Chapter 9

Measurement of Nitric Oxide (NO) Generation Rate by Chloroplasts Employing Electron Spin Resonance (ESR)

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Abstract

Chloroplasts are among the more active organelles involved in free energy transduction in plants (photophosphorylation). Nitric oxide (NO) generation by soybean (*Glycine max*, var ADM 4800) chloroplasts was measured as an endogenous product assessed by electron paramagnetic resonance (ESR) spin-trapping technique. ESR spectroscopy is a methodology employed to detect species with unpaired electrons (paramagnetic). This technology has been successfully applied to different plant tissues and subcellular compartments to asses both, NO content and generation. The spin trap MGD-Fe^{2+} is extensively employed to efficiently detect NO. Here, we describe a simple methodology to asses NO generation rate by isolated chloroplasts in the presence of either L-Arginine or nitrite (NO_2^-) as substrates, since these compounds are required for enzymatic activities considered as the possible sources of NO generation in plants.

Key words ESR, Chloroplasts, NO generation, NOS*like* activity, NO_2^-

1 Introduction

Electron spin resonance (ESR) spectroscopy is a methodology employed to measure species with unpaired electrons (paramagnetic) [1], such as free radicals and transition metals. Paramagnetism is related to the magnetic moment exerted by the unpaired electron and allows the use of ESR in a wide array of experimental conditions. This high degree of selectivity renders ESR useful even when working with complex biological systems [1].

NO is an inorganic free radical gaseous molecule with multiple roles in biological systems [2]. NO is itself paramagnetic with the free electron being shared between N_2 and O_2. The broader chemistry of NO involves a redox array of species with distinctive properties and reactivities: NO^+ (nitrosonium), NO^- (nitroxyl anion), and NO (NO radical). Neutral NO has a single electron in its 2p-π antibonding orbital and the removal of this electron forms NO^+ while the addition of one more electron to NO forms NO^- [3]. From a biological point of view the important reactions of NO are

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those with O_2 and its various redox forms and with transition metal ions. NO also reacts rapidly with O_2^- in aqueous solution, yielding peroxynitrite ($ONOO^-$) [4].

When discussing the chemistry and physiological effects of NO, it should be considered that NO is a highly diffusible second messenger that can elicit effects relatively far from its site of production. The concentration and therefore the source of NO are the major factors determining its biological effects [5]. At low concentrations (<1 μM) the direct effects of NO predominate. At higher concentrations (>1 μM), the indirect effects mediated by reactive nitrogen species (RNS) prevail. The direct effects of NO involve the interaction of NO with metal complexes or proteins leading to tyrosine nitration, selectively and reversibly, and it has been shown that there are $ONOO^-$ dependent and independent pathways for the nitration in vivo [6]. NO also is able to terminate lipid peroxidation [7]. The indirect effects of NO, produced through the interaction of NO with either O_2 or O_2^-, include nitrosation (when NO^+ is added to an amine, thiol, or hydroxy aromatic group), oxidation (when one or two electrons are removed from the substrate), or nitration (when NO_2^+ is added to a molecule) [5]. $ONOO^-$ acts as both, nitrating agent and powerful oxidant, to modify proteins (formation of nitrotyrosine), lipids (lipid oxidation, lipid nitration), and nucleic acids (DNA oxidation and DNA nitration) [8]. In summary, the potential reactions of NO are numerous and depend on many different factors. Thus, the relative balance between oxidative and nitrosative stress should be carefully evaluated for better understanding the complexity of NO biological effects.

