Analysis of Malondialdehyde, Chlorophyll Proline, Soluble Sugar, and Glutathione Content in *Arabidopsis* seedling

Zhijin Zhang, Rongfeng Huang*

Cold and Arid Regions Environmental and Engineering Research Institute, Chinese Academy of Sciences, Lanzhou, China
*For correspondence: rfhuang@caas.cn

[Abstract] The protocol has four sub-protocols, which are about the measurement of malondialdehyde, chlorophyll proline, soluble sugar, and glutathione content, respectively, in *Arabidopsis* seedlings by using spectrophotometer. These methods are simple, effective and reproducible, which will help the researchers who are not familiar with these approaches, quickly get reliable results.

A. Measurement of Malondialdehyde

Materials and Reagents

1. Thiobarbituric acid (TBA) (Sigma-Aldrich, catalog number: T5500)
2. Trichloroacetic acid (TCA) (Sigma-Aldrich, catalog number: T9159)
3. Malondialdehyde (MDA) (BOC Sciences, catalog number: 542-78-9)

Equipment

1. Centrifuge
2. Spectrophotometer

Procedure

Note: The experiment is done at room temperature (RT) except of specific indication.

1. 0.1 g leaf tissue (with similar age, and young expanded leaf may be better) is ground into powder with liquid nitrogen, and then put the powder into a tube containing 1 ml 0.1% (w/v) TCA and mix by inverting the tube to homogenize the leaf tissue.
2. Centrifuge homogenized samples at 10,000 *x g* for 10 min, and then transfer supernatant to a new tube.
3. 4 ml of 20% TCA containing 0.5% TBA was added to the supernatant and mixed well.

4. The mixture is boiled at 95 °C for 15 min and quickly cooled on ice (TBA can interact with MDA and results into red compound in acidic buffer, so the content of MDA can be calculated by measuring the density of the resulting red compound with spectrophotometer at 532 nm. The high temperature can accelerate the reaction and low temperature can inhibit it).
5. Centrifuge the mixture at 10,000 *x g* for 5 min, and then transfer supernatant to a new tube.
6. To generate a standard curve, a serial concentration of MDA is made: 1 μM, 2 μM, 5 μM, 10 μM, 20 μM and 50 μM (the volume of each dilution depends on the size of the cuvette of spectrophotometer).
7. Measure the optical density of standard samples from step 6 at 532 nm by spectrophotometer and make the standard curve to get the extinction coefficient (Figure 1).

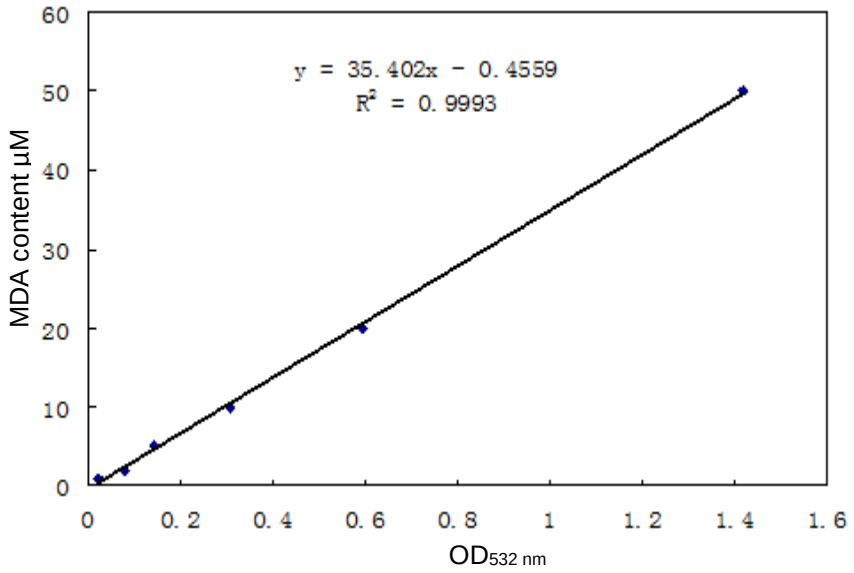

Figure 1. The standard curve of MDA

8. Measure the optical density of plant samples from step 5 at 532 nm and calculate the content of MDA according to the standard curve (Madhava Rao and Sresty, 2000; Baryla *et al*., 2000)

B. Measurement of chlorophyll

Materials and Reagents

1. Dimethyl formamide (DMF) (Sigma-Aldrich, catalog number: D4551)

Equipment

1. Centrifuges
2. Spectrophotometer

Procedure

Note: The experiment is done at room temperature.

