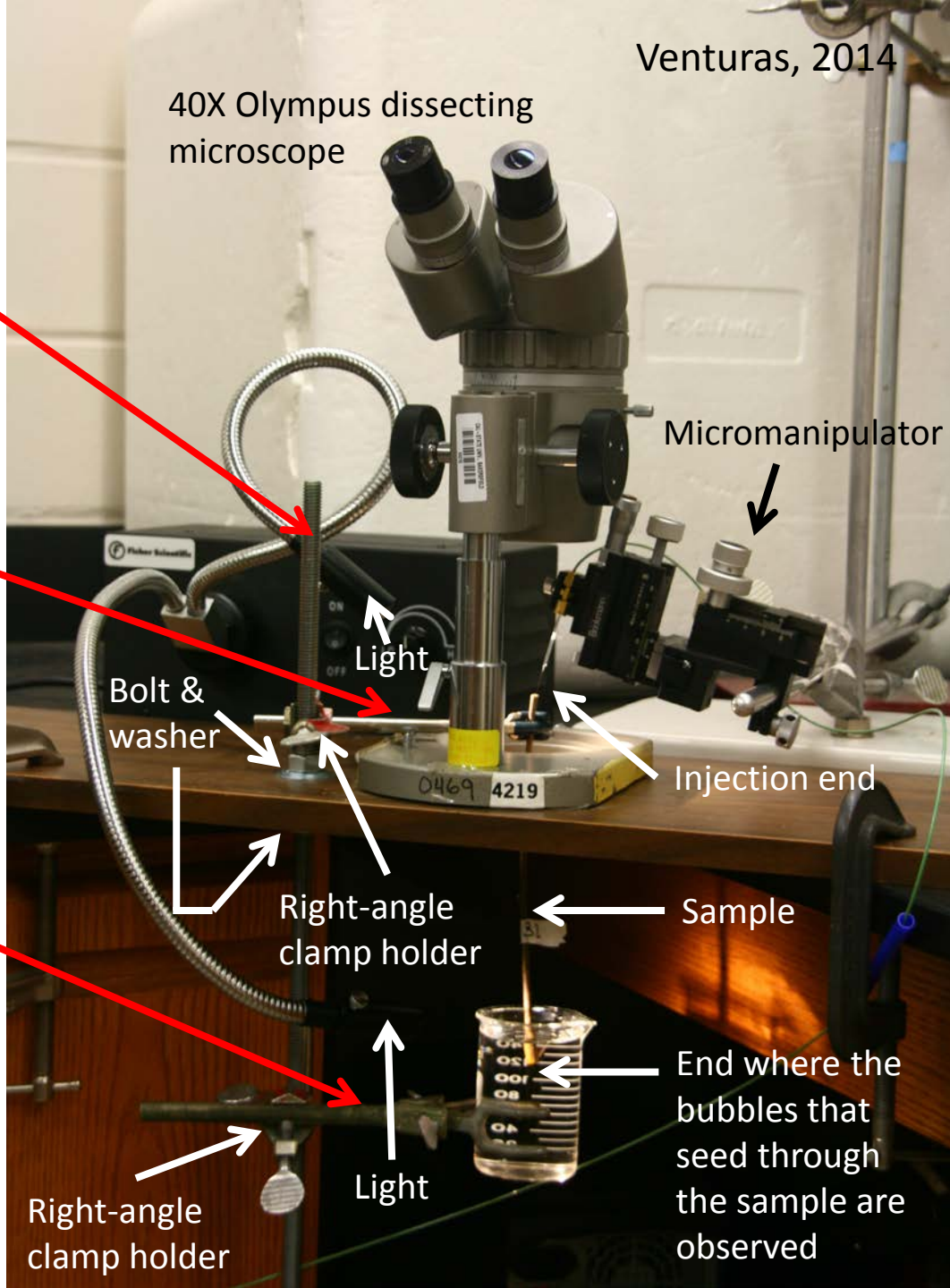
Detailed set-up 1

The threaded rod attached to the board acts as a support for the ring stand clamp that secures the sample and the beaker. The clamp may be raised or lowered along the threaded rod to adjust the distance between the beaker and injection site for different sample lengths.

Small three finger extensible ring stand clamp with rubber covered prongs that secures the sample in place.

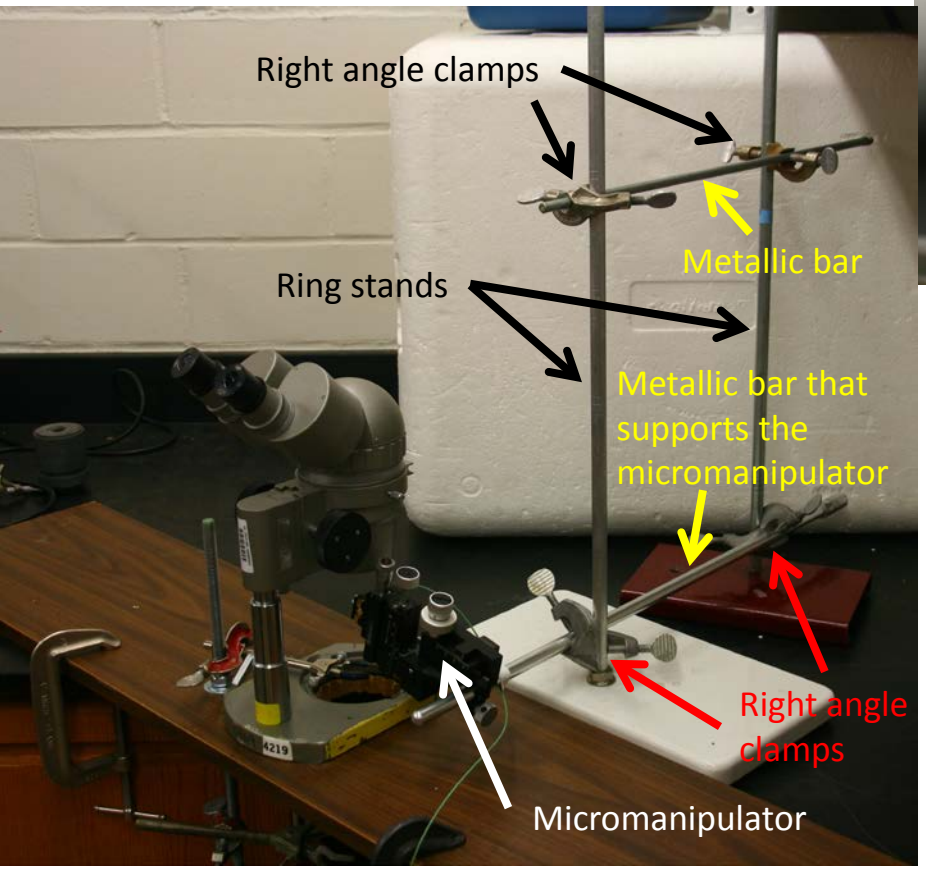
Large three finger extensible ring stand clamp with rubber covered prongs to support the beaker containing the degassed solution.

