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Plant Science 176 (2009) 662–668

Contents lists available at ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

Role of nitric oxide in soybean cotyledon senescence

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ARTICLE INFO

Article history:
Received 8 October 2008
Received in revised form 5 February 2009
Accepted 10 February 2009
Available online 20 February 2009

Keywords:
Soybean cotyledons
Lipid peroxidation
Nitric oxide
Protein nitration
Senescence

ABSTRACT

Senescence was studied in soybean (*Glycine max*, var ALM 4500) cotyledons from 10- and 25-day-old seedlings. Rejuvenated cotyledons, obtained by removing epicotyls from 5-day seedlings, showed a lower decrease in the content of photosynthetic pigments from day 10 to day 25, as compared to naturally senescent cotyledons. Unlike controls, rejuvenated cotyledons showed no changes in electrolyte leakage, chlorophyll *a*, and carotene content during the studied period. At day 10, nitric oxide (NO) content in rejuvenated cotyledons was higher than in naturally senescent cotyledons. The role of NO in senescence was assessed by exposing seedlings to sodium nitroprusside (SNP). SNP-treated cotyledons showed a constant NO content over the studied period of 4.0 ± 0.4 nmol g^{-1} FW, conserved photosynthetic pigment content and lower lipid radical content, as compared to naturally senescent cotyledons.

These results suggest that NO can protect against senescence-dependent alterations in cotyledons, because (i) exogenous NO can partially protect naturally senescent cotyledons and (ii) rejuvenated cotyledons, which do not show the senescent phenotype, have higher endogenous levels of NO.

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1. Introduction

Cotyledons have a short life span, since they gradually senesce with the progression of seedling development and die shortly after the appearance of differentiated leaves [1]. The major physiological function of cotyledons is to ensure the development of the growing seedling until differentiation of photosynthetically efficient leaves. Leaf senescence, described as an oxidative process [2], was considered as a coordinated degeneration program [3], but more recently Jansson and Thomas [4] suggested that apoptotic-like programmed cell death and senescence are regulated by different set of genes. Although cotyledon senescence is not fundamentally different from leaf senescence, organ specific differences with respect to the photosynthetic activity of these leaf organs during natural senescence have been reported [5].

Nitric oxide (NO) has emerged as a key signaling molecule in plants, involved in several biological processes, including stomatal closure, seed germination, root development [6–8], and expression of defense-related genes and programmed cell death [9]. NO was postulated to mediate the biological effects of primary signaling molecules such as hormones [10], and it was suggested that it plays a role in leaf senescence. Also, Hung and Kao [11] showed evidence of a protective effect of NO against senescence of rice leaves promoted by methyl jasmonate. Corpas et al. [12] reported that in senescent pea leaves endogenous NO generation was clearly reduced in the vascular tissues, and the NOS-like activity of peroxisomes was down regulated. Moreover, *Arabidopsis* transgenic plants expressing a NO degrading bacterial dioxygenase, degraded NO faster than wild type plants, and exhibited a yellowing phenotype similar to that observed in senescence [13]. Moreover, fumigation with 4 ppm NO gas attenuated the induced senescence, supporting the hypothesis that NO could act as a factor for delaying leaf senescence [13].

Epicotyl removal above the yellow cotyledons leads to subsequent rejuvenation [14], and this practice has been used as an approach for studying reversibility of the senescence process [9,15]. In this sense, Ananieva et al. [16] reported a full recovery of the polypeptide profile in the rejuvenated cotyledons. In addition, other parameters like photosynthetic rate and the photochemical efficiency of PSII, were also recovered. Thus, the rejuvenated cotyledons provide a frame of reference to compare changes in natural senescence, and to evaluate a possible role of NO in the senescence symptoms in the cotyledons.

The hypothesis presented here is that, besides reactive oxygen species, NO is involved in cotyledon senescence. To test the link between reactive species and senescence, the oxidative and nitrosative status of cotyledons were assessed over a 25-day

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Abbreviations: EPR, electronic paramagnetic resonance; MGD, sodium-N-methyl-D-glucamine dithiocarbamate; NO, nitric oxide; $ONOO^-$, peroxynitrite; POBN, α-(4-pyridyl-1-oxide)-N-*t*-butyl nitrone; SNP, sodium nitroprusside; TEMPOL, 4-hydroxy-2,2,6,6-tetramethyl piperidine-N-oxyl.

