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Quantification of Uric Acid or Xanthine in Plant Samples

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[Abstract] We developed this protocol to assay and quantify the content of uric acid or xanthine in various tissues of *Arabidopsis thaliana* mutant lines with defective urate oxidase or xanthine dehydrogenase1 and in their complementation and suppressor lines (Hauck *et al.*, 2014).

The protocol is based on a method developed by Invitrogen Life Technologies for measuring uric acid or xanthine in human serum (see References 2 and 3). That protocol though required two adaptions for its use in plant science. Firstly by heating the plant samples, the activity of urate oxidase and xanthine dehydrogenase in the wild type samples is eliminated. Wild type extracts always serve as the proper pigmentation background when calculating the standard curves of uric acid and xanthine. Secondly, all samples are measured with and without the addition of urate oxidase or xanthine dehydrogenase to correct for any H_2O_2 in the samples induced by previous stress.

The assay is based on the following pair of coupled reactions:

1) Uric acid + O_2 → Hydroxyisourate + H_2O_2 (urate oxidase reaction)

2) AR + H_2O_2 → Resorufin + O_2 (horse radish peroxidase reaction)

Accordingly for Xanthine:

1) Xanthine + H_2O + O_2 → Uric acid + H_2O_2 (xanthine oxidase reaction)

2) AR + H_2O_2 → Resorufin + O_2 (horse radish peroxidase reaction)

Materials and Reagents

1. Sea sand (*e.g.* Merck, catalog number: 107711) to facilitate mechanically homogenizing the samples
2. Liquid nitrogen
3. Horse radish peroxidase (HRP) (*e.g.* Sigma-Aldrich, catalog number: P8375)
4. Urate oxidase (UOX) (*e.g.* Sigma-Aldrich, catalog number: U0880)
5. Xanthine oxidase (XO) (*e.g.* SERVA Electrophoresis GmbH, catalog number: 38418)
6. Amplex® ultra red (AR) (Life Technologies, Invitrogen™, catalog number: A36006)
7. Uric acid (*e.g.* Sigma-Aldrich, catalog number: U2625)
8. Xanthine (*e.g.* Sigma-Aldrich, catalog number: X7375)
9. Dimethylsulfoxide (DMSO) (*e.g.* Sigma-Aldrich, catalog number: D4540)
10. Prepare 10 mM AR-stocks (see Recipes)

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11. Prepare stocks of HRP, UOX and XO (see Recipes)

Equipment

1. Standard flat-bottom microplates (*e.g*. Greiner, catalog number: 655161)
2. Spectrophotometer equipped for reading multiwell plates (*e.g.* MultiSkan Go, Thermo Fisher Scientific)
3. Heating block with shaker function
4. Ultracentrifuges for 20,000 *x g* (and ideally for 40,000 *x g*)
5. Rotator for grinding samples (*e.g.* Heidolph RZR 2020, but a simpler device may also do) with a tissue-grinder which fits neatly into a conically-tapered 1.5 ml tube (see Figure 1).

Figure 1. Tissue grinder for microtubes (Taylor Scientific)

6. Precision analytic balance, *e.g.* Mettler Toledo XS series, ideally equipped with an ErgoClip holder for 1.5 ml tubes
7. Heated magnetic stirrer
8. Micropipettes (1,000, 200, 100, 20 µl)
9. Multi-channel micropipette (range 50-200 µl)
10. 1.5-ml reaction tubes
11. 0.5 ml reaction tubes
12. 15 ml tubes (if dilutions of extracts become necessary)

Procedure

A. Prepare the extraction buffer and the stocks described in Recipes (takes ~ 1 h)

B. Reagents and equipment to prepare before measuring (~ 1 h)
   1. Prepare fresh stocks of 500 µM uric acid or 500 µM xanthine for standard curves (see Figure 2, Row A).
      Notes:
      a. *Add the uric acid or xanthine to ~200 ml extraction buffer (EB) in a beaker glass. Begin heating and stirring on a heating plate (set to ~100 °C). Use a pH-meter with thermometer to monitor temperature and pH. Heating will help to dissolve the*

