bio-protocol

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Quantitative Analysis of Redox Pool (NAD^+, NADH Content) in Plant Samples Under Aluminum Stress

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Abstract

Nicotinamide adenine dinucleotide (NAD) is an essential cofactor of numerous enzymatic reactions found in all living cells. Pyridine nucleotides (NAD^+ and NADH) are also key players in signaling through reactive oxygen species (ROS), being crucial in the regulation of both ROS-producing and ROS-consuming systems in plants. NAD content is a powerful modulator of metabolic integration, protein de-acetylation, and DNA repair. The balance between NAD oxidized and reduced forms, *i.e.*, the NADH/NAD^+ ratio, indicates the redox state of a cell, and it is a measurement that reflects the metabolic health of cells. Here we present an easy method to estimate the NAD^+ and NADH content enzymatically, using alcohol dehydrogenase (ADH), an oxido-reductase enzyme, and with MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) as the substrate and 1-methoxy PMS (1-Methoxy-5-methylphenazinium methyl sulfate) as the electron carrier. MTT is reduced to a purple formazan, which is then detected. We used *Arabidopsis* leaf samples exposed to aluminum toxicity and under untreated control conditions. NADH/NAD^+ connects many aspects of metabolism and plays vital roles in plant developmental processes and stress responses. Therefore, it is fundamental to determine the status of NADH/NAD^+ under stress.

Keywords: NAD^+, NADH, Stress, Aluminum, *Arabidopsis*, Redox status

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Background

Nicotinamide adenine dinucleotide (NAD) is an important coenzyme ubiquitously found in all living cells. The balance between the oxidized and reduced forms of NAD (the NADH/NAD^+ ratio) is crucial to cell survival. This ratio is an important component that indicates the redox state of a cell, important for major cellular processes like signal transduction and epigenetics, and reflects both the metabolic activities and the health of cells. NAD^+ is responsible for the transfer of electrons between molecules during metabolic processes; therefore, its levels are essential for maintaining normal cellular respiratory function. Furthermore, NAD functions in modulating cellular redox status and controlling signaling and transcriptional events (Awasthi *et al.*, 2019).

Depletion of NAD in cells is a major cause of cell death. Quantifying the generation and consumption of pyridine nucleotides, NADH and NAD^+, is important to monitor enzymatic reactions or screen the modulator or product of these enzyme reactions. Pyridine nucleotides are involved in other defense and signaling reactions, such as nitric oxide production and metabolism of reactive lipid derivatives. NAD status can alter photosynthesis and plant stress responses (Dutilleul *et al.*, 2003), suggesting that NAD content is a powerful modulator of metabolic integration (Dutilleul *et al.*, 2005). NADH and NAD^+ are also key players in signaling through reactive oxygen species (ROS) (Moller, 2001; Apel and Hirt, 2004; Mittler *et al.*, 2004; Foyer and Noctor, 2005). NAD-consuming reactions are of importance in stress conditions for signaling in interactions with ROS and other redox components. A balance in the rates of oxidation and reduction of these nucleotides is a prerequisite for the continuation of both catabolic and anabolic processes. Therefore, the NADH/NAD^+ ratio is a proxy for the metabolic state of plant cells, and determining its content under stress is fundamental for understanding stress response mechanisms.