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doi:10.1016/j.plantsci.2009.02.007

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period by measuring electrolyte leakage, lipid radical content, protein nitrotyrosines and endogenous NO content; along with physiological parameters such as chlorophyll and carotene content, fresh and dry weight. To further explore the role of NO, both a model of rejuvenation and the effect of exposure to an NO donor were employed on soybean cotyledons.

2. Materials and methods

2.1. Plant material, growing conditions and treatments

Soybean (*Glycine max* var. ALM 4500) seeds were placed in the dark at controlled temperature over water-saturated filter paper. After 48 h of imbibition, germinated seeds were transferred to pots containing vermiculite, and irrigated daily with Steinberg solution [17]. Seedlings were grown in a greenhouse chamber at 22–24 °C, with a photoperiod of 16 h, receiving 300 μmol m^{-2} s^{-1} of photosynthetically active radiation (PAR 400–700 nm) supplied by a bank of Philips 40-W daylight fluorescent light. The model of rejuvenation in cotyledons was performed by removing the epicotyls from seedlings 5 days after planting [18]. For NO treatment, 0.1 mM sodium nitroprusside (SNP) solutions prepared daily were sprayed over the seedlings. Cotyledons were excised from soybean seedlings at 10 or 25 days after planting, and employed for the assays. Fresh- (FW) and dry weight (DW) of intact soybean cotyledons were measured before and after exposure to 70 °C, until constant weight, respectively. The percentage water content in the cotyledons was calculated according to Eq. (1).

$$\text{Water content}\,(\%) = \left[\frac{\text{FW} - \text{DW}}{\text{FW}}\right] \times 100 \qquad (1)$$

2.2. Electrolyte leakage assay

Four cotyledons were excised from soybean seedlings, and placed in vials containing 20 ml of distilled water. The conductivity of the medium was measured immediately (L_0) and after 3 h of incubation at room temperature (L_3), employing a multi-parameter analyzer (Consort C831). To evaluate the maximal conductivity (L_m), the cotyledons were boiled for 10 min, and the conductivity was assayed at room temperature. Electrolyte leakage was calculated according to Eq. (2) [19].

$$\text{Electrolyte leakage}\,(\%) = \left[\frac{L_3 - L_0}{L_m \times \text{FW}}\right] \times 100 \qquad (2)$$

2.3. Chlorophyll and carotene content determination

Chlorophyll and carotenes were extracted from powdered soybean cotyledons in acetone 80% (v/v). After centrifugation, the content of photosynthetic pigments in the supernatant was spectrophotometrically evaluated by measuring the absorbance at 663, 647 and 470 nm according to Lichtenthaler [20].

2.4. Electronic paramagnetic resonance (EPR) detection of NO

Fresh cotyledons (0.5 g FW) were excised from seedlings, homogenized in 0.5 ml of 100 mM phosphate buffer, pH 7.4, and supplemented with the spin trap MGD_2–Fe^{2+} (10 mM MGD, 1 mM $FeSO_4$) [21]. 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 ECS 106 EPR spectrometer, operating at 9.5 GHz. Instrument settings include 200 G field scan, 83.886 s scan time, 327.68 ms time constant, 5.983 G modulation amplitude, 50 kHz modulation frequency and 20 mW microwave power. Quantification of the spin adduct (MGD_2–Fe^{2+}–NO) was performed using standard solutions of TEMPOL introduced into the same sample cell used for spin trapping. The TEMPOL solutions were standardized spectrophotometrically at 429 nm (ε = 13.4 M^{-1} cm^{-1}). The concentration of MGD_2–Fe^{2+}–NO adduct was obtained by double integration of the three-line spectra and compared with the TEMPOL spectra.

2.5. EPR detection of lipid radicals

Recently collected cotyledons were homogenized in 100 mM potassium phosphate buffer, pH 7.4, in the presence of the spin trap POBN (50 mM final concentration), and employed for lipid radical detection. EPR spectra were recorded at room temperature using a Bruker ECS 106 spectrometer, operating at 9.81 GHz with 50 kHz modulation frequency. EPR instrument settings for the experiments were as follows: microwave power, 20 mW; modulation amplitude, 1.232 G; time constant, 81.92 ms [22]. The content of the POBN-lipid radical adduct was quantified as described above.

