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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 different opinions on the importance and functions of NO in plants. Partly, this may be due to the difficulties in detecting and even more in quantifying NO. Here, we summarize protocols for detecting NO and quantifying nitrite concentration in *Arabidopisis* seedlings and for the NO real time measurement in biofilms formed by the plant growth promoting rhizobacteria *Azospirillum brasilense* (*A. brasilense*). NO in oxygen-containing aqueous solution 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 different tissues of plants, and 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. Sulfanilamide (Sigma-Aldrich, catalog number: S9251)
   Note: Working solution 1% (w/v) Sulfanilamide in 5% (v/v) phosphoric acid. Storage at 4 °C in dark.

6. N-(1-Naphthyl)ethylenediamine dihydrochloride (NED) (Sigma-Aldrich, catalog number: 33461)
   Note: Working solution 0.1% (w/v) NED in H_2O. Storage at 4 °C in dark.
7. Standard nitrite solution (Sigma-Aldrich, catalog number: 237213)
   Note: Working solution 100 µM sodium nitrite in Mili Q water.
8. Sodium phosphate dibasic (Sigma-Aldrich, catalog number: S0876)
9. Sodium phosphate monobasic (Sigma-Aldrich, catalog number: 0751)
10. Buffer A (100 mM phosphate buffer, pH 7.4) (see Recipes)

Equipment

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

Procedure

1. Grow *Arabidopsis* in Petri dishes containing ½ strength MS medium for 5 d and then transfer to treatment (100 mM NaCl) for 5 d or more (step 1, Figure 1).
2. Ground 100 mg of seedlings or dissect the plant into root and shoot to a powder under liquid nitrogen in a mortar and suspend samples in 300 µl of 100 mM sodium phosphate (pH 7.4) [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, load 50 µl of supernatant in a well of the Elisa plate by triplicated [step 2(c), Figure 1]. For protein using Bradford (1976), take 1 or 2 µl of supernatant by triplicated.
5. For nitrite Standard, prepare 1 ml of a 100 µM nitrite solution [step 2(c), Figure 1]. Dispense 50 µl of the 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 5-10 min at room temperature, protected from light.
8. Dispense 50 µl of the NED Solution to all wells (step 3, Figure 1).

9. Incubate at room temperature for 5-10 min, protected from light. A purple/magenta color will begin to form immediately, when the Griess reaction occurred (step 3, Figure 1).
10. Measure absorbance in a plate reader with a filter between 520 nm and 550 nm, a single measure for per well is sufficient. The measurement should be done within 30 min after step 9 (step 3, Figure 1). Color may fade after this time.
11. Use the standard curve to calculate the nitrite concentration in the samples. Refer the resulting data as nitrite per µg of protein. Use the equation (step 4, Figure 1) for nitrite calculation, replace the valor of "y" for the absorbance detected for the sample and then calculated the "x" valor, this valor correspond a nitrite concentration.

