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Research article

Chloroplast functionality has a positive effect on nitric oxide level in soybean cotyledons

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ABSTRACT

The subcellular localization of NO generation in soybean cotyledons, and the relationship between NO synthesis and *in vivo* chloroplast performance were studied. Employing the NO probe 4-aminomethyl-2′,7′-difluorofluorescein diacetate (DAF-FM DA) and fluorescence microscopy, a strongly punctuated fluorescence was detected in mesophyll cells. The co-localization of DAF-FM and chlorophyll fluorescence, in confocal laser microscopy images, indicated the presence of NO in the chloroplasts. NO visualization was dependent on light, seedling age, and chloroplast function throughout cotyledons lifespan. The addition of herbicides with action in chloroplasts (DCMU and paraquat) dramatically reduced the quantum yield of photosystem II (ϕ_{PSII}), and lead to images with absence of punctuated green fluorescence. Moreover, electron paramagnetic resonance signals corresponding to NO-spin trap adduct observed in cotyledon homogenates decreased significantly by the treatment with herbicides, as compared to controls. Neither chloroplast function nor NO content were significantly different in cotyledons from plants growing in the presence of ammonium or nitrate as the nitrogen source.

These findings suggest that chloroplasts are organelles that contribute to NO synthesis *in vivo*, and that their proper functionality is essential for maintaining NO levels in soybean cotyledons.

1. Introduction

Nitric oxide (NO) plays important roles in plants, where it is involved in physiological processes like stomatal closure [1], seed germination [2], root development [3], senescence [4], expression of defense-related genes and programmed cell death [5]. In addition, NO participates in plant responses to biotic and abiotic stresses, such as interactions with pathogenic microorganisms [6,7], wounding [8], salinity, drought, and hypoxia [9]. Although it is a key signaling molecule acting throughout the lifespan of plants, how plants or tissues regulate NO levels remains unclear, controversial and is currently a subject of study [10,11].

At least seven different pathways to produce NO in plants appear to exist, and they can be classified as either oxidative or reductive [11]. Briefly, nitrate reductase (NR), and mitochondrial or plasma membrane-associated NO production (NR:NiNOR system) are all reductive pathways and depend on nitrite as a primary substrate, whereas NO production from arginine (Arg), polyamine or hydroxylamine are among the oxidative pathways [11].

Nitric oxide synthases (NOS) are present in almost all known organisms except plants, where no NOS genes or enzymes have been identified yet. Even though NO plays a crucial role in plant physiology, higher plants seem to have lost the specific NOS in the course of evolution [10]. Recently, Foresi et al. [12] characterized the sequence, protein structure and biochemistry of an NOS from the green alga *Ostreococcus tauri*, that contains the main characteristics of animal NOS [12]. NO generation in this alga is dependent on irradiance and growth phase. This single-cell alga is of particular interest because it shares a common ancestor with higher plants, providing compelling evidence that an active NOS functions in a photosynthetic organism belonging to the plant kingdom [12].

Although Arabidopsis Nitric Oxide Associated 1 (*NOA*1), or *RIF1* [13] was reported to encode a protein with NOS activity [14], the biological role of AtNOA1/RIF1 is currently believed to be primarily associated with chloroplasts ribosome functions [15–17]. In *rif1* seedlings, not only chloroplast ultrastructure, but also the level of proteins encoded by the chloroplastic genome were affected [13],

Abbreviations: cPTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; DAF-FM DA, 4-aminomethyl-2′,7′-difluorofluorescein diacetate; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; EPR, electron paramagnetic resonance; PQ, 1,1′-dimethyl-4,4′-bipyridinium dichloride.

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suggesting that *NOA1/RIF1* might bind plastidial ribosomes and is required for the normal function and proper protein synthesis in plastids [16]. Recently, it has been reported that NO accumulation in Arabidopsis is independent of NOA1 in the presence of sucrose [18]. It is possible that the primary requirement for NOA1 activity is efficient chloroplast function to generate photosynthates. Provision of sucrose enables *noa1* to accumulate NO, and this fact raised the question why fixed carbon may be necessary for NO accumulation in Arabidopsis [18].

The first reports describing chloroplasts as an NO source dealt with tobacco leaf cells subjected to a fungal elicitor from *Phytophthora cryptogea* and a range of abiotic stressors [19,20], and these were followed by studies in Arabidopsis cell cultures exposed to iron overload [21]. Furthermore, Jasid et al. [22], employing isolated chloroplasts, evidenced NO generation *in vitro* that was inhibited by the Arg analogs L-NAME or LNNA, and also a nitrite-dependent NO generation that was reduced in the presence of DCMU, showing that soybean chloroplasts are able to produce NO *in vitro*, with the supplementation of adequate substrates.

It is clear that NO content in plants varies among tissues, depends on physiological status and also occurs as a generalized stress response. Previous studies have indicated a high NO production in cotyledons [23]. In addition, in soybean cotyledons NO content strongly depends on seedling age, showing a maximum value at around day 7 of seedling development [4,24]. Here, we explore the hypothesis that the content of NO in soybean cotyledons is related to chloroplast functionality *in planta*. Employing fluorescence microscopy and electron paramagnetic resonance (EPR) we show here that chloroplasts contribute to NO synthesis *in vivo*. Moreover, the level of NO in the whole tissue is related to chloroplasts functionality. Finally, as nitrate reduction could be involved in NO synthesis, we explore NO generation in plants grown with ammonium as the unique nitrogen source.

