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Detection of Nitric Oxide and Determination of Nitrite Concentrations in *Arabidopsis thaliana* and *Azospirilum brasilense*

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[Abstract] There is now general agreement that nitric oxide (NO) is an important and almost ubiquitous signal in plants. Nevertheless, there are still many controversial observations and differing opinions on the importance and functions of NO in plants. Partly, this may be due to the difficulties in detecting and quantifying NO. Here, we summarize protocols for detecting NO and quantifying nitrite concentration in *Arabidopsis* seedlings. We also present a method to measure NO in biofilms formed by the plant growth promoting rhizobacteria *Azospirillum brasilense* (*A. brasilense*). NO in oxygen-containing aqueous solutions has a short half-life that is often attributed to a rapid oxidation to nitrite. Here we detail the use of the fluorescent probe DAF-FM DA and the electrochemical method for directly detecting and quantifying NO, respectively, and the Griess reagent to indirectly detect NO through its oxidized nitrite form. These protocols could be useful in a variety of cell types and plant tissues, as well as for microorganisms.

Part I. *In vitro* determination of nitrite concentration

Materials and Reagents

1. Square Petri dishes (Deltalab, catalog number: 200204)
2. Multi-well plates (96 well) (Deltalab, catalog number: 900010)
3. 10-day-old *Arabidopsis* ecotype Columbia (Col-0)
4. Murashige and Skoog Basal Salt Mixture (MS) (Sigma-Aldrich, catalog number: M5524)
5. Sodium chloride (NaCl) (Sigma-Aldrich, catalog number: S9888)
6. Sulfanilamide (Sigma-Aldrich, catalog number: S9251)
   Note: The working solution is 1% (w/v) Sulfanilamide in 5% (v/v) phosphoric acid. Store at 4 °C in the dark.
7. N-(1-Naphthyl)ethylenediamine dihydrochloride (NED) (Sigma-Aldrich, catalog number: 33461)

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Note: The working solution is 0.1% (w/v) NED in H_2O. Store at 4 °C in the dark.

8. Standard nitrite solution (Sigma-Aldrich, catalog number: 237213)
   Note: The working solution is 100 µM sodium nitrite in Milli Q water.
9. Sodium phosphate dibasic (Sigma-Aldrich, catalog number: S0876)
10. Sodium phosphate monobasic (Sigma-Aldrich, catalog number: 0751)
11. Buffer A (100 mM phosphate buffer, pH 7.4) (see Recipes)

Equipment

1. Centrifuge (Thermo Fisher Scientific, model: Sorvall Legend Micro 17R)
2. Elisa plate reader (Metrolab 980 microplate reader)

Procedure

1. Grow *Arabidopsis* in Petri dishes containing ½ strength MS medium for 5 d and then transfer to treatment solution (100 mM NaCl) for 5 d or more (step 1, Figure 1).
2. Grind 100 mg of seedlings or dissect the plant into root and shoot to form a powder under liquid nitrogen in a mortar. Add 300 µl of 100 mM sodium phosphate (pH 7.4) to the samples [step 2(a), Figure 1].
3. Centrifuge samples at 10,000 *x g* for 15 min at 4 °C (step 2(b), Figure 1b).
4. Use the supernatant for nitrite and protein quantification: For nitrite quantification, load 50 µl of supernatant in a well of the Elisa plate in triplicate [step 2(c), Figure 1]. For protein quantification**,** perform the Bradford (1976) assay with 1 or 2 µl of supernatant in triplicate.
5. For the nitrite Standard, prepare 1 ml of a 100 µM nitrite solution [step 2(c), Figure 1]. Dispense 50 µl of Buffer A into the wells in rows B-H. Add 100 µl of the 100 µM nitrite solution to the remaining 3 wells in the first row. Immediately perform 6 serial two-fold dilutions (50 µl/well) in triplicate down the plate to generate the nitrite Standard reference curve (100, 50, 25, 12.5, 6.25, 3.13 and 1.56 µM). Do not add any nitrite solution to the last set of wells (0 µM) as this will serve as the blank measurement.
6. Allow the Sulfanilamide Solution and NED Solution to equilibrate to room temperature (15-30 min). Dispense 50 µl of the Sulfanilamide Solution to all experimental samples and wells containing the dilution series for the nitrite Standard reference curve [step 2(c), Figure 1].
7. Incubate for 5-10 min at room temperature, protected from light.
8. Dispense 50 µl of the NED Solution to all wells (step 3, Figure 1). An appropriate control assay without NED solution is necessary to detect any interference due to sample.
9. Incubate at room temperature for 5-10 min, protected from light. A purple/magenta color will begin to form, if the Griess reaction has occurred (step 3, Figure 1).