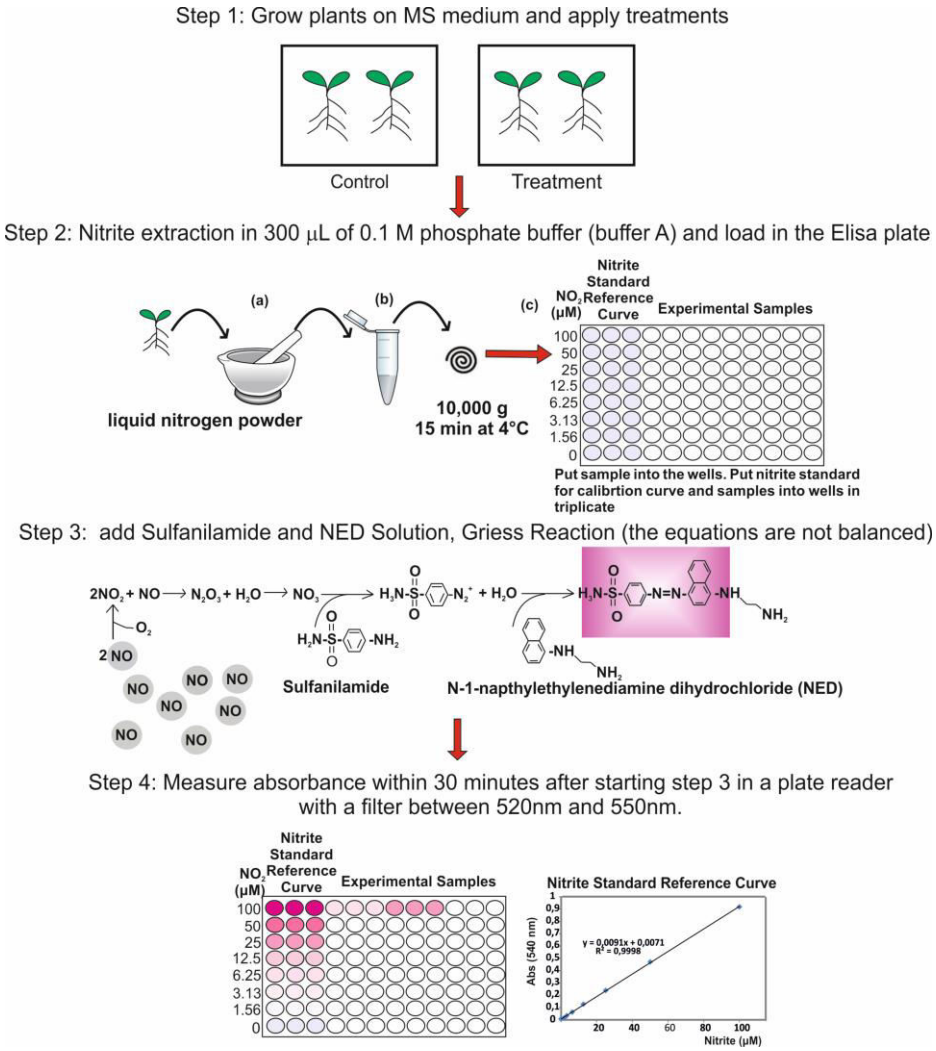

Figure 1. Nitrite determination by Griess assay in *Arabidopsis* seedlings

Recipes

1. Buffer A (100 mM phosphate buffer, pH 7.4)
   77.4 mM Sodium phosphate dibasic

22.6 mM Sodium phosphate monobasic

Storage at room temperature

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: Working solution 100 µM sodium nitrite in Mili Q water. Prepare freshly for 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 freshly for use.
22. Buffer B (see Recipes)
23. NFb-malic medium (see Recipes)

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 calibrating the sensor should be polarized for a few hours, preferably overnight immersed in calibration solution or Mili Q water and connected to 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 Zero button from NOMS software.
e. Add 10 µl of nitrite standard solution to a 20 ml calibration solution while stirring. Wait until the current reaches its maximum potential and begins to decline.
f. Zero the background again pressing Zero button from 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 on NOMS software by placing cursor on 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.

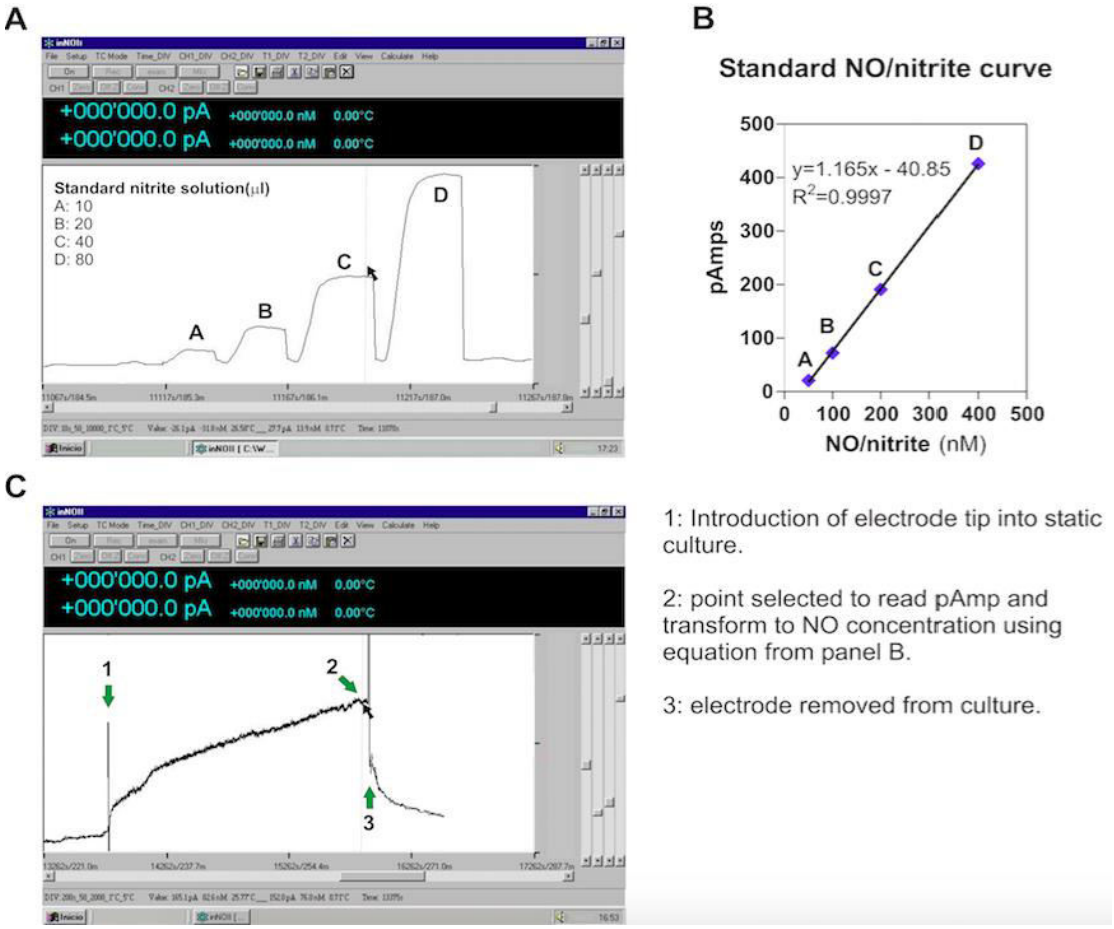

Figure 2. Construction of standard curve (A, B) and determination of NO in *A. brasilense* Sp245 biofilm sample (C) using a NO electrode

