Chapter 15

Determination of Reactive Oxygen Species in Salt-Stressed Plant Tissues

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Abstract

Reactive oxygen species (ROS) participate in signaling events that regulate ion channel activity and gene expression. However, excess ROS exert adverse effects that stem from their interaction with macromolecules. Thus, the assessment of the effects of salinity on ROS changes are central to understanding how plants respond and cope with this stress. ROS determination in salt-stressed plants poses specific challenges. On the one hand, salinity comprises osmotic and ion-specific effects which may, in turn, have different effects on ROS production. On the other hand, changes in ROS production may happen when tissues from salinized plants are subject to water potential (Ψ) changes when incubated in non-isosmotic solutions. This chapter provides detailed accounts of methods for ROS detection in tissues from salt-stressed plants and includes suggestions for avoiding artifacts when dealing with such tissues.

Key words: Reactive oxygen species, Hydrogen peroxide, Superoxide, Hydroxyl radical, Salt stress, Fluorescence, Tetrazolium salts

1. Introduction

Reactive oxygen species (ROS) are highly reactive oxygen derivatives comprising both oxygen radicals and certain nonradicals (1). Oxygen radicals are independent species with unpaired electrons and include singlet oxygen (O_2^1), superoxide radicals ($^{\bullet}O_2^-$) and the hydroxyl radical ($HO^{\bullet}$). Nonradical ROS include H_2O_2 and ozone (O_3), among others.

ROS are generated in most plant cell compartments (2) and controlled ROS production appears to be a general characteristic of expanding plant cells and organs (3), where ROS participate in signaling events (4, 5) that regulate ion channel activity (6) and gene expression (7). However, ROS levels generally increase in many plant parts under biotic (8) and abiotic (9) stress conditions,

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including salinity (10). Excess ROS exert adverse effects (oxidative damage) that stem from their interaction with macromolecules such as lipids, proteins, and nucleic acids.

The ROS balance response to salt stress is expected to differ among tissues (11) and cell compartments (12, 13). Thus, the assessment of the effects of salinity on ROS changes is central to understanding how plants respond and cope with this stress. Techniques for measuring ROS have been extensively reviewed (14), and only a few specific references on plant systems are mentioned here (15–18).

ROS determination in salt-stressed plants poses specific challenges (19). On the one hand, osmotic and ion-specific effects of salinity on ROS production may be different (19, 20), while on the other hand, changes in ROS production may occur when tissues from salinized plants are subject to water potential (Ψ) changes (21) when incubated in non-isosmotic solutions (20). Essentially, when assessing ROS production under saline conditions, it is necessary to include realistic estimates of apoplastic ion concentration in the incubation medium, and to take into account the plant tissue Ψ.

This chapter provides accounts of methods for ROS detection in tissues from salt-stressed plants that we have personally practiced. Qualitative, histochemical methods are described for detecting ROS by epifluorescence, H_2O_2 by electron microscopy, and, $^{\bullet}O_2^-$ with nitro blue tetrazolium (NBT). Quantitative assessment methods for H_2O_2, $^{\bullet}O_2^-$, and $HO^{\bullet}$ production are also detailed. A method for the obtention of apoplastic fluid to estimate ion concentration in this compartment is also included.

ROS detection by epifluorescence is based on the formation of a fluorescent compound from 2′,7′-dichlorofluorescin diacetate (DCFH-DA) when the acetate group is cleaved and nonfluorescent 2′,7′-dichlorofluorescin (DCFH) is oxidated to the fluorescent DCF product in a peroxidase-dependent reaction (22). Endogenous peroxidase activity is considered to be sufficient to sustain this reaction. The non-polar unreactive DCFH-DA can be taken up by the cells and subsequently deacetylated by endogenous esterases to render the polar DCFH (23), to which the cell membrane is supposedly impermeable. Alternatively, DCFH-DA may be first deacelytated by apoplastic esterases and it will then detect extracellular ROS.

