Xylenol Orange Assay to Determine Soluble Peroxides in Complex Plant Tissues

Adapted from Cheeseman et al. 2006: <https://doi.org/10.1093/jxb/erl004>

*Read this paper before starting. It is the best (most rigorous!!!) H2O2 study I’ve ever frikkin’ seen.*

*Remember: If the controls aren’t working, SOMETHING IS WRONG. See dissertation for descriptions of control tests, ProQuest Publication number:* ***30633857***

**REAGENTS NEEDED**

* NaH2PO4 buffer + 5mM KCN, pH 6.4 – ‘extraction buffer’ – Store at 4ºC
* 0.25 M xylenol orange dissolved in ddH2O (okay to store at room temperature, though long-term storage capabilities unknown)
* 500 µM Ferrous (II) ammonium sulphate + 200 µM sorbitol + 2% EtOH + 50 mM H2SO4 – ‘Pre-working solution’ (okay to store at room temperature, though long-term storage capabilities unknown) *It is HIGHLY recommended you buy a fresh batch of ferrous (II) ammonium sulphate hexahydrate since oxidation over time makes it go bad ☹ To be safe, make sure it’s < 1 year old.*
* 30% stabilized H2O2 solution, reagent grade – Store at 4ºC – use within 3 months of opening or so; wouldn’t use it after 6!

**MATERIALS NEEDED**

* LOTS of liquid nitrogen 🡨 for mortar and also to keep your tubes in
* Clean, pre-chilled mortar + pestle (mid – small size for grinding a few flower buds at a time)
* Tweezers, prongs, spatula – all chilled; these are for handling cold things!
* Gloves, and winter gloves to wear while grinding
* 15 mL falcon tubes 🡨 needed to perform the extraction in
* 96-well white plates (clear, flat-bottomed, non-binding) – greiner bio-one item #655903
* 1000 µL, 200 µL, 10 µL pipette tips and pipettes
* Tin/aluminum foil
* Shallow Tupperware that you can put ice in and place your plate on

**EQUIPMENT NEEDED**

* Laminar flow hood 🡨 tidy & decontaminated w/ EtOH
* Ice buckets + LOTS of ice 🡨 it is absolutely critical that all reagents, samples, and standards stay COLD
* Benchtop vortexer (put in hood)
* Centrifuge w/ temperature control (set to 4ºC) and 15 mL tube compatible buckets
* Synergy H1 Plate Reader, to read absorbance at 550 nm and 800 nm [Shachar-Hill Lab]

**HEADSPACE NEEDED**

* Healthy dose of paranoia
* Positive yet cautious attitude 😊

**EXTRA TIPS**

* **Keep EVERYTHING cold**
* **Be clean and sterile – pretend like you are extracting RNA!**
* **Keep tubes in liquid nitrogen until you are ready to grind, in order to prevent them from exploding!!!**
* **Store ALL samples IN LIQUID NITROGEN. H2O2 degrades even at -80ºC!**

1. Before you start, prepare space in your lab notebook for the extraction notes. Plan your plate (i.e., what wells will your standard be in? And your samples?), and what samples you are going to do.
2. Turn on the centrifuge, set temp to 4ºC.
3. Label two 15 mL tubes for each one of your samples. (one for extraction, one for supernatant collection after centrifugation)
4. Add 5 mL of extraction buffer to 1 of the 2 tubes for each sample. Keep cool on ice. You will be scraping your ground tissue right into this aliquoted buffer.
5. BEFORE GRINDING – Measure weight of each tube + the 5 mL buffer and write down. You MUST do this so that you can weigh the tubes again after vortexing in the buffer. *The difference between these two measurements is the weight of your sample – which we will need to normalize H2O2 / gram of fresh weight*
6. Next, make your standards in the extraction buffer. **[[SEE TABLE 1]]** This is to replicate the conditions of our samples in our standards as best we can, so they’re biochemical properties are comparable. **TIPS: \*\*Go slow, change tips frequently, do pumps with pipette liquids to mix solutions well – always vortex before exchanging liquids to be sure everything is homogenous, make sure all solutions stay cool! H2O2 should be reasonably stable on ice (since the KCN is less likely to volatilize).**
7. Obtain a shallow Tupperware or other container, and fill ~ halfway with packed ice. Line the top of the ice with clean aluminum foil. Be sure to make the surface as flat as possible. Then, take a clean, never-before-used 96-well plate and wipe the clear bottom with a kimwipe dabbed in 100% EtOH. You might want to examine the wells for any scratches – don’t use those wells! After the surface of the aluminum foil and bottom of your plate are clean, gently set the plate on the foil + ice. Place another sheet of foil over the plate to protect it from dust. Put the plate setup in the 4ºC until you are ready to add the working solution, standards, and samples.
8. Make your working solution (WS). Recipe for 10 mL: 8 µL of 0.25 M xylenol orange in ddH20 + 9,992 µL of ‘pre-working solution’. Vortex vigorously. Place on ice. I like to have at least 2 ice buckets – one for standards + WS and the other to hold my samples.
9. CAREFFULLY and QUICKLY remove your samples from the liquid nitrogen storage tank and put in a Styrofoam container full of liquid N if you haven’t already. You want to avoid thawing and warming at all possible – not moving fast enough when transferring to different pools of liquid nitrogen could also result in exploding of tubes and loss of samples ☹
10. After you have the samples you are grinding in a manageable group of liquid N (like in our Styrofoam container!) you are ready to begin the extraction! \*\*Recommendation: have a clean mortar and pestle for each genotype on hand. That way, you can dirty one up and set it aside. The key is to do this as quickly and carefully as possible. Alternatively, you could thoroughly wipe a mortar out with a handful of kimwipes and ethanol in between samples… but if you can do the first option, shoot for that!
11. Quickly remove the lid/cap of a sample and dump the tissues in the pre-chilled mortar. Grind the tissue to a fine powder with the pre-chilled pestle. Then, scrape out as much sample powder as quickly as possible directly into your tube + 5 mL buffer (using a clean spatula – pre-chill this too!). Try to do this in 10-15 seconds!
12. Vortex the sample vigorously, then place on ice.