Software

1. NOMS software

Procedure

1. Grow *A. brasilense* Sp245 (2 ml per well) on tissue 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 running in Buffer B followed by 15 min in NFb-malic medium.
3. Zero the background.
4. Immerse microelectrode 3-4 mm in the bacterial culture and start recording changes on current potential. Usually, 30-40 min recording time is needed per sample to measure NO production in *Azospirillum* static cultures.

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 concentration of NO, replace the value of "y" for the pA detected for the sample and then calculated the "x" value, this value correspond to NO concentration in nM.
   Note: The concentration of nitrite in a sample can be measured in vitro by injecting a certain volume of the sample (bacterial culture supernatants) 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 Mili Q water and 2 ml of 1 M sulfuric acid. After potassium iodine dissolved completely, add Mili 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 dissolved in 200 ml of deionized water yields 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride (pH 7.4), at 25 °C. Storage 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 root 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)

Note: Storage at room temperature.

7. 0.25 mM Potassium chloride (KCl) (Sigma-Aldrich, catalog number: P3911)
   Note: Storage at room temperature.
8. DAF-FM diacetate (DAF-FM DA) (Thermo Fisher Scientific, Molecular probes™, catalog number: D-23844)
   Note: Storage at -20 ºC.
9. Abscisic acid (Sigma-Aldrich, catalog number: A1049)
   Note: Storage 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. Take the seedlings with tweezer and transfer to microplate wells containing abscisic acid (ABA) 10 µM 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 washing 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).

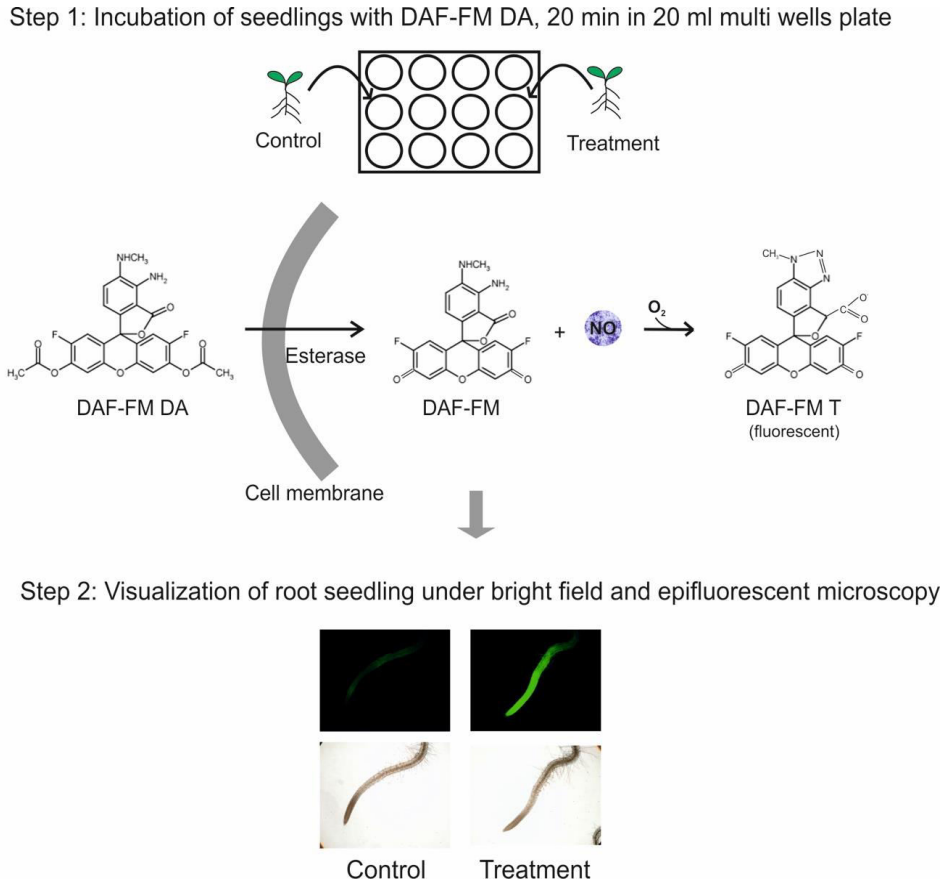

Figure 3. Nitric Oxide detection by DAF-FA DA in *Arabidopsis* root seedlings

Recipes

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

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