To detect NO, different methodologies have been developed employing spectrophotometric [9] and fluorescent techniques [10], an O_2 monitor, or ESR [11]. The O_2 monitor methodology was developed to measure the consumption of O_2 in liquid phase by NO. This method, often used for quantification of aqueous stock solutions of NO, is based on the reaction of NO with O_2, according to the reaction 1, where the consumption of O_2 is recorded.

$$4NO + O_2 + 2H_2O \rightarrow 4HNO_2 \quad [1]$$

ESR is one of the most powerful techniques for the detection and identification of biological radicals, being certainly the only method by which NO and its paramagnetic derivatives can be unambiguously identified [12]. Previous work has shown the capacity of ESR of detecting NO in the presence of exogenous traps in soybean embryonic axis [13] and cotyledons [14, 15], or sorghum embryonic axes [16]. Although no nitric oxide synthase (NOS) enzyme has been identified in plants, a NOS*like* activity has been extensively reported. Caro and Puntarulo [13] have determined a NADPH-diaphorase activity in homogenates from soybean

axes. Galatro et al. [17] and Jasid et al. [18] have assessed L-Arginine (L-Arg) dependent NO generation by soybean leaves, and soybean chloroplasts employing ESR. Moreover, NO generation by L-Arg-dependent NOS activity was described in isolated peroxisomes from pea leaves employing ESR [19].

Chloroplasts are key organelles in plant metabolism and they seem to be involved in NO production [15, 20–23]. Two independent pathways for NO generation in isolated chloroplasts from soybean plants have been described: (i) one dependent on L-Arg and NADPH (NOS*like*), and (ii) another dependent on nitrite (NO_2^-) [18] (Fig. 1). These NO generation sources were evaluated employing the spin trap sodium-*N*-methyl-D-glucamine dithiocarbamate $(MGD)_2$-Fe^{2+} (Fig. 2), and the required substrates and cofactors [18]. Galatro et al. [15] showed that chloroplasts contribute to NO synthesis in vivo employing both, confocal fluorescence microscopy, and EPR techniques. The level of NO in the soybean cotyledons was related to chloroplasts functionality. The detection of NO in coincidence with cotyledon maximum fresh weight, chlorophyll content, and quantum yield of PSII, supported the hypothesis of a strong link between NO levels and chloroplast functionality. Moreover, seedlings exposed *in vivo* to herbicides showed an impaired NO accumulation, and deleterious effects on chloroplast function (loss of photosynthetic capacity). The use of the herbicide DCMU (3-(3,4-dichlorophenyl)-1,1-dimethyl urea), that binds plastoquinone and blocks electron flow at the quinone acceptors of photosystem II, supports a role for the integrity of the photosynthetic electron chain in chloroplasts NO production *in vivo*, as has been previously observed by Jasid et al. [18] in isolated soybean chloroplasts.

In this chapter we describe a simple ESR methodology to asses NO generation rate by isolated chloroplasts in the presence of either L-Arg or NO_2^-.

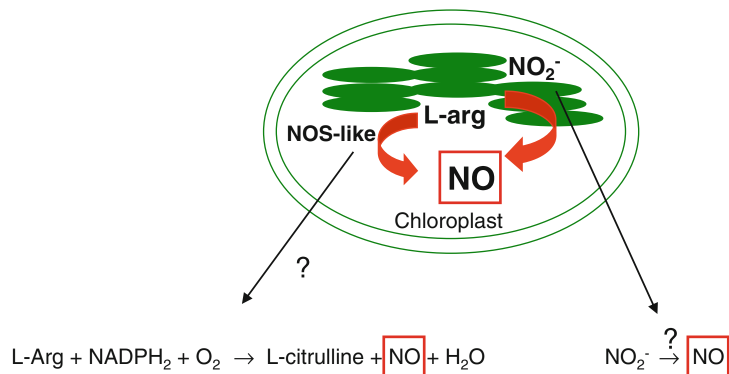

Fig. 1 Scheme of the main proposed sources of NO in chloroplasts. NOS*like*, nitric oxide-like activity, and NO_2^--dependent NO generation