2.6. Western blot analysis of nitrotyrosines

Cotyledons were powdered and suspended in 100 mM phosphate buffer, pH 7.4 (3 mg prot ml^{-1}). The protein content in the samples was measured according to Bradford [23]. The homogenate was mixed with an equal volume of sample buffer according to Laemmli [24] and incubated for 10 min at 95 °C. Proteins (25–50 μg per well) were loaded in 12% (w/v) acrylamide concentration mini-gels and electrophoresis was performed at room temperature under a constant voltage (120 V) for 2 h. Afterwards, the proteins were electro-transferred to nitrocellulose membranes at 130 V for 1 h. Blots were blocked with 3% (w/v) BSA dissolved in PBS-T [10 mM potassium phosphate buffer, pH 7.4, 150 mM NaCl, 0.1% (v/v) Tween 20], incubated overnight with the primary antibody (mouse anti-nitrotyrosine IgG, Chemicon International) dissolved in blocking buffer (1/4000), and washed several times with PBS-T. Membranes were then incubated for 2 h with the secondary antibody (goat anti-mouse IgG conjugated to horseradish peroxidase) prepared 1/10,000 in PBS-T with 1% (w/v) BSA and washed several times with PBS-T. Western blot assays were developed by using a chemiluminescence kit (Bio-Rad). Band intensity was assessed employing Scion Image for Windows.

2.7. Statistical analyses

Data in the text, figures and tables are expressed as means $\pm$ SE of three to six independent experiments, with two replicates in each experiment. Differences on measured parameters were tested for significance employing single-factor ANOVA, and the significantly different means were evaluated using the Bonferroni post-Test (GraphPad InStat for Windows, Version 3.0; GraphPad Software Inc.).

3. Results and discussion

Based on previous data [25], soybean cotyledons from 10-day seedlings were chosen as maximally developed cotyledons, while cotyledons from 25-day seedlings were considered as senescent organs. FW increased after planting, and cotyledons from 10-day-old seedlings exhibited maximum values (211 ± 6 mg cot^{-1}), whereas cotyledons from 25-day seedlings exhibited a loss of 20% in FW (Fig. 1A). Upon senescence, cotyledons lost cellular components and their DW fell from day 10 to day 25, representing a loss of 53% in dry matter (Fig. 1B). However, water content remained high during the studied period (88% and 93% for 10 and 25 days, respectively). Cotyledon senescence ends with cotyledon abscission from the

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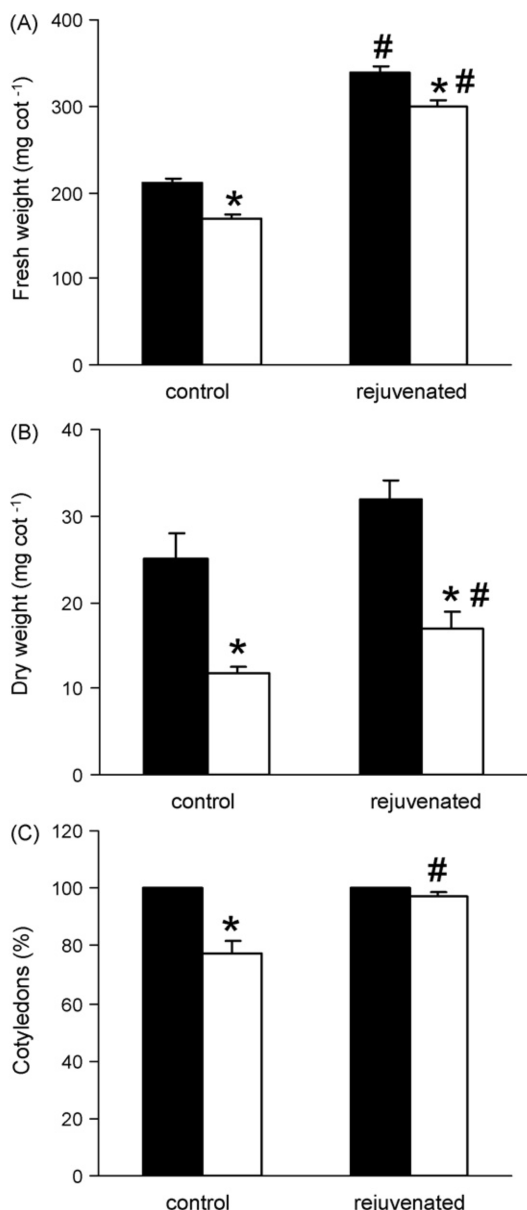