2. Results

2.1. NO localizes in chloroplasts of soybean cotyledons

To investigate NO production, 4-aminomethyl-2′,7′-difluorofluorescein diacetate (DAF-FM DA) was employed as a fluorescent probe [25]. DAF-FM DA is a membrane-permeant substance that reacts with a product of NO oxidation (N_2O_3) [26]. Cotyledons from 7-day old seedlings were excised, cut in thin sections, and loaded with DAF-FM DA. Microscopic observations showed a strongly punctuated fluorescence in mesophyll cells (Fig. 1Aa). When cotyledons were simultaneously incubated in the presence of cPTIO as NO scavenger, the images showed a much lower fluorescence signal, evidencing the specificity of DAF-FM DA for NO (Fig. 1Ab). Non-significant autofluorescence background was observed in tissues incubated without DAF-FM DA (Fig. 1Ac).

In order to gain insight into the subcellular localization of endogenous NO, 7-day old cotyledons were observed employing confocal laser microscopy after incubation in the presence of the same fluorescent probe, DAF-FM DA. Cotyledons exhibited fluorescence corresponding to NO detection (pseudocolored green, Fig. 1Ba), and the chlorophyll autofluorescence (in red, Fig. 1Bb and Supp. Fig. 1). The co-localization of DAF-FM and chlorophyll fluorescence is shown pseudocolored in yellow-orange (Fig. 1Bc)

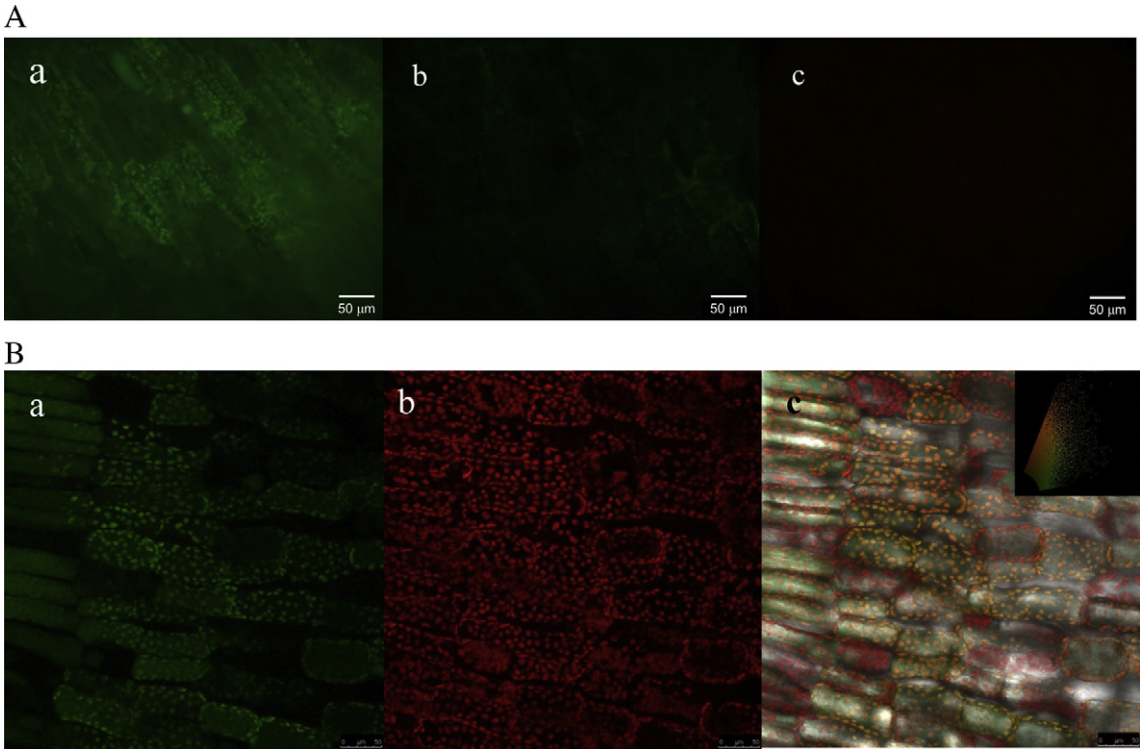

Fig. 1. NO detection in soybean cotyledons sections employing DAF-FM DA fluorescence. A. Fluorescence microscopy. Soybean plants were grown in vermiculite, daily irrigated with Steinberg solution and cotyledons were excised on day 7 after planting. a. Cotyledon sections loaded with 17 μM DAF-FM DA for 30 min and washed for 10 min. b. Cotyledon sections loaded with DAF-FM DA for 30 min in the presence of the specific NO scavenger cPTIO (400 μM), washed and observed. c. Cotyledon sections incubated for 30 min without DAF-FM DA (autofluorescence) (green color; $\lambda_{excitation} = 450–490$ nm and $\lambda_{emission} = 500–550$ nm). Images are representative of at least three independent experiments. B. Confocal laser microscopy. Cotyledons from 7-day old plants were incubated in the presence of 17 μM DAF-FM DA for 30 min ($\lambda_{excitation} = 488$ nm and $\lambda_{emission} = 500–525$ nm). a. Fluorescence corresponding to NO detection with DAF-FM (pseudocolored green). b. Chlorophyll autofluorescence ($\lambda_{excitation} = 488$ nm, $\lambda_{emission} = 670–730$ nm, pseudocolored red). c. Merged image showing co-localization of DAF-FM fluorescence and chlorophyll signals (shown in orange-yellow). Embedded scattergram in the upper right side of picture Bc was produced by the co-localization analysis software Leica SP-5. Images are representative of three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