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chemicals but additionally you will need to add a few drops of 1 M NaOH (do this under visual control) until all the uric acid or xanthine is dissolved. Some alkalinization (you will reach around pH 8.5-9) is necessary, because both chemicals dissolve poorly in water.

b. *Another method to prepare uric acid stocks has been described by Xinhua (2006) but was not employed by us.*

2. Label a set of three 1.5 ml tubes for each plant sample biological replicate you want to measure, you will need one tube for sample extraction (no. 1) and two to collect the supernatant of each sample extract (no. 2+3).

C. Harvesting plant samples

We routinely analyzed various plant parts like developing seeds (age 7 to 19 days post anthesis), mature dry seeds, seedlings (age 3 to 15 days after imbibition of the seeds on agar plates), leaves at bolting time (leaf age being defined by their position on the rosettes and root tissue. For more details see Figure 5 in Hauck *et al.* (2014).

Note that you need to include wild type samples to calculate the standard curves for the uric acid or xanthine concentration.

For each sample place the empty sample tube (no. 1) in the holder of the electronic balance and set the balance back to zero, then weigh in your sample. As a rule of thumb, use ~ 5-10 mg seeds, ~ 50-100 mg leaves, ~ 20 mg roots. Write down the weight for each sample. Close sample tube tightly and transfer it to liquid nitrogen.

D. Extraction procedure (~ 2 h)

1. Set up the rotator with the tissue-grinder, have EB, and a bottle of distilled water at hand.
2. To avoid any carry-over of metabolites we recommend to first homogenize the wild type samples, which do not contain any uric acid or xanthine unless you deal with leaves of stressed plants.
3. Take a sample out of the liquid nitrogen, carefully open the lid and add very little sea sand to facilitate the complete grinding and homogenization of the sample. To 100 mg leaves add 500 µl EB, to 5-10 mg seeds add 250 µl EB, then start grinding. Better hold on tight to those sample tubes! When the sample appears homogenous, close the tube´s lid and transfer it back to liquid nitrogen. Rinse the pistil thoroughly with distilled water and proceed to the next sample.
4. Set up the heating block (inset must be suitable for conically-tapered 1.5 ml tubes) to 95 °C. Transfer all samples back to room temperature, carefully open the lids, wait a couple of minutes, close lids and transfer all tubes to 95 °C heat block.
5. Open lids once more to avoid sudden popping-up which may cause loss of sample. Heat-shake for 10 min at 95 °C, subsequently centrifuge them for 15 min at room temperature at 20,000 x *g*.

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6. Carefully transfer the supernatants with a micropipette to the second set of tubes (no. 2), repeat as follows for each sample: add again same amount of EB as before, vortex, heat-shake and centrifuge as before, transfer supernatant to the tube (no. 2).
7. Finally, ultracentrifuge all the tubes (no. 2), ideally at 40,000 x *g* for 15 min. to thoroughly sediment tissue debris and transfer all supernatants finally to the third set of fresh tubes (no. 3).

   Note: As uric acid or xanthine accumulates during seed development in A.t. uox or xdh1 mutants, use 15 ml-tubes to make dilutions (e.g. 2x, 5x) of the final extracts (tubes no. 3) when measuring dry seeds.

   When initially measuring metabolite standards we found a linear relationship between absorption at 560 nm and concentrations of uric acid or xanthine up to 250 µM.

E. Preparing 50 µl of standards and samples (~ 30 min, per plate)

For uric acid (or xanthine) standards use the wild type extract of the same plant part like the samples you are measuring, because this will basically eliminate possible bias caused by pigments. Wild type extracts will generally not contain uric acid or xanthine (but leaf extracts of a stressed wild type plant may contain H_2O_2, so better use at least two different wild type extracts). The standards will give you the slope of the regression line you need to determine the metabolite content of your samples. The plate shown in Figure 2 was prepared as follows: A1→ 50 µl wild type extract (=> 0 µM), A2 → 45 µl wild type extract + 5 µl of 500 µM uric acid (=> 50 µM) *etc.*

Next 50 µl of each sample were pipetted into the respective wells on the left and right half of the plate (*e.g*. young leaves, plant 1 in B1, B2, B7, B8; old leaves, plant 3 in F5, F6, F11, F12 *etc.*).

