

Histochemical Detection of Superoxide and H₂O₂ Accumulation in *Brassica juncea* Seedlings

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[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 (H₂O₂) and superoxide (O₂⁻) 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 H₂O₂ in the presence of peroxidases and produces reddish brown precipitate. NBT reacts with O₂⁻ 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 CdCl₂ (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 H₂O₂ or O₂⁻, 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. H₂O₂ is visualized as reddish brown stain formed by the reaction of DAB with the endogenous H₂O₂ (Figure 1a). The O₂⁻ content is detected as dark blue stain of formazan compound formed as a result of NBT reacting with the endogenous O₂⁻ (Figure 1b).

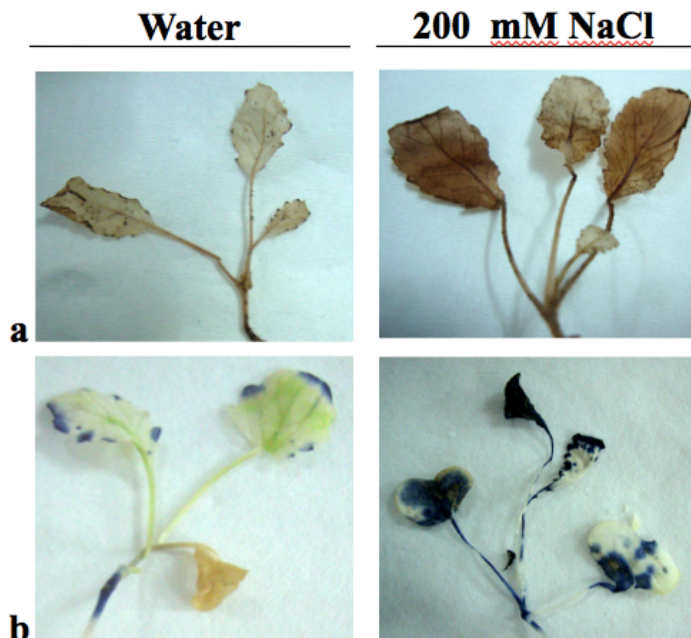


Figure 1. Detection of Hydrogen peroxide (a) and superoxide anion (b) accumulation in *Brassica juncea* seedlings exposed to 200 mM NaCl stress for 3 days. Seedlings grown in the presence of water served as the untreated control.

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.

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