



Low irradiance pulses improve postharvest quality of spinach leaves (*Spinacia oleracea* L. cv Bison)

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ABSTRACT

The aim of this work is to extend and improve the postharvest life of mature spinach leaves using clean technologies like the use of short pulses of light at low irradiance. After harvest spinach leaves were immediately sealed in polyethylene bags in the laboratory. These bags were placed in a dark chamber at 23 °C under continuous dark or with the application of light pulses (LP) consisting of 15 min each 2–6 h or 7 min each 2 h for 3 d. The chosen irradiance, 30 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD, corresponded to the light compensation point previously measured in spinach plants under greenhouse conditions. After the leaves were treated with LP for 3 d, all the samples were transferred to a chamber at 4 °C under continuous dark for another week. Senescence was triggered in leaves under continuous dark after 3 d of storage and delayed in those receiving LP. In addition ascorbic acid and glutathione contents were kept higher in LP-treated than in untreated spinach. These trends were conserved after storage under continuous dark and refrigeration for another week. When LP was applied in combination with 1-MCP the antioxidant capacity was further improved. These results demonstrate that short LP of low irradiance can be used to extend and to improve postharvest life of mature spinach leaves.

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1. Introduction

Spinach leaves constitute an important source of vitamins, minerals and fibers for human diet. Storage under dark conditions accelerates degradative processes visualized by chlorophyll loss and accompanied by the decrease of nutritional attributes such as vitamins or antioxidants (Hodges and Toivonen, 2008). In addition, thylakoid protein dismantling produces a loss of photosystem II operation that can be monitored by chlorophyll fluorescence (DeEll and Toivonen, 2000). All these changes are part of the leaf senescence syndrome (Nooden et al., 1997).

Several factors affect the nutritional properties during postharvest conservation. Among them, temperature and humidity constitute critical factors extending postharvest storage of many leafy vegetables (Cantwell and Kasmire, 2002). It has been demonstrated that storage under 0–5 °C improves spinach conservation delaying the development of senescence symptoms. To avoid leaf dehydration this vegetable is maintained in an atmosphere with high relative humidity (about 95%). Refrigeration and humidity are considered as the main storage conditions to be controlled during postharvest transport, storage and commercialization (Suslow

and Cantwell, 2011). Another ambient condition that keeps better spinach quality is their storage under light exposition (Toledo et al., 2003). In particular this light treatment has not been extensively studied. Although continuous low irradiance treatment was successfully used to extend spinach postharvest life, the potential use of light pulses (LP) with different lengths has not yet been explored. In addition, the hormone ethylene is another crucial factor controlling plant development speeding up the progression of senescence related changes. Although spinach produces ethylene at low rates, this vegetable is highly sensitive to this hormone (Suslow and Cantwell, 2011). It was recently observed that the progress of undesirable modifications in stored spinach can be delayed by inhibiting ethylene sensibility with application of 1-methylcyclopropene (1-MCP, Gergoff et al., 2010a). Finally, it is worth noting that the combined effects of these factors have not been exhaustively studied for spinach postharvest improvement.

Ascorbic acid (AA) and glutathione (GSH) are vital integrants of the antioxidant system in plants mediating the cross talk between bioenergetics processes with signaling pathways (Foyer and Noctor, 2011). Rapid degradation of these antioxidants takes place before the first senescence symptoms can be visualized (Gergoff et al., 2010b). In addition, AA is an important nutritional metabolite that humans do not have the capacity to synthesize (Chatterjee, 1973), constituting a vitamin (Vitamin C) (Szent-Gyorgyi, 1927). Furthermore, its concentration in plant tissues

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is extensively used to monitor postharvest quality of vegetables during transportation and storage. It is synthesized in plant mitochondria by the oxidation of L-galactone-1,4-lactone (L-Gall) feeding electrons into the electron transport chain. This reaction is catalyzed by L-Gall dehydrogenase (L-GallDH) localized in the inner mitochondria membrane (Bartoli et al., 2000). In plant tissues AA is present at very high concentrations playing multiple functions and it is well known by its central participation in the antioxidant defence (Noctor and Foyer, 1998). It reacts with highly reactive oxygen species protecting leaf tissues and transforming itself in the relatively unreactive monodehydroascorbate (MDHA) radical (Buettner and Jurkiewicz, 1996). Then, MDHA can be spontaneously disproportionated to dehydroascorbate (DHA) and AA. Both oxidized forms, MDHA and DHA, can be recovered by NAD(P)H or GSH in reactions catalyzed by MDHA and DHA reductases (MDHAR and DHAR), respectively (Foyer and Halliwell, 1976; Asada, 1999).

The conservation of freshness and nutritional properties of vegetables during their manipulation after harvest using clean and cheap technology is an important task (Kader, 2002). In this regard, the experiments carried out in the present work were designed to test the hypothesis that brief LP of low irradiance delay leaf senescence and improve postharvest spinach life. In addition, it was studied the spinach improvement when LP were applied in combination with ethylene inhibition during their dark storage.

2. Materials and methods

2.1. Plant material and treatments

The experiments were carried out using mature spinach leaves (*Spinacia oleracea* L. cv Bison) detached from two-month-old plants collected from the greenhouses at the Experimental Station JA Hirschhorn (National University of La Plata). Spinach leaves were sealed in low density polyethylene bags in the laboratory. Low density polyethylene film presents permeability rates to O₂ and CO₂ of 4000 and 20,000 cm³ m⁻² s⁻¹ at 1.0 Pa and 25 °C, respectively. In addition, its permeability to water vapor is 7.0 × 10⁻⁸ kg m⁻² s⁻¹ at 25 °C and 90% relative humidity.