1. 0.1 g leaf tissue is ground into powder with liquid nitrogen, and then homogenized with 1 ml 100% DMF.
2. Centrifuge homogenized samples at 10,000 *x g* for 10 min, and then gather the supernatant.
3. Measure the optical density of the supernatant at 664 nm and 647 nm, respectively.
4. Calculate the content of chlorophyll a and chlorophyll b by the following formulas (Sibley *et al.,*1996; Inskeep and Bloom, 1985; Aono *et al.,* 1993):
   a. [chlorophyll a] = 12.7 x A_{664} - 2.79 x A_{647}
   b. [chlorophyll b] = 20.7 x A_{647} - 4.62 x A_{664}
   c. [chlorophyll a + chlorophyll b] = 17.90 x A_{647} + 8.08 x A_{664}

C. Measurement of Proline

Materials and Reagents

1. Sulphosalicylic acid (DingGuo, catalog number: DS094)
2. Proline (Sigma-Aldrich, catalog number: 858919)
3. Ninhydrin (Sigma-Aldrich, catalog number: 151173)
4. Acetic acid (DingGuo, catalog number: DS002)
5. Orthophosphate (Sigma-Aldrich, catalog number: P2023)
6. Toluene (Sigma-Aldrich, catalog number: 650579)
7. Ninhydrin reagent (see Recipes)

Equipment

1. Centrifuges
2. Spectrophotometer

Procedure

Note: The experiment is done at room temperature (RT) except of specific indication.

1. To generate a standard curve, a serial concentration of Proline is made in 3% sulphosalicylic acid: 1 μM, 10 μM, 50 μM, 100 μM, 150 μM, 200 μM, 300 μM, 1 ml for each dilution.
2. Each 500 μl standard solution is added with 500 μl acetic acid and 500 μl ninhydrin reagent in 5 ml tube and boil for 45 min, and then cooled in ice for 30 min.
3. Add equal volume toluene to each sample and vibrate for 1 min, and then centrifuge at 1,000 *x g* for 5 min.
4. Measure the optical density of toluene solution at 520 nm by spectrophotometer and make the standard curve (Figure 2).

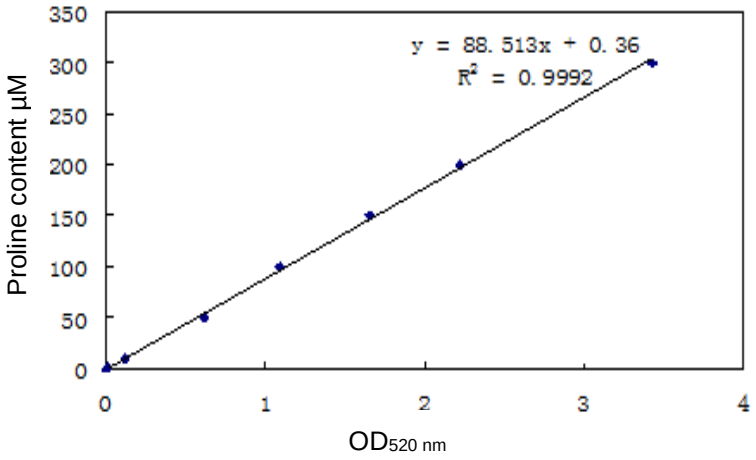

Figure 2. The standard curve of proline

5. 0.5 g plant sample is ground into powder with liquid nitrogen, and then homogenized with 2 ml of 3% sulphosalicylic acid in tube.
6. Centrifuge homogenized samples at 5,000 *x g* for 5 min, and then collect the supernatant
7. The supernatant is treated as steps 2 and 3, and measure the optical density of samples as step 4, and then calculate the content of praline using the standard curve from step 3 (Bates *et al*., 1973; Lattanzioa *et al.,* 2009).

Recipes

1. Ninhydrin reagent
   2.5 g ninhydrin is successively added to 60 ml Glacial Acetic acid and 40 ml 6 M orthophosphate, and then dissolved at 70 °C. After cool down, the reagent can be stored in brown bottle at 4 °C for less than 24 h.