Subcellular H_2O_2 detection by electron microscopy is based on the formation of cerium perhydroxide crystals that are deposited after the reaction of $CeCl_3$ with endogenous H_2O_2 (24). Positive staining is detected in electron micrographs as the formation of electron-dense deposits. In vivo H_2O_2 production can be measured as the formation of a pink adduct that results from the reaction between H_2O_2, 4-aminoantipyrine (4-AAP) and 3,5-dichloro-2-hydroxybenzene sulfonic acid (DCHBS) in a peroxidase (POX)-dependent reaction (25).

The qualitative determination of $^{\bullet}O_2^-$ with NBT depends on the formation of a blue insoluble formazan precipitate that results from the reaction between $^{\bullet}O_2^-$ and NBT. Tetrazolium compounds that form soluble formazans can be used to quantify $^{\bullet}O_2^-$ production. The reaction between $^{\bullet}O_2^-$ and Na, 3′-[1-[(phenylamino)-carbonyl]-3, 4-tetrazolium](4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) produces a soluble formazan that can be measured spectrophotometrically (26).

$HO^{\bullet}$ production can be quantified by spectrofluorometry as the formation of hydroxyl benzoate resulting from the reaction of $HO^{\bullet}$ and benzoate (BZ) (27).

As mentioned above, realistic estimates of apoplastic ion concentration should be included in the incubation media. The methods to obtain apoplastic fluid usually involve tissue centrifugation at low speed and a check for symplastic contamination by determining the glucose 6-P dehydrogenase (G6PDH) activity (3, 28).

2. Materials

The methods described below are for either intact seedlings or excised plant parts (2, 13, 19, 29). See Note 1 for tissue manipulation tips and Note 2 for the comments on the composition of incubation solutions.

All solutions should be prepared using distilled or deionized water and analytical grade reagents. Solutions can be prepared at room temperature and stored at 4°C (unless otherwise indicated).

2.1. Obtaining the Apoplastic Fluid and Estimating the Cytoplasmic Contamination and Na^+ Concentration

1. Net bags.
2. Falcon and Eppendorf tubes.
3. Spectrophotometer (UV lamp).
4. Atomic emission spectrophotometer or flame photometer.
5. Unrefrigerated centrifuge.
6. Reagents for determining G6PDH activity (30) and protein concentration (31).

2.2. Localized ROS Detection in Intact Seedlings Using 2′,7′-Dichlorofluorescin and Epifluorescence (See Note 3 on Specificity and Controls)

1. Intact plants or excised plant sections.
2. Petri dish.
3. Microwave oven.
4. Thermometer.
5. Epifluorescence or stereoscopic microscope with excitation filter BP 450–490 and emission filter LP 520 and attached camera. Alternative: confocal microscope with a 405 nm diode laser as source of excitation light and acquisition through a 515/30 BP filter (green channel).

6. Solutions and inclusion medium.
   (a) *2',7'-dichlorofluorescin diacetate (DCFH-DA)*. For 1 mL of 25 mM DCFH-DA in ethanol, weigh 12 mg DCFH-DA into a 1.5 mL Eppendorf tube. Add 1 mL absolute ethanol and mix. Wrap the tube with aluminum foil. If not used immediately, this solution can be stored frozen at –20°C for up to 3 months.
   (b) *Potassium phosphate buffer*. For 50 mL of 20 mM potassium phosphate buffer, pH 6, weigh 23 mg KH_2PO_4 and 118 mg KH_2PO_4 and transfer to a glass beaker containing 40 mL water. Mix and adjust pH with HCl (see Note 4). Make up to 50 mL with water.
   (c) *KI control*. Weigh 1.66 g KI and add to the buffer solution above before adjusting to the final volume.
   (d) *Agar mixture*. Weigh 1 g agar. Add 90 mL of 20 mM potassium phosphate buffer to a 200 mL graduated glass beaker. Mix and place in a microwave oven and heat at maximum power setting for 1 min. Return the glass beaker to room temperature. Add 0.2 mL 25 mM DCFH-DA solution and wrap with aluminum foil. Make up to 100 mL. The final solution is 1% (w/v) agar, in 20 mM phosphate buffer pH 6, and 50 µM DCFH and should be prepared immediately before use (see Notes 5 and 6).