While performing measurements safety goggles should be used. We place a Pexiglass panel between the system and the observer as added protection.

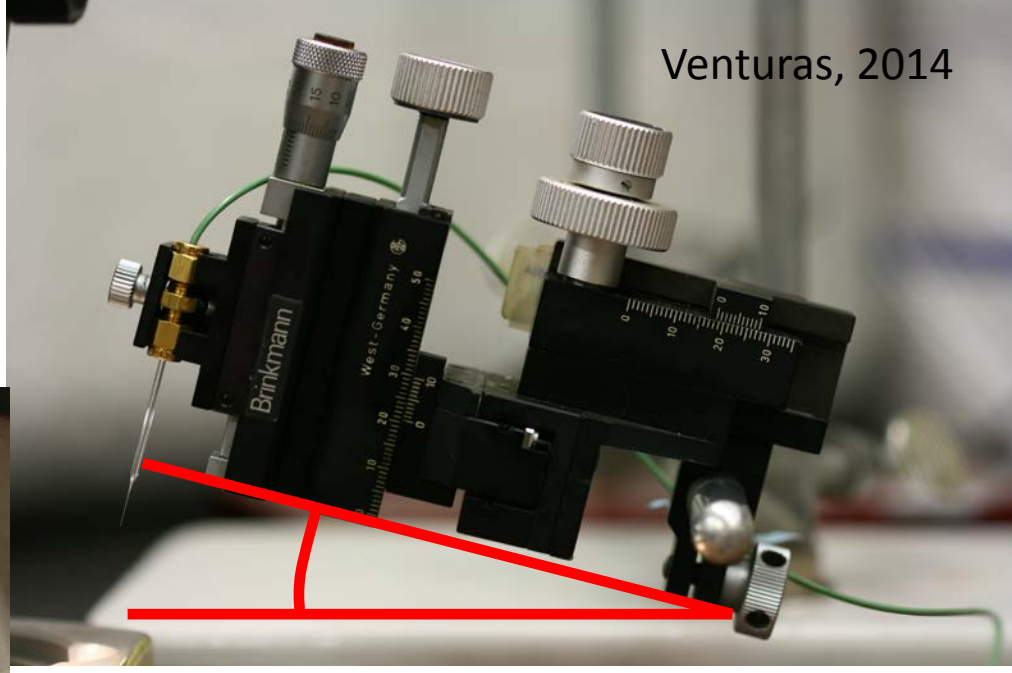


Detailed set-up 2

We use 2 ring stands, 4 right angle clamp holds and 2 cross bars to attach the micromanipulator since this reduces vibrations during manipulation. Additionally, this system allows the micromanipulator to slide sideways when inserting a sample or replacing the capillary.



If the sample end is slightly tilted so that the capillary axis is equal to the vessel axis, the insertion of the capillary is facilitated.

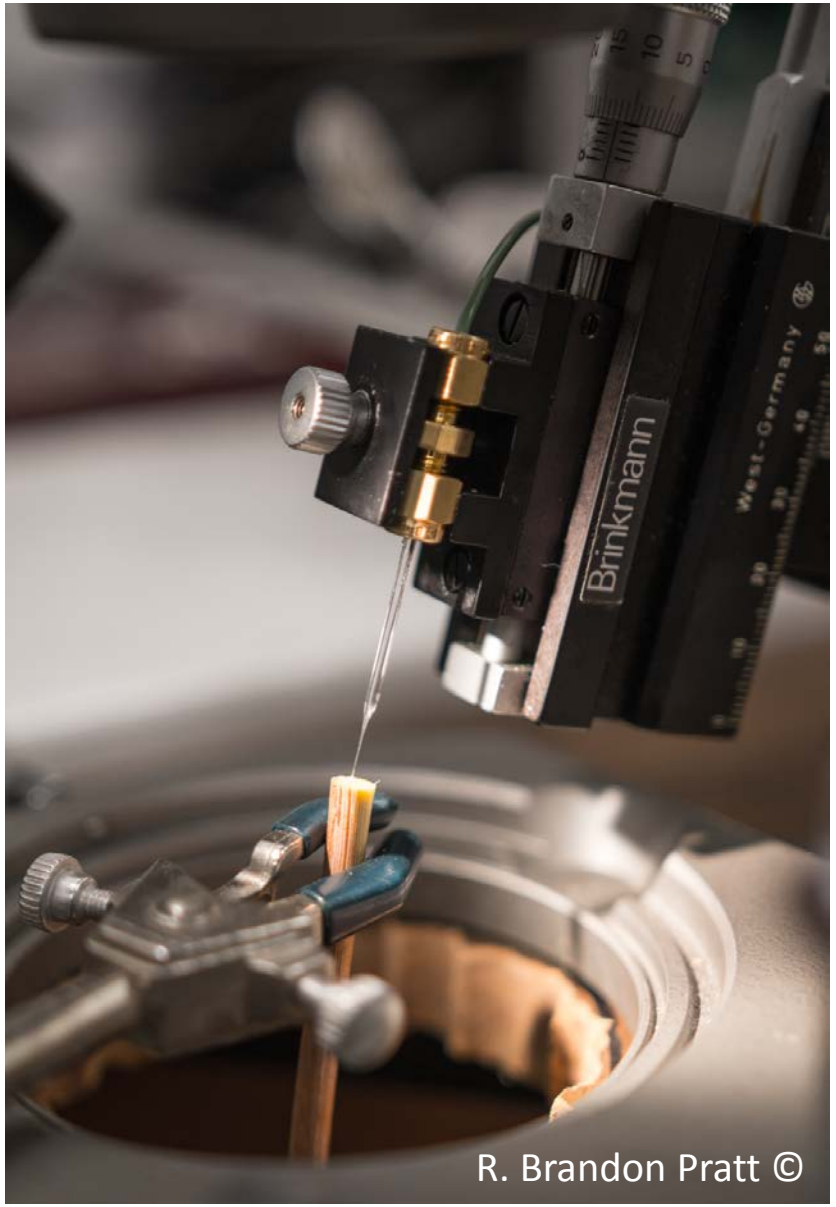


If the micromanipulator is set at a slight angle it enables seeing the capillary tip and the vessels that it will be inserted into at the same time through the dissecting scope.

Detailed set-up 3

Venturas, 2014

A hole drilled in the plank or table allows the sample to be placed vertically for inserting the capillary and performing measurements.



We use the heat setting at 925 and the pull setting at 990.