indicating the presence of NO in the chloroplasts. Quantitative co-localization analysis was performed for the merged Fig. 1Bc, and the overlap coefficient according to Manders gave a value of 0.6905, calculated with the Leica SP-5 software [27].

Since the fluorescence in chloroplasts due to NO generation is dependent on light intensity [20], photographs were taken in the same conditions after illumination of samples for 20 s in the fluorescence microscope. The same time of illumination was employed for controls of autofluorescence and co-incubation with chemicals. After illumination of cotyledon, signal bleaching was not apparent (Supp. Fig. 2), rather, DAF-FM signal increased after illumination. Excitation with blue or green light were employed with identical results (data not shown), and cytosol fluorescence was not significantly enhanced as a consequence of illumination, ruling out a direct effect of 450 nm light on the fluorescent probe.

2.2. Changes in NO generation throughout cotyledons lifespan

Since a large part of cellular NO localizes in chloroplasts (Fig. 1Bc), and chloroplasts undergo important physiological changes during cotyledon ontogeny [28] which may affect their functions, endogenous NO generation was analyzed throughout cotyledon lifespan. Development of soybean cotyledons was followed by measuring fresh weight, total chlorophyll content and the quantum yield of photosystem II (ϕ_{PSII}) (Fig. 2). Fresh weight increased after planting, reached a maximum value at around day 7, and started to decline thereafter, probably as a result of dry matter remobilization first and, later, due to senescence. Total chlorophyll content and PSII quantum yield peaked on day 5, remained more or less constant until day 14, and then declined, probably as a result of senescence (Fig. 2). At the end of the experiment (day 19), 53% of cotyledons had fallen from plants.

Confocal microscopy of cotyledons from 7 to 14 days old plants showed green fluorescence corresponding to NO detection in correlation with high levels of chlorophyll autofluorescence, whereas cotyledons with non functional (i.e., day 1) or senescent (day 19) chloroplasts showed a lower green fluorescence, consistent with reduced chloroplast functionality (Fig. 3). Overall, NO detection in chloroplasts correlated well with PSII quantum yield (Figs. 2 and 3, Supp. Fig. 3).

2.3. Endogenous NO generation was affected by herbicides acting on chloroplasts

The effect on NO generation of herbicides with a primary action on chloroplasts was analyzed. Soybean plants were allowed to grow in control conditions until day 5 after germination, when they were irrigated with DCMU (0.4 g m^{-2}) in the nutrient solution or sprayed with a PQ solution (93 μM). After 48 h of treatment no differences in fresh weight were found in DCMU or PQ treated cotyledons as compared to the control group (293 ± 10 mg cot^{-1}). On the other hand, quantum yield of photosystem II (ϕ_{PSII}), evaluated *in vivo* on day 7 after germination, showed that photosynthetic electron transport was dramatically inhibited as a consequence of both herbicide treatments (Table 1).

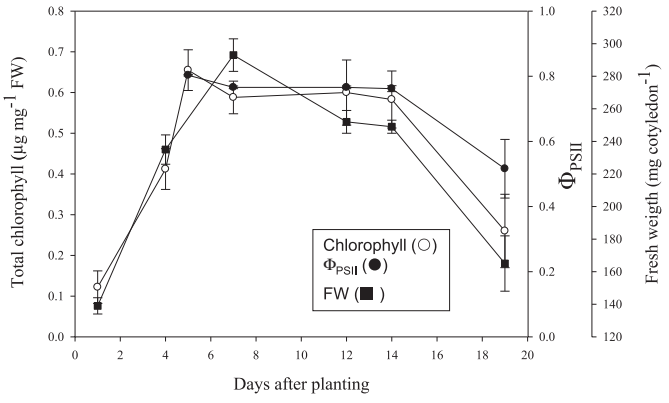

Fig. 2. Changes in physiological parameters during cotyledons lifespan. Total chlorophyll content, quantum yield of photosystem II photochemistry (ϕ_{PSII}), and fresh weight were evaluated in cotyledons from soybean plants grown in vermiculite and daily irrigated with Steinberg solution.

NO level was analyzed employing the fluorophore DAF-FM DA, as described before. Absence of punctuated green fluorescence was observed under both treatments, indicating that NO presence in chloroplasts was prevented by the treatment with either one of the herbicides (Fig. 4A). In autofluorescence controls from plants exposed to PQ, highly fluorescent zones were observed (data not shown), however after loading with DAF-FM DA no punctuated fluorescence was observed.