**Repeat steps 11 -12 until all samples are extracted**

1. Weigh the tubes + 5 mL buffer + ground sample. Record new weight. This weight minus the weight in step 5) is the fresh weight of that sample.
2. Place the samples in a symmetrical (balanced) fashion in the 4ºC large centrifuge. Centrifuge on max speed for 10 minutes. **TIP: You can fill a 15 mL tube with 5 mL of ddH20 to use as a balance if needed.**
3. After centrifugation, carefully remove the tubes from the centrifuge and put them back on ice. Try not to disturb the separation between supernatant and plant debris. There might be some specks and floaties – this is okay. Just try to avoid them as best you can in the next step.
4. Take as much of the supernatant as you can from the centrifuged tubes and place it in the clean, pre-labeled and chilled (on ice) tube for that sample.
5. Retrieve your plate set up in the fridge; add 100 µL of cold WS to all the wells (at least the ones you plan on using).
6. Add 100 µL of each standard to their appropriate wells – at least 4 technical replicates are recommended!
7. Add 100 µL of each sample to their appropriate wells – at least 4 technical replicates are recommended!

**TIP: mix the solutions slowly and carefully in steps 18) and 19) by pipetting up and down. Try to avoid air bubbles. Loading the plate will take some time, maybe 5-10 minutes, but that’s okay; best to remain focused and not get confused than do things too quickly. While it should be done as soon as possible, there is wiggle room since the rxn is slow – especially when it’s on ice.**

1. Once the plate is loaded, place the sheet of tin foil back over it and head to the Shachar-Hill lab to use the plate reader. Your plate should still be on ice! Keep it cool! When you get to their lab, slip the plate set up into their 4ºC fridge. Let the rxn incubate in there for *at least* 30 minutes. 60 minutes may be more optimal since by then, the rxn should be complete. Choose a time to measure it and stick with that time for all subsequent plates for other reps and time points.
2. While the plate incubates, turn on the plate reader and computer. The plate reader will probably make some noises and retract the plate holder in and out a couple times. Select the Gen5 / plate reader user. Double click the Gen5 software icon.
3. Select the appropriate protocol, or create a new one. You want an endpoint reading, two absorbance readings (@ 550 nm and 800 nm). No need for shaking or path length correction, or messing with the temp control.
4. The plate reader is ready when the plate holder is out and the software knows the temp inside the plate reader (indicated by the plate reader icon at the top of the software window, should be a temp next to it, and not ‘???’)
5. Once the plate has finished incubation in the fridge and the plate reader is good to go, you’re ready for the reading! Carefully remove your plate from the 4ºC fridge and set a timer for 30 seconds before reading. Before placing the plate in the plate reader, check that there is no condensation on the bottom of the plate. If there is, use a kimwipe and 100% ethanol to quickly wipe it clean, being careful not to warm the plate through touch.

Yeah it seems paranoid, but please for the love of God you must do this.

1. Export the data to an excel sheet when prompted. Save the data, and put it on an external drive so you can take it away!
2. Plot your standard curve and use the equation to figure out the concentrations of soluble peroxides in your samples 😊 Normalize by fresh sample weights.