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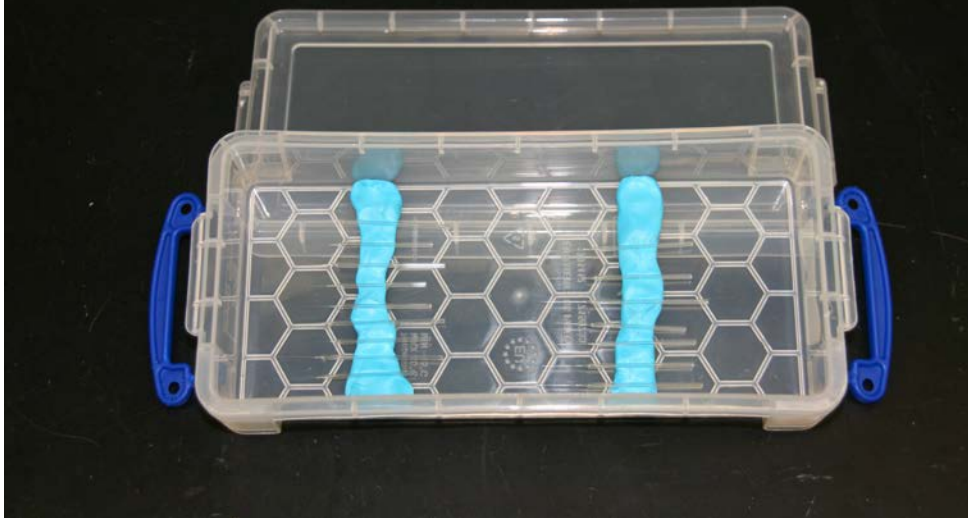
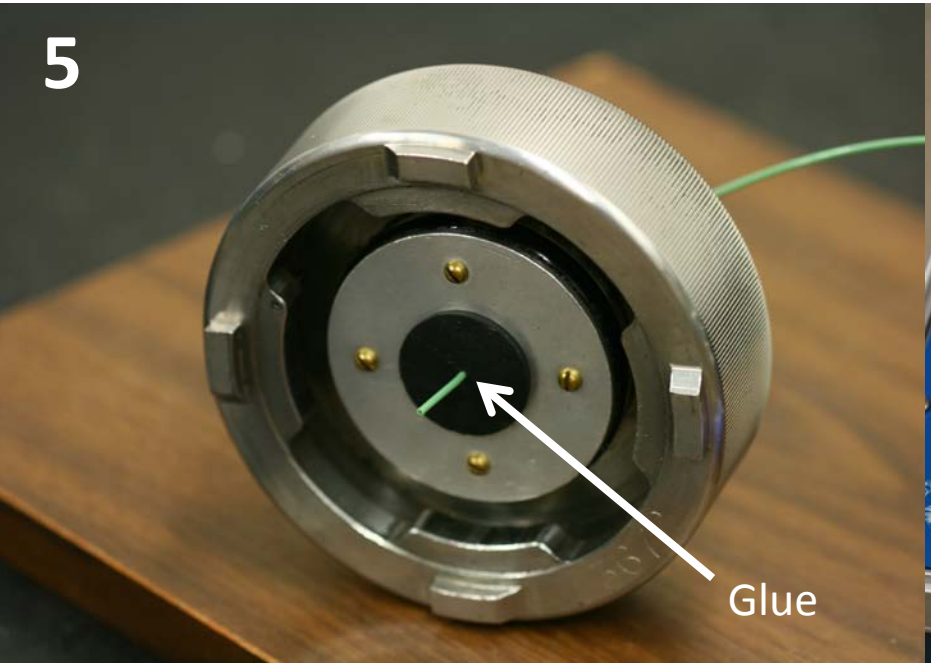
We found that the ideal length of pulled capillaries was 4 – 5 cm. To achieve this we did not pull the capillaries in the center. We obtained one segment of the right length and a second longer one, which we pulled again.

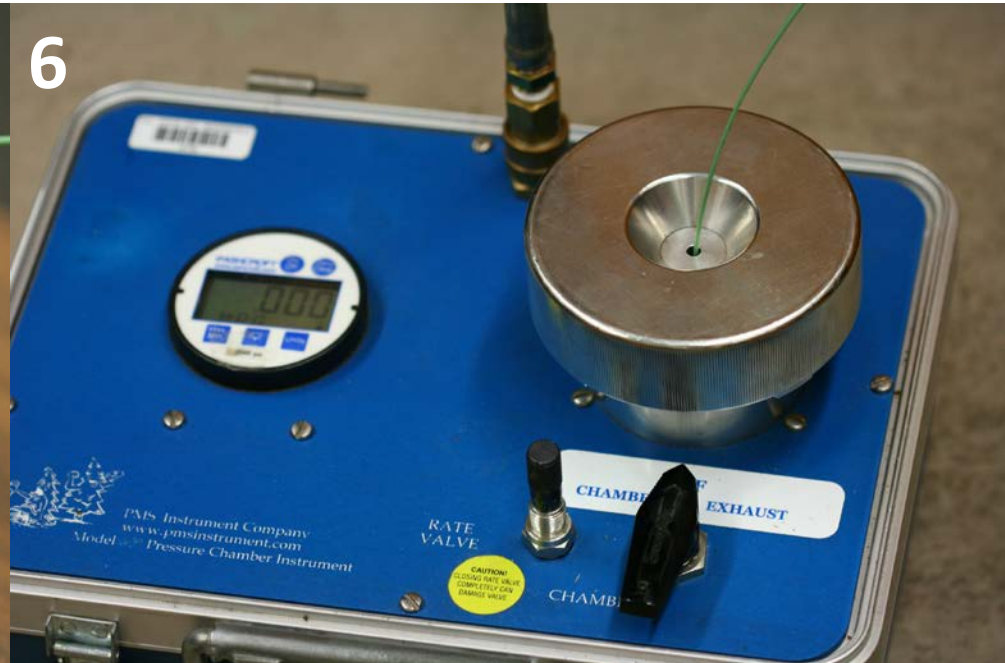
A good system for storing the pulled capillaries is using a plastic container and putty.



Pressure chamber assembly

Connect the pressure chamber to the N_2 tank (1). Insert a wet paper towel in the chamber in order to avoid pit membrane desiccation (3). Add a volume reducer in the chamber for not wasting as much N_2 and having better control on pressure increase velocity (4). Thread the PEEK tubing through the rubber grommet and secure it place with glue (5). Place the lid on the chamber (6). **Important:** When you finish the measurements remember to remove the paper towel from the chamber.