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10. Measure absorbance in a plate reader with a filter between 520 nm and 550 nm. A single measurement for each well is sufficient. The measurement should be done within 30 min after step 9 (step 3, Figure 1), because the color may fade after this time.
11. Use the standard curve to calculate the nitrite concentration in the samples. Calculate the concentration as nitrite per µg of protein. Use the equation (step 4, Figure 1) for nitrite calculations. The “y” value is the absorbance detected for each sample and the “x” value, is the nitrite concentration.

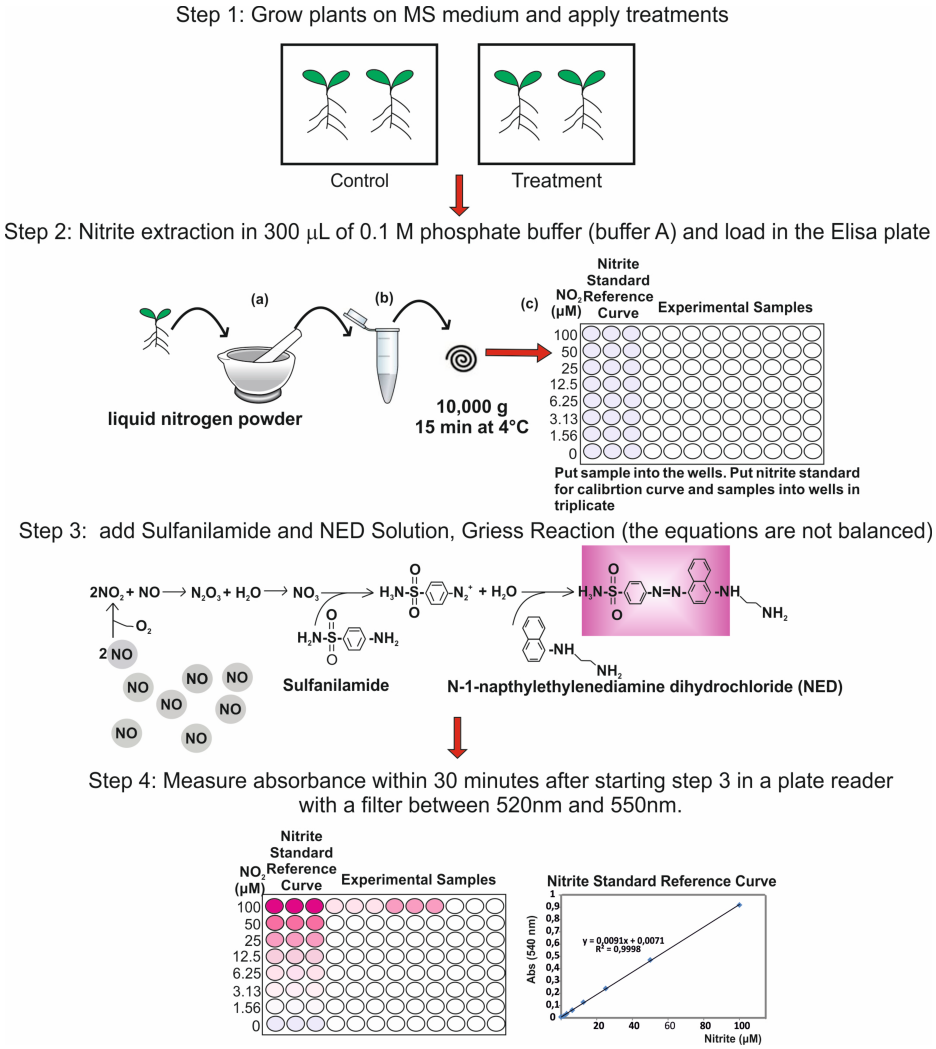

Figure 1. Nitrite determination by Griess assay in *Arabidopsis* seedlings

Recipes

1. Buffer A (100 mM phosphate buffer, pH 7.4)
   77.4 mM Sodium phosphate dibasic
   22.6 mM Sodium phosphate monobasic
   Store at room temperature

