Chapter 2

Chemiluminescence Detection of Nitric Oxide from Roots, Leaves, and Root Mitochondria


Abstract

NO is a free radical with short half-life and high reactivity; due to its physiochemical properties it is very difficult to detect the concentrations precisely. Chemiluminescence is one of the robust methods to quantify NO. Detection of NO by this method is based on reaction of nitric oxide with ozone which leads to emission of light and amount of light is proportional to NO. By this method NO can be measured in the range of pico moles to nano moles range. Using direct chemiluminescence method, NO emitted into the gas stream can be detected whereas using indirect chemiluminescence oxidized forms of NO can also be detected. We detected NO using purified nitrate reductase, mitochondria, cell suspensions, and roots; detail measurement method is described here.

Key words Chemiluminescence, Mitochondria, Nitric oxide, Cell suspensions, Hydroponics

1 Introduction

Nitric oxide is a free radical molecule that plays an important role in plant growth development and stress [1]. In recent years nitric oxide roles in plants have been emerging. Various enzymes have been characterized for their ability to generate NO [2, 3]: cytosolic nitrate reductase (NR), mitochondria nitrite NO reductase, the plasma membrane nitrite: NO reductase (PM-NiNOR), xanthine oxidoreductase, NO synthase-like enzyme, polyamine (PA), and hydroxylamine (HA)-mediated pathways. In order to interpret NO roles, it is vital to know how much NO is produced from each enzyme system and its spatial and temporal generation pattern is also very important. Despite extensive research, accurate method to measure NO concentration is currently unavailable.

NO stability depends on its concentration. For instance, at 20 μM concentration NO has a half-life approximately 160 s whereas at 100 μM concentration half-life of NO is about 8 s [4]. NO
concentration varies according to specific stress and developmental stage. For instance under anoxia roots produce very high amount of NO continuously whereas during development cells produce minute amount of NO; therefore methods should be sensitive enough to measure NO at low and higher concentrations. In some cases NO production restricted to specific cells for instance during stomatal closing NO production increases in guard cells [5].

There are various methods that have been developed such as diamino fluorescence diacetate (DAF) method, chemiluminescence, electron spin resonance (ESR), mass spectrometry, Griess reagent assay, oxyhemoglobin assay, NO electrodes, laser-based infrared spectrometry (LAPD), and arginine-to-citrulline assay [6, 7, 31]. Each method has some advantages and disadvantages; for instance chemiluminescence is the most widely accepted method for measurement of NO; it can measure NO from pico moles to nano moles range (Table 1) but the disadvantage is that it can measure only NO emitted into gas stream [17]. The scavenged NO cannot be detected by this method. For detection of scavenged NO indirect chemiluminescence method is needed [18]. Another method called as laser-based photoacoustic detection of NO can measure NO very precisely but disadvantage is similar to chemiluminescence that it can measure only NO emitted into gas phase. The membrane inlet mass spectrometry (MIMS) is another method where NO emission can be measured very precisely, but the disadvantage is high cost and maintenance of the device; moreover NO that is emitted into gas phase only can be measured [19]. Another method to measure NO is using NO electrode [20, 21]. The advantage of this method is that NO can be measured in extract and cell suspensions treated with various elicitors or any abiotic stress. Disadvantage is that it can measure NO in gas phase only [22]. Electron spin resonance (ESR) is another method to measure NO in pico molar range. The advantage is that it measures NO very precisely, and the disadvantage is that it can measure NO that is in oxidized forms and measurement of emitted NO is not possible by this method [23]. Oxyhemoglobin assay is another method in which oxyhemoglobin (HbO₂) reaction with NO leads to production of methemoglobin (MetHb) and nitrate (NO₃). This method can measure NO in nano molar range [24]. The advantage is that it can be easily done but the disadvantage is that it can measure only trapped NO; NO emitted into gas phase is not possible to detect [25]; another disadvantage is that reactive oxygen species and low pH can interfere with measurement.

By using direct and indirect chemiluminescence one can measure both oxidized and reduced forms of NO.