Additionally, total NO content was evaluated in cotyledons employing EPR spin trapping. As expected, control cotyledons showed the characteristic three line spectrum corresponding to the adduct $(MGD)_2$–Fe–NO, in agreement with the EPR spectrum obtained from standard solutions of 5 mM $(MGD)_2$–Fe–NO prepared from S-nitrosoglutathione (GSNO) and the spin trap (Fig. 4B). The additional line in the EPR spectra of soybean cotyledons corresponds to the reaction of MGD with Cu present in the samples [29]. On the other hand, lower signals corresponding to $(MGD)_2$–Fe–NO adduct were observed in cotyledons from plants treated with DCMU or PQ (Fig. 4B). In order to rule out a direct effect of herbicides on the nature of the EPR signal from the $(MGD)_2$–Fe–NO adduct, *in vitro* incubations employing PQ and DCMU were carried out. No interference from PQ or DCMU was detected (Supp. Fig. 4).

Although other subcellular sources of NO generation cannot be ruled out employing this methodology, these results indicate a lower NO generation in cotyledons when their chloroplast electron transport chain is impaired, and are consistent with the microscopic observations with DAF-FM DA (Fig. 4A).

2.4. NO generation and the source of nitrogen

Since nitrate may be a substrate for NO synthesis [11] we analyzed the influence of the nitrogen source on NO generation in soybean cotyledons. Plants were grown in the presence of ammonia as the unique source of nitrogen. In this condition, plants can synthesize aminoacids and proteins but nitrate reduction pathways are severely down-regulated. Nitrite concentration was evaluated in cotyledons from control plants growing with complete nutritive solution (8 ± 1 nmol g^{-1} FW), while in ammonium-growing plants nitrite content was below the detection limit. Seven days after germination total chlorophyll content, fresh weight, and quantum efficiency of photosystem II were measured, and no significant differences were observed between control and ammonium-supplied plants (Table 2).

Cotyledons from plants growing with ammonium as the exclusive source of nitrogen showed an NO accumulation in chloroplasts (assessed by DAF-FM DA-dependent fluorescence) non-significantly different from those plant cotyledons grown with complete nutrient solution (Fig. 5A, Supp. Fig. 5), in agreement with the values of NO content obtained by EPR in cotyledon homogenates (Fig. 5B)".

Additionally, the effect of blocking the photosynthetic electron transport chain on ammonium-supplied cotyledons was studied. Cotyledons were treated with DCMU 48 h before the measurements. The NO fluorescence, visualized employing DAF-FM DA, was dramatically decreased as compared to cotyledons growing with ammonium but not exposed to DCMU. Likewise, the EPR signals

