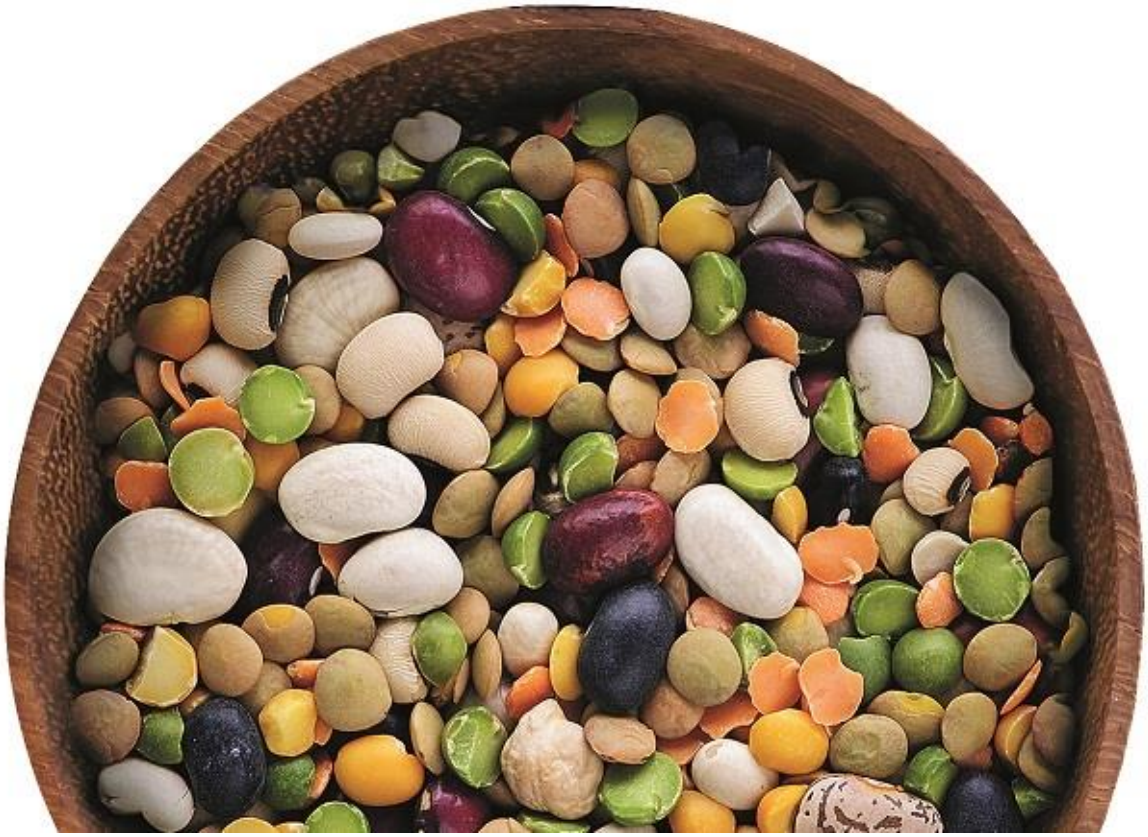
TRansition paths to sUstainable
legume-based systems in Europe

Antioxidant assay 5: Superoxide dismutase (SOD)

Date: September, 30th 2018



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research and innovation programme under grant agreement No 727973

Content

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1 Summary Information

1.1 Partner Summary

SOP Code	EU_TRUE_SOP_014
TRUE Partner Acronym	AUA
Primary Author	Ntatsi, Georgia (*ntatsi@aua.gr*)
Other Authors	Savvas, Dimitrios
Linked Reference and Hyperlink (if available)	Yong He, Zhujun Zhu Jing Yang, Xiaolei Ni and Biao Zhu, 2009. Grafting increases the salt tolerance of tomato by improvement of photosynthesis and enhancement of antioxidant enzymes activity. Environmental and Experimental Botany Volume 66, Issue 2: 270-278 *http://dx.doi.org/10.1016/j.envexpbot.2009.02.007* K.V.M. Rao, T.V.S. Sresty, 2000. Antioxidant parameters in the seedlings of pigonpea (*Cajanus cajan* (L.) Millspaugh) in response to Zn and Ni stresses. Plant Sci., 157, 113-128 *https://www.ncbi.nlm.nih.gov/pubmed/10940475*
Associated files to use with the SOP [and function]	NA

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1.2 SOP Summary

Title

Antioxidant assay 5: Superoxide dismutase (SOD)

Brief description

Superoxide dismutase catalyses the breakdown of superoxide radical anion and provides the first line of defence against oxygen toxicity. It therefore provides a good indication as to whether a plant is under stress or not. Superoxide dismutase (EC 1.15.1.1) designates the variety of metalloproteins containing Cu^{2+}, Zn^{2+}, Mn^{3+} or Fe^{3+} as an active metal cofactor that catalyse the dismutation of the superoxide radical anion into oxygen and hydrogen peroxide. Cu/ZnSOD is located in the cytosol and chloroplast of the plant cell, MnSOD is in the matrix of the mitochondria and peroxisomes.