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Part II. *In vivo* detection of NO

Method 1. Electrochemical detection of NO

Materials and Reagents

1. Multi-well plates (24 wells flat bottom) (Sigma-Aldrich, Corning® Costar®, catalog number: CLS3527)
2. 20 ml borosilicate glass vial (Thermo Fisher Scientific, catalog number: 033377)
3. *Azospirillum brasilense* Sp245 strain
4. Standard nitrite solution (Sigma-Aldrich, catalog number: 237213)
   Note: The working solution is 100 µM sodium nitrite in Milli Q water. Prepare fresh for each use.
5. Potassium iodide (Sigma-Aldrich, catalog number: 746428)
6. Sulfuric acid (Merck Millipore Corporation, catalog number: 100732)
7. DL-Malic acid (Sigma-Aldrich, catalog number: 240176)
8. Potassium phosphate dibasic (K_2HPO_4) (Sigma-Aldrich, catalog number: P3786)
9. Magnesium sulfate heptahydrate ($MgSO_4{\cdot}7H_2O$) (Sigma-Aldrich, catalog number: 230391)
10. Sodium chloride (NaCl) (Sigma-Aldrich, catalog number: S7653)
11. Calcium chloride hydrate ($CaCl_2{\cdot}H_2O$) (Sigma-Aldrich, catalog number: 202940)
12. Ethylenediaminetetraacetic acid ferric sodium salt (Fe-EDTA) (Sigma-Aldrich, catalog number: E6760)
13. Potassium hydroxide (KOH) (Sigma-Aldrich, catalog number: P1767)
14. Potassium nitrate (KNO_3) (Sigma-Aldrich, catalog number: P8291)
15. Sodium molybdate dehydrate ($NaMoO_4{\cdot}2H_2O$) (Sigma-Aldrich, catalog number: 331058)
16. Manganese (II) sulfate monohydrate ($MnSO_4$) (Sigma-Aldrich, catalog number: M7634)
17. Boric acid (H_3BO_3) (Sigma-Aldrich, catalog number: B6768)
18. Copper (II) sulfate pentahydrate ($CuSO_4{\cdot}5H_2O$) (Sigma-Aldrich, catalog number: C8027)
19. Zinc sulfate heptahydrate ($ZnSO_4{\cdot}7H_2O$) (Sigma-Aldrich, catalog number: Z0251)
20. Phosphate buffered saline, pH 7.4 (Sigma-Aldrich, catalog number: P4417)
21. Calibration solution (see Recipes)
    Note: Prepare fresh for each use.
22. Buffer B (see Recipes)
23. NFb-malic medium (see Recipes)

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Equipment

1. Nitric Oxide Measuring System (NOMS) (*e.g.,* Innovative Instruments Inc., model: inNO-T-II System)
2. NO-specific sensor (*e.g.,* Innovative Instruments Inc., model: amiNO-2000)
3. Sensoready (Innovative Instrument) device