Fig. 1. Changes in physiological parameters in naturally senescent and rejuvenated soybean cotyledons. Measurements were performed at 10 (■) or 25 (□) days of development. (A) Fresh weight. (B) Dry weight. (C) Percentage of non-fallen cotyledons. Data are expressed as means ± SE of three independent experiments, with two replicates in each experiment. *Significantly different from values for 10-day cotyledons developed under the same growing conditions; $p < 0.05$ (GraphPad InStat for Windows Version 3.0; GraphPad Software Inc.). #Significantly different from the values for naturally senescent cotyledons at the same time of development; $p < 0.05$ (GraphPad InStat for Windows Version 3.0; GraphPad Software Inc.).

seedling. No cotyledon abscission was observed 10 days after planting in naturally senescent seedlings, but seedlings lost 21% of their cotyledons 25 days after planting, confirming that at that time the senescent phenotype is established (Fig. 1C).

Rejuvenated cotyledons obtained by cutting the epicotyl on day 5 after planting, as described in Section 2, increased their FW with the same profile as naturally senescent cotyledons. However, FW in rejuvenated cotyledons was significantly higher than in control cotyledons even though they were excised from seedlings at the same developing time (Fig. 1A). At day 25 after germination, rejuvenated cotyledons also showed a higher DW as compared to control cotyledons (Fig. 1B), and water content remained high over the studied period (90% and 94% for 10 and 25 days, respectively). Removal of epicotyls from seedlings caused a significant decrease in cotyledon abscission at day 25, as compared to 25-day control seedlings (Fig. 1C).

Leaf yellowing is considered the first visible symptom of senescence [26,27]. Chlorophyll content in cotyledons has been taken as an index of chloroplast development and its decline could be correlated with a general disassemble of the structure of chloroplasts. A decline in net photosynthesis rate has been reported for soybean and zucchini senescing cotyledons [28]. In the present study a significant decline in total chlorophyll (Fig. 2A), chlorophyll *a* (Fig. 2B) and *b* (Fig. 2C), and carotene content (Fig. 2D) was found in naturally senescent cotyledons from 25-day seedlings as compared to that measured in cotyledons from day 10. In agreement with previous works describing the reversibility of the senescence process [14,15,28,29], rejuvenated cotyledons from 25-day seedlings showed higher total chlorophyll content, as compared to naturally senescent cotyledons from seedlings at the same age (Fig. 2A). Chlorophyll *a* and carotene content did not change with age in rejuvenated cotyledons (Fig. 2B, D). On the other hand, chlorophyll *b* showed a significant decrease at day 25 as compared to 10-day rejuvenated cotyledons (Fig. 2C). Thus, the physiological parameters evaluated in soybean cotyledons, such as FW and DW, were significantly higher in rejuvenated cotyledons than in naturally senescent cotyledons. Moreover, rejuvenated cotyledons remained in the seedling 25 days after germination and also conserved intact the photosynthetic pigments, confirming that this is an appropriate model for comparing senescence-related changes.

Electrolyte leakage was evaluated as an index of membrane integrity in intact excised cotyledons. In naturally senescent cotyledons relative conductivity was $3.1 \pm 0.5\ g^{-1}$ FW at day 10 and it increased to $4.6 \pm 0.8\ g^{-1}$ FW at 25 days after planting. Rejuvenated cotyledons exhibited a lower electrolyte leakage than control cotyledons over the complete studied period, and non-significantly different values were measured in relative conductivity between day 10 and day 25 (1.5 ± 0.2 and $1.7 \pm 0.2\ g^{-1}$ FW for 10 and 25 days after germination, respectively). These results showed that degradation of cellular structures such as membranes, caused by senescence-related oxidative stress [30] was avoided in rejuvenated cotyledons.

The extent of lipid peroxidation in soybean cotyledons was assessed by EPR, employing POBN as spin trap (Fig. 3A). The content of lipid radicals, expressed on a fresh weight basis, was similar for 10- and 25-day-old control cotyledons. However, rejuvenated cotyledons showed a lipid radical content significantly lower than control cotyledons at day 25 (Fig. 3B). Data from lipid radical quantification are in agreement with the high degree of conservation achieved in membranes from rejuvenated cotyledons as compared to naturally senescent.