Assembling the capillary to the PEEK tube

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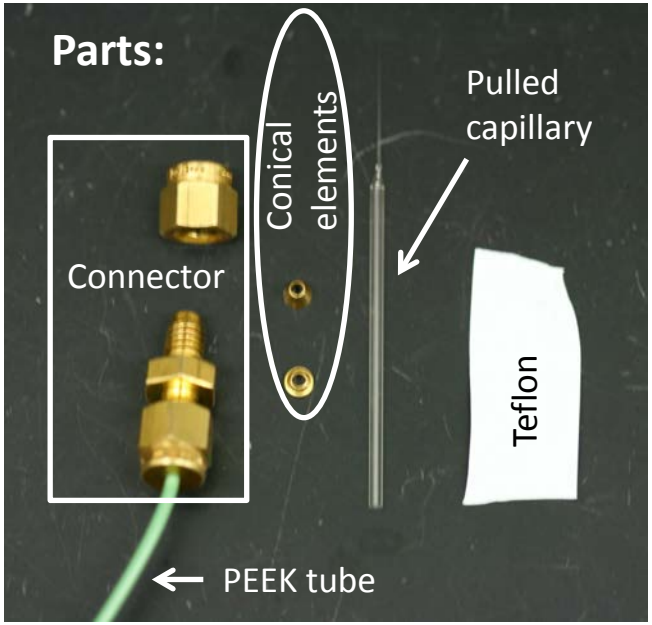
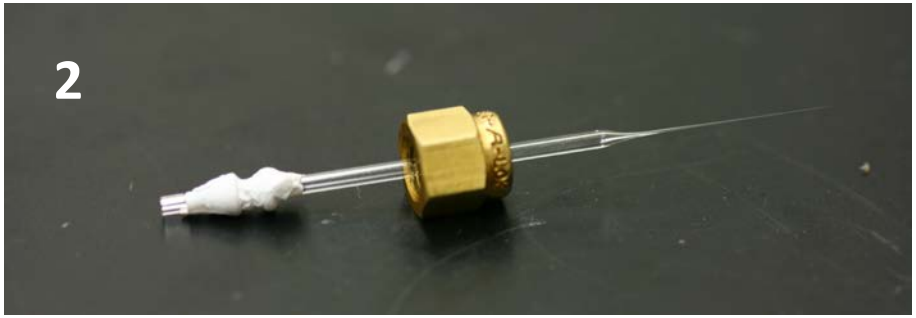
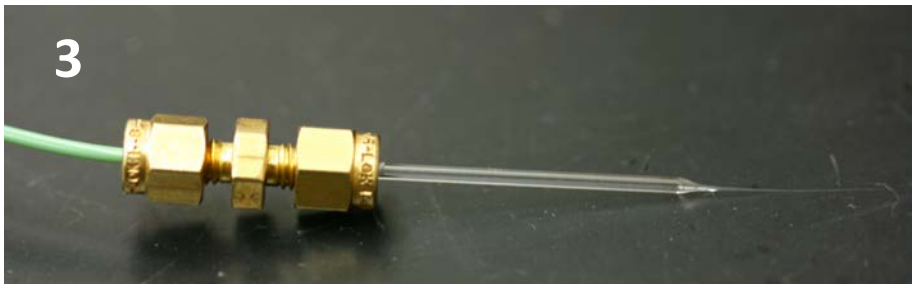


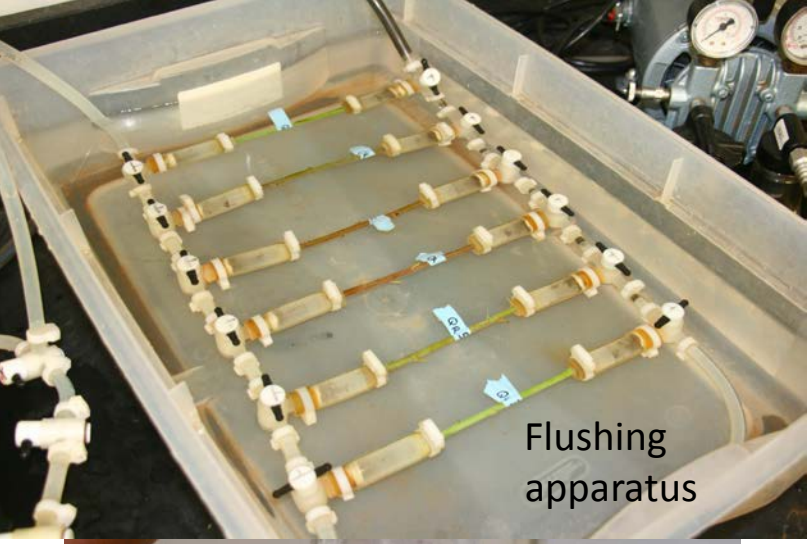
1. Thread the capillary though the bolt and conical elements.
2. Wrap approximately a 1.5 cm segment of Teflon around the conical elements.
3. Screw the bolt in place. You must apply enough pressure for the capillary not to shoot out when pressure is injected, however, if you tighten it too much the capillary will brake.

Note: The PEEK tube is attached to the connector in the same manner.