D. Measurement of soluble sugar

Materials and Reagents

1. Ethanol (DingGuo, catalog number: DS023)
2. Glucose (DingGuo, catalog number: DS063)
3. Anthrone (SCRC, catalog number: 30015014)
4. H_2SO_4 (Sigma-Aldrich, catalog number: 339741)
5. Thiourea (Amresco, catalog number: M222)
6. Chloroform (Sigma-Aldrich, catalog number: C2432)
7. Anthrone reagent (see Recipes)

Equipment

1. Centrifuges
2. Spectrophotometer
3. Shaker

Procedure

Note: The experiment is done at room temperature (RT) except of specific indication.

1. To generate a standard curve, a serial concentration of glucose is made: 1 μM, 10 μM, 50 μM, 100 μM, 150 μM, 200 μM, 300 μM, 5 ml for each concentration of glucose solution.
2. 50 μl of each diluted glucose solution is mixed with 4.95 ml anthrone reagent and then boiled for 15 min.
3. Measure the optical density of glucose standards at 620 nm by spectrophotometer to generate a standard curve.
4. 0.1 g dried sample is ground into powder with liquid nitrogen, and then homogenized with 2 ml 80% ethanol in shaker at 200 rpm in 50 ml tube for 1 h.

5. Centrifuge at 6,000 *x g* for 10 min, and then transfer as much supernatant as possible into a new 5 ml tube.
6. Add equal volume of chloroform, completely mix, and then centrifuge at 12,000 *x g* for 10 min.
7. The aqueous part is transferred to a new tube and repeat steps 2 and 3 to measure the optical density of the sample. The content of soluble sugar is calculated according the standard curve made at step 3 (Mandre *et al.*, 2002; Jin *et al*., 2007).

Recipes

1. Anthrone reagent
   72% H_2SO_4
   500 mg/L anthrone
   10 g/L thiourea

E. Measurement of Glutathione

Materials and Reagents

1. Trichloroacetic acid (TCA) (Sigma-Aldrich, catalog number: T9159)
2. Polyvinylpolypyrrolidone (PVPP) (Sigma-Aldrich, catalog number: P6755)
3. 2-amino-2-(hydroxymethyl)-1,3-propanediol (TRIS) (DingGuo, catalog number: DH350)
4. 5,50-dithio-bis (2-nitrobenzoic acid) (DTNB) (DingGuo, catalog number: DH499)
5. Glutathione reductase (GR) (Merck KGaA, catalog number: 359960)
6. Glutathiol (GSSG) (Solarbio, catalog number: G8690)
7. Reaction solution (see Recipes)

Equipment

1. Centrifuges
2. Spectrophotometer

Procedure

Note: The experiment is done at room temperature (RT) except of specific indication.

1. To generate a standard curve, a serial concentration of GSSG is made: 0.5, 1, 2, 5, 10, 20 μM, 2 ml for each dilution of GSSG.

2. 100 µl of each GSSG standard made at step 1 is added to 3 ml of Reaction solution and incubated for 15 min. Then add 100 mM DTNB to a final concentration of 10 mM and incubate at 25 °C for 15 min.
3. Measure the optical density of each sample at 412 nm by spectrophotometer, and make standard curve with a function of the concentration of GSSG standard and the optical density of each GSSG standard.
4. 0.5 g of *Arabidopsis* leaves is ground in liquid nitrogen.
5. The samples are homogenized with 1 ml extract solution and mixed completely by inverting the tube.
6. The mixture is centrifuged at 10,000 *x g* at 4 °C for 10 min, and then the supernatant is transferred to a new tube.
7. 100 µl supernatant is treated as described at step 2, and the optical density of the supernatant is measured at 412 nm as described at step 3, and calculate the GSSG content of the sample according the standard curve.
8. 100 µl supernatant is mixed with 3 ml 500 mM Tris–HCl (pH 8.0) buffer containing 10 mM DTNB and incubated at 25 °C for 15 min. The optical density is then measured at 412 nm. The Glutathione (GSH) content is determined by the same standard curve as described at step 3 with the following formula: [GSH] = 2 x [standard curve].
9. The total glutathione content = [GSH] + [GSSG] (Huang *et al.,* 2005; Chen *et al*., 2011)

Recipes

1. Reaction solution
   500 mM Tris–HCl (pH 8.0) buffer
   GR (1 U for each 3 ml reaction solution)
   1 mM EDTA
   3 mM $MgCl_2$
   150 µM NADPH
2. Extract solution
   0.1% TCA (pH 2.8)
   1 mM EDTA
   1% (w/v) PVPP

Acknowledgments

This protocol is adapted from Huang *et al*. (2005) as well as other works mentioned in the reference list.

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