F. Prepare two kind of working solution (~ 15 min)

Each sample will be measured simultaneously in two different set-ups: Firstly with UOX (or XO) and secondly without UOX (or XO) to determine the amount of H_2O_2 already contained in the sample. The absorption values of reaction 2 will then be subtracted from those of reaction 1. Generally the right half of your plate will stay quite colorless, except when measuring extracts of stressed leaves.

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Table 1. Composition of the working solution (WS)

# assays/wells (including standards)	10	20	30	35	40	45	50	55	60
Working solution (WS) consists of:									
Amplex red (AR), µl	5	10	15	17.5	20	22.5	25	27.5	30
HRP, µl	2	4	6	7	8	9	10	11	12
UOX (or XO), µl	4 (2	8 4	12 6	14 7	16 8	18 9	20 10	22 11	24 12)
Extraction buffer (EB), ml	0.5	1.0	1.5	1.75	2.0	2.25	2.5	2.75	3.0

Table 1 will help you to prepare the working solutions.

E.g. to measure the plate shown in Figure 2, you will need (2 x 4 + 16 x 2) x 50 µl = 40 x 50 µl = 2.0 ml (better to prepare 2.5 ml) of working solution with UOX (or XO), and 16 x 2 x 50 µl = 1.6 ml (prepare 2.0 ml) of working solution without UOX (or XO). KEEP THE WS PROTECTED FROM LIGHT (*e.g.* wrap into aluminum foil).

G. Setting up a 96-well microtiter plate

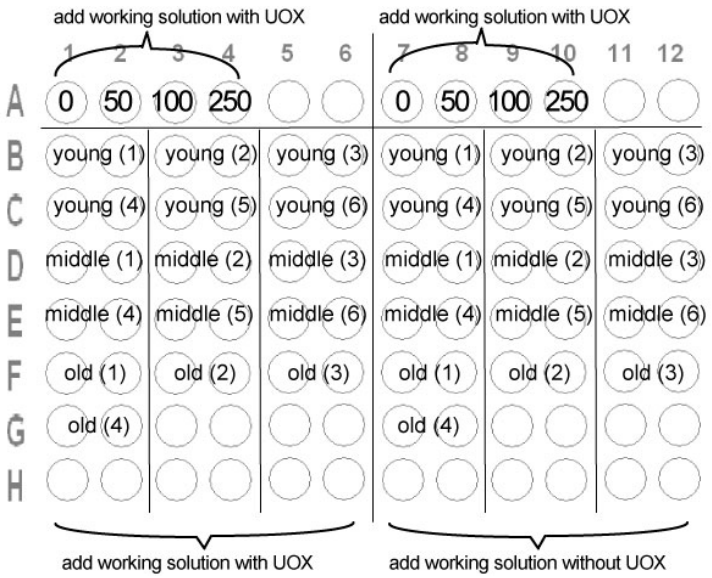

Figure 2. Setup example of a 96-well plate arranged to assay the content of uric acid in leaves of different age (Rows B-G) in four to six biological replicates, each in duplicates (column 1/2, 3/4, 5/6 *etc)*. The above setup delivered the data shown in the EXCEL-sheet.

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1. Uric acid standard in duplicate (A1-4, A7-10): Row A contains varying concentrations of uric acid in 50 µl of a wild type extract + 50 µl working solution (WS) with UOX.
2. Samples: Columns 1-6 contain 50 µl sample extract + 50 µl WS with UOX, columns 7-12 contain 50 µl sample extract +50 µl WS without UOX.