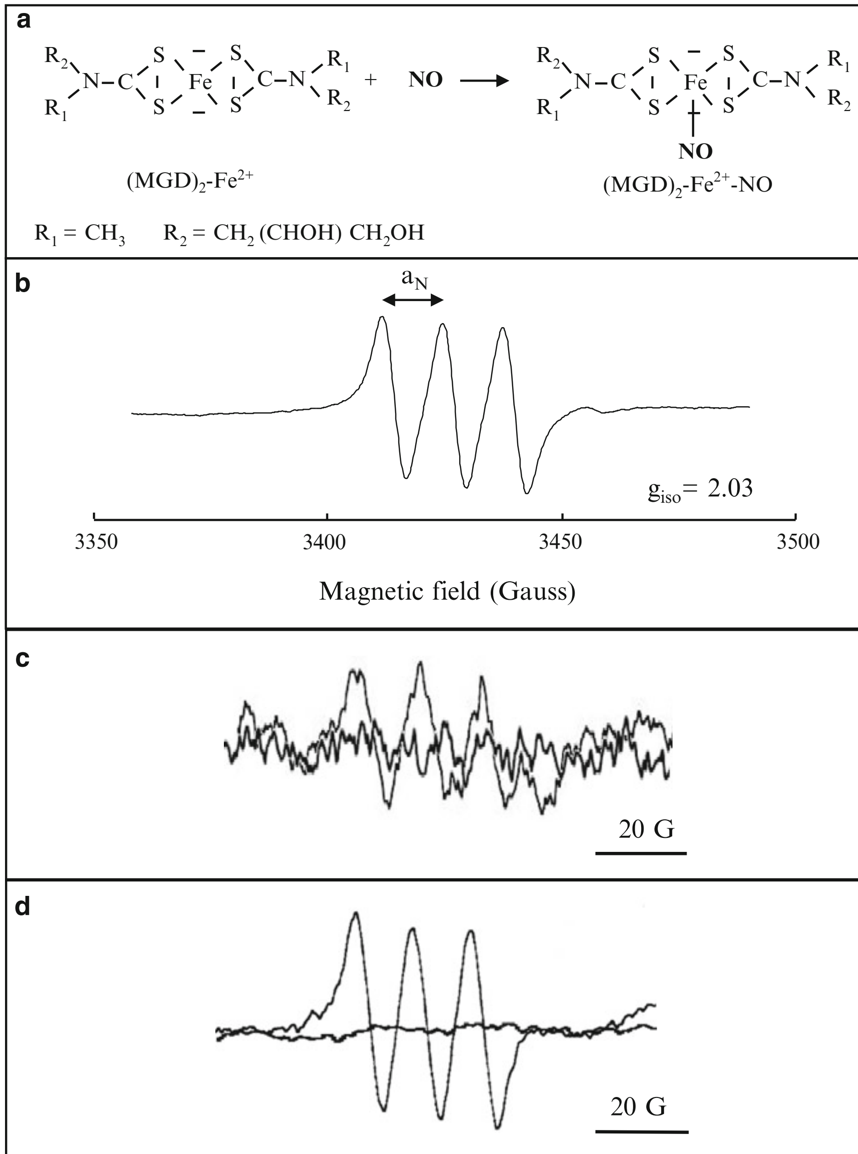

Fig. 2 ESR-spin trapping detection of NO. (**a**) Chemical reaction between $(MGD)_2$-Fe^{2+} complex and NO to form $(MGD)_2$-Fe^{2+}-NO adduct. (**b**) Typical EPR spectra of $(MGD)_2$-Fe^{2+}-NO adduct obtained employing a standard solution of 0.1 mM *S*-nitrosoglutathione (GSNO) mixed with the spin trap. (**c**) L-Arg-dependent NO generation in isolated chloroplasts: ESR spectra of the $(MGD)_2$-Fe^{2+}-NO adduct from soybean chloroplasts incubated for 10 min in the presence of MGD-Fe (10:1 mM), 1 mM Arg, 1 mM $CaCl_2$, 5 mM $MgCl_2$, and 0.1 mM NADPH, overlapped to the spectrum obtained with the reaction media in absence of chloroplasts. (**d**) NO_2^--dependent NO generation by isolated chloroplasts: ESR spectra of the $MGD)_2$-Fe^{2+}-NO adduct from soybean chloroplasts incubated for 3 min in the presence of MGD-Fe (10:1 mM) and 1 mM $NaNO_2$ overlapped to the spectrum obtained with the reaction media in absence of chloroplasts. Taken and modified from Puntarulo et al. [29] and Jasid et al. [18]