Evaluation of physiological parameters such as FW, DW, and the content of photosynthetic pigments showed that rejuvenated cotyledons maintain metabolic activity unchanged. Moreover, membranes are more protected in rejuvenated cotyledons than in naturally senescent cotyledons. Thus, it could be expected that antioxidative mechanisms are enhanced in rejuvenated cotyledons in order to protect them against physiologically produced oxidants.

EPR has been employed to determine the presence of NO in plant tissues. This methodology, combined with spin trapping, is highly specific but has a high detection limit, however it has been successfully employed in several conditions (e.g. during germination) [8,31]. In this sense, it has been reported that cotyledons produce high amounts of NO [32]. NO content in soybean cotyledons strongly depends on the seedling age, showing a maximum value at around day 7 of seedling development [25]. Naturally senescent cotyledons from 10-day-old seedlings showed the typical triplet signal of the $(MGD)_2$–Fe^{2+}–NO adduct, when

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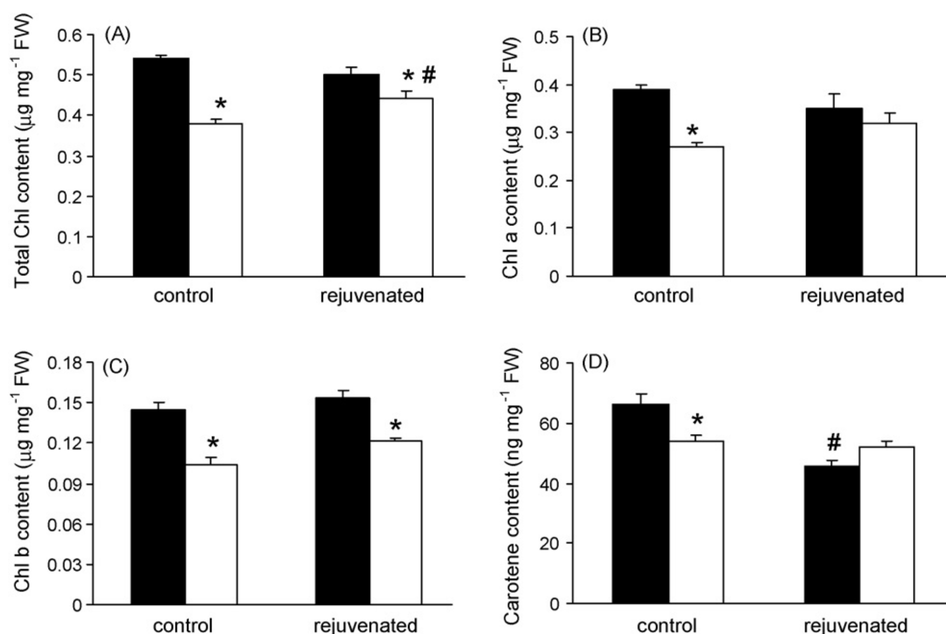

Fig. 2. Changes in photosynthetic pigments in naturally senescent and rejuvenated soybean cotyledons. (A) Total chlorophyll content. (B) Chlorophyll *a* content. (C) Chlorophyll *b* content. (D) Carotene content. Values were measured in cotyledons 10 (■) or 25 (□) days after planting. Data are expressed as means ± SE of three independent experiments, with two replicates in each experiment. *Significantly different from values for 10-day cotyledons developed under the same growing conditions; $p < 0.05$ (GraphPad InStat for Windows Version 3.0; GraphPad Software Inc.). #Significantly different from values for naturally senescent cotyledons at the same time of development; $p < 0.05$ (GraphPad InStat for Windows Version 3.0; GraphPad Software Inc.).

mixed with the spin trap solution (Fig. 4), while no signal from the adduct was detected in cotyledons excised from 25-day seedlings. Quantification of EPR signals from 10-day cotyledons was not performed due to a low signal/noise ratio. Ten days after planting rejuvenated cotyledons showed an NO content (11 ± 3 nmol NO g^{-1} FW) significantly higher than that found in naturally senescent cotyledons (Fig. 4). However, no EPR-signal was obtained in rejuvenated cotyledons from 25-day-old seedlings (Fig. 4). Changes in NO levels were also described in pea seedlings, where it was found that the synthesis of NO, through a constitutive nitric oxide synthase activity, depends on the plant organ and developmental stage of the seedlings [33]. The finding of a higher NO concentration in 10-day rejuvenated cotyledons as compared to control suggests that the steady state concentration of NO also depends on other factors different from seedling age, probably related to signals from other part of the plant or an altered nitrogen metabolism. Moreover, an increased NO synthesis can be triggered by abiotic stress conditions, among them mechanical wounding, as it was previously reported [42]. The increased steady state concentration of NO could be related to the preservation of cellular structures such as membranes [35], and the preservation of chlorophyll in rejuvenated cotyledons, as it was previously proposed [11,36].

