bio-protocol

Published: Mar 05, 2022

A Quick Method to Quantify Iron in *Arabidopsis* Seedlings

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Abstract

Iron (Fe) is an indispensable micronutrient for plant growth and development. Since both deficiency, as well as a surplus of Fe, can be detrimental to plant health, plants need to constantly tune uptake rates to maintain an optimum level of Fe. Quantification of Fe serves as an important parameter for analyzing the fitness of plants from different accessions, or mutants and transgenic lines with altered expression of specific genes. To quantify metals in plant samples, methods based on inductively coupled plasma-optical emission spectrometry (ICP-OES) or inductively coupled plasma-mass spectrometry (ICP-MS) have been widely employed. Although these methods are highly accurate, these methodologies rely on sophisticated equipment which is not always available. Moreover, ICP-OES and ICP-MS allow for surveying several metals in the same sample, which may not be necessary if only the Fe status is to be determined. Here, we outline a simple and cost-efficient protocol to quantify Fe concentrations in roots and shoots of *Arabidopsis* seedlings, by using a spectroscopy-based assay to quantify Fe^{2+}-$BPDS_3$ complexes against a set of standards. This protocol provides a fast and reproducible method to determine Fe levels in plant samples with high precision and low costs, which does not depend on expensive equipment and expertise to operate such equipment.

Keywords: *Arabidopsis*, Iron quantification, Spectrophotometry, BPDS, Plant Nutrition

This protocol was validated in: Plant Physiol (2021), DOI:10.1093/plphys/kiab329

Background

Iron (Fe) is an essential micronutrient, which is involved in numerous biochemical and physiological processes in plants. A deficiency or an excess of Fe in plants limit plant growth and cause severe losses in crop yield and quality. Imbalances of Fe levels in plants also affect the homeostasis of other nutrients (Schmidt *et al.*, 2020). Therefore, the determination of Fe concentrations in plant tissues is mandatory for the assessment of the nutritional status of the plant and serves as an important parameter for studying Fe-related genes. Currently, inductively coupled plasma (ICP) spectrometry-based methods are considered the gold standard for determining Fe levels. Although these methods are highly sensitive and reliable, their accessibility is rather limited due to costly equipment, specialized training required for the operation of such equipment, and the requirement of generally large amounts of material, which may render the analysis difficult in cases where sample size is limited. Therefore, a simple, reliable, and easily accessible method to measure Fe levels at high accuracy is a valuable alternative to ICP-based methods.

Here, we describe a method for quantifying total Fe in roots and shoots of plant seedlings. The method is based on a protocol established for the analysis of Fe concentration in *Plantago lanceolata* L. leaves (Schmidt, 1996), which we have adapted and optimized for convenient and routine analysis of the more commonly used model plant *Arabidopsis thaliana*. The method involves wet acid digestion of dried plant samples with nitric acid and hydrogen peroxide, to solubilize Fe and reduction of Fe(III) complexes by hydroxylammonium chloride, followed by a colorimetric assay based on the ability of the chelating agent bathophenanthroline disulfonate (BPDS) to form red-colored complexes with ferrous Fe. The resulting $Fe^{2+}(BPDS)_3$ complex has a distinct absorbance band at 535 nm under acid conditions, allowing for the quantification of Fe concentrations by interpolation from a standard curve. BPDS has been widely used for the determination of Fe levels in a variety of samples (Pré and Benlatrèche, 1977; Tangerås, 1983; Hirayama and Nagasawa, 2017; Freinbichler *et al.*, 2020). The present protocol was optimized for *Arabidopsis thaliana* samples, and has been used for quantifying Fe concentrations as low as 50 µg/g dry weight sample or less (Tsai *et al*., 2018; Gautam *et al*., 2021), but can be modified and applied to determine Fe levels in samples from other plant species.

