Histochemical Detection of Superoxide and H_2O_2 Accumulation in *Brassica juncea* Seedlings

Deepak Kumar[1], Mohd Aslam Yusuf[1], Preeti Singh[1], Meryam Sardar[2] and Neera Bhalla Sarin[2*]

[1]School of Life Sciences, Jawaharlal Nehru University, New Delhi, India; [2]Department of Biosciences, Jamia Millia Islamia, New Delhi, India

*For correspondence: neerasarin@rediffmail.com

[Abstract] Plant cells continually produce reactive oxygen species (ROS) as a by-product of aerobic metabolism. Increased production of ROS occurs under unfavorable conditions imposed by various abiotic and biotic factors. Accumulation of ROS is damaging to various cellular components and macromolecules including plasma membrane, nucleic acids, and proteins and eventually leads to cell death. In this protocol, we describe the histochemical detection of hydrogen peroxide (H_2O_2) and superoxide (O_2^-) anion, two of the most important ROS, in *Brassica juncea* seedlings by using 3, 3'-Diaminobenzidine (DAB) and Nitrotetrazolium blue chloride (NBT) as the chromogenic substrate. DAB is oxidized by H_2O_2 in the presence of peroxidases and produces reddish brown precipitate. NBT reacts with O_2^- to form a dark blue insoluble formazan compound. The protocol can be used in other plant species and for different plant tissues.

Materials and Reagents

1. *Brassica juncea* seedlings
2. Vermiculite
3. Absolute ethanol
4. 60% Glycerol
5. Aluminium foil
6. Test tubes
7. Measuring cylinder
8. Distilled water
9. Nitrotetrazolium blue chloride (NBT) (Sigma-Aldrich, catalog number: N6639)
10. 3,3'-Diaminobenzidine (DAB) (Sigma-Aldrich, catalog number: D8001)
11. Sodium phosphate buffer (see Recipes)
12. NBT staining solution (see Recipes)
13. DAB staining solution (see Recipes)

Equipment

1. Paper towel
2. Weighing balance
3. Aluminium foil
4. Magnetic stirrer
5. pH meter
6. Water bath

Procedure

1. *Brassica juncea* seedlings grown on vermiculite for 15 days at 22 °C, 75 % relative humidity, and a photoperiod of 16 h light and 8 h dark are treated with a stress inducing agent [for *e.g.* 200 mM NaCl (for salinity stress), 200 mM mannitol (for drought stress), or 20 mM $CdCl_2$ (for heavy metal stress) for 3 days]. The untreated seedlings grown under the same conditions serve as the experimental control.
2. Remove the seedlings from the pot and wash them with distilled water to remove any extraneous material associated with the tissues.
3. Place the seedlings in test tubes and immerse them in DAB or NBT staining solution for detection of H_2O_2 or O_2^-, respectively. Wrap the tubes with aluminium foil and keep overnight at room temperature.
4. Drain off the staining solution from the test tubes.
5. Remove the chlorophyll for proper visualization of the stain. This is done by immersing the seedlings in absolute ethanol and heating in a boiling water-bath for 10 min (or more if necessary, with intermittent shaking).
6. Transfer the seedlings on a paper towel saturated with 60% glycerol.
7. Photograph the stained seedlings against a contrast background for proper documentation. H_2O_2 is visualized as reddish brown stain formed by the reaction of DAB with the endogenous H_2O_2 (Figure 1a). The O_2^- content is detected as dark blue stain of formazan compound formed as a result of NBT reacting with the endogenous O_2^- (Figure 1b).

