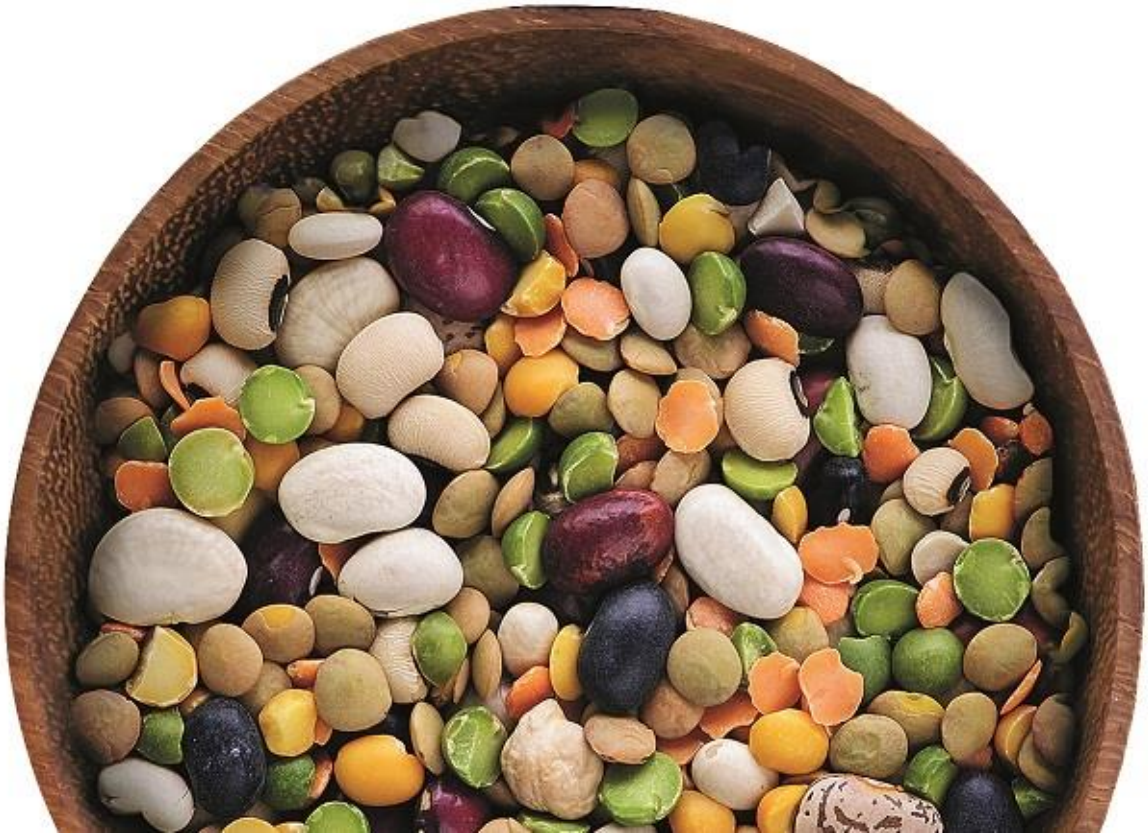
TRansition paths to sUstainable legume-based systems in Europe

Antioxidant assay 1: Ascorbate peroxidase (APX)

Date: September, 30th 2018



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Content

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1 Summary Information

1.1 Partner Summary

SOP Code	EU_TRUE_SOP_010
TRUE Partner Acronym	AUA
Primary Author	Ntatsi, Georgia (*ntatsi@aua.gr*)
Other Authors	Savvas, Dimitrios
Linked Reference and Hyperlink (if available)	Nakano, Y., Asada, K., 1981. Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant and Cell Physiology, Volume 22, Issue 5, August 1981, Pages 867-880 *https://doi.org/10.1093/oxfordjournals.pcp.a076232*
Associated files to use with the SOP [and function]	NA

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1.2 SOP Summary

Title

Antioxidant assay 1: Ascorbate peroxidase (APX)

Brief description

Ascorbate (AsA) is a major antioxidant and free-radical scavenger in plants and is considered to be of paramount importance as an electron donor for H_2O_2 detoxifications *via* ascorbate peroxidase (APX) in plant cells (Ahmad *et al.*, 2010)

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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 an 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 Ascorbate peroxidase

The ascorbate peroxidase (APX) activity was measured in the leaf-samples in accordance to the method of Nakano and Asada (1981), with a few modifications due to ascorbate oxidation and therefore the decrease in absorbance at 290 nm .

200 µL of reaction mixture containing **25 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbate, 0.1 mM EDTA and 0.1 mM hydrogen peroxide** and 10 µL plant extract were used. The reaction was started by adding the hydrogen peroxide and the absorbance at 290 nm was measured over five minutes (7-14 minutes better) with the Synergy HT 96- position microplate spectrophotometer (Biotek, Winooski, USA). The absorbance of the mixture before hydrogen peroxide was added served as blank. To transfer the data in an excel format, the programme KC4 was used. For the calculation of APX-activity the absorbance coefficient of reduced ascorbate (**E = 2.8 mM*cm^{-1}**) was used (Nakano and Asada, 1981).