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2 Protocol Steps

1st stage: Enzyme Extraction

0.1 g of each pulverized, frozen sample (leaf and root samples) was homogenised with ice-cold **25 mM HEPES buffer (pH 7.8)**. The HEPES buffer contained **0.2 mM EDTA, 2 mM ascorbate and 2 % (w/v) polyvinylpyrolidon (PVP)**. The homogenates were centrifuged at 4 °C and 14,000 rpm. The supernatants obtained were used for enzyme analysis. The procedure for enzyme extraction was carried out at 0-4 °C.

Reagents

To prepare **1 L** of the necessary reagents, the following equation was used:

$$m = C * V * M \qquad (equation\ 1)$$

with m = weight (g)
C = molarity (mol/L)
V = volume to be prepared (L)
MB = molecular weight (g/mol)

- **25 mM HEPES buffer (pH 7.8)**

  Weigh 5.9575 g of HEPES (MB = 238.30) in a 1 L volumetric flask and adjust to 1 L with H_2O (m = 0.025 * 1 * 238.30). Adjust the pH to 7.8 using either a diluted HCl or NaOH solution.

- **0.2 mM EDTA**

  Weigh 0.0745 g of EDTA (MB = 372.24) in a 1 L volumetric flask and adjust to 1 L with H_2O (m = 0.0002 * 1 * 372.24).

- **2 mM ascorbate (AsA)**

  Weigh 0.352 g of Ascorbate (MB = 176.12) in a 1 L volumetric flask and adjust to 1 L with H_2O (m = 0.002 * 1 * 176.12).

- **2% (W/V) polyvinylpyrolidon PVP**
  - 2 g in 100 mL

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2nd stage: Measuring Superoxide Dismutase (SOD)

Superoxide dismutase (SOD) activity was assayed according to Rao and Sresty (2000) with small modifications.

200µL of reaction mixture containing **100 mM phosphate buffer (pH 7.8), 100 mM methionine, 1 mM nitroblue tetrazolium (NBT), 0.1 mM EDTA, 10µl leaf extract or 25 µL root extract and 2 µM riboflavin** started the reaction. The samples were incubated at room temperature under a lamp for 15 minutes before measuring the absorption of NBT at **560 nm**, with Anthos Zenyth 240 96-well microplate absorbance reader spectrophotometer (Anthos Mikrosysteme GmbH, Germany). A reaction mixture without the plant extract was used as control; while a non-irradiated complete reaction mixture served as blank. The ADAP BASIC Software for device control and data transfer to PC was used.

Reagents for the preparation of the reaction mixture:

- **100 mM phosphate buffer (pH 7.8)**

1M K_2HPO_4
Since the molar mass of K_2HPO_4 is 174.1760 g/mol, a 1 M solution would be 174.1760 g in 1L.

1M KH_2PO_4
Since the molar mass of KH_2PO_4 is 136.0857 g/mol, a 1 M solution would be 136.0857 g in 1L.

Preparation of 1L of 0.1 M = 100 mM potassium phosphate buffer at 25 °C

Desired pH	Volume of 1M K_2HPO_4 (mL)	Volume of 1M KH_2PO_4 (mL)
7.0	90.8	9.2

Hence, for the preparation of 100 mL of 0.1 M = 100 mM potassium phosphate buffer at 25 °C

Desired pH	Volume of 1M K_2HPO_4 (mL)	Volume of 1M KH_2PO_4 (mL)
7.0	9.08	0.92

Add the volume indicated in the table above in a 100 mL volumetric flask and adjust to 100 mL with H_2O.

- **13 mM methionine**

  To prepare 10 mL of a 13 mM solution of methionine, weigh 19,397 g of methionine (MB = 149.21) in a 10 mL volumetric flask and adjust to 10 L with H_2O (m = 0.013 * 10 * 149.21 from equation 1).