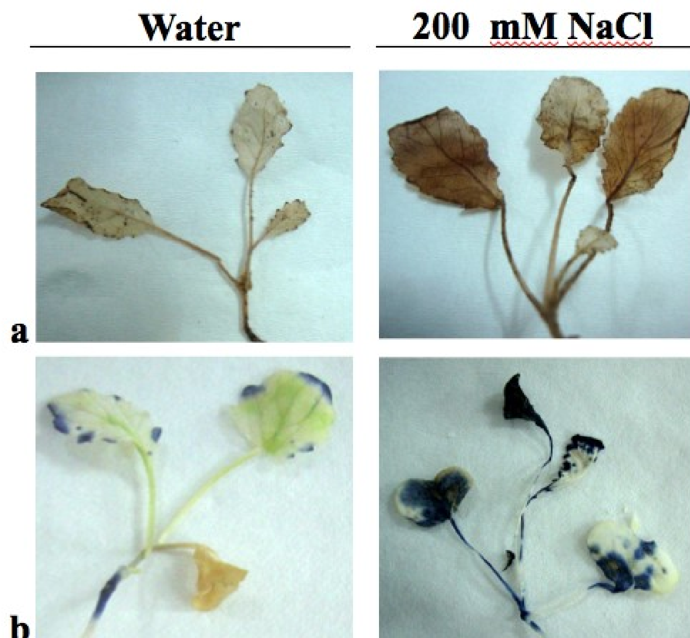

Figure 1. Detection of Hydrogen peroxide (a) and superoxide anion (b) accumulation in *Brassica juncea* seedlings exposed to 200 mM NaCl stress for 3 days. Seedlings grown in the presence of water served as the untreated control.

Recipes

1. Sodium phosphate buffer (pH 7.5)
   a. Prepare 1 M Sodium phosphate monobasic (NaH_2PO_4) solution by dissolving 12 g NaH_2PO_4 in a final volume of 100 ml distilled water.
   b. Prepare 1 M Sodium phosphate dibasic (Na_2HPO_4) solution by dissolving 14.2 g Na_2HPO_4 in a final volume of 100 ml distilled water.
   c. Mix 16 ml 1 M NaH_2PO_4 and 84 ml 1 M Na_2HPO_4 solutions and make the volume to 2 L with distilled water for preparing 50 mM sodium phosphate buffer (pH 7.5).
2. DAB staining solution
   a. Dissolve 50 mg DAB in approximately 45 ml distilled water in an amber colored bottle.
   b. Adjust the pH to 3.8 using 0.1 N HCl while mixing properly on a magnetic stirrer. On complete dissolution the solution will be clear and light brown in color.
   c. Make the volume to 50 ml to get 1 mg/ml solution.
   d. DAB staining solution should be prepared freshly before use.
3. NBT staining solution
   a. In an amber colored bottle, dissolve 0.1 g NBT in 50 mM sodium phosphate buffer (pH 7.5) and make the volume to 50 ml to get a 0.2% solution.

b. Mix the solution thoroughly using a magnetic stirrer.

c. NBT staining solution should be prepared freshly before use.

Acknowledgments

The protocol was implemented in a project that was partially funded by the Council of Scientific and Industrial Research (CSIR) Grant No. 38/1126/EMR-II. DK and PS acknowledge the financial support from CSIR and University Grants Commission (UGC). MAY was a UGC-Dr. D.S. Kothari Postdoctoral Fellow during the course of this project. Research in the laboratory of NBS is supported by U.G.C.-C.A.S., U.G.C.-R.N.W., Department of Science and Technology (D.S.T.)-F.I.S.T., and D.S.T.-PURSE. The authors would like to acknowledge Thordal-Christensen *et al.* (1997) and Jabs *et al.* (1996) (kindly see the reference section) whose work was adapted in the present protocol.

References

1. Jabs, T., Dietrich, R. A. and Dangl, J. L. (1996). Initiation of runaway cell death in an *Arabidopsis* mutant by extracellular superoxide. *Science* 273(5283): 1853-1856.
2. Kumar, D., Yusuf, M. A., Singh, P., Sardar, M. and Sarin, N. B. (2013). Modulation of antioxidant machinery in alpha-tocopherol-enriched transgenic *Brassica juncea* plants tolerant to abiotic stress conditions. *Protoplasma* 250(5): 1079-1089.
3. Thordal-Christensen, H., Zhang, Z., Wei, Y. and Collinge, D. B. (1997). Subcellular localization of H_2O_2 in plants. H_2O_2 accumulation in papillae and hypersensitive response during the barley-powdery mildew interaction. *Plant J* 11: 1187-1194.