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Procedure of total sugar content, proline, chlorophyll a, b, total, SOD, POD, H2O2 and APX

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ABSTRACT

Analysis of physiological characteristics was carried out at the Physiology and 77 Protection Laboratory of the Unit Research Sungei Putih, Galang, Deli Serdang, North 78 Sumatra. Characteristics observed were total sugar (µM), proline (mg/L), chlorophyll a, b, total (µg/mL), hydrogen peroxidase (µmol/g), ascorbate peroxidase (unit/mg), superoxide dismutase (unit/mg), and peroxide dismutase (unit/mg). Herewith we describe the protocol used in our paper entitled "Physiological Characters of IRR 400 Series Rubber Clones (*Hevea brasiliensis* Muell. Arg.) on Drought Stress", to analyze total sugar content, proline, chlorophyll a, b, total, SOD, POD, H2O2 and APX

1 Total sugar content

Reactor:

1. Anthrone reagent 0.1 g anthrone added to 100 ml sulfate solution
2. Sulfuric acid solution: sulfuric acid: distilled water (100:29)
3. TCA 2.5%: 2.5 gr TCA dissolved in 100 ml of distilled water
4. Standard 2 mM sucrose:

    - Stock I (40 mM): 69 mg sucrose dissolved in 5 ml TCA 2.5%
    - Stock II (2 mM): 1 ml stock I plus 19 ml TCA 2.5%

Procedure

1. Samples taken 150 micro liters plus 2.5% TCA to a total of 500 micro liters
2. Add 3 ml of anthrone reagent and vortex
3. heated by immersing in boiling water for 15 minutes
4. Cooled by immersing in water
5. Absorbance is measured at a wavelength of 627 nm

Standard curve creation

1. A standard solution series is made as follows:

A standard solution series

1. Add 3 ml of anthrone reagent and vortex. Then the procedure is the same as above.

Reference
M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith, “Colorimetric Method for Determination of Sugars and Related Substances,” pp. 350–356

2 Proline

The frozen plant material is homogenized in 3% aqueous sulfosalicylic acid (0.01g/0.5ml) and

the residue is removed by centrifugation at 12000 rpm for 10 min.
1 ml of homogizad tissue reacts with 1 ml of ninhydrin acid and 1 ml of glacial acetic acid in a test tube for 1 hour at 100oC and the reaction is stopped in an ice bath
The reaction mixture is extracted with 2 ml of toluene, mixed vigorously and left at room temperature for 30 minutes until the separation of the two phases
The toluene containing chromophore (1 ml upper phase) is warmed to room temperature and its optical density is measured at 520 nm using toluene for blank.
Proline levels are determined from a standard curve using D-Proline
References

L. Bates, “Short Communication: Rapid determination of free proline for water stress studies,” *Plant Soil*, vol. 39, pp. 205–207, 1973.
H. Koca, M. Bor, F. Özdemir, and I. Türkan, “The effect of salt stress on lipid peroxidation, antioxidative enzymes and proline content of sesame cultivars,” *Environ. Exp. Bot.*, vol. 60, no. 3, pp. 344–351, 2007, doi: 10.1016/j.envexpbot.2006.12.005.
M. R. Amirjani, “20.pdf,” *Am. J. Plant Physiol.*, vol. 5, no. 6, pp. 350–360, 2010, [Online]. Available: ISSN 1557-4539.
S. Gyawali *et al.*, “Seedling, early vegetative, and adult plant growth of oilseed rapes (Brassica napus L.) under saline stress,” *Can. J. Plant Sci.*, vol. 99, no. 6, pp. 927–941, 2019, doi: 10.1139/cjps-2019-0023.

3 Chlorophyll a, b and total

Crushed fresh leaves as much as 0.1 gram with mortal, and use 80% acetone as much as 10 ml, the extract is filtered with filter paper and transferred into the vial, the absorbance was measured at a wavelength of 645 nm and a 663 nm.Chlorophyll a, chlorophyll b and total chlorophyll are calculated using the formula

- Chlorophyll a = (12.7 x A663) – (2.69 x A645) 10
- Chlorophyll b = (22.9 x A645) – (4.68 x A663) 10
- Total chlorophyll = (8.02 x A663) + (20.2 x A645) 10

Reference

G. A. and J. P. G. Hendry, *Methods in Comparative plant ecology. A laboratory manual*. 1993.

4 SOD

Extract buffer was prepared by mixing 10 ml of 1 mM EDTA solution with 50 ml of phosphate buffer pH 7.6.Then add distilled water up to 100 ml.0.1 g of the leaves were crushed with 0.1 g of PVP and liquid nitrogen until it became powder and then put into a tube containing 1 ml of extract buffer. Centrifuged at 10,000 rpm and 4^{O}C for 10 minutes.Methionine 13 mM solution was prepared by weighing 19.3973 mg of methionine and dissolving it in 10 ml of distilled water.0.1 mM EDTA solution was prepared by weighing 1.1167 mg of EDTA and dissolving it in 30 ml of distilled water.The 75 mM NBT solution was prepared by weighing 0.61323 mg of NBT and dissolving it in 10 ml of distilled water.A 2 mM riboflavin solution was prepared by weighing 0.00752 mg of riboflavin and dissolving it in 10 ml of distilled water.Prepare a test tube.Added 750 µL buffer extract, 50 µL methionine solution, 50 µL NBT, 150 µL EDTA and

100 µL enzyme extract.Enzyme extract (sample) was not added to the blank solution.Then brought to light conditions 15 watts.Added 50 µL of riboflavin and allowed to stand for 15 minutes.Added 350 µL of sterile distilled water.The solution was stirred and then read with a UV/VIS spectrophotometer at a wavelength of 560 nm.