Materials and Reagents

1. 96-well plate (Tarsons Product, India)
2. 50 mL centrifuge tubes (Tarsons Product, India)
3. 1.5/2 mL tubes (Tarsons Product, India)
4. Root sample of *Arabidopsis* genotype Col-0
5. Double distilled water
6. Planton box (Tarsons Product, catalog number: 020080, size: 75 × 75 × 100mm)
7. Sodium hypochlorite (NaOCl) (Himedia Laboratories, catalog number: PCT1311-5X50M)
8. Calcium chloride ($CaCl_2$) (Himedia Laboratories, catalog number: PCT0004-500G)
9. Aluminum chloride ($AlCl_3$) (Merck, catalog number: 8010810100)
10. Nicotinamide adenine dinucleotide (NAD) (Sigma-Aldrich, catalog number: NAD100-RO-1G)
11. Nicotinamide adenine dinucleotide hydrogen (NADH) (Sigma-Aldrich, catalog number: 10107735001-500MG)
12. Magnesium sulphate heptahydrate ($MgSO_4·7H_2O$) (Himedia Laboratories, catalog number: RM684-5KG)
13. Manganese (II) Sulphate pentahydrate ($MnSO_4·5H_2O$) (FUJIFILM Wako Pure Chemical Corporation, catalog number:139-00825)
14. Ferrous sulphate heptahydrate ($FeSO_4·7H_2O$) (Himedia Laboratories, catalog number: GRM3917-500G)
15. Zinc sulphate hepta hydrate ($ZnSO_4·7H_2O$) (Himedia Laboratories, catalog number: PCT0118-1KG)
16. Copper (II) sulphate pentahydrate ($CuSO_4·5H_2O$) (Himedia Laboratories, catalog number: RM630-500G)
17. Potassium nitrate (KNO_3) (Himedia Laboratories, catalog number: RM1401-500G)
18. Boric acid (H_3BO_3) (Himedia Laboratories, catalog number: MB007-1KG)
19. Sodium phosphate monobasic anhydrous (NaH_2PO_4) (Himedia Laboratories, catalog number: MB183-500G)
20. Ammonium molybdate tetrahydrate ($(NH4)_6Mo_7O_{24}·4H_2O$) (Sigma-Aldrich, catalog number: 431346)
21. Cobalt (II) chloride hexahydrate ($CoCl_2·6H_2O$) (Himedia Laboratories, catalog number: PCT0103-500G)
22. EDTA, disodium salt hydrate (Na_2EDTA) (Sigma-Aldrich, catalog number: E5134)
23. Sodium nitrate ($NaNO_3$) (Himedia Laboratories, catalog number: GRM1184-500G)
24. Sodium phosphate monobasic dihydrate ($NaH_2PO_4·2H_2O$) (Sigma-Aldrich, catalog number: 71505)
25. Sodium phosphate dibasic dodecahydrate ($Na_2HPO_4·12H_2O$) (Sigma-Aldrich, catalog number: 71649)
26. Calcium chloride dihydrate ($CaCl_2·2H_2O$) (Himedia Laboratories, catalog number: MB034-500G)
27. Sodium hydroxide pellets (NaOH) (Himedia Laboratories, catalog number: MB095-500G)
28. Hydrochloric acid (HCl) (Himedia Laboratories, catalog number: AS004-2.5L)

29. Tris base (Sigma-Aldrich, catalog number: T1503)
30. Bicine (Sigma-Aldrich, catalog number: B3876)
31. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Sigma-Aldrich, catalog number: 1.11714-1G)
32. 1-Methoxy-5-methylphenazinium methyl sulfate (1-methoxy PMS) (Sigma-Aldrich, catalog number: M8640-100MG)
33. Alcohol dehydrogenase (ADH), from Yeast (Sigma-Aldrich, catalog number: A7011)
34. 0.2 M NaOH solution
35. Modified MGRL solution (see Recipe 1)
36. Bicine/NaOH buffer (see Recipe 2)
37. 1 M Tris-HCl (see Recipe 3)
38. 10 M Ethanol (see Recipe 4)
39. 80 mM EDTA-2Na (see Recipe 5)
40. ADH solution (see Recipe 6)
41. Reaction Mixture (see Recipe 7)
42. NAD standard (see Recipe 8)
43. NADH standard (see Recipe 9)

Equipment

1. Weighing balance (Sartorious, 0.1 mg–220 g)
2. Pipettes/multi-channel pipette (Gilson, Pipettman, 2-2020-200 and 100–1000 µL)
3. pH meter (pH Tutor, Eutech Instrument)
4. Centrifuge (*Eppendorf 5424 Microcentrifuge*)
5. Magnetic stirrer with hot plate (Tarsons Product, India)
6. Micro pestle (Tarsons Product, India)
7. Autoclave (Equitron, Equitron Medica Pvt. Ltd., India)
8. Water bath (Equitron unstirred water bath, Equitron Medica Pvt. Ltd., India)
9. pH test paper (Himedia Laboratories, India)
10. Microtiter plate reader (SUNRISE microplate reader, TECAN)
11. Nylon mesh (100 µM pore size)
12. Fuji film plastic mounts, 35 mm (Fuji photo Co. Ltd. Japan)