2.3. Subcellular H_2O_2 Detection by Electron Microscopy

Positive staining is detected in electron micrographs as the formation of electron-dense deposits. A treatment with KI is an appropriate control.

The solutions and procedures indicated do not include the processing of the plant material for transmission electron microscopy observation, detail is provided until the fixation step. From there on, use standard transmission electron microscopy techniques to embed, slice, mount, shade, and observe the tissue.

1. Small plant sections (approximately 3 mm or smaller), briefly rinsed after cutting to remove wound-induced ROS.
2. Electron microscopy embedding and mounting materials and equipment.
3. Transmission electron microscope.
4. Solutions.
   (a) *$CeCl_3$*. For a 5 mM $CeCl_3$ solution in 5 mM MOPS buffer, pH 7.2, weigh 62 mg $CeCl_3$ and 58 mg MOPS and transfer to 40 mL water in a glass beaker. Mix and adjust pH with NaOH (see Note 6). Make up to 50 mL with water. Transfer to a 50 mL Falcon tube wrapped with aluminum foil. Prepare and use the same day, do not store.
   (b) *KI control*. Weigh 1.66 g KI and add to the solution above before adjusting to final volume.

(c) *Sodium phosphate buffer*. For 100 mM sodium phosphate buffer pH 7.0 solution, weigh 0.584 g $NaH_2PO_4 \cdot H_2O$ and 1.547 g $Na_2HPO_4 \cdot 7H_2O$ and transfer to a glass beaker containing approximately 70 mL water. Mix and adjust pH with HCl (see Note 4). Make up to 100 mL with water.

(d) *Glutaraldehyde*. For 2.5 % glutaraldehyde in sodium phosphate buffer solution, add 40 mL sodium phosphate buffer solution to a 100 mL graduated glass beaker. Weigh 1.25 g glutaraldehyde and transfer to a glass beaker containing approximately 40 mL 100 mM sodium phosphate buffer pH 7.0. Mix, make up to 50 mL with sodium phosphate buffer solution, and store.

2.4. Determination of $^{\bullet}O_2^-$ with Nitro Blue Tetrazolium

Controls for this technique must include an effective $^{\bullet}O_2^-$ dismutating catalyst such as superoxide dismutase (SOD) or Mn salts.

1. Plant parts or tissue sections.
2. Falcon or Eppendorf tubes.
3. Vacuum pump.
4. Vacuum desiccator.
5. Glass slides or plates for mounting.
6. Camera.
7. Solutions.

   (a) *Nitro blue tetrazolium (NBT)*. To prepare 50 mL of 122 μM NBT solution, weigh 5 mg NBT and transfer to a glass beaker containing approximately 40 mL water. Mix and make up to 50 mL with water. Transfer to a 50 mL Falcon tube wrapped with aluminum foil. For salinized plants, weigh an appropriate amount of NaCl or sorbitol (depending on whether the material to be tested is Na^+-including or not) and add before making up to final NBT solution volume (see Note 2). If not used immediately, solutions can be stored frozen at –20°C for up to 3 months.

   (b) *$MnCl_2$*. For a 10 mM $MnCl_2$ solution, weigh 63 mg $MnCl_2$ and transfer to a glass beaker containing approximately 40 mL water. Mix and make up to 50 mL with water.

   (c) *9:1 ethanol and glycerin*. Make up enough to completely submerge the stained tissue and sustain 30 min boiling.

2.5. Spectrophotometric Determination of In Vivo H_2O_2 Production

Use a KI treatment as a control for this technique.