The measurement of NO by chemiluminescence is usually performed by a NO analyzer (CLD 770 AL ppt, Eco-Physics, Switzerland). The principle for measuring NO is the measurement
<table>
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<td><em>Nicotiana benthamiana</em></td>
<td>Excised leaves</td>
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<td>Nitrate supplementation [8]</td>
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<td><em>Hordeum vulgare</em></td>
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<td>3</td>
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<td><em>Nicotiana tabacum</em></td>
<td>Suspension culture cells</td>
<td>0.38 nmol/gFW/h 0.79 nmol/gFW/h</td>
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<td>5</td>
<td><em>Helianthus annuus</em></td>
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<td>170 nmol/gFW/h</td>
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<td><em>Glycine max</em></td>
<td>Leaves</td>
<td>10 μmol/gFW/h</td>
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<td>7</td>
<td><em>Nicotiana tabacum</em></td>
<td>Leaves</td>
<td>40 nmol/gFW/h</td>
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<td>Roots</td>
<td>9 nmol/gFW/h</td>
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<td></td>
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<td>WT Root mitochondria</td>
<td>&lt;1 nmol/mg protein/h 3.5 nmol/mg protein/h</td>
<td>0.5 mM Nitrite/1 mM NADH/air 0.5 mM Nitrite/1 mM NADH/anoxia [13]</td>
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<td>WT</td>
<td>&lt;1 nmol/mg protein/h 1 nmol/mg protein/h</td>
<td>0.5 mM Nitrite/1 mM NADH/20 μM myxothiazol</td>
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<td>Root</td>
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<td>&lt;1 nmol/gFW/h 12 nmol/gFW/h</td>
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<td>Root</td>
<td>17 nmol/gFW/h</td>
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<td>Leaf mitochondria</td>
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<td>NR-free <em>nia</em> 30 double mutant Root segments</td>
<td>&lt;1 nmol/gFW/h</td>
<td>Nitrite/air</td>
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<td>&lt;1 nmol/gFW/h</td>
<td>Nitrite/anoxia</td>
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<td>&lt;1 nmol/mg protein/h</td>
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<td>7 nmol/mg protein/h</td>
<td>Ammonia/tungstate/anoxia</td>
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<td>3 nmol/gFW/h</td>
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<td>40 nmol/gFW/h</td>
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<td>&lt;1 nmol/gFW/h</td>
<td>0.5 mM Nitrite/2.5 mM SHAM/anoxia</td>
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<td>NR-free <em>nia</em> double mutant Root mitochondria</td>
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<td>1.5 nmol/mg protein/h</td>
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<td>&lt;1 nmol/mg protein/h</td>
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<td>NR-free <em>nia</em> 30 Leaf mitochondria</td>
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<td>0.5 mM Nitrite/anoxia</td>
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<td>WT Root segments</td>
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<td>0.5 mM Nitrite/1.5 mM KCN</td>
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<td><em>Arabidopsis thaliana</em> Leaves</td>
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<td><em>Pisum sativum</em> Roots</td>
<td>14.4 ± 0.05 nmol/gFW/h</td>
<td>NOS activity [15]</td>
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<td>Stems</td>
<td>37.8 ± 0.22 nmol/gFW/h</td>
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<td>Leaves</td>
<td>7.2 ± 0.02 nmol/gFW/h</td>
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<td><em>Nicotiana tabacum</em></td>
<td>Leaves with petioles</td>
<td>0.01–0.05</td>
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<td>Cell suspensions</td>
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<td>Reoxygenation</td>
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<td>NiR deficient <em>Nicotiana tabacum</em> mutant “clone 271”</td>
<td>Leaves</td>
<td>1–23</td>
<td>Dark-Light-dark transient</td>
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<td>5–16</td>
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<td><em>Nicotiana tabacum</em> cv. Xanthi and cv. Gatersleben</td>
<td>Cell suspensions</td>
<td>0.83 ±0.31</td>
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<td>35.76 ±13.01</td>
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<td>0.75 ±0.31</td>
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<td>0.36 ±0.10</td>
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<td>0.28 ±0.10</td>
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<td>0.07 ±0.03</td>
<td>Ammonium medium/air+10 μM myxothiazol+2.5 mM SHAM+2 mM KCN</td>
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<td>28.62 ±10.29</td>
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<td>15.52 ±6.71</td>
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<td>0.81 ±0.52</td>
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<td>0.57 ±0.09</td>
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<td>0.53 ±0.09</td>
<td>Nitrate medium/air+10 μM myxothiazol</td>
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<td>0.37 ±0.14</td>
<td>Nitrate medium/air+2.5 mM SHAM</td>
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(continued)
### Table 1 (continued)

