Detection of Hydrogen Peroxide by DAB Staining in Arabidopsis Leaves

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Summary of Q&A (updated on Sept. 19, 2016)

(To increase readability of the Q&A section and avoid redundant questions to be asked to our authors, useful information from the Q&A section is summarized by Bio-protocol Editors and published below after authors' review.)

1. Experimental procedure

Q#1. *What are the reasons for adding Tween 20 and Na_2HPO_4 to DAB solution?*

A. Tween 20 aids in the even dispersal and uniform infiltration of DAB. A precisely buffered and pH maintained DAB solution maintains its stability for longer during the assay. Both these features contribute to the robustness and reproducibility of this protocol

Q#2. *Is it normal that the DAB solution develops a white precipitate when added into the base?*

A. I wouldn't expect a white precipitate. Could something be precipitating in the buffer from before? Incomplete acidifying of the DAB has led to light brown precipitation in our hands. If that is what you're seeing, a low level of that should not affect the experiment. In addition to this, after publishing this protocol, I realized that using the DAB tetrachloride salt prevents the need for the acidification step. With this, I never saw any DAB precipitation again. Such search for 3,3'-Diaminobenzidine tetrahydrochloride (the one by Santa Cruz sc-209686 works fine. I'm quite sure the one from Sigma will be fine too).

Q#3. *How much water and DAB could be used to make a 1 L DAB solution?*

A. To make 1 L DAB solution, I would recommend adding 900 ml sterile distilled water to 1 g DAB, then add proportional Tween-20 and top up with 200 mM Na_2HPO_4. Also, try to use acidified DAB instead of just DAB, then you don't need to do the acidification step in my protocol.

Q#4. *Should I increase the incubation time in DAB and by how much, considering that my staining is a bit faint after infiltration incubated in DAB for 4 hours?*

A. Yes, incubating longer (5 - 8 hours) is OK. Did you de-stain efficiently? Did you ensure that the vacuum infiltration was correctly carried out and the vacuum pressure was relatively strong (this will enable the stain to be taken up by the leaves)? Was your DAB prepared fresh and was the DAB staining solution quite homogenous? It is difficult to precisely answer your question as several aspects can impact the strength of your stain, but if you keep all these points (and of course several others in your experimental design), then hopefully you'll be able

to see a stronger stain. Does your positive control definitely expect higher stain / ROS production?

Q#5. *Does Ethanol: Acetic Acid: Glycerol=3:1:1 mean that I should put 3 portion ethanol, 1 portion acetic acid and 1 portion glycerol in 5 portion water?*

Ethanol: Acetic Acid: Glycerol=3:1:1 means 3 portion ethanol, 1 portion so on. Don't make it in water. Take 300 ml Ethanol, add 100 ml Acetic acid and 100 ml glycerol, and use this as the bleaching solution.

2. Data analysis

Q#6. *How to photograph the leaves on a plain white background under uniform lighting?*

A. Depends on what you want to look at. If you want an overview of the distribution and overall intensity of the DAB stain, then just place the de-stained leaf on a plain white background under uniform lighting and you should get a clear image of the stain.
If you want to look at it under the microscope, just mount the leaf as you would normally on a glass slide and view under a light microscope. As the stain requires no phase contrast or fluorescence detection, it is simple to adjust the plane according to what cells you wish to see.

Q#7. *How to determine the concentration of hydrogen peroxide, by the color of stains or by the number of stains?*

A. Both the intensity and distribution of the stain can be used to determine the extent of DAB staining, which is consequently proportional to hydrogen peroxide accumulation levels. Both of these can be worked out using a variety of bio-imaging software available such as ImageJ or CellProfiler. A bit of effort is required to set up these methods and the best advice I can give is reading the tutorials on these software to learn their capability, then playing around with high- resolution photographs of your DAB stains. However, DAB is not a specifically quantitative stain in the protocol that I have described, and I do not know of a way to quantify the actual, accurate concentration of H2O2 with our methods. For that, you can use other methods such as xylenol orange or luminol detection.