2 Materials

2.1 Chloroplasts Isolation Reagents

2.1.1 Isolation Buffer

50 mM HEPES (*N*-[2-Hydroxyethyl] piperazine-*N′*-[2-ethane-sulfonic acid]), pH 7.6, 330 mM sorbitol, 2 mM EDTA (Ethylenediaminetetraacetic acid), 1 mM $MgCl_2$, 0.05 % (w/v) bovine serum albumin (BSA), 5 mM ascorbic acid, and protease inhibitors (40 μg/mL phenylmethylsulfonyl fluoride (PMSF), 0.5 μg/mL leupeptin, 0.5 μg/mL aprotinin). To prepare 500 mL (final volume) weigh 5.958 g of HEPES, 30 g of sorbitol, 0.38 g of EDTA, 0.250 g of BSA, and 9.52 mg of $MgCl_2$. Add 400 mL of distilled water, mix employing a magnetic stirrer, and adjust the pH to 7.6 employing 1 M NaOH solution. Complete the volume to 500 mL.

This buffer can be stored at 4 °C. PMSF, protease inhibitors and ascorbic acid must be added previous to chloroplast isolation (*see* **Note 1**).

2.1.2 Wash and Resuspension Buffer

50 mM HEPES, pH 8.0, 330 mM sorbitol. To prepare 200 mL of buffer weight 2.383 g of HEPES, 12 g of sorbitol, add 150 mL of distilled water, mix employing a magnetic stirrer and adjust the pH to 8.0 employing 1 M NaOH solution. Complete the volume to 200 mL. Store the buffer at 4 °C.

2.1.3 Percoll Cushion

50 mM HEPES, pH 8.0, 330 mM sorbitol, 30 % [v/v] Percoll. To 12 mL of Percoll add 50 mM HEPES buffer, pH 8.0, 330 mM sorbitol (wash and resuspension buffer) up to 40 mL final volume.

2.2 NO Detection Reagents

2.2.1 Spin Trap Solution

100 mM potassium phosphate buffer pH 7.4, containing the spin trap (20 mM MGD, 2 mM $FeSO_4$). The suspension of isolated chloroplast will be supplemented with equal volume of 100 mM potassium phosphate buffer pH 7.4 containing the spin trap. Weight MGD (MW 293.34, 7.3 mg/mL, 25 mM) and dissolve it in 100 mM potassium phosphate buffer, pH 7.4. To prepare a 10 mM $FeSO_4$ solution in 0.01 N HCl, weight 13.9 mg of $FeSO_4{\cdot}7H_2O$ (278.02 g/mol) and dissolve it in 5 mL of 0.01 N HCl. Add 200 μL 10 mM $FeSO_4$ to 800 μL MGD solution (final concentration will be 20 mM MGD, 2 mM $FeSO_4$, *see* **Note 2**).

2.2.2 L-Arg-Dependent NO Generation Rate

100 mM potassium phosphate buffer containing the spin trap (10 mM MGD, 1 mM $FeSO_4$), 1 mM L-Arg, 1 mM $CaCl_2$, 5 mM $MgCl_2$, 0.1 mM NADPH, and chloroplasts. Prepare the following concentrated solutions of L-Arg (50 mM, in 100 mM potassium phosphate buffer pH 7.4), $CaCl_2$ (50 mM, in distilled water), $MgCl_2$ (100 mM, in distilled water), and NADPH (10 mM, in distilled water). These solutions will be added to the buffer containing the chloroplasts at the moment of the measurement as it is described in the Method section (*see* **Note 3**). All the solutions can be stored at −20 °C, except for the NADPH solution that should be conserved at −70 °C.

2.2.3 NO_2^--dependent NO generation rate

100 mM potassium phosphate buffer, pH 7.4, containing the spin trap (10 mM MGD, 1 mM $FeSO_4$), 1 mM $NaNO_2$, and the suspension of chloroplasts. Prepare a concentrated solution of $NaNO_2$ (100 mM, in distilled water). This solution will be added to the buffer containing the chloroplasts at the moment of the measurement, as it is described in the Method section.