1. Plant sections.
2. Falcon or Eppendorf tubes.

3. Vacuum pump.
4. Vacuum desiccator.
5. Spectrophotometer (visible light wavelength).
6. Solutions.
   (a) *4-aminoantipyrine (4-AAP)*. For a 5 mM 4-AAP solution, weigh 10 mg AAP and transfer to a 15 mL Falcon tube containing 5 mL water. Mix, make up to 10 mL with water, and store.
   (b) *Reaction mixture*. 100 μM 4-AAP, 1 mM 3, 5-dichloro-2-hydroxybenzene sulfonic acid (DCHBS), 0.06 mg^{-1} mL^{-1} horseradish POX (HPOX). Add 40 mL water to a 100 mL graduated glass beaker. Weigh 13 mg DCHBS, 3 mg HPOX, and transfer to the glass beaker. Add 1 mL 5 mM 4-AAP solution. Mix and make up to 50 mL with water. Transfer to a 50 mL Falcon tube wrapped with aluminum foil. For the NaCl treatment, weigh an appropriate amount of NaCl or sorbitol and add before mixing (see Note 2). For the H_2O_2 specificity control, add 1.66 g KI to the reaction mixture before adjusting to the final volume.
   (c) *Ethanol-glycerin*. For 9:1 ethanol-glycerin solution, add 45 mL ethanol and 5 mL glycerine to a glass beaker and mix to homogeneity. Transfer to a 50 mL Falcon tube.

2.6. Spectrophotometric Determination of In Vivo $^{\bullet}O_2^-$ Production

Suitable controls for this technique must include an effective $^{\bullet}O_2^-$ dismutating agent such as SOD or a Mn salt.

1. Falcon or Eppendorf tubes.
2. Vacuum pump.
3. Vacuum desiccator.
4. Spectrophotometer (visible light wavelength).
5. Solutions.
   (a) *$MnCl_2$*. For a 10 mM $MnCl_2$ solution, weigh 63 mg $MnCl_2$ and transfer to a glass beaker containing approximately 40 mL water. Mix and make up to 50 mL with water.
   (b) *3'-[1-[(phenylamino)-carbonyl]-3, 4-tetrazolium](4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) solution*. For 0.5 mM XTT, weigh 17 mg XTT and transfer to a glass beaker containing approximately 40 mL water. Mix and make up to 50 mL with water. Transfer to a 50 mL Falcon tube wrapped with aluminum foil. For the NaCl treated plant material, weigh an appropriate amount of NaCl or sorbitol and add before mixing (see Note 2). For the $^{\bullet}O_2^-$ specificity control, add 0.5 mL of the $MnCl_2$ solution or SOD (50 μm mL^{-1}) before making up to the final volume. If not used immediately, solutions can be stored frozen at –20°C for up to 3 months.

2.7. Fluorometric Determination of In Vivo $HO^{\bullet}$ Production

1. Falcon or Eppendorf tubes.
2. Vacuum pump.
3. Vacuum desiccator.
4. Spectrofluorometer.
5. *Benzoate (BZ) solution*. For a 2.5 mM BZ solution, weigh 18 mg BZ and transfer to a glass beaker containing water. Mix and make up to 50 mL with water. Transfer to a 50 mL Falcon tube wrapped with aluminum foil. For the salt-treated plants, weigh an appropriate amount of NaCl or sorbitol and add before mixing (see Note 2). Prepare and use the same day, do not store.

3. Methods

3.1. Obtaining the Apoplastic Fluid and Estimating the Cytoplasmic Contamination and Na^+ Concentration

1. Prepare a 15 mm long section from a plastic tube that can fit into a 50 mL Falcon tube, depending on the size of the plant material. Seal one end with plastic net. Introduce a net bag inside the tube. The net pores should be smaller than the surface of the plant segments (see next point).
2. Carefully introduce approximately 100 excised, rinsed leaf or root segments (10 mm in length) in the net bag. Make sure they are all positioned parallel to the tube side. Seal the net bag loosely to prevent the segments from moving.
3. Centrifuge the tubes for 1 min at $1{,}000 \times g$ to remove any rinsing solution.
4. Centrifuge again for 10 min at $2{,}000 \times g$ to collect the apoplastic fluid. Store the apoplastic fluid at –20°C.
5. Use the apoplastic fluid to check G6PDH activity, protein concentration, and Na^+ concentration. Several batches of apoplastic fluid will be necessary to carry out all assays. Compare apoplastic G6PDH activity on a protein concentration basis with that of whole tissue homogenate. It should not be higher than 5 % of whole tissue activity when the apoplastic fluid is not contaminated with cytosol.
6. Dilute uncontaminated apoplastic fluid samples with an appropriate water volume to determine Na^+ concentration by atomic emission spectrophotometry or flame photometry. See comments on estimation of apoplastic ion concentrations in Note 7.