Sample preparation


Flushing apparatus

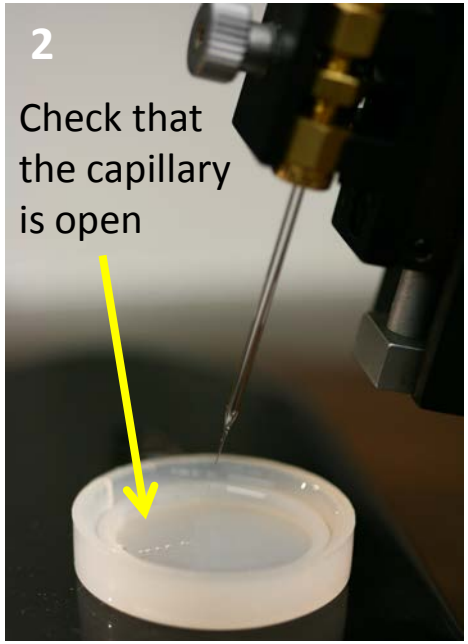
Vacuum infiltration

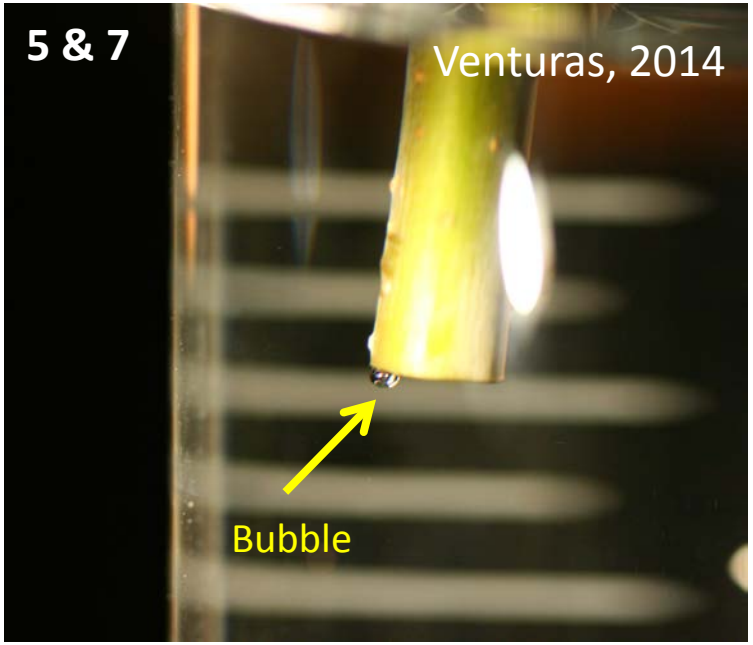
Samples should be hydrated because drying may change pit membrane properties by causing pectins or cellulose microfibrils to shrink making pit pores larger and more vulnerable to air seeding. You should consider removing any embolism within vessels by flushing or vacuum infiltrating stems prior to performing single vessel air injections. For details on how to construct a flushing apparatus visit Anna L. Jacobsen webpage: http://www.csub.edu/~ajacobsen/Flushing%20Methods.pdf

We flush the samples for 1 hour with a ultra-filtered (0.1 µm filter) 20 mM KCl degassed solution. Samples can also be vacuum infiltrated for 1 hour with the same solution.

Since cycles of embolism and refilling can cause cavitation fatigue (Hacke *et al.* 2001), samples should be harvested by cutting under water or cutting large branches in air and then excising the measurement segment under water in the lab. If samples cannot be immediately measured they can be stored for a few days in a plastic bag covered with wet paper towels if they are refrigerated (+5ºC). Do not freeze nor dry samples before performing measurements on them since this can potentially damage the pit membranes.

Reference: Hacke *et al.* (2001) Plant Physiology 125: 779-786





For performing measurements:

1. Cut the end of the capillary (we use a razor) where the diameter is slightly smaller than the diameter of the vessel that is going to be injected. Attach the capillary to the tubing system as already shown.
2. Make sure that the capillary is open by checking if bubbles are formed when the capillary is immersed in water and the pressure chamber is pressurized.
3. Connect the capillary to the micromanipulator and, looking through the dissecting scope, use the micromanipulator controls to insert the capillary in the selected vessel.
4. Seal the capillary in place in the vessel with fast drying glue. A tooth pick might help in this process. Depending on the species vessel connectivity you might have to seal the whole cross-section of the injection end to avoid air seeding and pressure loss through the injection end (see additional notes in consecutive pages).
5. Check it the injected vessel is open. We apply low pressure (0.05 MPa) and if we see bubbles coming out the opposite end of the sample we assume that it is open, i.e. there are no terminal vessel elements present within the injected vessel through the measured sample length. In such case, we remove the capillary and inject another vessel after removing the glue. If the vessel is not open at both ends we proceed to step 6.
6. We increase very slowly the pressure in the chamber (at a rate of 0.01 MPa/10 s).
7. When bubbles are observed coming out of the other end the pressure is registered as the air-seed pressure.

Polarity of samples

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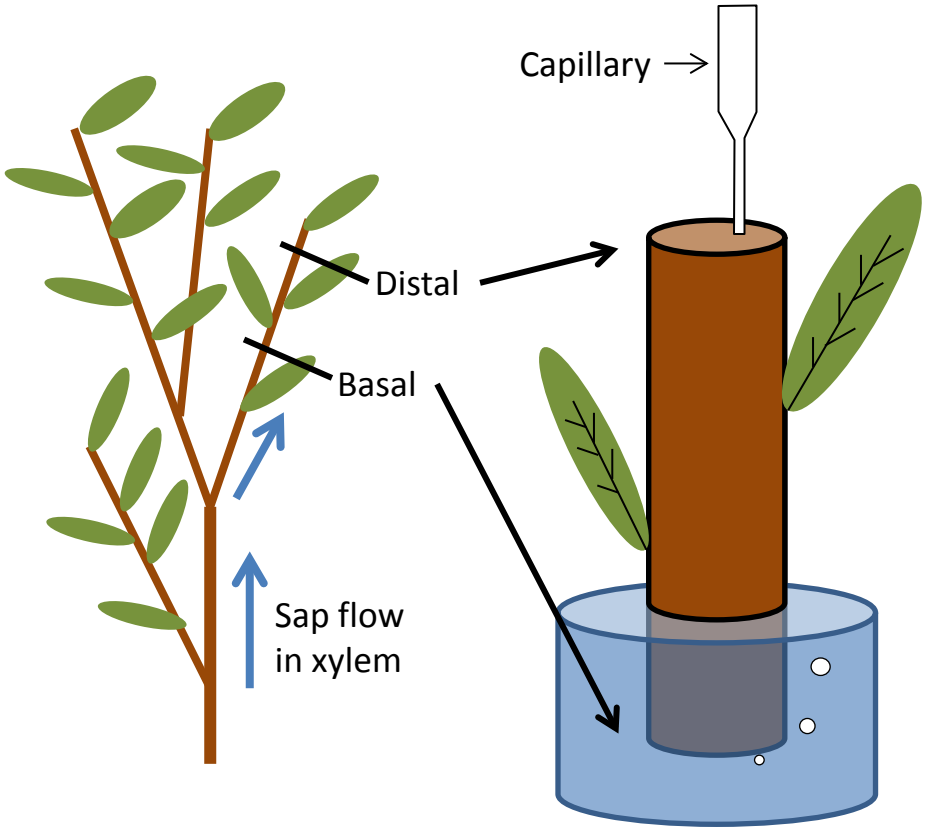
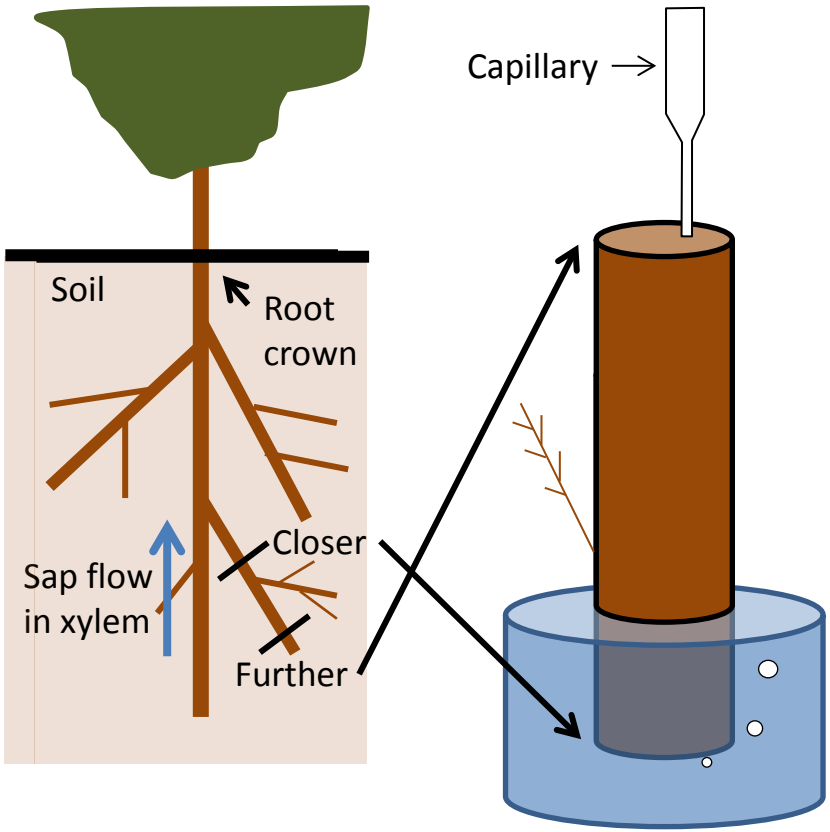
Stem samples