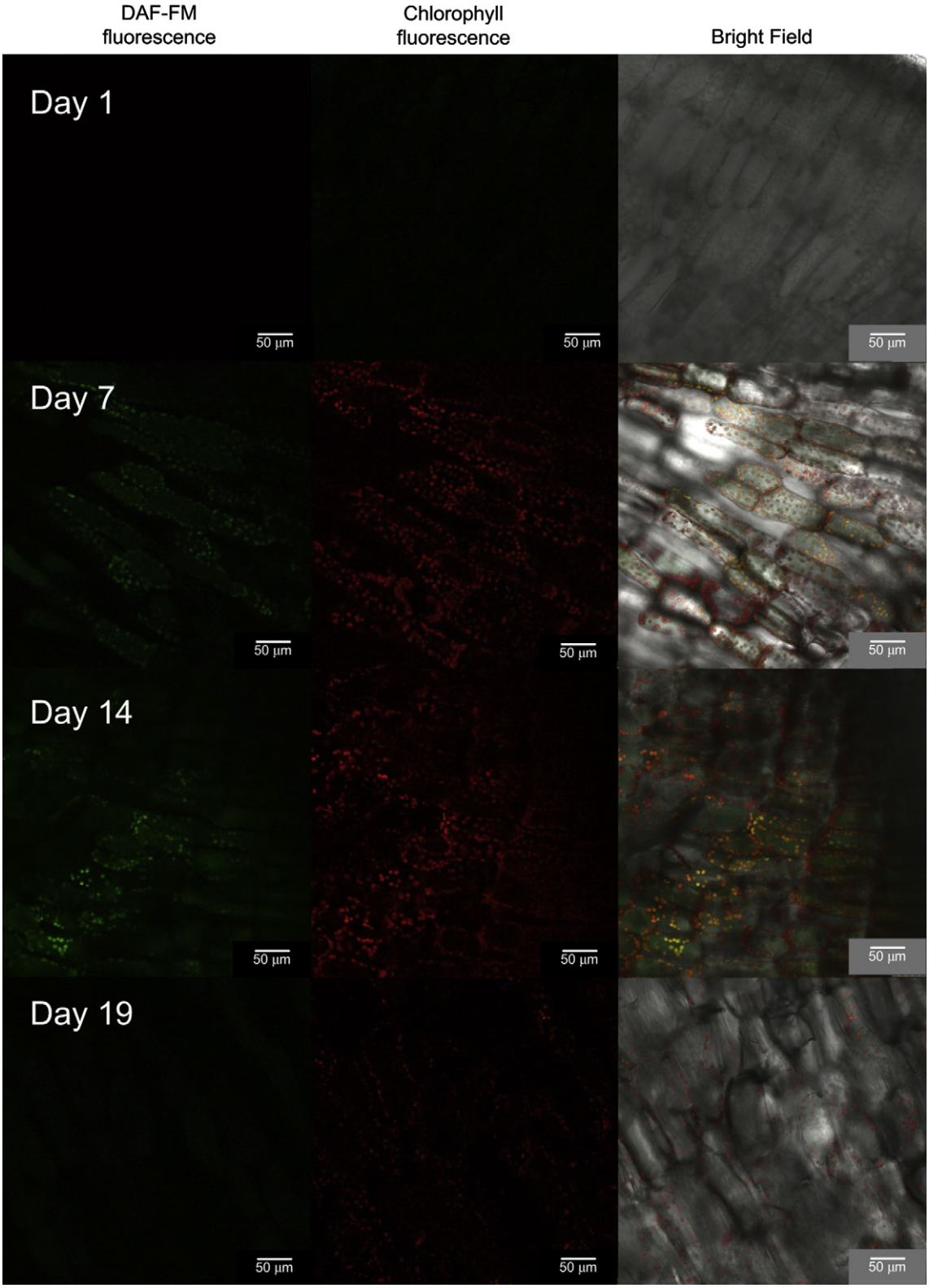

Fig. 3. Confocal laser microscopy for NO detection in soybean cotyledons throughout seedling development. Left panel, NO visualization employing DAF-FM in cotyledons excised from plants on days 1, 7, 14, and 19 after germination. Tissues were loaded with 17 µM DAF-FM DA for 30 min, washed and observed after illumination for 20 s. Middle panel, chlorophyll autofluorescence from cotyledons of 1, 7, 14, and 19 days of development, respectively. Right panel, merged images showing co-localization of green fluorescence corresponding to NO detection and chlorophyll signals (shown in orange-yellow) from cotyledons of 1, 7, 14, and 19 days of development, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Chloroplast functionality and NO detection by EPR. Quantum yield of photosystem II (ϕ_{PSII}) and EPR signal height of NO detection in soybeans treated with herbicides acting on chloroplasts.

	ϕ_{PSII}	Signal height (AU)
Control	0.765 ± 0.003	20 ± 3
+DCMU	0.034 ± 0.005[a]	2.3 ± 0.3[a]
+PQ	0.27 ± 0.06[a]	2.0 ± 0.5[a]

Plants were grown in Steinberg solution. DCMU and Paraquat were added 48 h before the measurements.

[a] Significantly different from control plants. ANOVA, $p < 0.05$. Quantum efficiency of photosystem II (ϕ_{PSII}) was evaluated in 7-day cotyledons from control plants, plants treated with DCMU (0.4 g m^{-2}) in the nutrient solution, or sprayed with PQ (93 μM) 48 h before measurements. EPR signal height corresponds to the difference between the maximum and minimum of the signal recorded at lowest field during NO measurement. For quantum yield of photosystem II (ϕ_{PSII}), data are expressed as means ± SE of three independent experiments, with at least six replicates in each experiment. For EPR measurements, data are expressed as means ± SE of three independent experiments, with at least two replicates in each experiment.

showed almost no NO in cotyledons from ammonium-fed plants treated with DCMU (Fig. 5B).

3. Discussion

One of the major limitations in studying NO and other reactive nitrogen species in biological systems is the lack of appropriate tools to monitor their production *in vivo*. Fluorescence, especially in combination with microscopy, is suitable for *in vivo* analyte sensing [30], and the fluorescent probe DAF-FM DA has been extensively used for this purpose in plant tissues [18,31–33]. In order to gain insight into the subcellular localization of NO endogenously produced by soybean cotyledons from 7-day-old seedlings, we employed fluorescence and confocal laser microscopy. In tissues loaded with the membrane-permeant probe DAF-FM DA, fluorescence was observed as a punctuated pattern under an epifluorescence microscope, whereas the confocal images enabled us to localize the fluorescence in chloroplasts. It is important to point out that fluorescence was observed after illumination of samples with the microscope lamp. The dependence on light irradiation for NO emission was previously observed in tobacco leaf peelings [20], and NO fluorescence increased after increasing irradiance in the green alga *O. tauri* [12]. Due to the dark incubation of samples with the

Table 2
Fresh weight, total chlorophyll, and quantum yield of photosystem II (ϕ_{PSII}) in soybean cotyledons growing under different nitrogen sources.

	Control	NH_4^+	NH_4^+ + DCMU
Fresh weight (mg)	292 ± 10	289 ± 8	277 ± 18
Chlorophyll (μg mg^{-1} FW)	0.59 ± 0.04	0.61 ± 0.06	0.57 ± 0.03
Φ_{PSII}	0.765 ± 0.003	0.767 ± 0.009	0.055 ± 0.008[a]

Plants were grown in Steinberg solution, or in Steinberg solution in the presence of ammonium as nitrogen source, with or without DCMU applied 48 h before the measurements.

[a] Significantly different from control and NH_4^+ supplied plants, ANOVA, $p < 0.05$.