Sensor calibration

a. Before calibration, the sensor should be polarized for a few hours (preferably overnight) by immersing it in calibration solution or Milli Q water and connect to the Sensoready device.
b. Turn on PC and open NOMS software.
c. The sensor is calibrated by a chemical reaction for NO production based on the conversion of nitrite to NO in acidic solution in the presence of iodide ion. The reaction has a molar ratio 1:1, meaning that the amount of NO produced equals the amount of nitrite added. In this protocol, we used the term “NO/nitrite” concentration to unify both.
   Note: Other methods such as using the NO donor (±)-S-Nitroso-N-acetylpenicillamine (SNAP) and NO saturated solutions can be assayed (Allen et al., 2003).
d. Immerse the tip of the sensor in the calibration solution. Zero the background using the “Zero” button in the NOMS software.
e. Add 10 µl of nitrite standard solution to 20 ml of calibration solution while stirring. Wait until the current reaches its maximum potential and begins to decline.
f. Zero the background again by pressing the “Zero” button in the NOMS software.
g. Repeat steps 5-6 at least three more times with adding 20, 40 and 80 µl of nitrite standard solution, respectively.
h. Measure the peak height of each addition with the NOMS software by placing the cursor on the peak (panel A, Figure 2). Plot current (pA) vs. concentration of NO/nitrite (nM) to make a reference curve (panel B, Figure 2).
   Note: The final volume in the vial is 20 ml, and the NO concentration range is 0-400 nM.

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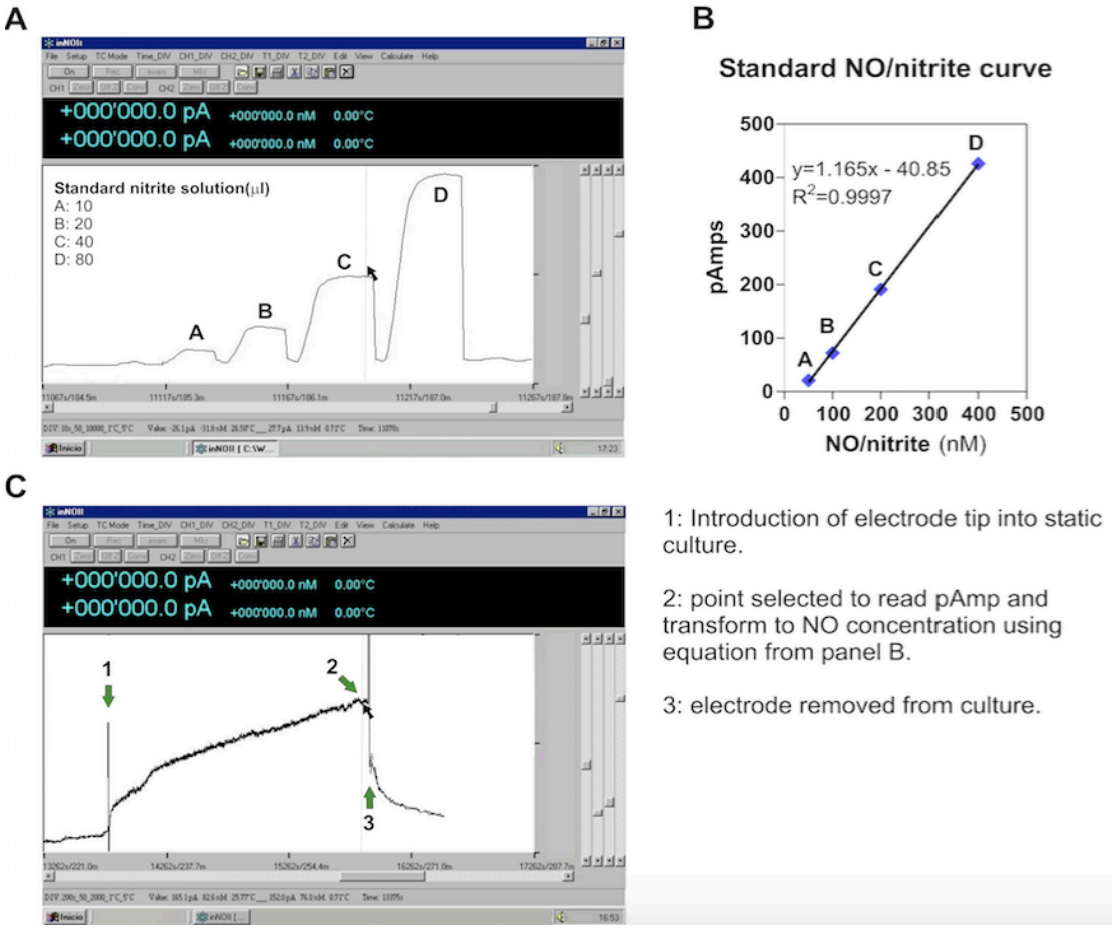