Reagents for the preparation of the reaction mixture:

- **25 mM potassium phosphate buffer (pH 7.0)**

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	61.5	38.5

Hence, for the preparation of 100 mL of 0.025 M = 25 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	1.5375	0.9625

- **0.5 mM ascorbate**

  To prepare 100 mL of a 0.5 mM solution of ascorbate, use 8.806 mg of Ascorbate (MB = 176.12) (m = 0.0005 * 100 * 176.12 from equation 1).

- **0.1 mM H_2O_2**

  First determine the molarity (C) of your commercial H_2O_2 solution (mol/g).

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Using the equation 2 below:

$$C = (m/MB) * V \quad \textit{(equation 2)}$$

with m = weight (g) = D * % solution
MB = Molecular weight (g/mol)
V = Volume (L)

The molarity of a 30% solution of H_2O_2 can be calculated using:

$$C = (D * \% \text{ solution}) / MB * 1{,}000 \quad \textit{(equation 3)}$$

MB = 34.01
% of solution = 30
Density = 1.1 g/cm^3 = 1,100 g/L

Hence the molarity of 30 % solution of H_2O_2 is 9.79 M or 9,790 mM (1.1 * 30/100) /34.01 *1,000).

To prepare 1 L of a 0.1 mM solution, use the following equation:

$$C1V1 = C2V2$$

with C1 = 9,790 mM
C2 = 0.1 mM
V2 = 1,000 mL

$$V1 = 0.1*1{,}000/9{,}790 = 0.0102 \text{ mL}$$

Similarly, to prepare a 100 mL of a 10 mM solution, use 0.102 mL of your H_2O_2 commercial solution (V1 = 10*100/9.790)
Then prepare a diluted solution of 0.1 mM (1 mL of 10 mM into 100 mL H_2O) http://www.graphpad.com/quickcalcs/molarityform.cfm

- **0.1 mM EDTA**

  To prepare 100 mL of a 0.1 mM solution of EDTA, use 3.7224 mg of EDTA (MB = 372.24) (m = 0.0001 * 100 * 372.24 from equation 1 above).

Preparation of 100 mL of the reaction mixture

Solution	Stock Solution Molarity	Quantity of stock
25 mM K_2HPO_4	1 M K_2HPO_4	1.5375 mL
25 mM KH_2PO_4	1 M KH_2PO_4	0.9625 mL
0.5 mM ascorbate		8.806 mg
0.1 mM H_2O_2	10 mM	1 mL

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0.1 mM EDTA		3.7224 mg
H_2O		96.5 mL
Total Volume		**100 mL**

3rd stage: Calculations

First with the absorbance measured, calculate the C using equation 4:

$$\mathbf{C = \Delta A / (\varepsilon * d) = \Delta A / [2.8 * 10^3 * d]} \quad (\textit{equation 4})$$

with C = molarity of ascorbate (mol/L)
ΔA = Absorption Difference
e = Extinction absorbance coefficient of reduced ascorbate:
- (ε = 2.8 mM*cm^{-1} = 2.8 *10^3 L/mol*cm)

D = Light path (cm) (0.1 cm plastic plate, 0.2 cm glass plate)

To calculate the ascorbate peroxidase activity, use equation 5 below:

$$\mathbf{U\ (mol/g/min) = (V * C) / (\Delta t * FM)} \quad (\textit{equation 5})$$

with U = Activity (mol min-^{1} gr^{-1}))
V = Assay volume (L)
Δt = t1- t0 = (time between measurements) (min)
FM = weight of the fresh material (g)

Or by substituting C in equation 5 for equation 4:

$$\mathbf{U = (V * C) / (\Delta t * FM) = [V\ (L) * C] / [\Delta t * FM]}$$

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

$$\mathbf{U'\ (mol\ gr^{-1}\ protein\ min^{-1}] = U / Protein\ content\ (g)}^1 \quad (\textit{equation 6})$$

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

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Reference

Parvaiz Ahmad, Cheruth Abdul Jaleel, Mohamed A. Salem, Gowher Nabi & Satyawati Sharma (2010) Roles of enzymatic and nonenzymatic antioxidants in plants during abiotic stress, Critical Reviews in Biotechnology, 30:3, 161-175, DOI: 10.3109/07388550903524243

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3 Linked SOPs

SOP Code	SOP Function
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_014	Antioxidant assay 5: Superoxide dismutase (SOD)
EU_TRUE_SOP_015	Bradford protein assay

4 Disclaimer

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6 Citation

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