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<tr>
<th>S. No.</th>
<th>Plant type</th>
<th>Tissue</th>
<th>NO emission rates</th>
<th>Condition</th>
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<tbody>
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<td>0.32 ± 0.07 nmol/gFW/h</td>
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<td>Nitrate medium/air + 2 mM KCN</td>
<td>Nitrate medium/air + 10 μM myxothiazol + 2.5 mM SHAM + 2 mM KCN</td>
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<td>24.05 ± 4.68 nmol/gFW/h</td>
<td>28.83 ± 9.40 nmol/gFW/h</td>
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<td>28.37 ± 5.47 nmol/gFW/h</td>
<td>30.73 ± 9.23 nmol/gFW/h</td>
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<td>1.35 ± 0.72 nmol/gFW/h</td>
<td>13 Nicotiana tabacum cv. Xanthi</td>
<td>Nitrate medium/anoxia + 10 μM myxothiazol + 2.5 mM SHAM + 2 mM KCN</td>
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<td>0.01–0.05 nmol/gFW/h</td>
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<td>Nitrate medium + water</td>
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<td>0.4–1.0 nmol/gFW/h</td>
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<td>Ammonium medium + cryptogein</td>
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<td>0 nmol/gFW/h</td>
<td>0.1–22 nmol/gFW/h</td>
<td>Nitrite + cPTIO/air</td>
<td>Nitrite + cPTIO/anoxia</td>
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<td>0.1–18.97 nmol/gFW/h</td>
<td>0.1–0.2 nmol/gFW/h</td>
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<td>Nitrite + cryptogein</td>
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<td>0.0–0.2 nmol/gFW/h</td>
<td>0.1–0.6 nmol/gFW/h</td>
<td>Nitrite + cryptogein + cPTIO</td>
<td>Nitrite + cryptogein + cPTI</td>
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<table>
<thead>
<tr>
<th>Plant</th>
<th>Tissue</th>
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<th>Chemical Conditions</th>
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<tr>
<td><em>Pisum sativum</em></td>
<td>Leaf slices</td>
<td>0, 80, &lt;1</td>
<td>0.5 mM Nitrite/air, nitrate/anoxia</td>
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<td>Leaf mitochondria</td>
<td>&lt;1</td>
<td>0.5 mM Nitrite/1 mM NADH/air/anoxia</td>
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<td>Root segments</td>
<td>&lt;1, 24</td>
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<td></td>
<td>Purified root mitochondria</td>
<td>&lt;1, 4.5, 2, 1</td>
<td>0.5 mM Nitrite/1 mM NADH/air/anoxia, myxothiazol, SHAM/anoxia</td>
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<tr>
<td><em>Hordeum vulgare</em></td>
<td>Leaf slices</td>
<td>0, 130, &lt;1</td>
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<td>Leaf mitochondria</td>
<td>&lt;1</td>
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<td>&lt;1, 9</td>
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<td>Purified root mitochondria</td>
<td>&lt;1, 15, 6, 3</td>
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</tbody>
</table>

NO Detection by Chemiluminescence
of light emission resulting from the reaction between nitric oxide (NO) and ozone (O). In this method NO is measured directly. The reactions are described as follows:

\[
\begin{align*}
\text{NO} + \text{O}_3 & \rightarrow \text{NO}_2 + \text{O}_2 \quad (1) \\
\text{NO} + \text{O}_3 & \rightarrow \text{NO}_2^* + \text{O}_2 \quad (2) \\
\text{NO}_2^* & \rightarrow \text{NO}_2 + h\nu \quad (3)
\end{align*}
\]

In the first reaction nitric oxide reacts with ozone and forms some portion of nitrogen dioxide and oxygen; in the second reaction NO reaction with ozone leads to generation of nitrogen dioxide in excited state. Deactivation of NO\(_2^*\) leads to emission of photons. Amount of light emitted is proportional to amount of NO.