Figure 2. Construction of standard curve (A, B) and determination of NO in *A. brasilense* Sp245 biofilm sample (C) using a NO electrode

Software

1. NOMS software

Procedure

1. Grow *A. brasilense* Sp245 (2 ml per well) on tissue culture plates for 2 d in NFb-malic medium under static conditions to allow biofilm formation.
2. Immediately before use, stabilize the microelectrode for 15 min in Buffer B followed by 15 min in NFb-malic medium.
3. Zero the background.
4. Immerse microelectrode 3-4 mm into the bacterial culture and start recording changes of current potential. Usually, 30-40 min of recording time is needed per sample to measure NO production in *Azospirillum* static cultures.

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5. Enter the obtained current value in the standard curve to establish NO concentration of the samples. Use the equation (panel B, Figure 2) to transform the current values to a concentration of NO. The “y” value is the pA detected for the sample and the “x” value corresponds to NO concentration in nM.
   Note: The concentration of nitrite in a sample can be measured in vitro by injecting bacterial culture supernatant into an acid/iodide solution in which nitrite is converted to NO and then detected by the sensor.

Recipes

1. Calibration solution
   Weigh and dissolve 20 mg of potassium iodide in 15 ml of Milli Q water and 2 ml of 1 M sulfuric acid. After potassium iodine has dissolved completely, add Milli Q water to make 20 ml of solution.
   Note: Once this solution becomes light yellow, due to the formation of iodine in the solution, discard and prepare a new solution.
2. Buffer B
   PBS was prepared according to product specifications. One tablet of PBS was dissolved in 200 ml of deionized water to yield 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride (pH 7.4), at 25 °C. Store at room temperature.
3. NFb-malic medium [modified as in Arruebarrena *et al.* (2013)]
   For 1 L NFb-malic medium pH 6.5 with nitrate as N source: 3.7 g Malic acid, 5 ml K_2HPO_4 10% (w/v), 2 ml $MgSO_4{\cdot}7H_2O$ 10% (w/v), 1 ml NaCl 10% (w/v), 2 ml $CaCl_2{\cdot}H_2O$ 1% (w/v), 2 ml Micronutrients solution, 4 ml Fe-EDTA 1.64% (w/v), 4.5 g KOH, 1.39 g KNO_3.
   For 200 ml of micronutrients solution: 200 mg $NaMoO_4{\cdot}2H_2O$, 235 mg $MnSO_4$, 280 mg H_3BO_3, 8 mg $CuSO_4{\cdot}5H_2O$, 24 mg $ZnSO_4{\cdot}7H_2O$.

Method 2. NO Fluorometric assay

Materials and Reagents

1. Square Petri dishes (Deltalab, catalog number: 200204)
2. Multi-well plates (12 or 24 wells) (Biofil®)
3. Microscopic glass slides (Deltalab, catalog number: D100001) and cover slips (Deltalab, catalog number: D102440)
4. Five-day-old Arabidopsis seedlings, ecotype Columbia (Col-0)
5. Murashige and Skoog Basal Salt Mixture (MS) (Sigma-Aldrich, catalog number: M5524)
6. 1 mM Calcium chloride ($CaCl_2$) (Sigma-Aldrich, catalog number: 449709)

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Note: Store at room temperature.

7. 0.25 mM Potassium chloride (KCl) (Sigma-Aldrich, catalog number: P3911)
   Note: Store at room temperature.
8. DAF-FM diacetate (DAF-FM DA) (Thermo Fisher Scientific, Molecular probes™, catalog number: D-23844)
   Note: Store at -20 ºC.
9. Abscisic acid (Sigma-Aldrich, catalog number: A1049)
   Note: Store at -20 ºC.
10. Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, catalog number: D8418)
11. 5 mM MES buffer (pH 5.7) (Sigma-Aldrich, catalog number: M3671)
12. DAF-FM diacetate (DAF-FM DA) stock solution (see Recipes)
13. Buffer C (see Recipes)