Procedure

1. Surface sterilize viable *Arabidopsis* seeds in a 1.5 mL centrifuge tube with 1% Sodium hypochlorite for 3 min and rinse five times in autoclaved distilled water. Carry out all procedures inside a laminar flow hood to avoid contamination. Keep the rinsed seeds at 4°C for vernalization in dark conditions. After 2 days, place the vernalized seeds on the nylon mesh mounted on Fujifilm plastic mounts, and allow them to float on a Planton box already filled with modified MGRL solution (Recipe 1) in aseptic conditions, at 20 ± 2°C, with a photoperiod of 14 h, and with a photon flux density of 220 µmol m^{-2} sec^{-1} (PAR). After 5 days, with one hand decant the modified MGRL and replace with the treatment solution (10 µM $AlCl_3$ solution containing 100 µM $CaCl_2$, pH 5.0); while replacing the solution, hold the Fujifilm plastic mounts with the mesh bearing the seedlings on the other hand, using forceps. Harvest samples (whole plant tissue) for the redox pool assay at 6 and 12 h after the beginning of the treatment (Figure 1).
2. Grind samples (whole plant tissue, 100 mg) in liquid nitrogen with a micro pestle in a 1.5 mL centrifuge tube, and then extract with 1 mL of 0.2 N HCl. Centrifuged the homogenate at 16,000 × *g* and 4°C for 10 min; make multiple aliquots of the supernatant (0.2 mL each) for replicates.

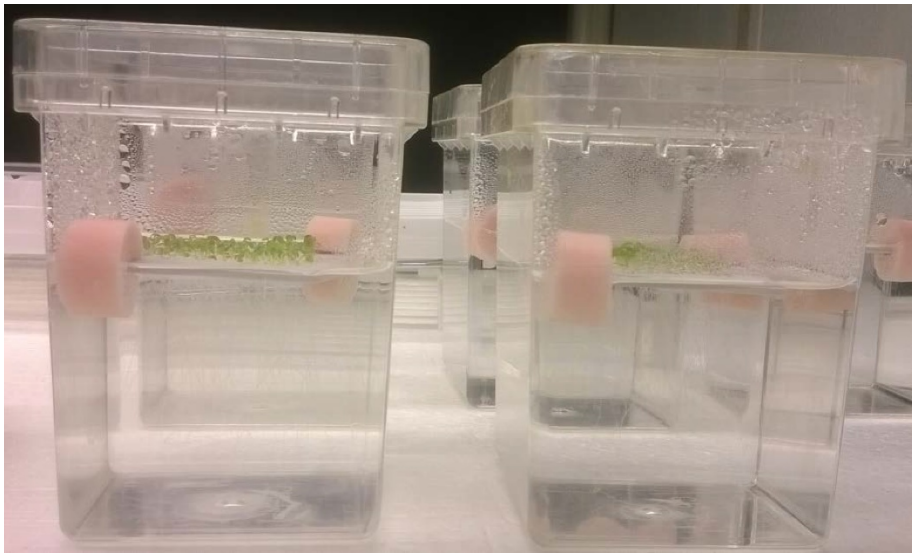

Figure 1. *Arabidopsis* plant grown in aseptic condition on MGRL hydroponic solution.

3. For the NAD^+ assay, incubate 0.2 mL of extract in boiling water (98–100°C) for 1 min, and then cool it rapidly and neutralize it by adding 20 µL of 0.2 M NaH_2PO_4 (pH 5.6), followed by the stepwise addition of 0.2 M NaOH aliquots. Vortex the sample after each addition and check pH with pH indicator paper. The final pH should be between 5 and 6, which requires approximately 0.16 mL of 0.2 M NaOH.
4. To measure NADH, extract leaf samples as for NAD^+ but with 0.2 M NaOH as the extraction medium, and neutralize the heated supernatant aliquot with 0.2 N HCl to a final pH of 7–8 for all samples. This requires approximately 0.14 mL of 0.2 N HCl. Vortex the sample after each addition and verify pH with pH indicator paper.
5. Prepare the enzymatic reaction mixture as follows:
    a. Add MTT and 1-Methoxy PMS in separate tubes and dissolve in water (prepare these solutions at room temperature) (see Recipe 7).
    b. Add 2 mL of 1 M Bicine/NaOH Buffer, 0.4 mL of 1 M tris, 1 mL of 80 mM EDTA, and 1 mL of 10 M ethanol in a 50 mL centrifuge tube (see Recipe 7).
    c. Add the dissolved MTT and 1-Methoxy PMS to the 50 mL centrifuge tube, adjust the final volume to 20 mL, and incubate in a water bath at 25°C until further use (see Recipe 7). This solution will act as the reaction mixture.
    d. Prepare the ADH solution and keep it on ice (see Recipe 6).
    e. Add 40 µL of each standard sample (see Recipe 8 for NAD^+, 9 for NADH), plant sample (from step 3 for NAD^+ and from step 4 for NADH), and blank sample (40 µL water) to a 96-well plate.
    f. Add ADH (4 µL) to the reaction mixture (156 µL) and gently mix.
    g. Add 160 µL of enzymatic reaction mixture into each sample well of the 96-well plate and immediately measure the absorbance using a microtiter plate reader.
    h. Set the parameter for measurement of absorbance as: measurement filter, 570 nm; and kinetics, 10 measurements at 1 min intervals, shaking for 5 s before every reading.
    i. Plot the standard graphs of NAD^+ and NADH in a Microsoft Excel spreadsheet and further evaluate the plant sample contents (Figure 2).