Reference

M. M. Bradford, “A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding,” *Anal. Biochem.*, vol. 72, no. 1–2, pp. 248–254, 1976, doi: 10.1016/0003-2697(76)90527-3.

5 **POD**

Grind the leaves as much as 0.1 g with PVP and liquid nitrogen until they become powder and then put into a tube containing 1 ml of CaCl2.Then centrifuged at 10,000 rpm and 40C for 10 minutes.A 0.5 M CaCl2 solution was prepared by dissolving 5.55 g of CaCl2 in 100 ml of distilled water.Solution A was prepared by dissolving 1.458 g of phenol and 0.045 g of 4-Dimethylaminoantipyrine in 90 ml of distilled water.MES buffer was prepared by dissolving 0.293 g of MES in 75 ml of distilled water and then dividing it into three parts of 25 ml each.The solutions were optimized at pH 5.5;6.0;6.5 by adding 1 M NaOH. Then the peroxidase activity was tested with a UV/VIS spectrophotometer.HEPES buffer was prepared by dissolving 0.357 g of HEPES in 75 ml of distilled water and then dividing into three parts of 25 ml each.The solutions were optimized at pH 7.0;7.5;8.0 by adding 1 M NaOH. Then the peroxidase activity was tested with a UV/VIS spectrophotometer.The peroxidase activity test of HEPES and MES, the highest result was MES buffer solution pH 5.5 with peroxidase activity 0.79.Solution B was prepared by mixing MES solution pH 5.5 with 1.5 ml of 30% H2O2 solution with a final concentration of 0.01 M. Prepare a test tube and then add 1.4 ml of solution A and 1.5 ml of solution B.Enter the sample as much as 200 µL.Enzyme extract (sample) was not added to the blank solution.The solution was read with a UV/VIS spectrophotometer at a wavelength of 510 nm at 0 minutes and 2 minutes.

Reference

P. C. Ashbrook, “Standard Operating Procedures,” *J. Chem. Heal. Saf.*, vol. 21, no. 5, p. 29, 2014, doi: 10.1016/j.jchas.2014.07.006.

6 **Protein**

0.1 g of the leaves were crushed with 0.1 g of PVP and liquid nitrogen until it became powder and then put into a tube containing 1 ml of extract buffer.Then centrifuged at 10,000 rpm and 4^{o}C for 10 minutes.Bradford's reagent was prepared by dissolving 0.02 g of CBB G-250 in 10 ml of 95% ethanol and 20 ml of phosphoric acid.This solution was stirred in the dark, filtered using filter paper and then added 150 ml of distilled water.The standard protein solution was prepared by dissolving 0.01 g of BSA in 10 ml of distilled water to obtain a 1000 ppm BSA stock solution.Dilute BSA by taking 0.5 ml of BSA stock and adding 4.5 ml of distilled water so that the level is 100 ppm.Then the dissolved protein standard was measured by adding 0.1 ml of standard solution with 5 ml of Bradford reagent.A test tube was prepared and 100 µL sample was added, 2.5 ml Bradford was added and incubated for 10-60 minutes in the dark and then read with a spectrophotometer with a wavelength of 595 nm.

Reference

M. M. Bradford, “A rapid and sensitive method for the quantitation of microgram quantities of

protein utilizing 672 the principle of protein-dye binding," Anal. Biochem., vol. 72, no. 1–2, pp. 248–254, 1976, doi: 10.1016/0003-673 2697(76)90527-3.

7 H2O2

Put 1 ml TCA (Tricloroacetic acid/CCl3COOH) 0.1% (w/v)
(0.108 g TCA + 108 ml aquades) into the tube
Grind the leaves as much as 0.1 g + 0.1 g PVP 1% + liquid nitrogen (into the tube)
then centrifuged at 12000 rpm for 15 minutes
Take 200 µl enzyme extracts
Added with 0.5 ml BP 10 mM pH 7 (V1= 100 ml, 100 mM BP; V2= 1000 ml, 10 mM BP)
Added 1 ml of KI M (Mr = 166) (17,928 g KL + 108 ml distilled water)
Blank: 1.5 ml H2O2 + BE 200 H2O2
Activity measurements were calculated with a UV/VIS spectrophotometer at a wavelength of 390 mm.

Reference
M. M. Bradford, "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding," *Anal. Biochem.*, vol. 72, no. 1–2, pp. 248–254, 1976, doi: 10.1016/0003-2697(76)90527-3.

8 APX

1 ml of 0.5 mM ascorbic acid (inserted in the tube)
0.1g of crushed leaves + 0.1 g of 1% PVP + liquid nitrogen (inserted in a tube)
Centrifuge at 12000 rpm for 15 minutes
Take 100 microliters of enzyme extract
Add 300 µl of 50 mM BP pH 7 (V1+ 100 ml, 100 mM BP; V2=200 ml, 50 mM BP)
Add 400 µl 0.5 mM of ascorbic acid C6H8O6 (Mr=176) (0.0088 g ascorbic acid + 100 ml distilled water) Add Add 300 µl of 0.1 mM EDTA (1.1167 mg + 30 ml of distilled water)
Add 400 µl of 0.1 mM H2O2 3% (Mr=34.0147)
Blank: BP + ascorbic acid + EDTA + BE 100 micro liter
APX activity was measured using a UV/VIS spectrophotometer every 10 seconds for 1 minute at a wavelength of 290 nm.
APX is calculated by the formula:
Enzyme activity APX = Absorbance/minute/ε x total Vtotal/Vsample divided by the fresh weight of the sample

Reference
Y. Nakano and K. Asada, "APX Nakano & Asada 1981.pdf," Plant Cell Physiol., vol. 22, no. May, pp. 867–880, 2018.