2.3 ESR Equipment

Bruker ER 070 spectrometer (Karlsruhe, Germany) operating at 17–19 °C (room temperature).

3 Methods

3.1 Chloroplast Isolation

All procedures should be carried out at 4 °C in an ice bath.

1. Add 100 mL of isolation buffer to 10 g of soybean leaves. Homogenize the plant material in a blender employing short periods of blending (1 or 2 s) to disaggregate the tissue.
2. Filter the homogenate through two layers of Mira cloth.
3. Centrifuge the filtrate 5 min at 1500 × *g* at 4 °C (40 mL tubes).
4. Discard the supernatant and resuspend the pellets gently in isolation buffer (1 mL).
5. Load this suspension (1 mL) in 10 mL of Percoll cushion and centrifuge at 4000 × *g* for 10 min (*see* **Note 4**).
6. Collect the pellet containing the intact chloroplasts employing a Pasteur pipette, in 2 mL Eppendorf tubes (no more than 1 mL per Eppendorf), add wash and resuspension buffer up to 2 mL, and centrifuge for 5 min at 1500 × *g* (4 °C). Suspend the pellet in 2 mL of the same buffer.
7. Repeat the washing procedure and suspend all the final pellets in the same buffer (i.e., 0.5 mL). Final protein and chlorophyll concentration will depend on the performance of the isolation procedure and the intactness of chloroplasts fraction obtained, as only intact chloroplast pass through the Percoll cushion (it can be obtained around 1–2 mg protein mL^{-1}) (*see* **Note 5**).

3.2 ESR Measurement of NO Generation Rate

3.2.1 L-Arg-Dependent NO Generation Rate

1. To asses L-Arg-dependent NO generation rate by chloroplasts, the organelles have to be subjected to osmotic shock in HEPES buffer 50 mM, pH 8.0, by the lack of sorbitol in the medium. The chloroplasts suspension (150 μL) should be centrifuged for 5 min at 1500 × *g* (4 °C). Discard the supernatant and resuspend the chloroplasts in 150 μL of 50 mM HEPES buffer, pH 8.0. Mix this volume of membrane disrupted chloroplasts (1 mg protein mL^{-1}) with an equal volume of 100 mM phosphate buffer pH 7.4, containing the spin trap (20 mM MGD, 2 mM $FeSO_4$).

2. Add the appropriate cofactors: to 270 μL of chloroplasts suspension mixed with the spin trap solution, add 6 μL of Arg 50 mM, 15 μL of 100 mM $MgCl_2$, 6 μL 50 mM $CaCl_2$, and finally 3 μL 10 mM NADPH, and incubate up to 10 min at room temperature. The final concentration will be: 1 mM Arg, 1 mM $CaCl_2$, 5 mM $MgCl_2$, 0.1 mM NADPH in 300 μL of the suspension containing the chloroplasts and 10:1 mM (MGD-Fe) (*see* **Note 6**).
3. Transfer the sample to bottom-sealed Pasteur pipettes to record the EPR spectra at room temperature (18 °C) employing the following instrument settings: microwave frequency 9.5 GHz, 200 G field scan, 83.9 s sweep time, 328 ms time constant, 5.983 G modulation amplitude, 50 kHz modulation frequency, 20 mW microwave power, and 3400 G center field. The scan number must be adjusted according to the NO generation rate of the sample.
4. This procedure for assessing NO generation rate will be repeated at different time points (i.e., 10, 20, and 30 min) after starting the reaction by the addition of NADPH.
5. Quantification of the spin adduct could be performed using an aqueous solution of TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl). The concentration of the $(MGD)_2$-Fe^{2+}-NO adduct is obtained by double integration of the three lines and cross-checked with the TEMPOL spectra. The amount of TEMPOL spins in the EPR cavity range from 0.5 to 5 nmol (standard curve) because 50 μL of a 10–100 μM solution of TEMPOL are added to the cavity employing the bottom-sealed Pasteur pipettes (*see* **Note 7**).