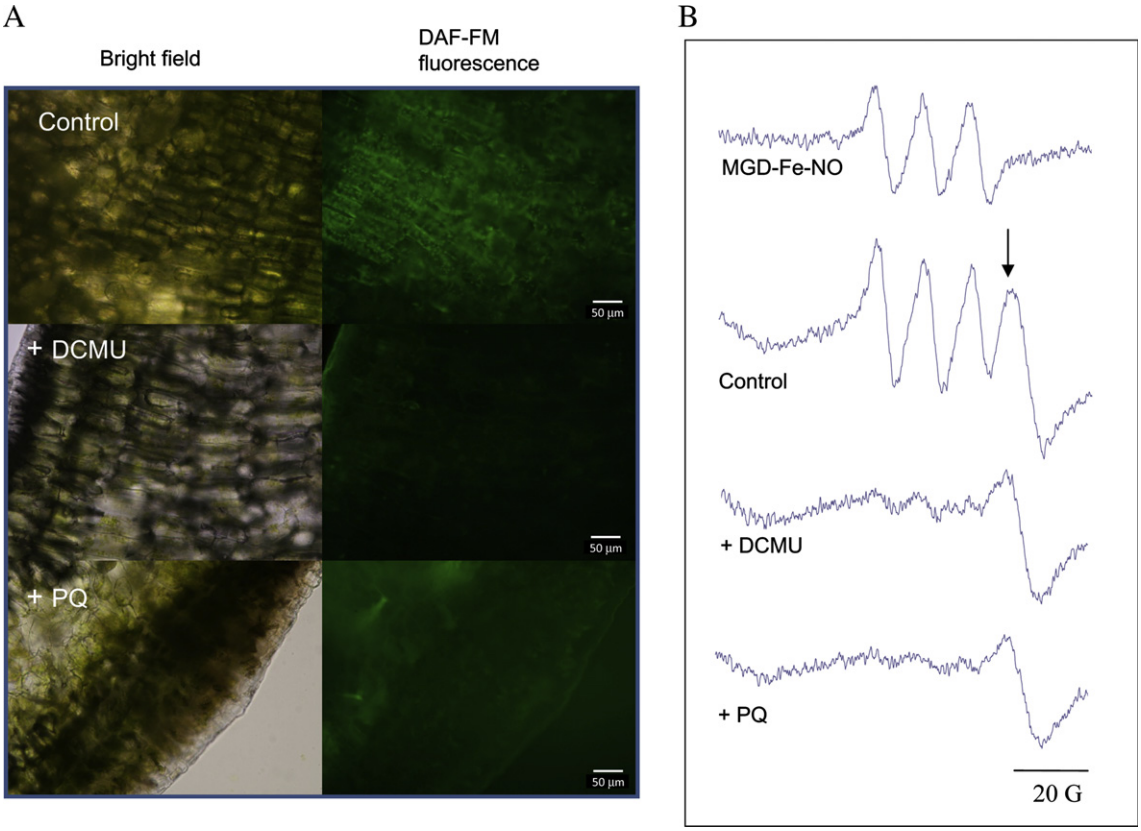

Fig. 4. NO content in soybean cotyledons treated with herbicides acting on photosynthetic electron transport. A. NO detection employing DAF-FM DA fluorescence in soybean cotyledons. Cotyledons were cut in thin sections and loaded with 17 μM DAF-FM DA for 30 min, washed and observed after illumination ($\lambda_{excitation}$ = 450–490 nm, $\lambda_{emission}$ = 500–550 nm). Left panel, Bright-field images of cotyledons from control plants, and treated with DCMU and PQ respectively. Right panel, DAF-FM fluorescence of control cotyledons, and cotyledons treated with DCMU and PQ respectively. B. Typical EPR spectra of MGD–Fe–NO adduct. Samples were homogenized in the presence of MGD–Fe as spin trap. From top to bottom: Standard solution of 5 mM S-nitrosoglutathione (GSNO) mixed with the spin trap. Control cotyledons from 7-day-old plants, the arrow indicates the signal corresponding to MGD–Cu adduct. Cotyledons treated with DCMU 48 h before the measurements. Cotyledons treated with PQ 48 h before the measurements. Images and EPR signals are representative of three independent experiments.