Q#8. *How to determine whether the H_2O_2 production or dark brown color is caused by elicitors or by wounding like the syringe injection?*

A. This is where experimental skill comes in. It took me and several other members of the lab, as well as a large number of collaborators and additional colleagues many, many attempts to get it right. The thing to get right is not to wound the leaf, which can happen with "heavy hands" or mishandling. It is very common with students and first-timers. Over time, a good experimenter will learn the amount of pressure that is required to correctly infiltrate the leaf tissue. Applying

water 30 minutes below on the leaf surface that you will be infiltrating has helped as well. It enables the liquid to be dispersed more easily into the apoplast. What is important to keep in mind is NOT to force the liquid in, as this will damage the leaf surface. With practice and correct positioning of the syringe, you will slowly learn that with the right gentle finger pressure, the liquid actually fills very passively and easily into the tissue.

Q#9. *Can I assume that the brown coloration is in response to pathogen attack, so all brown stains are produced by the pathogen presence, given that DAB is oxidized by H_2O_2?*

A. DAB is oxidized by hydrogen peroxide, which in turn leads to the dark brown precipitate/color you see. Hydrogen peroxide is not necessarily only produced by pathogen attack (i.e. the oxidative burst) - This is where you may be having a problem. ROS is produced due to all kinds of stresses on plant tissue - so if your samples are stressed for any reason, there may be background ROS (and hence the brown stain). The key is to minimize any other stress so the hydrogen peroxide produced by the pathogen attack is relatively higher than basal levels, and this difference is observed by staining.

Q#10. *How to handle ImageJ software and Cellprofiler with light microscopic images?*

A. The principle behind using ImageJ or CellProfiler is essentially the same. Since I prefer CellProfiler, I would recommend trying that (http://www.cellprofiler.org/). You will notice that they have extensive tutorials and FAQs that can be applied to any type of image analysis, not just ROS staining with DAB. Briefly, CellProfiler has a tool with which you can manually identify regions of interest on your leaf. In the case of DAB, you would use pixel intensity. Over a certain pixel intensity threshold, you would set the identification as "DAB stain". Next, you can feed as many images as you like into that pipeline and it will sort based on the threshold you set. With this, you can get a quantitative assessment of the amount of staining in your tissue.

3. Protocol suitability for other plant tissues

Q#11. *Would it be good to make the staining with leaf discs for oil palm leaves instead of using the complete leaf?*

A. Using leaf discs is actually a more common way to do DAB (and most types of ROS) staining. I just did whole leaves because it is easy with Arabidopsis as they're small, similar sized and easy to fit into 12 and 6 well plates. I would encourage you to use leaf discs for you oil palm leaves. The bigger the better, so 0.5 cm diameter minimum I would suggest as I guess your leaves are quite big. There will be mechanical stress when you cut the leaves (ideally with a cork borer), but the way to overcome this is to allow your leaf discs to equilibrate and stabilize in sterile water for about 12 - 24 hours (without any agitation). Then you can proceed to do the

DAB assay on those discs as described above. Also make a note of the new DAB I recommend to use.

Q#12. *Whether this method can be applied to apple leaves?*

A. There is no reason why not. I have not tried with Apple leaves. This protocol would definitely need to be modified based on your tissue though, and they're several places where it can be modified if you think about it in a smart way. For e.g. rice and wheat leaves are tougher than Arabidopsis, thicker, with more waxy cuticle etc. Therefore, you need to ensure the vacuum infiltration is effective. This is how the leaf tissue takes up the stain. For e.g. I modified that part of the protocol to use a tougher/stronger vacuum pump. You can also play around with the duration of staining and de-staining. There are additional ways to de-stain a leaf, you can boil for longer, you can change the de-stain solution more times etc. So, in summary, yes you should be able to adapt this protocol for apple leaves.

Q#13. *Whether this protocol can stain Arabidopsis roots?*

A. The DAB stain has been used by several other researchers to stain *Arabidopsis* root tissue. My protocol, however, is designed for leaf tissue; hence the destain step. Since roots do not have chlorophyll, in principle you can modify this protocol but remove the destain step.

Author's update:

It is possible to buy a DAB tetrachloride salt, which enables it to dissolve much better in an aqueous solution. Using this version of DAB, it is possible to avoid the acidifying step.