

Detection of Hydrogen Peroxide by DAB Staining in *Arabidopsis* Leaves

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[Abstract] In this protocol, the *in situ* detection of hydrogen peroxide (one of several reactive oxygen species) is described in mature *Arabidopsis* rosette leaves by staining with 3,3'-diaminobenzidine (DAB) using an adaptation of previous methods (Thordal-Christensen *et al.*, 1997; Bindschedler *et al.*, 2006; Daudi *et al.*, 2012). DAB is oxidized by hydrogen peroxide in the presence of some haem-containing proteins, such as peroxidases, to generate a dark brown precipitate. This precipitate is exploited as a stain to detect the presence and distribution of hydrogen peroxide in plant cells. The protocol can be modified slightly to detect hydrogen peroxide in different types of plant tissue.

Materials and Reagents

1. *Arabidopsis* plants
2. DAB non-acidified powder (Sigma-Aldrich, catalog number: D8001)
3. Tween 20 viscous liquid molecular biology grade (Sigma-Aldrich, catalog number: P9416)
4. Sodium phosphate (Na_2HPO_4) electrophoresis grade (Sigma-Aldrich, catalog number: S3397)
5. Aluminum foil
6. HCl
7. Na_2HPO_4
8. Ethanol
9. Acetic acid
10. Glycerol
11. DAB staining solution (see Recipes)
12. 200 mM Na_2HPO_4 (see Recipes)
13. Bleaching solution (see Recipes)

Equipment

1. *Arabidopsis* growth chamber
2. Magnetic stirrer and stirring bar
3. pH meter
4. 1 ml needleless syringes
5. 12-well microtiter plate
6. Dessicator
7. Shaker
8. Water bath
9. 50 ml falcon tube

Procedure

A. Preparation of DAB staining solution

1. In 50 ml falcon tube, add 50 mg DAB and 45 ml sterile H₂O for a final 1 mg ml⁻¹ DAB solution.
2. Add small magnetic stirrer and reduce pH to 3.0 with 0.2 M HCl (to dissolve DAB).
3. Cover tube with aluminium foil since DAB is light-sensitive.
4. Add 25 µl Tween 20 (0.05% v/v) and 2.5 ml 200 mM Na₂HPO₄ to the stirring DAB solution.
5. This will generate a 10 mM Na₂HPO₄ DAB staining solution and will pull the pH back up again.

Note: Sometimes the DAB will still not fully dissolve, but usually very high levels of homogeneity in the solution are achieved. The DAB solution is only good for the day, made fresh.

B. Staining leaves with DAB solution

6. Grow *Arabidopsis* plants under normal conditions, or as per your requirements.
7. Select rosette leaves on plants that are just pre-bolting (3-4 weeks typically).
8. Apply treatment of choice, for example wounding or pathogen infiltration.
9. As an example, we typically apply 100 µl of microbial elicitor solution dissolved in water (e.g. 0.5 µM Flg22) injected directly into the leaf via a 1 ml needleless syringe. Flg22 is a bacterial peptide epitope that is commonly used to trigger innate immune responses in plants.
10. Ensure that similar, mature rosette leaves are selected for your treatment of choice.
11. Sample at least 3 leaves per plant from 6 independent plants (biological replicates).
12. It is good practice to repeat the entire experiment at least once to generate robust data.

13. Allow plants to incubate depending on treatment. We typically leave the plants for 1 h if we are studying biotic stress responses following Flg22 infiltration for example.
14. Sample the leaves at the desired time point by manually removing each leaf from the plant and placing in a 12-well microtiter plate. It is ok to place two or three leaves in one well.
15. Apply 2 ml of the DAB staining solution to the leaf or leaves in the well. Adjust the volume to ensure that leaves are immersed.
Note: It may be difficult to judge because these leaves are naturally hydrophobic, but if there is enough liquid for the total leaf tissue volume, then shaking the plate in the following steps will ensure that all parts of the leaf are in contact with the solution.
16. Apply 2 ml of 10 mM Na₂HPO₄ as the control treatment for replicate leaves.
17. Ensure that the DAB solution is taken up by the leaf by gently vacuum infiltrating the leaves. This is achieved by placing the 12-well plates in a dessicator and applying gentle vacuum for 5 min.
18. Cover the 12-well plate with aluminium foil (since DAB is light-sensitive).
19. Place the plate on a standard laboratory shaker for 4-5 h at 80-100 rpm shaking speed.
Note: 4 h is optimum time for the DAB stain to develop for many biotic interactions where the level of hydrogen peroxide produced is quite high. It is perfectly fine to increase this incubation time if the interaction being studied produces lower levels of sustained hydrogen peroxide production. We have tried up to 8 h staining with no detrimental effects observed in the assay.
20. Following the incubation, remove the foil and replace the DAB staining solution with bleaching solution (ethanol: acetic acid: glycerol = 3:1:1).
21. Place the 12-well plate carefully in a boiling water bath (~90-95 °C setting on water bath is OK) for 15 min. This will bleach out the chlorophyll but leave the brown precipitate formed by the DAB reacting with the hydrogen peroxide. The time can be adjusted by ± 5 min depending on the appearance of the leaves (they should be completely devoid of chlorophyll).
22. After 15 ± 5 min of boiling, replace the bleaching solution with fresh bleaching solution and allow to stand for 30 min. Samples at this stage can be stored at 4 °C for up to 4 days with no detrimental effects observed in our hands.
23. Leaves can be directly visualized for DAB staining. Photographs are recommended on a plain white background under uniform lighting.

Recipes

1. DAB staining solution (please see procedure 1)

2. 200 mM Na₂HPO₄
pH > 6.8
3. Bleaching solution
Ethanol: acetic acid: glycerol = 3:1:1

Acknowledgments

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