## 2 Materials

### 2.1 Plant Material and Cultivation

1. In this method plants such as tobacco, pea, and barley were used.
2. Hydroponic trays.
3. Air pumps.
4. Air diffusers.
5. Plant growth chamber with controlled conditions such as temperature, humidity, and light.
6. Hydroponic solution contains 1 mM NH\(_4\)NO\(_3\), 250 \(\mu\)M CaCl\(_2\), 100 \(\mu\)M FeEDTA, 1 mM MgSO\(_4\), 100 \(\mu\)M H\(_3\)BO\(_3\), 1.5 \(\mu\)M CuSO\(_4\), 50 \(\mu\)M KCl, 10 \(\mu\)M MnSO\(_4\), 0.1 \(\mu\)M Na\(_2\)MoO\(_4\), 100 \(\mu\)M Na\(_2\)SiO\(_3\), 2 \(\mu\)M ZnSO\(_4\), and 1 mM KH\(_2\)PO\(_4\) (see Notes 1 and 2).

### 2.2 Mitochondria Isolation

1. Mortar and pestle.
2. Sucrose.
3. Tetra-sodium pyrophosphate.
4. Polyvinylpyrrolidone.
5. Ethylenediaminetetraacetic acid KH\(_2\)PO\(_4\).
7. Ascorbic acid.
9. Miracloth (for filtration).
10. MgCl\(_2\).
11. HEPES.
12. Percoll density gradient.

### 2.3 Chemiluminescence Detection

1. Pure air and nitrogen gas.
2. Transparent cuvette.
3. Rubber tubes.
4. Charcoal column 1 m long, 3 cm internal diameter, particle size 2 mm.
5. Pure nitric oxide gas for calibration.
6. Flow controllers (FC-260, Tylan General, Eching, Germany).
7. Orbitor shaker.
8. Blue tag.
9. Razor blade.

2.4 Indirect Chemiluminescence Detection

1. 50 mM Vanadium (III) chloride in 1 M HCl.
2. 50 mM HEPES (pH 7.4).
3. 1–5 U of NOS, 1 mM l-arginine, 1 mM MgCl₂, 0.1 mM NADPH, and 12 μM BH₄.

2.5 Description of the Analyzer CLD 770 AL ppt

Figure 1 shows various components of the analyzer. Despite the fact that the CLD 770 AL ppt analyzer contains two reaction chambers in the small pre-chamber, NO reacts with ozone. The actual chemiluminescence reaction occurs in the main reaction chamber where NO reacts with ozone and generation of NO chemiluminescence signal generation takes place.

Fig. 1 Steps in direct and indirect chemiluminescence detection of nitric oxide. (a) Oxygen passes into ozone generator. (b) Ozone generator generates ozone and passes into reaction chamber. (c) Cells/root slices/leaf slices emit NO and pass into reaction chamber. (d) Injection of extracts into hot acidic VCLIII. (e) Reaction chamber where NO reacts with ozone and generation of NO chemiluminescence signal generation takes place. (f) Signal detector translates into ppb of NO.
chamber. The reaction between NO and ozone takes place under low pressure. An external vacuum pump generates a reaction chamber with low pressure of 15 mbar. This low pressure is sufficient to drive both the gas sample and the ozone into the chamber. A photomultiplier collects the light and converts and amplifies current pulses. A microprocessor calculates the NO signal in the range of ppb. A custom-made software based on Visual Designer (PCI-20901SS, Ver. 4.0, Tuscon, Arizona, USA) was used to process the NO signal.

3 Methods

3.1 Plant Cultivation

1. Surface sterilize seeds with a mixture of Tween detergent (0.05 %) and H$_2$O$_2$ (0.015 %) and wash four times with distilled water. Water seeds and keep on wet filter paper for 48 h.