Materials and Reagents

1. Butter paper
2. Tissue paper/Kimwipes
3. 15 mL screw cap conical tubes (Sarstedt, catalog number: NC1377856)
4. 96-well microplate (Greiner CELLSTAR®, catalog number: 655180)
5. Nitric acid (65%) (Merck, catalog number: 1.00456.1000, store at room temperature)
6. Hydrogen peroxide solution (30%) (Merck, catalog number: 1.07209.1000, store at room temperature)
7. ddH_2O
8. Bathophenanthrolinedisulfonic acid disodium salt hydrate (Sigma, catalog number: B1375-5G, store at room temperature)
9. Sodium acetate (Sigma, catalog number: S8750-500G, store at room temperature)
10. Hydroxylamine hydrochloride (Alfa Aesar, catalog number: A15398.36, store at room temperature)
11. Iron (III) chloride hexahydrate (Merck, catalog number: 1.03943.0250, store at room temperature inside a dry cabinet)
12. Assay solution (see Recipes)
13. Fe standard solutions (see Recipes)

Equipment

1. Vannas scissors
2. Drying oven (Hot air oven, PREMA®)
3. Vortex mixer (Vortex-Genie 2, Scientific Industries)
4. Heat block (Elite Dry Bath Incubator, Major Science)
5. Microplate spectrophotometer (Power Wave XS2, BioTek Instruments, Agilent Technologies)

Software

1. Gen5™ BioTek Instruments (Agilent Technologies)
2. GraphPad Prism version 9

Procedure

A. Sampling

1. Sow seeds on Estelle and Somerville (ES) nutrient media (Estelle and Somerville, 1987), and stratify for at least two days in the dark at 4°C, before transferring to a growth chamber. Grow seedlings at 21–22°C under continuous illumination (50 µmol m^2 s^{-1}) for two weeks.
2. To separate seedlings into shoots and roots for sample harvesting, use Vannas scissors to cut seedlings on the media at hypocotyl junctions.
3. For shoot samples, collect 25–30 shoots of two-week-old seedlings in butter paper.
4. For root samples, collect 30–36 roots of two-week-old seedlings in butter paper. Before placing roots into butter paper, make sure that the roots don't contain any residual media, which also contains Fe. Remove any media from the roots with tissue paper/Kimwipes, followed by washing twice with ddH_2O. Then gently dry roots with tissue paper/Kimwipes.
5. Dry samples in an oven for at least two days at 60–65°C.
6. Determine the dry weight of each sample.
7. After weighing, transfer the samples into 15 mL conical tubes.

B. Sample digestion

1. Add 225 µL of nitric acid (65%) into each tube and screw the caps tightly.
2. Incubate for 6 h at 95°C on a heat block, vortexing the tubes every 1–2 h. Make sure that samples do not stick to the walls of the tubes and no precipitates are present, as these could affect measurements.
3. Carefully unscrew the caps of the tubes, add 150 µL of H_2O_2 (30%) into each tube, and screw the caps back tightly.
4. Incubate for 2 h at 56°C on a heat block, and vortex every 30–60 min to make sure no precipitates are formed, as this could also affect measurements.
5. Add 225 µL of ddH_2O. Fully digested samples should be pale yellow in color, and ideally should have no white precipitates.
6. For the ease of pipetting in the following steps, carefully transfer the digested samples into 1.5 mL microcentrifuge tubes. At this point, samples are ready to be immediately used for the Fe quantification in procedure C, or can be stored at 4°C in the dark for up to 1 month for later analysis.

C. Standard and sample preparation, and spectroscopic reading

1. Prepare the assay solution (see Recipe 1) and Fe standard solutions (see Recipe 2).
2. Add 5 µL of standards/samples in microplate wells. Run three technical replicates for each standard/sample.
3. Add 245 µL of assay solution to each well with standard/sample.
4. Cover the microplate and keep it in the dark to incubate at room temperature for 5 min before spectrophotometric measurement. Following incubation, the standards will turn pink/red, with increasing color saturation as the Fe level increases **(Figure 1A)**, and the samples will turn light yellowish-pink/orange-red depending on the Fe content.
5. Measure absorbance at 535 nm. Use the 0 Fe standards as blanks to zero the plate reader.

Data analysis

1. Create a standard curve in an Excel sheet by plotting the average blank-corrected 535 nm absorbance values vs. the concentration of standards. Make sure that the coefficient of determination (R^2) value is ≥0.990.
2. Use the equation derived from the standard curve to calculate the Fe concentrations in the samples. **Table 1** shows the calculation of Fe concentrations in root samples.
3. Divide the Fe concentrations of the samples by their respective dry weight (DW), to obtain the final Fe concentration per g DW (**Table 1**).
4. Plot the final graph in Excel or any suitable software. The representative plot shown in **Figure 1B** was generated using GraphPad Prism 9. The sample data shown only represents one biological replicate, with three technical replicates. A complete experiment should have at least three biological replicates.