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- **75 µM NBT nitroblue tetrazolium**

  To prepare 10 mL of a 75 µM solution of NBT nitroblue tetrazolium, weigh 0.6132 mg of NBT nitroblue tetrazolium in a 10 mL volumetric flask and adjust to 10 L with H_2O (MB = 817.65) ($m = 75.10^{-6} * 10 * 817.65$ from equation 1).

- **2 µM riboflavin**

  To prepare 10 mL of a 2 µM solution of riboflavin, use 7.528 µg of riboflavin (MB = 376.38) ($m = 2.10^{-6} * 10 * 376.38$ from equation 1).

Stock solutions preparation:

Solution	Stock Solution	Quantity	H_2O
100 mM K_2HPO_4	1 M K_2HPO_4	9.08 mL	90 mL
	1 M KH_2PO_4	0.92 mL	
13 mM methionine		19.3973 mg	10 mL
75 µM NBT nitroblue tetrazolium		0.61324 mg	10 mL
2 µM riboflavin		7.5276 µg	10 mL
0.1 mM EDTA		0.37224 mg	10 mL

Once the reagents have been prepared, there are two ways of introducing them into the wells:

1. **Addition of each solution separately**
   For each well separately:

SOD	Volume of stock solution	Blank	Measurement
100 mM Potassium Phosphate Buffer	100 mL	10 µL	10 µL
13 mM methionine	10 mL	20 µL	20 µL
75 µM NBT nitroblue tetrazolium	10 mL	10 µL	10 µL
2 µM riboflavin	10 mL	5 µL	5 µL
0.1 mM EDTA	10 mL	10 µL	10 µL
Extract		-------	10 µL
H_2O		145 µL	135 µL
Total Volume		**200 µL**	**200 µL**

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2. Preparing a reaction mixture solution for 50 samples

Attention: Immediate use after preparation otherwise the solution will be useless (turns from yellow into brown)

SOD	Volume of stock solution	Reaction mixture For blank[a]	Reaction mixture for measurement[b]
100 mM Potassium Phosphate Buffer	100 mL	10 µL * 50 samples =500 µL	500 µL
13 mM methionine	10 mL	1000 µL	1000 µL
75 µM NBT nitroblue tetrazolium	10 mL	500 µL	500 µL
2 µM riboflavin	10 mL	250 µL	250 µL
0.1 mM EDTA	10 mL	500 µL	500 µL
Water	------	7.250 µL	6.750 µL
Extract	-------	----------	10 µL from each sample
Total Volume	**-------**	**10 mL**	**10 mL**

[a] 200 µL in each well; [b] (190 µL in each well) + (10 µL of extract)

3rd stage: Calculations

One unit of SOD is defined as the volume of extract that caused inhibition of the photo-reduction of NBT by 50%. First with the absorbance measured, calculate the units of activity (C) using equation 4:

$$C = (0.5 * A_{conc}) / A_{ext} \qquad \textit{(equation 2)}$$

with C = Units of activity (units)
A_{conc} = Absorbance of the control (without extract)
A_{ext} = Absorption of sample with extract

To calculate the superoxide dismutase activity (U), use equation 3 below:

$$U = C / FM \qquad \textit{(equation 3)}$$

- U = Activity (units /g FM)
- FM = gram of the fresh material (g)

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From this, you can also calculate the superoxide dismutase activity *per* protein (U') by dividing U by the protein content in grams (Equation 6).

$$\mathbf{U' \ (units\ gr^{-1}\ protein] = U/\ Protein\ content\ (g)^{1}}$$

[1] *See EU_TRUE_SOP_015 on how to determine Protein Content.*

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3 Linked SOPs

SOP Code	SOP Function
EU_TRUE_SOP_010	Antioxidant assay 1: Ascorbate peroxidase (APX)
EU_TRUE_SOP_011	Antioxidant assay 2: Catalase (CAT)
EU_TRUE_SOP_012	Antioxidant assay 3: Guaiacol peroxidase (G-POD)
EU_TRUE_SOP_013	Antioxidant assay 4: Glutathione reductase (GR)
EU_TRUE_SOP_015	Bradford protein assay

4 Disclaimer

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6 Citation

Please cite this report as follows:

Ntatsi, G., Savvas, D. (2018) Standard Operating Procedure 014: Antioxidant assay 5: Superoxide dismutase (SOD). Developed by the EU-H2020 project TRUE ('Transition paths to sustainable legume-based systems in Europe'), funded by the European Union's Horizon 2020 Research and Innovation programme under Grant Agreement Number 727973. Available online at: *www.true-project.eu*

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