2. Grow Nicotiana tabacum cv. Gatersleben or nia plants in a vermiculite/sand mixture (2 parts vermiculite/1 part sand) for 2–3 weeks and from then transfer to hydroponic system for a further 3–4 weeks.

3. In case of barley and pea plants germinate seeds on filter paper after 2–3 days’ growth transfer them directly to hydroponic system.

4. Use plants of growth stage 5–7 weeks for the experiments.

5. During the sand/vermiculite water plants with full-strength nutrient solution twice a week. The full-strength nutrient solution for “nitrate”-grown plants should contain 5 mM KNO$_3$, 1 mM CaCl$_2$, 1 mM MgSO$_4$, 0.025 mM NaFe-EDTA, 1 mM K$_2$HPO$_4$, 2 mM KH$_2$PO$_4$, and trace elements according to [26].

6. Adjust pH to 6.3 with KOH and transfer the vermiculite/sand plants into hydroponic tanks, each containing 15 plants in 8.0 l nutrient solution, and place in growth chambers with artificial illumination (HQI 400 W, Schreder, Winterbach, Germany) at a photon flux density of 300 μmol m$^{-2}$ s$^{-1}$ (PAR) and a day length of 16 h.

7. Maintain the day/night temperature regime of the chamber at 25/20 °C, respectively.

8. Flush the root medium with pre-moisturized air, using four 100 mm long micro-ceramic diffusers per tank (Europet Benelux, Gemert, The Netherlands).

3.2 Isolation of Mitochondria

1. All mitochondrial isolation steps should be carried out at 4 °C (see Note 3).

2. Roots and leaves need to be chopped with a sharp razor blade into approximately 0.5 cm slices and 2 g of slices/10 ml solu-
tion and placed in 50 ml plastic measuring cylinder and ground the tissue using Ultra Turrax.

3. Filtrate the homogenate using one layer of Miracloth and four layers of nylon mesh (80–100 μm).

4. Centrifuge the filtrate at $2000 \times g$ for 10 min. Discard the pellet.

5. Centrifuge the supernatant at $12,000 \times g$ for 30 min, and discard the supernatant.

6. The pellet should be dissolved by passing soft paintbrush/application of slight pressure with pipette on the pellet or by repeatedly rinsing the pellet with a small volume of medium using a Pasteur pipette. Finally suspend the pellet in 2 ml of suspension buffer.

7. Place the mitochondrial suspension on the discontinuous Percoll gradient. Required concentrations of gradients should be prepared by mixing specific concentration of Percoll in a Percoll buffer [27, 28]. More specifically—the first layer (from below) contains 3 ml of 60 % Percoll (v/v) and then overlay with 4 ml 45 % (v/v) and then overlay with 4 l of 28 % (v/v) Percoll and then on the top with 4 ml of 5 % (v/v) Percoll. Load Percoll gently with Pasteur pipette at 40° angle.

8. The mitochondrial fraction appears at the interface between 45 and 28 % (v/v). Gently remove the layer with a Pasteur pipette, place it in a 50 ml centrifuge tube that contains 15 ml of suspension buffer, and centrifuge at $18000 \times g$ for 15 min. Discard the supernatant, resuspend the pellet in 15 ml suspension buffer, and centrifuge again at $18,000 \times g$ for 15 min.

9. Yellowish brown pellet containing the root mitochondria can be seen at the bottom of the tube.

### 3.3 Methods to Check Activity and Integrity of the Mitochondria

1. Monitoring mitochondrial activity: State 3/state 4 ratio is referred to as the respiratory control ratio (P:O). State 3 respiration means ADP-enhanced respiration. State 4 means respiration in the complete absence of the ATP synthesis. State 4 can be achieved by adding the ATP synthase inhibitor oligomycin (1 μg/ml). Oxygen uptake measurements for checking state 3/state 4 ratio can be done using oxygen electrode or by using the Microx TX2 oxygen-sensing device (PreSens Precision Sensing). A respiratory control ratio (P:O) of 3 means that mitochondria are well coupled; values lower than this indicate that they are only loosely coupled and that membranes are eventually damaged.
2. Peroxisomal contamination can be checked by adding 1 mM H$_2$O$_2$ to the mitochondrial suspension. Rates of oxygen evolution are proportional to the peroxisomal content.