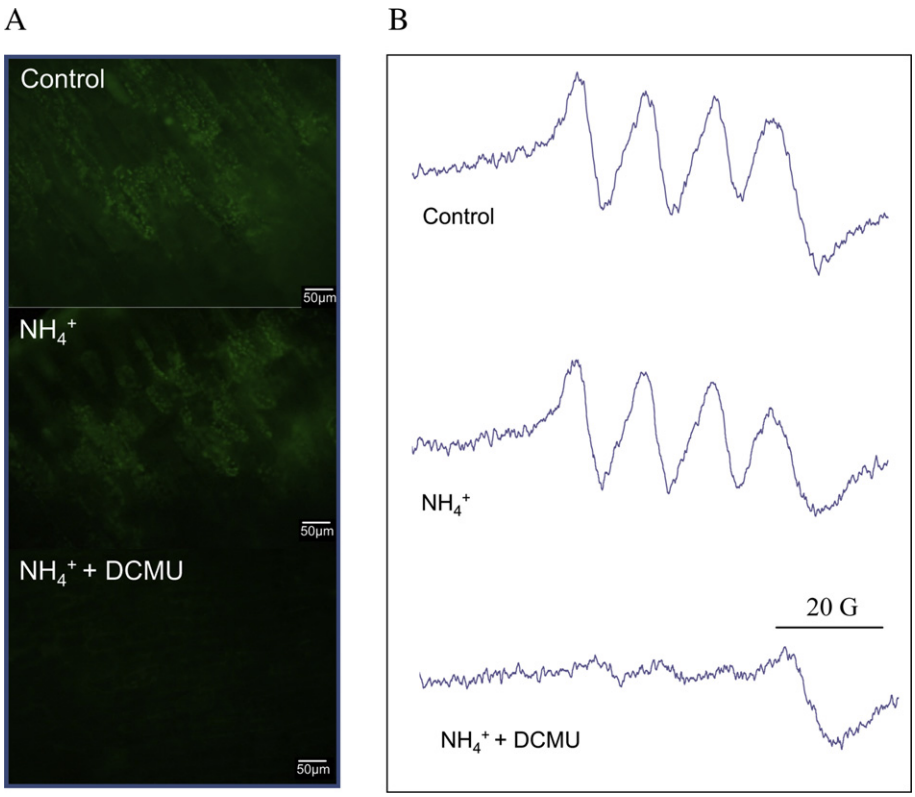

Fig. 5. NO content in soybean cotyledons growing under different nitrogen sources. A. NO detection by fluorescence microscopy in soybean cotyledons growing under different nitrogen sources. Cotyledons were excised from plants on day 7 after germination. Tissues were loaded with 17 μM DAF-FM DA for 30 min, washed and observed after illumination ($\lambda_{excitation}$ = 450–490 nm, $\lambda_{emission}$ = 500–550 nm). Control cotyledons (Steinberg solution) (top). Cotyledons growing in the presence of ammonium as the unique nitrogen source (middle). Cotyledons growing in the presence of ammonium as the unique nitrogen source but treated with DCMU 48 h before the measurements (bottom). B. Total NO content in soybean cotyledons evaluated by EPR spin trapping. Typical EPR signals from 7-day-old cotyledons from: control plants (top); plants growing in the presence of ammonium as nitrogen source (middle), and cotyledons growing in the presence of ammonium as nitrogen source and treated with DCMU 48 h before the measurements (bottom). Images and EPR signals are representative of three independent experiments.

fluorescent probe, the requirement for light could be related to the full restoration of light dependent functions in chloroplasts.

Even though DAF-FM DA has been widely used in plant tissues and its specificity is assumed, caution must be taken when working with compartments having low pH or high concentrations of reducing agents [34]. To assess NO specificity of intracellular DAF staining, we examined the effect of an NO scavenger, cPTIO. In our experimental conditions cPTIO significantly reduced the observed fluorescence. It is worthy of note that some discrepancies can be observed after the use of cPTIO as scavenger, where an increase in the observed fluorescence was reported probably due to the relative concentrations of cPTIO, NO and biological oxidants, bearing in mind that N_2O_3 is the actual species reacting with DAF [34].

Here, we tested the hypothesis that NO generation is related to chloroplast function. In this regard, the detection of NO in coincidence with cotyledon maximum fresh weight, chlorophyll content, and quantum yield of PSII, supports the hypothesis of a strong link between NO levels and chloroplast functionality. In addition, seedlings exposed *in vivo* to herbicides showed deleterious effects on chloroplast function (loss of photosynthetic capacity), and as a consequence an impaired NO accumulation was observed employing different methodological approaches. Even though both, DCMU and PQ interfere with chloroplasts function, their modes of action are different. It could not be ruled out that part of the effects of PQ on NO levels could be due to the generation of superoxide anion which consumes NO to form peroxinitrite ($ONOO^-$). Under the experimental conditions used in the present work, the significantly decreased detection in NO could be ascribed to either a lower NO generation rate or to a higher consumption rate of NO to form $ONOO^-$. However, the results obtained after the exposure to DCMU, that binds plastoquinone and blocks electron flow at the quinone acceptors of photosystem II, support a role for the requirement of the integrity of the photosynthetic electron chain in chloroplasts NO production. These results are consistent with the importance of chloroplasts for NO generation in soybean cotyledons, both as a result of the active synthesis of NO in the organelle ([22], Fig. 1) and/or because of an indirect requirement of some chloroplast products for NO synthesis in other areas of the plant, as it was above described [18].

Soybean cotyledons growing in the presence of ammonia, physiologically non-distinguishable from control (nitrate-fed) cotyledons, showed also a similar NO accumulation evaluated by both methodologies, fluorescence microscopy and EPR, indicating that cotyledons are able to produce similar amounts of NO independently of the source of nitrogen supplied. These results led us to propose that different sources of NO could operate for NO accumulation in soybean cotyledons, e.g., nitrite- and Arg-dependent sources [11]. Thus, it is likely that under different conditions, for example the lack of a substrate, one pathway could result more operative to maintain NO generation to support the required NO levels in the cell to allow a normal function and development. In this sense, NO production in Arabidopsis plants following pathogen attack may result from the interplay of Arg- and nitrite-dependent pathways [35].

Additionally, the fact that blocking the photosynthetic electron transport chain on ammonium-supplied cotyledons affects NO level, supports the hypothesis that some products of the electron

transfer chain of chloroplasts, e.g., NADPH, or at least a correct electron transfer process are needed for NO synthesis in chloroplasts. It would be possible that NO production could also be based on a yet unknown complex of separately encoded peptides or proteins that have a similar enzymatic activity as the animal NOS [10].