We inject the vessels in the distal end of the stem sample. This way we avoid injecting the capillary in a vessel that goes to the petiole of a leaf or side branch if there are any on the sample.



Root samples

We suggest that root segments should be injected in the end further away from the root crown of the plant. That way we avoid injecting a vessel that might go to a side root if there is any present.



Both stem and root samples should be flushed in the same direction as sap flows through the xylem of these organs.

Additional notes:

Venturas, 2014

Beaker solution

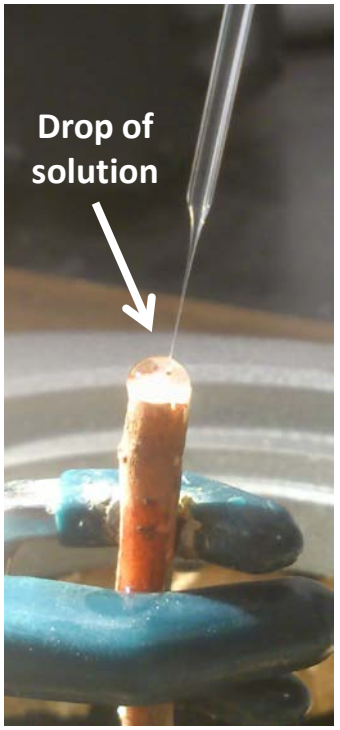
There are two main reasons for using a filtered (0.1 µm filter) 20 mM KCl degassed solution in the beaker where the sample end will be immersed for observing air seeding: (i) it avoids that air comes out of solution creating bubbles on the surface of the beaker or sample making it more difficult to observe and determine when air bubbles come through the sample; (ii) if water evaporates from the injection end while performing measurements or trying to insert the capillary it will be replaced by the degassed solution in which the opposite end of the sample is immersed.

Maximum injection pressure

We currently apply pressures ranging from 0 to 5 MPa with relative safety. However, we find that at pressures higher than 5 MPa the chances of the capillary breaking or shooting out of the tube connector are quite large.

Securing the capillary in the vessel

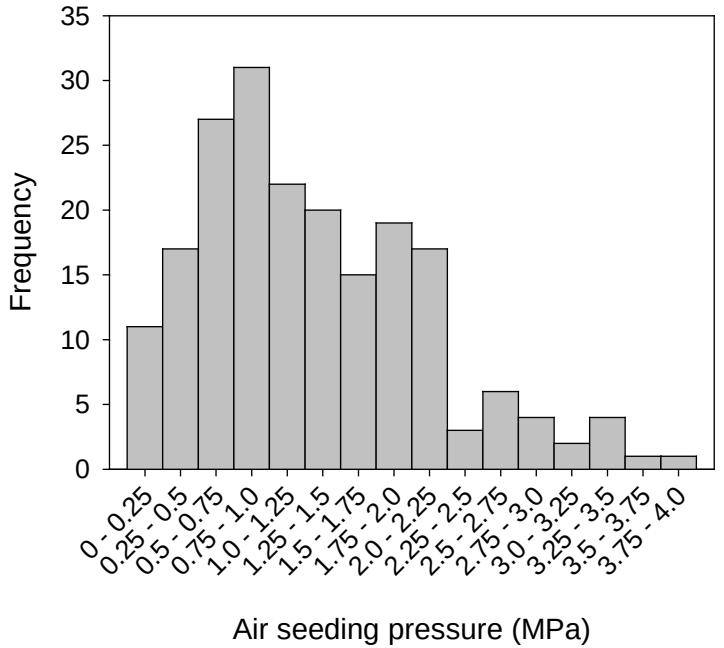
We use fast drying glue to secure the capillary in the injected vessel and to avoid pressure seeding and coming though the injection end of the sample. If air comes out of the injection end there will be a pressure drop in the sample, and we would probably over estimate the pressure required for air seeding through the vessel file and bubbles coming out of the sample end immersed in the beaker solution. Depending on the connectivity of the vessels of the species you are sampling, it may be necessary to cover the whole injection end with glue, or only a section of it. To determine if you have a good seal and no air is seeding through the injection end of the sample you can place a drop of solution on it when the glue has dried. Bubbles should not form in this drop if the end is properly sealed. The advantage of not covering the whole injection end with glue is that it is easier to inject multiple vessels from the same sample whit out having to remove the glue. We recommend using GEM Teflon covered razor blades for removing the glue from the injection when necessary for inserting a capillary in another vessel of the same sample.

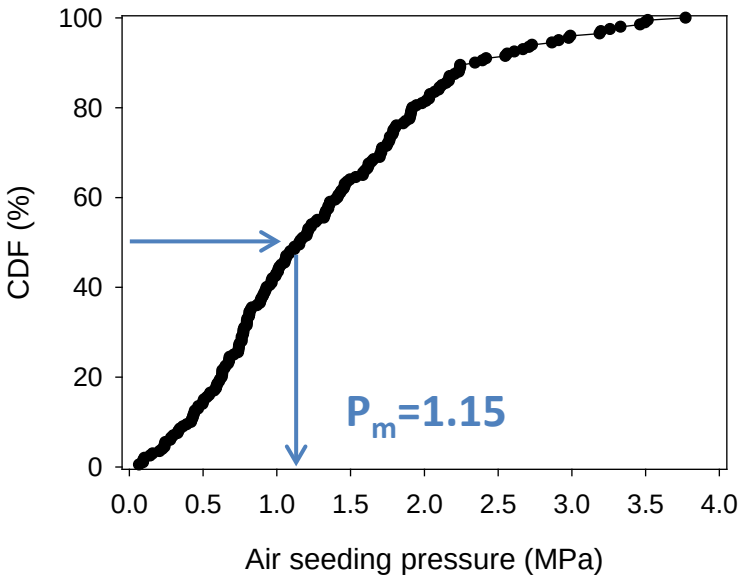


Data analysis (1)