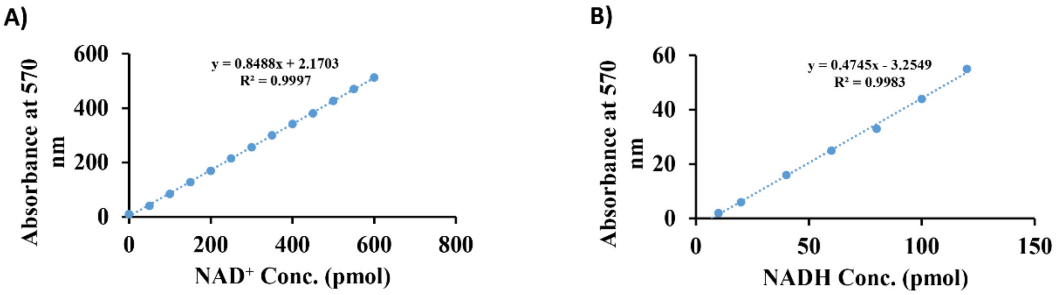

Figure 2. Standard curve for NAD⁺ (A) and NADH (B). Absorbance measured at 570 nm.

Data analysis

All analysis and graph plotting was done using Microsoft Office Excel 2016 spreadsheets. Each experiment was repeated thrice and the data presented are mean ± standard error (SE). Significance was tested with one-way ANOVAs. Duncan's multiple range test (DMRT) was performed for comparison among the set of experiments (Figure 3).

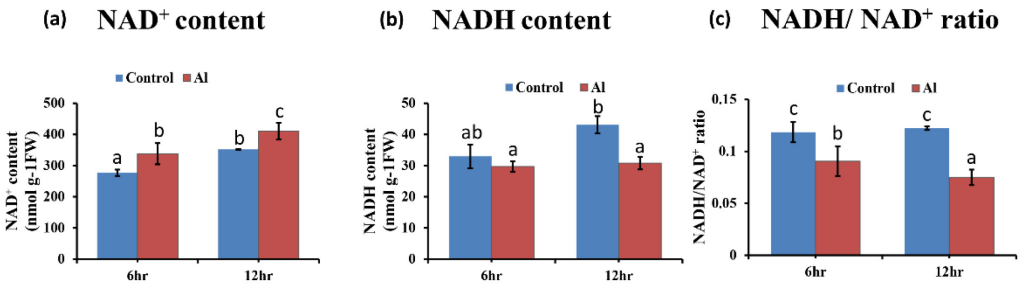

Figure 3. Example of NAD⁺ and NADH content and their ratio in *Arabidopsis* WT (Col-0) root samples. Absolute quantification of NAD⁺ and NADH and their ratio using a microtiter plate reader coupled enzyme assay in different replicates (a, b, and c). Values are means ± SE (n = 3) of three separate experiments. Means denoted by the same letter were not significantly different at $P < 0.05$ according to Duncan's multiple range test.