3.2.2 NO_2^--Dependent NO Generation Rate

1. Mix 150 μL of intact isolated chloroplasts (1 mg prot mL^{-1}) with equal volume of 100 mM potassium phosphate buffer pH 7.4 containing the spin trap (20 mM MGD, 2 mM $FeSO_4$). To 297 μL of this suspension, add 3 μL of 100 mM $NaNO_2$, and incubate up to 10 min at room temperature under ambient light conditions. The final concentration will be 1 mM $NaNO_2$, and 10:1 mM (MGD-Fe) in a final volume of 300 μL.
2. Transfer the sample to a bottom-sealed Pasteur pipette to record the EPR spectra at room temperature (18 °C) employing the instrument settings previously described (*see* **Note 8**).

This procedure for assessing NO generation rate will be repeated at different time points (i.e., 10, 20, and 30 min) after starting the reaction by the addition of $NaNO_2$.

4 Notes

1. Protease inhibitors and ascorbic acid must be added at the moment of performing the isolation procedure. Prepare a solution of 10 mg/mL of aprotinin, and 10 mg/mL of leupeptin in 100 mM potassium phosphate buffer, pH 7.4. Aliquots of protease inhibitors solutions (10 mg/mL) can be stored at −20 °C. To 100 mL of 50 mM HEPES, pH 7.6, 330 mM sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, and 0.05 % (w/v) bovine serum albumin, add 5 μL of each protease inhibitor solution, 4 mg of PMSF, and 0.088 g of ascorbic acid. Mix the solution.
2. When MGD and Fe solutions are mixed the final solution turns brown. MDG and Fe solutions should be mixed immediately before the measurement. The MDG solution should be freshly prepared. However, the solution of 10 mM $FeSO_4$ in 0.01 N HCl could be previously prepared and stored at −20 °C.
3. NADPH concentration in the stock solution should be checked by measuring the absorbance at $\lambda = 340$ nm ($\varepsilon = 6.22$/mM/cm). To prepare a 10 mM NADPH solution dissolve 50 mg of NADPH (MW 833.35) in 6 mL of distilled water. The absorbance at $\lambda = 340$ nm should be recorded to assess the concentration. Aliquots of this solution could be stored at −70 °C.
4. Depending on both, the percoll gradient and the plant material, slight adjustments should be performed on the centrifugation speed (i.e., 4000–5000 × *g*), and time (10–12 min).
5. The protein content in the final suspension of chloroplasts could be measured according to Bradford [24]. The intactness of chloroplasts could be determined as ferricyanide-dependent O_2 evolution according to Edwards et al. [25]. The purity of the obtained fraction could be analyzed by biochemical assays, such as the hydroxypyruvate reductase activity for assessing peroxisomal contamination [26], the phosphoenolpyruvate carboxylase activity, a cytosolic marker [27], and the fumarase activity as a marker of mitochondrial presence [28].
6. To explore the efficiency of classic known NOS inhibitors on the obtained enzymatic activity the incubations should be performed in the presence of 5 mM L-NAME (N_{ϖ}-nitro-L-Arg methyl ester hydrochloride) or L-NNA (N_{ϖ}-nitro-L-Arg) since they are Arg analogs. Controls with boiled chloroplasts (exposed 20 min to 100 °C), in absence of NADPH, and in absence of chloroplasts should be added to the experimental protocol. The signal of the basal system, consisting in buffer containing the spin trap without any addition, should be measured under the same conditions.

7. TEMPOL is a stable free radical and may be used as a standard to quantify the free radical adduct generation rate. TEMPOL solutions are standardized spectrophotometrically at $\lambda = 429$ nm ($\varepsilon = 13.4$/M/cm according to Jasid et al. [18]).
8. The inclusion in the experimental protocols of control samples without NO_2^-, or employing 1 mM $NaNO_3$ instead of 1 mM $NaNO_2$, boiled chloroplasts (20 min to 100 °C), and without chloroplasts must be considered. The supplementation with herbicides acting in chloroplasts, such as 3-(3,4 dichlorophenyl)-1,1- dimethyl urea (DCMU) (1 μM), may be employed to evidence if the intactness of electron transfer chain of chloroplasts is necessary for the NO generation (*see* ref [18]).

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