One of the molecular footprints left by the reactions of reactive nitrogen species with biomolecules is the nitration of protein tyrosine residues. The levels of protein 3-nitrotyrosines were higher in 10-day cotyledons than in cotyledons from 25 days seedlings, both in naturally senescent and rejuvenated cotyledons (Fig. 5). However, protein nitration in rejuvenated cotyledons was significantly higher than that found in naturally senescent cotyledons from 10-days seedlings (Fig. 5). These results showed a correlation between NO level in tissues and the nitration of protein tyrosine, indicating that protein 3-nitrotyrosines are biomarkers of *in vivo* NO-dependent metabolism. Two mechanisms have been proposed for biological nitration, the peroxynitrite ($ONOO^-$) and the heme-peroxidase pathway [37]. This post-translational modification can alter protein function [38,39], in particular tyrosine nitration may interfere with tyrosine phosphorylation of key proteins involved in plant signaling [40]. The presence of protein nitrotyrosines is often associated with pathological or stress conditions. In olive plants under salt stress conditions an increase of NOS activity and protein tyrosine nitration was described [41], in tobacco BY-2 cells treated with a fungal elicitor the induction of tyrosine nitration in proteins was demonstrated [34], and recently protein tyrosine nitration showed to be differentially modulated depending on the type of abiotic stress [42]. However, other studies showed a correlation between NO levels and protein nitrotyrosines irrespective of the presence of oxidative stress conditions [43].

The comparison between SDS–PAGE analysis of cotyledon total soluble proteins and the pattern of nitrated proteins suggests that this post-translational modification is directed to specific proteins. At least two proteins of around 29 kDa, one of 35 kDa and one of 80 kDa seems to be specifically nitrated in rejuvenated cotyledons. Taking into account the broad range of functions of NO in plants, the study of the signals that regulate NO synthesis, the pathways of NO synthesis in cotyledons, and the targets of post-translational protein modifications, are questions that need to be addressed.

NO acts as a negative factor in the senescence process [11,13]. In this sense, treatments with cytokinin, light and nitrate, which stimulate the expression or activity of the enzyme nitrate reductase [44,45], led to the enhancement of NO production in plants [46,47], and retarded the progress of plant senescence [27]. NOS activity appears to represent an enzymatic factor which influences plant senescence, since dark-induced leaf senescence occurs faster in Atnos1 knockout mutants as compared to wild type plants [48]. To further analyze the role of NO during the senescence of soybean cotyledons, seedlings were daily sprayed with an NO donor solution (0.1 mM sodium nitroprusside, SNP), from day 5 to day 25. NO content was evaluated in SNP treated cotyledons. The integration of the EPR spectra showed that the NO content was higher than in naturally senescent cotyledons, and remained unchanged at days 10 or 25 after germination (4.0 ± 0.4 nmol NO g^{-1} FW), confirming the effectiveness of the treatment with the NO donor. Parameters of growth were evaluated in SNP treated and compared to naturally senescent cotyledons. As it was mentioned above, 21% of the cotyledons had fallen from

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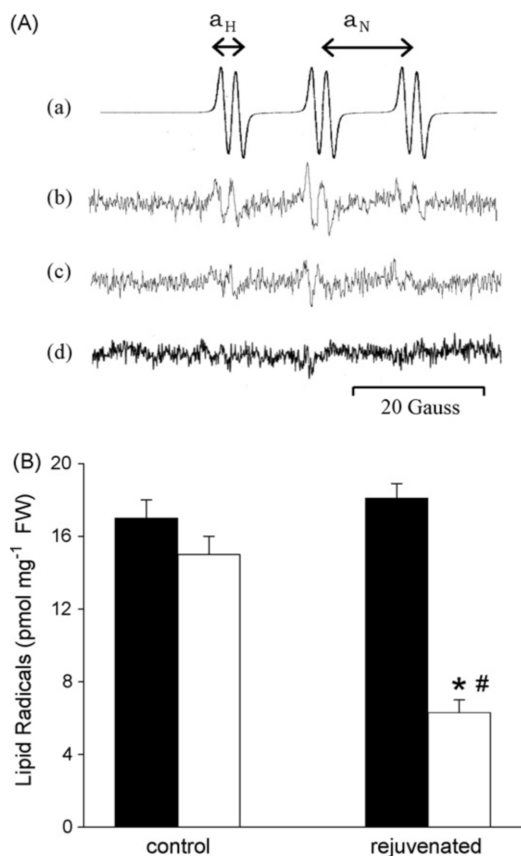