H. Setting up the scanner and reading the plate (~20 min per plate)

1. Before you start the reaction by adding the WS, make sure you can access the photometer for the next 15-20 min. Do the read-out as soon as possible after starting the reaction (by adding the WS), because O_2 and light will bias your assay (see Abstract).
2. Set the photometer to absorbance at 560 nm and read-out in 1-2 min-intervals so you can graphically follow the reaction.
3. Ideally use a multi-channel pipette to add 50 µl WS with UOX (or XO) to the left-side wells and the standards, or 50 µl WS without UOX (or XO) to the right-side wells. Each well on the left side then contains 50 µM AR, 20 milliunits HRP and 20 milliunits UOX (or 2 milliunits XO) in 100 µl. Right-side wells will contain 50 µM AR and 20 milliunits HRP only.
4. Aim at pipetting without introducing air-bubbles to the wells. Remove air-bubbles by pricking them with a dry pipet tip or a needle, then insert the plate into the photometer and read out the plate repeatedly until an endpoint of the reaction after approximately 15-20 min. is reached, indicated by the absorbance reaching a plateau.

I. Calculating the uric acid/xanthine content of your sample

Copy-paste the read-out to the provided EXCEL-Sheet, which then does steps I1-5 of the calculations for you.

Note: Refer to Figure 2 to match the plate´s read-out with the corresponding columns of the EXCEL-Sheet.

1. From the duplicates with/without UOX or XO calculate the arithmetic means of the absorption values, M_W and M_{WO}, for each sample.
2. Then subtract M_{WO}, the absorption value of reaction 2 (without UOX/XO) from M_W, the absorption of reaction 1 (with UOX/XO).

   Note that this operation also gets rid of the y-axis shifts caused by background.
3. From the uric acid or xanthine standards calculate the slope S [in 1/µM] of the regression line.
4. For each individual assay the metabolite concentration is $C_A = (M_W - M_{WO})/S$ [in µM] (with the assumption there was no dilution).
5. Considering the extraction volume V_E [in µl] the amount of metabolite in a given sample is $N_E = C_A \times V_E$ [in mol]. It was contained in P mg of the sample and therefore the metabolite concentration of that sample is $C_P = N_E / P$ [in µmol/g].

   We provide an EXCEL-sheet we routinely used: Rows 30-39 show a typical absorption

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readout of the photometer at 11-20 min. Row 39 contains the plateau value after 20 min and is the only photometric data used for the subsequent calculation. The cells with the orange filling highlight the calculation steps I1-5. For our purposes we further distinguished fresh weight, fw, from dry weight, dw (we kept reference samples for 2 days at 60 °C in a heating cabinet) of the samples and added the moisture contained in the fresh leave samples (fw-dw) to the extraction volume (see column L).

Representative data

The provided EXCEL-sheet contains the read-out of the plate set-up shown in Figure 2. Use it by pasting your own read-out data into row 39.

Notes

This protocol was developed over the course of several months and found reliable to measure uric acid and xanthine concentrations up to 250 µM in a single assay, because that is the range of a linear relationship between absorption and metabolite concentration (refer to the EXCEL-sheet). When assaying dry seeds, which accumulate the metabolites heavily in the respective enzyme mutants, it may be necessary to make 3- or 5-fold dilutions in a 15-ml tube, before loading the wells.

Recipes

1. Prepare 10 mM AR-stocks
   Add 400 µl DMSO to a 1 mg-vial of purchased AR, mix thoroughly and store as 50 µl aliquots at -20 °C.
   One vial is sufficient for 800 wells (= 800 single reactions)
2. Prepare stocks of HRP, UOX and XO
   Prepare ~500 ml of 0.1 M extraction buffer from an 0.5 M Tris/HCl pH 7.5 buffer stock.
   Then use the extraction buffer to prepare stocks of
   100 Units/ml HRP
   50 Units/ml UOX
   10 Units/ml XO
   from the purchased enzyme preparations (Reagents 3-5), and store them in 50 µl aliquots at -20 °C. Used aliquots can be stored at 4 °C for several weeks.

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