Recipes

1. MGRL solution

Sr. No.	Chemical constituents	Solution Stock Conc.	Solution Final conc.	Required volume for the preparation of 1 L solution, pH 5.8
1	$MgSO_4 \cdot 7H_2O$	0.15 M	0.03 mM	200 µL
2	$Mn\ SO_4 \cdot 5H_2O$	1.03 mM	0.206 µM	200 µL
3	$FeSO_4 \cdot 7H_2O$	0.86 mM	0.172 µM	200 µL
4	$ZnSO_4 \cdot 7H_2O$	0.1 mM	0.02 µM	200 µL
5	$CuSO_4 \cdot 5H_2O$	0.1 mM	0.02 µM	200 µL
6	KNO_3	0.3 M	0.06 mM	200 µL
7	H_3BO_3	3.0 mM	0.6 µM	200 µL
8	$(NH4)6Mo_7O_{24} \cdot 4H_2O$	2.4 µM	0.48 nM	200 µL

9	$CoCl_2 \cdot 6H_2O$	13 µM	2.6 nM	200 µL
10	Na_2EDTA	6.7 mM	1.34 µM	200 µL
11	$NaNO_3$	0.4 M	80 µM	200 µL
12	Na-PO_4 (pH 5.8) $NaH_2PO_4 \cdot 2H_2O$ $Na_2HPO_4 \cdot 12H_2O$	 0.175 M 0.175 M	 0.035 mM 0.035 mM	200 µL
13	$CaCl_2 \cdot 2H_2O$	1 M	200 µM	200 µL

Prepare adequate amounts of nutrient solution according to sample size and plant species; adjust pH to 5.8.

2. 1 M Bicine/NaOH (pH 8.0) Buffer

a. Dissolve 16.317 g of Bicine (MW = 163.17 g/mol]) in 75 mL of distilled water
b. Adjust to pH 8.0 using 10 N NaOH
c. Fill to final volume of 100 mL with dH_2O
d. Filter sterilize (recommended) or autoclave
e. Store at 4°C

3. 1 M Tris-HCl

a. Dissolve 12.1 g Tris Base (TRIZMA) in 70 mL of distilled water and add concentrated HCl to pH 8.0
b. Fill up to volume 1 L with distilled water
c. Store at room temperature.

4. 10 M Ethanol

For the preparation of this solution, take 58.4 mL of absolute Ethanol and make up to 100 mL with distilled water.

5. 80 mM EDTA-2Na

a. The dissolve 29.77 g of Na_2EDTA in 80 mL of distilled water and adjust the pH to 8.0 with NaOH
b. Adjust volume to 100 mL with distilled water, stir vigorously on a magnetic stirrer, and store at 4°C for longer storage.
c. Adjust the pH of the solution to 8.0 by the addition of NaOH to completely dissolve the Na_2EDTA.

6. ADH solution

Add 8 mg of ADH to a 1.5 mL tube and dissolve in 1 mL of bicine/NaOH. After dissolving, keep on ice for immediate use.

7. Reaction Mixture preparation

Chemicals constituents	**Total**	**20 mL**	**Final concentration**
MTT	mg	3.48 (dissolve in 6 mL of water)	0.42 mM
1-Methoxy PMS	mg	3.72 (dissolve in 6 mL of water)	0.55 mM
1 M Bicine/NaOH	mL	2	0.1 M
1 M Tris	mL	0.4	20 mM
80 mM EDTA-2Na	mL	1	4 mM
10 M EtOH	mL	1	0.5 M
H_2O (MilliQ)	mL	3.6	

8. NAD standard: NAD^+ standard

Standard curve (pmol/mL)	**blank**	**50**	**100**	**150**	**200**	**250**	**300**	**350**	**400**

1 µM NAD^+(µL)	0	5	10	15	20	25	30	35	40
H_2O (MilliQ) (µL)	100	95	90	85	80	75	70	65	60

Take 40 µL of sample from each concentration.

9. NADH standard: NADH standard

Standard curve (pmol/mL)	**blank**	**10**	**20**	**40**	**60**	**80**	**100**	**120**	**140**
100 nM NADH (µL)	0	10	20	40	60	80	100	12 (1 µM stock)	14
H_2O (MilliQ) (µL)	100	90	80	60	40	20	0	88	86

Take 40 µL of sample from each concentration.

Acknowledgments

This protocol was adapted from Hampp *et al.* (1984) and Takita *et al.* (1999).

Competing interests

The authors declare no conflicts of interest or competing interests.

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