Equipment

1. Bright field and fluorescent microscope Eclipse E200 microscope (Nikon Corporation) (http://www.nikon.com/)

Procedure

1. Grow Arabidopsis in Petri dishes in ½ strength MS medium for 4 to 5 d. Use tweezers to transfer the seedlings to microplate wells containing 10 µM ABA in ½ strength liquid MS medium for 2 h.
2. Replace the ABA solution with 1 ml Buffer C containing 10 µM DAF FM DA (step 1, Figure 3).
3. Incubate seedlings at room temperature protected from light for 20 min, followed by a wash with 1 ml of fresh Buffer C for 20 min.
4. Mount seedlings on glass slides and cover with cover slips. Samples are visualized under bright field and epifluorescent microscopy (excitation 490 nm; emission 525 nm) (step 2, Figure 3).

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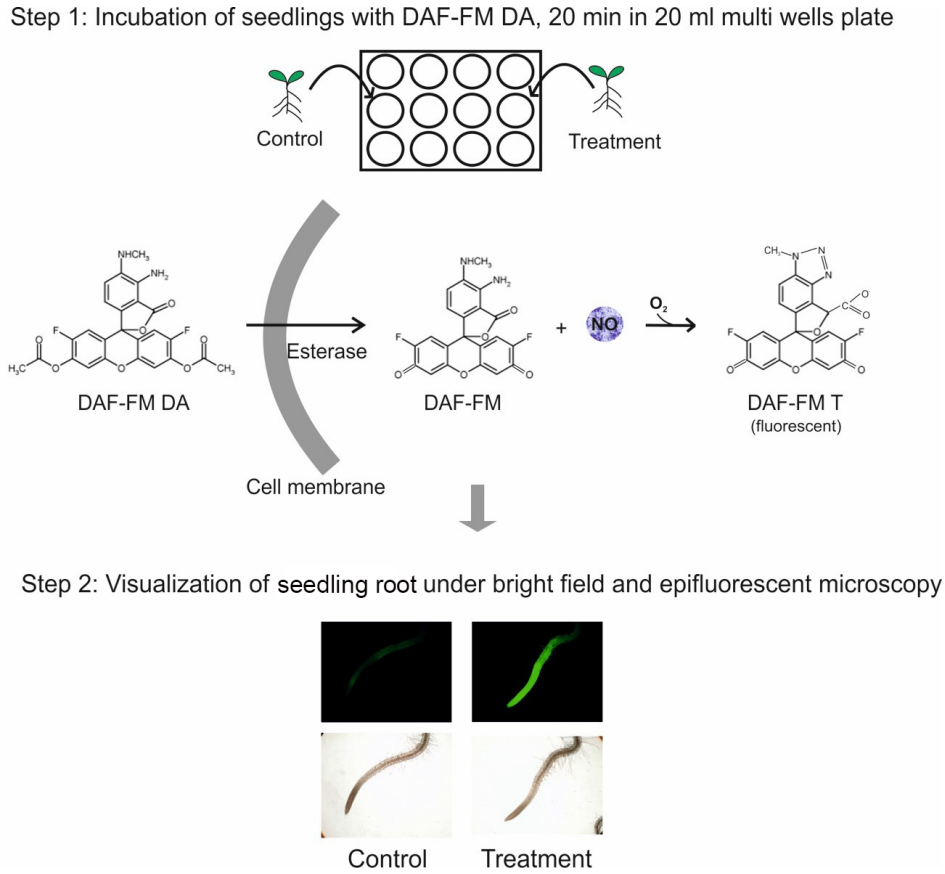

Figure 3. Nitric Oxide detection by DAF-FA DA in *Arabidopsis* seedling root

Recipes

1. DAF-FM diacetate (DAF-FM DA) stock solution
   5 mM DAF-FM DA in dimethyl sulfoxide (DMSO)
2. Buffer C
   Store at room temperature
   5 mM MES buffer, adjusted with KOH to pH 5.7
   1 mM $CaCl_2$
   0.25 mM KCl

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