Venturas, 2014

The data you obtain with this method is the pressure required for air to be seeded through a vessel or a file of vessels. The results can be represented as a histogram that represents the frequency of vessels seeding at given pressure intervals or as a cumulative distribution function (CDF) which represents the proportion of vessels that seed below a given pressure. The histogram and corresponding CDF of a 200 point simulated dataset are here represented:

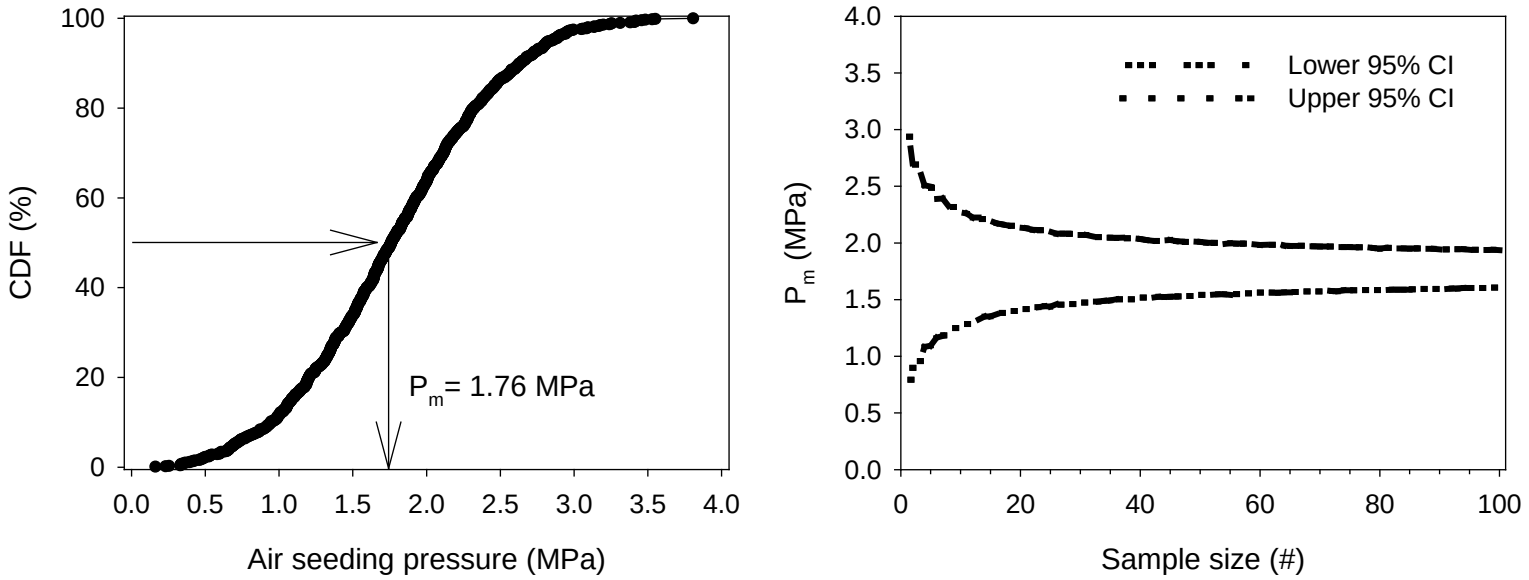




A parameter that can be very useful for interpreting the results is the pressure at which 50% of the vessels are air seeded (P_m). We can calculate P_m as the median of our sample. This allows us to incorporate the data of vessels that air seed at pressures greater than the pressure that our system can hold (e.g. vessels that seed above 5 MPa if that is the maximum pressure we can reach). The minimum sample size for obtaining a robust P_m estimate depends on the shape of our CDF. The Weibull distribution is usually the best fit for single vessel air injection CDF. Bootstrapping allows to estimate the minimum sample size required for obtaining a good estimate of P_m. Simulations show that this value is typically above 30 vessels per treatment (see figure in next page).

Data analysis (2)

Venturas, 2014

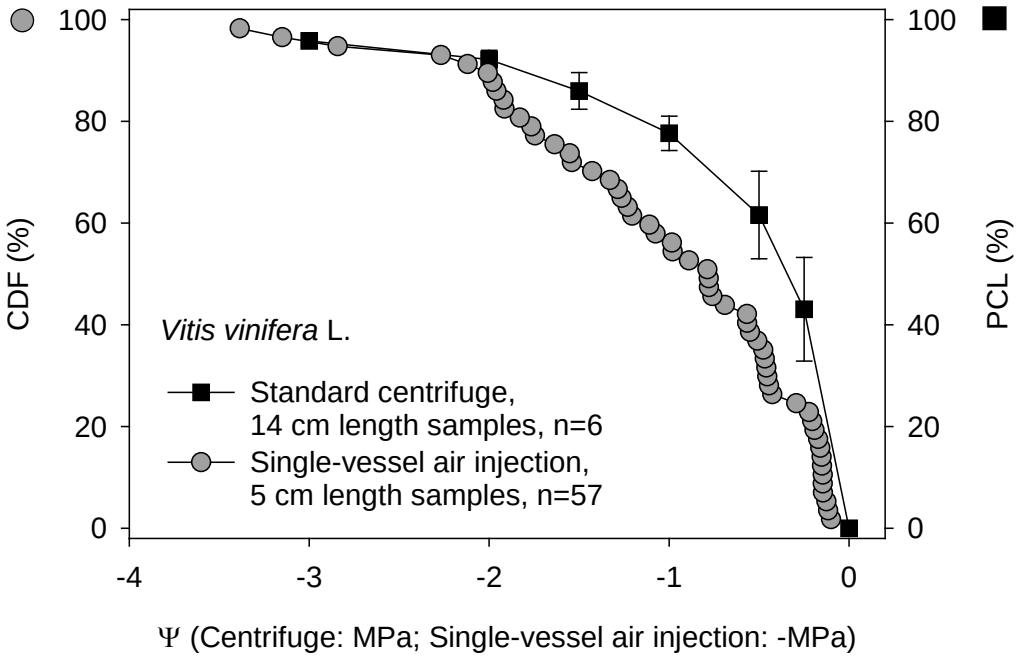


In the figure above, the left panel shows a CDF corresponding to a Weibull (alpha=3, beta=2) with P_m=1.76 MPa. The right panel shows the 95% confidence intervals (CI) for P_m of this Weibull curve. 10,000 subsamples were obtained from the Weibull for each sample size and the P_m calculated for each one of them. The P_m 2.5 and 97.5 percentiles for each sample size represent the 95% CI. As it can be observed, the larger the sample size is the P_m confidence interval is smaller. For this simulated dataset for a sample size n=5 the P_m 95% CI is (1.09, 2.50), for n=30 P_m 95% CI is (1.47, 2.07), and for n=100 P_m 95% CI is (1.60, 1.93).