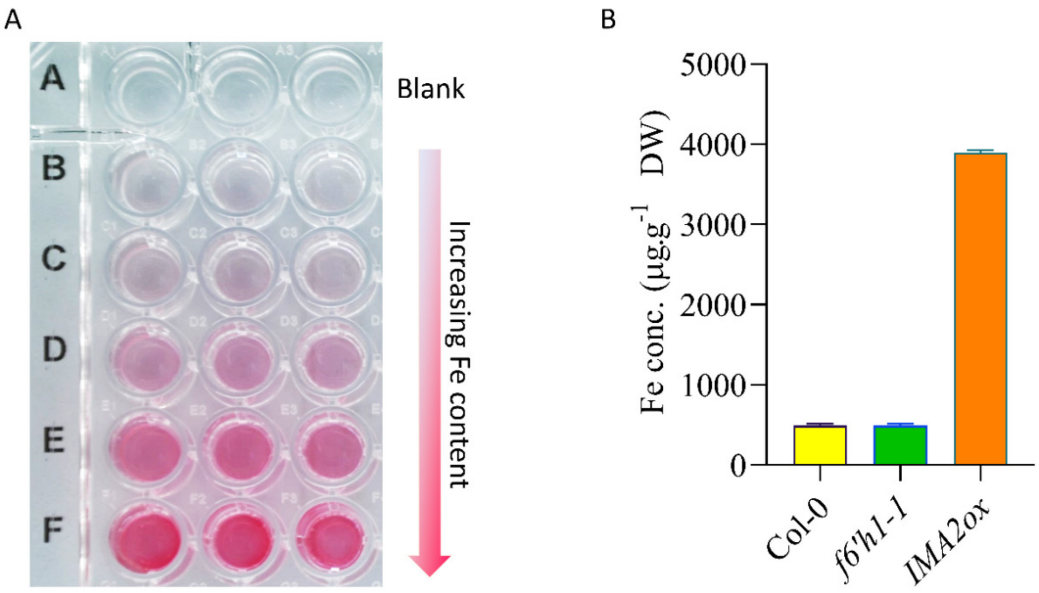


Figure. 1 Fe content-dependent color pattern of $Fe^{2+}(BPDS)_3$ complexes and their spectrophotometric quantification.
A. Representative figure showing the change in color of $Fe^{2+}(BPDS)_3$ complex across an increasing Fe content gradient. Equal volume of assay solution was added in a 96 well plate microplate containing different amounts of Fe (0, 2.5, 5, 10, 20, and 40 µg) in three technical repeats. The microplate was kept in the dark to incubate at room temperature for 5 min, a photo was captured immediately afterwards. **B.** Graph showing the Fe concentrations in different samples (genotypes). Wild-type (Col-0) plants, a coumarin-deficient mutant (*f6'h1-1;* Schmid *et al.*, 2014), and an Fe over-accumulating genotype (*IMA2ox*) (Gautam *et al.*, 2021) grown on

nutrient media containing 50 µM of Fe-EDTA are used to represent the Fe quantification. The data represent standard deviation of three technical replicates from the spectrophotometer reading. DW, dry weight.

Table. 1 Calculations to quantify Fe in root samples.

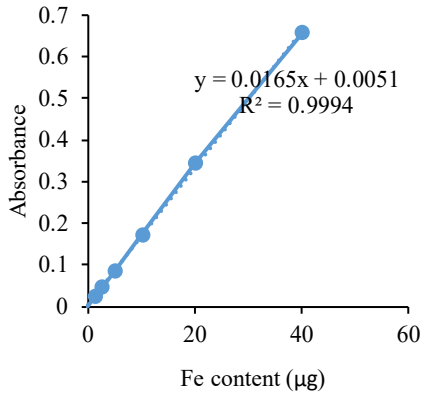
Spectrophotometric readings of the standards were used to plot a calibration curve. The Fe concentrations in the samples were calculated using the equation derived from the slope. Wild-type (Col-0) plants, a coumarin-deficient mutant (*f6'h1-1*; Schmid *et al.*, 2014), and an Fe over-accumulating genotype (*IMA2ox*; Gautam *et al.*, 2021) grown on nutrient media containing 50 µM Fe-EDTA are used to represent Fe quantification. The data represent the standard deviation of three technical replicates from the spectrophotometer reading. The microplate layout used to make spectrophotometric readings is shown at the top. STD, standard; S, sample; Abs., absorbance; DW, dry weight; Avg., average; SD, standard deviation.