Fig. 3. Lipid radicals during natural senescence and in rejuvenated cotyledons. (A) a: Computer-simulated spectrum using hyperfine splitting characteristic of POBN lipid radical adducts, a_N = 15.8 G and a_H = 2.6 G. b: Typical EPR spectrum from cotyledon homogenates from 25-day-old seedlings. c: Typical EPR spectrum from rejuvenated cotyledon homogenates from 25-day-old seedlings. d: Spectrum of POBN alone. (B) Lipid radical content in cotyledons. Quantification was performed by double integration of the EPR signals. Measurements were performed at 10 (■) or 25 (□) days of seedling development. Data are expressed as means ± SE of three independent experiments, with two replicates in each experiment. *Significantly different from values for 10-day cotyledons developed under the same model growing conditions; $p < 0.05$ (GraphPad InStat for Windows Version 3.0; GraphPad Software Inc.). #Significantly different from values for naturally senescent cotyledons at the same time of development; $p < 0.05$ (GraphPad InStat for Windows Version 3.0; GraphPad Software Inc.).

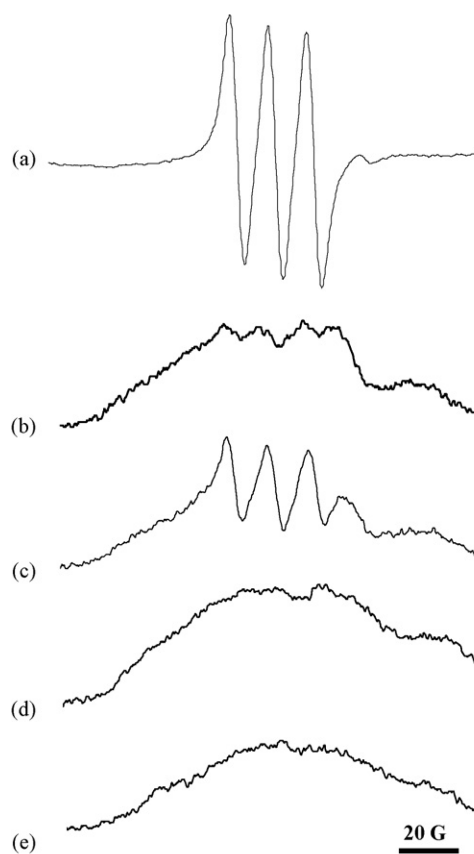

Fig. 4. EPR detection of NO in soybean cotyledons using MGD_2–Fe^{2+} as spin trap. MGD_2–Fe^{2+}–NO adduct spectrum (1 scan) corresponding to an NO donor solution (100 μM S-nitrosoglutathione) (a). Spectra (8 scans) corresponding to cotyledons from 10-day-old seedlings (b), rejuvenated cotyledons from 10-day-old seedlings (c), cotyledons from 25-day-old seedlings (d), rejuvenated cotyledons from 25-day-old seedlings (e). Measurements were performed at room temperature.

seedlings at day 25, whereas SNP-treated seedlings showed a 10% of cotyledon abscission at the same stage of development. A significantly higher DW was found in 25-day SNP-treated cotyledons as compared to control cotyledons (15.1 ± 0.1 and 11.8 ± 0.6 mg^{-1} cotyledon, respectively), whereas no significant differences in FW was found in SNP-treated cotyledons as compared to control ones. Relative conductivity, measured 25 days after planting, was slightly lower in SNP treated cotyledons as compared to cotyledons from control seedlings (4.1 ± 0.2 and 4.6 ± 0.8, respectively). The analysis of photosynthetic pigments showed that the decrease in total chlorophyll and carotenes, observed in naturally senescent cotyledons from day 10 to day 25, was significantly lower in SNP-treated cotyledons (Table 1). Lipid radical content evaluated in SNP-treated cotyledons was significantly lower than that found in control cotyledons excised from 25-day-old seedlings. While naturally senescent cotyledons experimented no change in the content of lipid radicals during the studied period, SNP-treated cotyledons showed a decrease as time progressed (Table 1). Measurements of electrolyte leakage and lipid radical quantification suggest that SNP treatment protected cotyledon membranes, in agreement with previous reports showing that SNP-treatment alleviated the increase of ion leakage in calluses of reed [49].