3.2. Localized ROS Detection in Intact Seedlings Using 2′,7′-Dichlorofluorescin and Epifluorescence

1. Measure the temperature of the agar mixture. When it reaches 30°C, pour it on a Petri dish and submerge the plant material (see Notes 8 and 9). Incubate in the dark.
2. After 15–30 min, place the Petri dish under an epifluorescence or confocal microscope to observe DCF fluorescence (see Note 10). Briefly check the material and take a picture (see Notes 11 and 12).

3.3. Subcellular H_2O_2 Detection by Electron Microscopy

1. Place the plant sections in an Eppendorf tube and completely submerge in the $CeCl_3$ solution. Infiltrate by applying gentle vacuum pulses for 1 min. Incubate for 3 h.
2. Remove and fix the plant material in glutaraldehyde solution for 24 h at 4°C.
3. Process for electron microscopy.

3.4. Histochemical Determination of $^{\bullet}O_2^-$ with Nitro Blue Tetrazolium

1. Place the plant sections in an appropriate tube and add sufficient NBT solution to cover completely (see Notes 13 and 14). To infiltrate, place the open tubes in a desiccator, and apply vacuum pulses, gently releasing the vacuum between each pulse. Usually, 8–10 pulses, 10 s each, are sufficient for infiltrating the tissue.
2. Incubate for 2 h at 30°C in the dark. Comments about incubation time in Note 10 also apply for this technique.
3. Remove the plant material from the NBT solution and place in beaker or glass tube with an appropriate volume of the 9:1 ethanol-glycerin solution. Place the vessel in a water bath and boil for 10 min or until the green color has faded almost completely.
4. Mount the material on a glass slide or sheet and cover with glass slips.
5. Photograph.
6. See Note 15 for tips on quantifying staining intensity.

3.5. Spectrophotometric Determination of In Vivo H_2O_2 Production

1. Submerge the plant material completely (see Notes 14 and 15) in the reaction solution. Infiltrate by applying gentle vacuum for 1 min. Incubate for 2 h at 30°C in the dark.
2. Collect the incubation medium and centrifuge at $10{,}000 \times g$, for 5 min.
3. Measure the absorbance at 515 nm.
4. Transform 515 nm absorbance readings into the H_2O_2 concentration using the molar extinction coefficient $2.6 \times 10^4\ M^{-1}\,cm^{-1}$.

3.6. Spectrophotometric Determination of In Vivo $^{\bullet}O_2^-$ Production

1. Submerge the plant material completely in the XTT solution (see Notes 14 and 15). Infiltrate by applying gentle vacuum for 1 min. Incubate for 5 h at 30°C in the dark.
2. Collect the incubation medium and centrifuge at $10{,}000 \times g$, for 5 min.
3. Measure the absorbance of the incubation medium at 470 nm.
4. Use the $^{\bullet}O_2^-$ molar extinction coefficient at 470 nm ($2.16 \times 10^4\ M^{-1}\,cm^{-1}$) to transform absorbance values into $^{\bullet}O_2^-$ concentration.

3.7. Fluorometric Determination of In Vivo HO• Production

1. Submerge the plant material completely in the BZ solution (see Notes 14 and 15). Infiltrate by applying gentle vacuum for 1 min. Incubate for 7 h at 30°C in the dark.
2. Collect the incubation medium and centrifuge at 10,000 × *g*, for 5 min.
3. Measure the fluorescence using a spectrofluorometer at 407 nm emission after excitation at 305 nm.
4. Run blanks without BZ in parallel to correct for unspecific fluorescence.
5. Prepare a BZ calibration curve by measuring a series of BZ dilutions (from 0 to 2.5 mM).
6. Transform the fluorescence values of the biological samples into HO• molar concentration using the calibration curve values.