	Microplate layout					
	1	**2**	**3**	**4**	**5**	**6**
A	Blank	Blank	Blank	S1	S1	S1
B	STD1	STD1	STD1	S2	S2	S2
C	STD2	STD2	STD2	S3	S3	S3
D	STD3	STD3	STD3			
E	STD4	STD4	STD4			
F	STD5	STD5	STD5			
G	STD6	STD6	STD6			

	Absorbance (Abs.)								
	1	**2**	**3**	**4**	**5**	**6**		DW(g) of roots	Samples
A	0	0	-0.001	0.039	0.036	0.038	Blank 535	0.004	Col-0/S1
B	0.022	0.031	0.021	0.041	0.043	0.044	Blank 535	0.0046	*f6'h1-1*/S2
C	0.043	0.044	0.051	0.28	0.28	0.284	Blank 535	0.0043	*IMA2ox*/S3
D	0.087	0.085	0.086				Blank 535		
E	0.174	0.171	0.173				Blank 535		
F	0.352	0.339	0.344				Blank 535		
G	0.626	0.666	0.68				Blank 535		

Fe content	Avg. abs.	x = (y - 0.0051)/0.0165		
0	0.000	2.05	1.87	1.99
1.25	0.025	2.18	2.30	2.36
2.5	0.046	16.66	16.66	16.90
5	0.086			
10	0.173			
20	0.345			
40	0.657			

Fe conc. (µg/g DW)		
Samples	Avg.	SD
Col-0	493.43	0.09
f6'h1-1	494.95	0.09
IMA2ox	3893.35	0.14



Notes

1. This method can be used to quantify Fe in samples grown on both soil and nutrient media.
2. Steps B to C need to be carried out inside a fume hood with proper protection, including corrosion resistant gloves, safety glasses, lab coat, and shoes.
3. The sample volume should be reduced or the reagent volumes should be adjusted for complete digestion of samples.
4. Use only high-grade acid-resistant falcon, PTFE, or Teflon™ tubes for acid digestion. Make sure to tightly cap the tubes during the wet acid digestion steps.
5. Although the volume of the digested sample is only 600 µL, a 15 mL Falcon tube is used for safety reasons, and to avoid damaging the tubes due to pressure from gas build-up during the heat assisted digestion steps.

Recipes

1. Assay solution (for 100 mL)

No.	Reagent	Working concentration	Amount to add
1.	Bathophenanthrolinedisulfonic acid disodium salt hydrate (MW: 590.53 g/mol)	1 mM	0.06 g
2.	Sodium acetate (MW: 82.03 g/mol)	0.6 M	4.92 g
3.	Hydroxylamine hydrochloride (MW: 69.49 g/mol)	0.48 M	3.34 g
4.	ddH_2O	-	adjust to 100 mL

Note: Prepare fresh assay solution in a light-shield bottle and store at 4°C until use.

2. Fe standard solutions

Prepare a 50 mM $FeCl_3$ stock solution (50 mM $FeCl_3$ contains 2.79 µg/µL of Fe). With this stock solution, prepare Fe standards in 1.5 mL microcentrifuge tubes as follows:

Standards	Blank	STD1	STD2	STD3	STD4	STD5	STD6
Fe content (µg)	0	1.25	2.5	5.0	10.0	20.0	40.0
Amount of stock to add (µL)	0	0.45	0.90	1.79	3.58	7.17	14.34
Nitric acid (65%)	225 µL into each tube						
H_2O_2 (30%)	150 µL into each tube						
ddH_2O	225 µL into each tube						

Acknowledgments

This work was supported by a grant from the Ministry of Science and Technology to W.S. (grant No.: 108-2311-B-001-033-MY3). The data used to create the representative graph and table has been extracted from previously published work (Gautam *et al.*, 2021).

Competing interests

The authors declare no conflict of interest.

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