3. Cytosolic contamination can be checked by measuring a cytosolic marker such as phosphoenolpyruvate carboxylase (PEPC) activity.

4. Thylakoid contamination can be checked by measuring the chlorophyll content in leaf mitochondria.

5. Western blots can be done by using antibodies against various marker enzymes of subcellular compartments, e.g., against peroxisomal protein KAT2 (3-ketoacyl-CoA thiolase-2) for checking peroxisomal contamination. Chloroplast contamination can be checked by antibody against large subunit of Rubisco [29].

### 3.4 Preparation of Root Segments

1. Wash roots with autoclaved deionized water.
2. On a clean glass plate spread root system.
3. Remove excess of water from root system by placing a blotting paper on the top of root system.
4. Cut lowest part of 2–3 cm lowest part of roots including root tips were cut into 3–5 mm segments, and place in a glass vessel containing 10 ml 20 mM HEPES-KOH, pH 7.0, 0.5 mM KNO$_2$, and 50 mM sucrose.

### 3.5 Preparation of Leaf Segments

Rinse freshly harvested leaves with deionized water and cut into 1 cm long and 1–3 mm wide segments excluding the midrib portion.

Vacuum infiltrate the leaf segments (1 g FW) for 2–3 min in 10 ml of 25 mM HEPES-KOH pH 7.4 and 0.5 mM CaSO$_4$.

Wash the segments and subsequently suspend in the same buffer for the NO measurements.

### 3.6 Measurement of Nitric Oxide

1. Place the vials containing root segments or leaf slices (1 g FW in 10 ml buffer) or mitochondrial suspensions (1.5–2.5 mg protein in 8 ml buffer) in a glass cuvette (1.0 l air volume) mounted on a rotary shaker (150 U min$^{-1}$).
2. Pull a constant flow of measuring gas (purified air or nitrogen) of 1.6 l min$^{-1}$ through the cuvette, and through a cold moisture trap and subsequently through the chemiluminescence detector (CLD 770 AL ppt, Eco-Physics, Dürnten, Switzerland, detection limit 20 ppt; 20-s time resolution) by a vacuum pump connected to an ozone destroyer.
3. Make the measuring gas (air or nitrogen) NO free by conducting it through a custom-made charcoal column (1 m long, 3 cm internal diameter, particle size 2 mm).
4. Carry out calibration with NO-free air (0 ppt NO) and with various concentrations of NO (1–35 ppb) adjusted by mixing the calibration gas (500 ppb NO in nitrogen, Messer Griesheim, Darmstadt, Germany) with NO-free air. Use flow controllers (FC-260, Tylan General, Eching, Germany) to adjust all gas flows.

5. Maintain air temperature of the cuvette at 25 °C. Customize the cuvette lid to allow the injection of various solutions through a serum stopper directly into the sample without opening the cuvette or interrupting shaking cycles.


3.7 Nitrate/Nitrite Trace Analysis (“Indirect chemiluminescence”)

1. Inject 100–500 μl of the respective into a reducing reaction mixture [50 mM vanadium (III) chloride in 1 M HCl] at 90 °C, under continuous stirring.

2. Pass the emitted gas via 100 ml of 1 M KOH to protect the analyzer from HCl carried over (see Note 4).

3. Calculate the production of NO by subtracting the 0 time value which represents nonenzymatic NO production from suspension medium and cell components [30].

4. Measure NO from recombinant iNOS as a positive control. This iNOS assay must contain 50 mM HEPES (pH 7.4), 1–5 U of NOS, 1 mM L-arginine, 1 mM MgCl₂, 0.1 mM NADPH, and 12 μM BH₄.

4 Notes

1. While growing plants on hydroponics regularly check pH.

2. Change nutrient solution at least three times a week.

3. Always perform all steps of mitochondria isolation at 4 °C.

4. While doing indirect chemiluminescence always watch the setup. Overheating of vanadium (III) chloride can damage setup and instrument.

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References


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