Data analysis (3)

Venturas, 2014

It is important to realize that the CDF obtained from single vessel air injection is different from the percentage loss of hydraulic conductivity (PLC) obtained by other methods such as the standard centrifuge technique (Alder *et al.* 1997). The problem is to know how much does each injected vessel contribute to stem flow. Nevertheless we could expect to obtain a similar shape in these curves if the sample is short enough as to represent the seeding pressure of one single vessel, especially if sample size is high enough and random. Therefore, under certain conditions P_m should be similar to the pressure that causes 50 % conductivity loss (P_{50}).

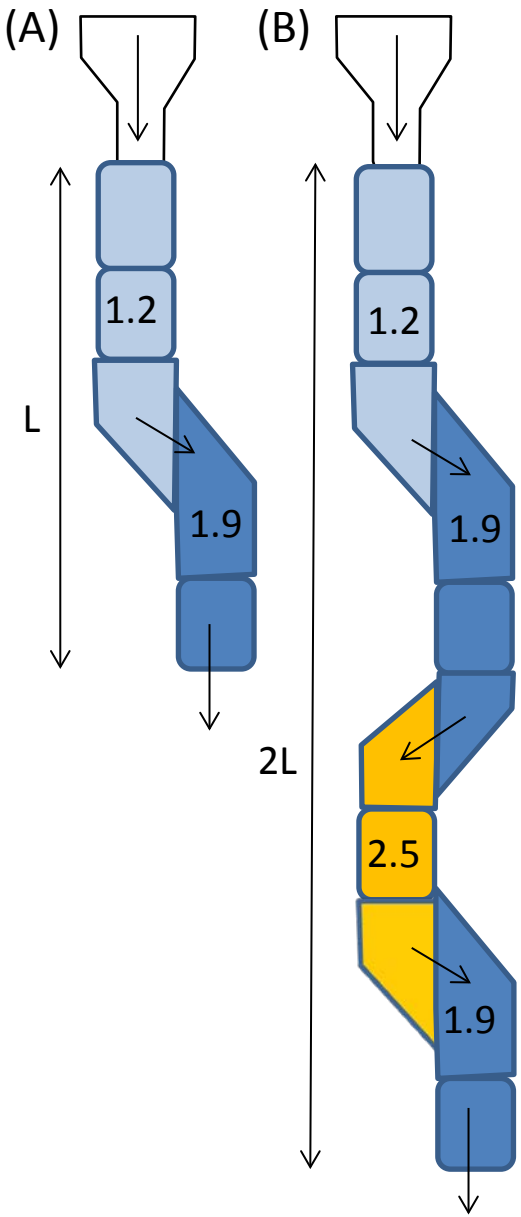


Sample standardization

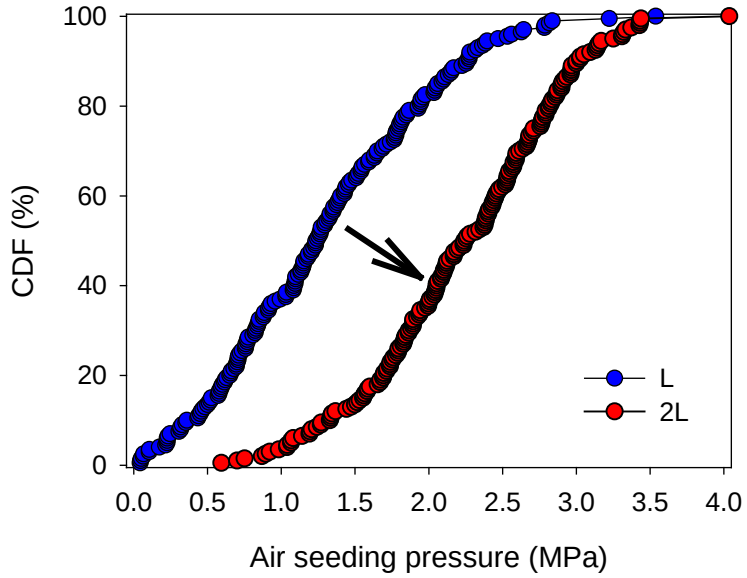
It is very important that samples are correctly standardized when comparing treatments, i.e. as many sources of variation as possible should be controlled (e.g. sample diameter, sample length, season of collection, exposure, plant vigor, age of the ring containing the vessels to be injected, location,…)

Sample length effect

Venturas, 2014

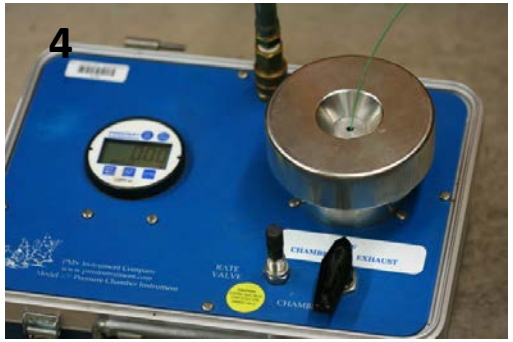


The longer a sample is, greater are the chances that air has to seed a greater number of vessels in a file before reaching the opposite end of the sample. This is represented in the sketch on the left, where the number within each vessel represents the pressure required for seeding that vessel. (A) In the sample of length L air would only need to seed one vessel an the pressure required would be 1.9 MPa. (L) The sample of length 2L air would have to seed 3 vessels and the pressure required would be 2.5 MPa. This can result in a shift of the vulnerability curve where longer samples would present higher resistance to cavitation:



Therefore the length of samples has to be selected adequately. Shorter samples will have greater percentage of vessels open at both ends, but will result in a curve that will better represent the single vessel air injection seeding pressures. Determining the vessel length distribution and mean vessel length with the silicon injection technique might help towards selecting the adequate sample length.

Danger! Take precautions!



This technique is potentially dangerous since you are working with a pressure chamber, high pressures and glass capillaries. Seek adequate training with these systems. Use protection items such as safety goggles (1) and a Plexiglass panel placed between the observer and the sample while applying pressure (2). Remember to close the high pressure air or N_2 tank when you finish preforming measurements. Release the gas contained in the tube that connects the tank to the pressure chamber before disconnecting it (3, 4).

Acknowledgements

I would like to thank Dr. Anna L. Jacobsen and Dr. R. Brandon Pratt for their comments on previous drafts since they have helped raise the quality of this document.

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