Taken as a whole, the results presented here showed that NO was detected as an endogenous metabolite in soybean cotyledons at day 10 after germination, but was undetectable after 25 days. The rejuvenated cotyledons exhibited a dramatic difference in the steady state concentration of NO at day 10 after germination with

Table 1
Photosynthetic pigments and lipid radical content in SNP-treated cotyledons from 10- and 25-day seedlings.

	10-Day cotyledons		25-Day cotyledons		Δ (day 25–day 10)	
	Control	SNP	Control	SNP	Control	SNP
Total chlorophyll (μg mg^{-1} FW)	0.54 ± 0.01	0.50 ± 0.01	0.38 ± 0.01	0.41 ± 0.01	0.16	0.09[a]
Carotene (ng mg^{-1} FW)	66.5 ± 0.3	59.2 ± 0.9	54.0 ± 0.2	58.0 ± 0.3[b]	2.8	1.5[a]
Lipid radicals (pmol mg^{-1} FW)	17 ± 1	18 ± 1	15 ± 1	7.5 ± 0.7[b]	2.0	10.5[a]

Data are expressed as means ± SE of three independent experiments. Δ represents the change in parameters evaluated at 25 and 10 days for control and SNP treated cotyledons.
[a] Significantly different from natural senescent cotyledons at the same age; $p < 0.05$ (GraphPad InStat for Windows Version 3.0; GraphPad Software Inc.).
[b] Significantly different from the change (day 25–day 10) for natural senescent cotyledons; $p < 0.05$ (GraphPad InStat for Windows Version 3.0; GraphPad Software Inc.).

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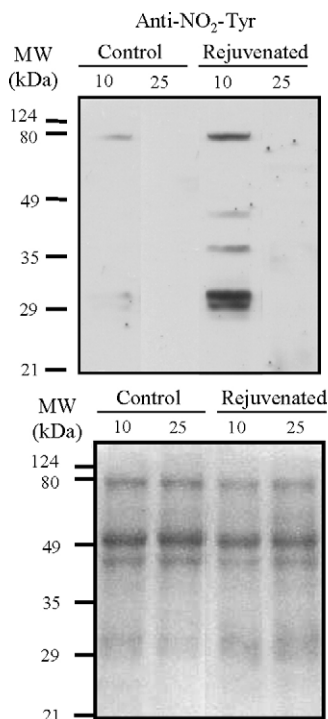

Fig. 5. Upper panel: nitrotyrosines in soybean cotyledons. Nitration of protein tyrosines in cotyledons from 10 to 25 days after germination. Cotyledons (50 μg proteins per well) were loaded in 12% (w/v) acrylamide concentration mini-gels. A mouse monoclonal anti-nitrotyrosine IgG (Chemicon International, dilution 1/4000) was employed. Lower panel: polypeptide profile of total soluble proteins. Cotyledon samples from 10 and 25 days after germination (50 μg proteins per well) were resolved on 12% (w/v) acrylamide gels followed by Coomassie brilliant blue staining.

respect to naturally senescent cotyledons, which correlates with high levels of protein nitrotyrosines and also with more conserved membranes and photosynthetic pigments. The exogenous NO application resulted in an effective incorporation by cotyledons, since an increased and constant NO content was detected in SNP-treated cotyledons. Exposure to the NO donor partially revert the senescent phenotype in naturally senescent cotyledons. Thus, the data presented here suggest a protective role for NO during senescence. The mechanism through which NO may counteract plant senescence could be related to the ability of this molecule to interrupt the chain reactions leading to lipid peroxidation, the preservation of photosynthetic pigments by a direct effect on chlorophyll biosynthesis or by modifying the activity or the turnover of proteins by post-translational modifications. Nitration of proteins in particular could have an important impact on plant cell signaling.

Acknowledgements

This study was supported by grants from the University of Buenos Aires, Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT) and CONICET. J.J.V. is a fellow from the University of Buenos Aires. M.S., S.P. and A.G. are career investigators and S.J. is a fellow from CONICET.

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