Localized NO production in chloroplasts could have a protective function, since NO can act as an antioxidant preserving lipids, proteins (including D1) and nucleic acids from photooxidative damage [22,36]. Exogenously applied NO affects photosynthesis, however, there are some contradictory reports probably due to the use of different NO-donors [37]. Initial studies reported a decrease in photosynthesis by NO [38]. Later an important action site of NO was shown to be PSII, where NO can inhibit electron transport [39,40]. NO could act as a regulator of photosynthetic electron transport since it causes inhibition of PSII electron transport and reduced Fv/Fm and qP values [37]. Increased production of NO in chloroplasts in response to relatively high irradiance could help to down-regulate photosynthetic electron transport and, thereby, decrease the risk of photooxidation under conditions where photochemical activity could exceed ATP and NADPH demand by the carbon reductive pathway. A similar mechanism of regulation has been described in mammalian mitochondria, where endogenous mitochondrial NO is able to regulate mitochondrial respiration [41].

Overall, these findings strongly suggest that chloroplasts are organelles that contribute to NO synthesis *in vivo*, and that their proper functionality is essential for maintaining NO levels in soybean cotyledons.

4. Materials and methods

4.1. Plant material, growing conditions and treatments

Soybean (*Glycine max* cv. ADM 4800) seeds were placed in the dark at controlled temperature (26–28 °C) over water-saturated filter paper. After 48 h of imbibition, germinated seeds were transferred to pots containing vermiculite, and irrigated daily with Steinberg solution (control plants, [NO_3^-] 3.93 mM) [42], or Steinberg with ammonia as nitrogen source ([NH_4^+] 3.93 mM), where indicated. Seedlings were grown in a growth chamber at 22–24 °C, with a photoperiod of 16 h, and 300 μmol m^{-2} s^{-1} of photosynthetically active radiation (PAR 400–700 nm) supplied by a bank of Philips 40-W daylight fluorescent lights. Nitrite concentration was determined in homogenates previously centrifuged at 10,000 g for 20 min, as previously described [43]. In some experiments, DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea, was added on day 5 by watering the pots (0.4 g m^{-2}) to allow root uptake of the herbicide, and cotyledons were collected on day 7 of growth. In other cases, plants were sprayed with 93 μM Paraquat (PQ), 1,1′-dimethyl-4,4′-bipyridinium dichloride, on day 5 and cotyledons were collected on day 7.

4.2. Chlorophyll content

Cotyledons were powdered with liquid nitrogen and chlorophyll was extracted in acetone 80% (v/v) for 1 h. After centrifugation, chlorophyll content in the supernatant was evaluated spectrophotometrically by measuring the absorbance at $\lambda = 663$, 647 and 470 nm according to Lichtenthaler [44].

4.3. Chlorophyll fluorescence measurements

Chlorophyll fluorescence measurements were performed in soybean cotyledons employing a modulated fluorescence system (FMS2; Hansatech Instruments, UK). In the presence of background illumination, the quantum yield of PSII (ϕ_{PSII}) was measured from the ratio of variable (F_v') to maximum (F_m') fluorescence in light-adapted cotyledons.

4.4. NO detection by electronic paramagnetic resonance (EPR)

Fresh cotyledons were excised from seedlings and homogenized in 100 mM phosphate buffer pH 7.4 (1 g ml^{-1}), supplemented with the spin trap MGD_2–Fe^{2+} (10 mM MGD, 1 mM $FeSO_4$) [45]. The homogenates were immediately transferred to Pasteur pipettes for EPR spin trapping measurements. The spectra were recorded at room temperature (18 °C) with a Bruker ER 070 spectrometer (Karlsruhe, Germany), with the instruments settings described previously [4].

4.5. NO detection by fluorescence microscopy

Thin cotyledon sections were incubated for 30 min at 25 °C, in darkness, with 17 μM DAF-FM DA (Sigma) prepared in 100 mM phosphate buffer (pH 7.4) with or without 400 μM cPTIO. Cotyledons were rinsed in the same buffer for 10 min. Observations were performed at $\lambda_{excitation} = 450$–490 nm and $\lambda_{emission} = 500$–550 nm [31]. The fluorescence of the segments was monitored with an Olympus BX51 fluorescence microscope. Cotyledon sections incubated in 100 mM phosphate buffer (pH 7.4) were used as controls to assess autofluorescence levels.

4.6. NO detection by confocal laser scanning microscopy

Cotyledon sections were incubated as described previously. After washing, the sections were embedded in perfluorodecalin and mounted for examination with a confocal laser scanning microscope system (Leica SP-5), using standard settings for DAF-FM green fluorescence ($\lambda_{excitation} = 488$ nm; $\lambda_{emission} = 500$–525 nm) and chlorophyll autofluorescence ($\lambda_{excitation} = 488$ nm; $\lambda_{emission} = 670$–730 nm). Background fluorescence in the DAF-FM channel, routinely negligible, was controlled with unstained cotyledon sections.

4.7. Statistical analyses

Data in the figures are expressed as means ± SE of three independent experiments. Differences in measured parameters were tested for significance employing single-factor ANOVA, and the significantly different means were evaluated using the Bonferroni post-test (StatView for Windows, Version 5.0; SAS Institute Inc.).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.plaphy.2013.01.019.

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