Detection and Measurement of ROS in Tobacco Leaves

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[Abstract] Leaf metabolism produces hydrogen peroxide (H_2O_2) at high rates, high level H_2O_2 accumulation can cause oxidative stress. This protocol describes a method for determining H_2O_2 concentration in tobacco leaves. In this method all extractions were performed with $HClO_4$, neutralized, and pretreated with ascorbate oxidase to eliminate ascorbate interferences. H_2O_2 content was determined using a colorimetric assay spiked with an internal control. Interfering peroxides were determined in parallel using a negative control treated with catalase and subsequently subtracted.

Materials and Reagents

1. Tobacco leaves (*Nicotiana tabaccum*, Wisconsin 38)
2. $HClO_4$
3. Polyvinylpolypyrrolidone (PVP)
4. K_2CO_3 in 0.3 M phosphate buffer (pH 5.6)
5. Ascorbate oxidase (Sigma-Aldrich, catalog number: A0157)
6. Phosphate buffer (pH 6.5)
7. Catalase (Sigma-Aldrich, catalog number: C3155)
8. H_2O_2
9. Horseradish peroxidase (Sigma-Aldrich, catalog number: 77332)
10. 3-(dimethylamino) benzoic acid (DMAB)
11. 3-methyl-2-benzothiazoline hydazone (MBTH)
12. Liquid nitrogen
13. 0.1 M phosphate buffer (pH 6.5)

Equipment

1. DU-800 Spectrophotometer (Beckman Coulter)

Procedure

1. The seeds of tobacco plants were allowed to germinate on MS medium, and then the plants were transferred to soil and grown for two weeks in a growth chamber at 25 ± 1 °C with PPFD of 100 µmol/m^2/s, a relative humidity of 75-80%, and a photoperiod of 12/12 h light/dark. For H_2O_2 content determination, tobacco leaves (50 mg) were ground with mortar and pestle to a fine powder in liquid nitrogen and the powder was extracted in 2 ml 1 M $HClO_4$ including 5% insoluble PVP.
2. The homogenate was centrifuged at 12,000 *x g* for 10 min and the supernatant was neutralized with several microliters 5 M K_2CO_3 to pH 5.6. The homogenate was centrifuged at 12,000 *x g* for 1 min to remove $KClO_4$.
3. To eliminate the interference of ascorbate, the supernatant was incubated for 10 min with 1 U/ml ascorbate oxidase at room temperature to oxidize ascorbate.
4. Preparing reaction mixture: the reaction mixture consisted of 0.05 M phosphate buffer (pH 6.5); 3.3 mM DMAB; 0.07 mM MBTH and 50 ng/ml horseradish peroxidase. The reaction was initiated by addition of an aliquot (50 µl) of the sample.

   Reaction mixture (1 ml)

Stock	**Volume**	**Final conc.**
0.1 M phosphate buffer (pH 6.5)	500 µl	0.05 M
33 mM DMAB	100 µl	3.3 mM
0.7 mM MBTH	100 µl	0.07 mM
horseradish peroxidase (0.1 U/µl)	1 µl	0.1 U
sample	50 µl	
ddH_2O		to 1,000 µl

   The absorbance change at 590 nm (ΔOD1) per minute was monitored at 25 °C for 1-5 min. The value represents the total peroxides including H_2O_2 in the sample.
5. To eliminate the interference of other peroxides, an aliquot of the supernatant pre-incubated with ascorbate oxidase as mentioned in step 3 was then incubated for 10 min with 1 U/m catalase at room temperature to catalyze H_2O_2 decomposition. The interference of other peroxides was then determined using the same procedure as described in step 4 (ΔOD2).

6. The absorbance change at 590 nm caused by H_2O_2 in sample was calculated as ΔOD1-ΔOD2.
7. H_2O_2 content in the sample was quantified by reference to an internal standard: in each determination in step 4, a parallel aliquot was assayed with addition of 2 nmol H_2O_2 to the reaction mixture, and the absorbance change at 590 nm (ΔOD3) was monitored at 25 °C.
8. The final H_2O_2 concentration was calculated as: (ΔOD1-ΔOD2)/ (ΔOD3-ΔOD1) × 2 nmol/50 μl × total volume of the extraction/total fresh weight of the tobacco leaves.

Recipes

1. 0.1 M phosphate buffer (pH 6.5)

pH	1 M K_2HPO_4 (ml)	1 M KH_2PO_4 (ml)
6.5	30.4	69.6

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