4. Notes

1. For any ROS determination method, it is of the utmost importance to manipulate the tissue with great care, avoiding any pressure while handling it, as this may induce injury-related ROS production (19). When working with explants such as leaf segments, it is very important to rinse them briefly after excision to remove any ROS produced as a result of the injury.
2. It is recommended that the osmotic potential of infiltration and incubation solutions be as similar as possible to the tissues Ψ, which can be measured psychrometrically. The choice of a suitable organic osmotic agent for that purpose is contingent on its action as a ROS scavenger (32). Note that the organic solute sorbitol is a weak scavenger for $^{\bullet}O_2^-$ (19, 33) and HO• (34).

   For salt-treated plant material, it is important to estimate apoplastic Na^+ and include it in the incubation solutions. When studying roots obtained from hydroponically grown plants, the ion composition in the root apoplastic solution can be expected to approximately reflect that in the nutrient solution. However, this will not be the case in soil-grown roots. In other plant organs, the composition of the apoplastic solution will depend on the ion balance control exerted along the xylem and the transpiration stream (35). Therefore, an assessment of the apoplastic ion concentration should be performed and the estimated concentration included in the staining or assay solutions (36). Average tissue ion estimates will not necessarily

reflect ion concentration in different compartments (10) and estimates of ion concentration from one plant part should obviously not be extrapolated to the subject tissue because significant differences in ion distribution within a plant are likely to exist (37).

3. Although DCFH is usually assumed to react mainly with H_2O_2, it can also become fluorescent in the presence of other oxidants (22), as was critically reviewed by (14). Appropriate controls, including a H_2O_2 scavenger such as KI (38), are essential for interpreting the results.
4. To adjust the pH, use a series of HCl solutions (e.g., 1 and 0.1 N), rather than concentrated HCl. This avoids sudden drops in pH.
5. The required volume of the agar solution depends on the size of the plant material; the solution should cover it evenly.
6. Use a series of NaOH solutions (e.g., 1 and 0.1 N) to avoid a sudden rise in pH.
7. Details on how to calculate apoplastic fluid dilution after infiltration can be found in (39). Although these methods can render only an approximate idea of the ion concentration in the apoplastic solution due to the very small volumes obtained and dilutions induced by infiltration, it is still preferable to obtain information from these procedures rather than to incubate the tissues without correcting for the estimated apoplastic ion concentration.
8. Note that the plant material is introduced before the medium has completely cooled. Keep the plant material submerged using dissection needles. When the agar begins to solidify, remove the needles.
9. If non-excised shoots are to be observed, roots can be kept in an appropriate nutrient medium, with or without NaCl, in a beaker or another vessel, alongside the Petri dish.
10. The incubation time depends on the type of plant material used. An estimated period is suggested above. For each material, try a series of increasing incubation periods until the right fluorescence intensity (sharp, not too high) can be observed.
11. It is important not expose the plant material to the microscope's light for too long. This exposure will increase the fluorescence intensity, produce a loss of detail at high light intensity, and will affect any comparisons between treatments. Expose for only a few minutes to check the material and then take a picture.
12. Fixed exposure times are essential if different treatments are being compared. Increasing the exposure time will also increase fluorescence [see, for example, ref. (20)].

13. Completely cover the plant material with the staining solution but use the lowest possible volume, increasing the ratio between solution and plant material will diminish staining quality.
14. Larger leaves can be rolled and placed in a Falcon tube, with due caution not to induce injuries or folds. Observe bubbles coming up to make sure air is drawn out and infiltration is effective.
15. Although this is essentially a qualitative technique, if the same incubation time is used for several simultaneous treatments, differences in blue color intensity will reflect differences in $^{\bullet}O_2^-$ production. Blue color intensity can be measured with an image processing software after splitting the images into blue, green, and red stacks. Color intensity in the blue channel can then be measured as luminance (Lu), (scale 0–255 from the darkest to the lightest) and transformed to optical density (OD), $OD = \log(Lu)^{-1}$.

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