Three leaves were placed in each bag (0.30 m × 0.40 m) using three replicates per treatment and sample time. Then, samples were placed in a chamber under continuous dark or with the application of LP at 23 °C during 3 d. The LP treatment used in the former experiments consisted of 15 min of 30 μmol m⁻² s⁻¹ PPFD each 2 h or 6 h (2 h- and 6 h-15 min LP, respectively). After the LP treatments, all the bags were transferred to a chamber under continuous dark at 4 °C for another week. The irradiance used for LP treatments corresponded to the light compensation point previously measured in spinach plants under greenhouse conditions. Only adaxial surface was exposed to light treatment. Samples were taken at 3 different times: harvest, 3 d after harvest and 10 d after harvest.

In the second set of experiments two modifications were made: (1) LP treatment consisted of 7 min (instead of 15 min) of 30 μmol m⁻² s⁻¹ PPFD each 2 h (2 h-7 min LP); and (2) LP treatment was applied in combination or not with 1 μL L⁻¹ 1-MCP (Smart FreshSM). Ethylene inhibitor treatment was performed immediately after spinach harvest placing about 400 g of leaves in an air tight 40 L container under darkness at 23 °C for 6 h. 1-MCP release was triggered with 25 mL of distilled water injected through a rubber septum placed in the container top. Control leaves received a similar treatment but without 1-MCP (Gergoff et al., 2010a). After 3 d under darkness at 23 °C, receiving or not 2 h-7 min LP combined or not with 1-MCP, all samples were stored under continuous darkness at 4 °C for another week. The trials described consisted on at least 5 independent experiments.

All reagents were purchased at Sigma–AldrichTM or otherwise are indicated.

2.2. Leaf senescence parameters

Chlorophyll content was measured using a SPAD-502 Chlorophyll Meter (Minolta, Japan) as the average of 8–10 determinations from each leaf. Potential quantum yield of Photosystem II (Fv/Fm) was measured with a Fluorescence Modulated System (FMS2, Hansatech Instruments, Ltd., Norfolk, UK) after incubation of leaves in darkness for at least 30 min as previously described (Gómez et al., 2008).

2.3. Antioxidant contents

About 100 mg (Fresh weight) of leaves were ground in 1 mL of 6% (w/v) TFA (Carlo Erba Reagents) for the determination of AA and DHA contents. Then leaves were centrifuged at 13,000 × g for 5 min and supernatants used for the measurements. AA was determined with a HPLC system equipped with a LC-10Atvp (Shimadzu, Japan) solvent delivery module, a C-18 column (Varian Chromsep 100 mm × 4.6 mm), and detected at 265 nm using an UV–Vis SPD-10Avp detector (Shimadzu, Japan). Measurements were made isocratically using as running buffer 100 mmol L⁻¹ potassium phosphate (Mallinkrodt Baker) pH 3.0. Total AA was measured after reducing DHA with 10 mmol L⁻¹ DTT (Bartoli et al., 2006). GSH and its oxidized form (glutathione disulfide, GSSG) contents were determined in leaves homogenized in 3% (w/v) TCA. Homogenates were centrifuged as described above and supernatants used for the assays. Thiol groups were detected spectrophotometrically at 412 nm in a reaction mixture consisted on 0.5 mmol L⁻¹ 5,5'-dithiobis 2-nitrobenzoic acid, 500 U L⁻¹ glutathione reductase and 0.2 mmol L⁻¹ NADPH. GSSG was measured after removing GSH with 2-vinylpyridine (1:50, 2-vinylpyridine:sample) (Griffith, 1980). The redox state for both antioxidants was calculated as: (oxidized content/total content) × 100.

2.4. Enzyme assays

Homogenates were performed at 4 °C grounding leaves in a 100 mmol L⁻¹ bicine–NaOH buffer pH 7.5, containing 1 mmol L⁻¹ EDTA, 10% (v/v) glycerol, 4 mmol L⁻¹ cysteine and protease inhibitors (250 μmol L⁻¹ PMSF and 2 μmol L⁻¹ leupeptin) (Bartoli et al., 2006). Then samples were centrifuged at 13,000 × g for 10 min and supernatants used for the activity assays. Enzyme activities were measured with a UV-160 A spectrophotometer (Shimadzu, Japan). MDHAR and DHAR were determined as described by De Gara et al. (2000) with minor modifications. DHAR activity was measured in a reaction mixture consisted of 50 mmol L⁻¹ phosphate buffer pH 6.5, 0.2 mmol L⁻¹ DHA and 2.5 mmol L⁻¹ GSH. In the case of MDHAR the media included 50 Tris–HCl pH 7.8, 1 mmol L⁻¹ AA, 0.2 mmol L⁻¹ NADH and approximately 0.2 U mL⁻¹ ascorbate oxidase. Catalase was measured as described by Aebi (1984) following the degradation of H₂O₂ at 240 nm in a media including 50 mmol L⁻¹ potassium phosphate buffer pH 7.0, 0.2% triton X-100 and 15 mmol L⁻¹ H₂O₂. Aliquots of about 50–100 μg of leaf protein were added for the enzyme determinations.

The capacity to synthesize AA was assayed as the *in vivo* activity of L-GallDH measuring AA accumulation after the incubation of leaf discs in 2 mmol L⁻¹ L-Gall or water for 3 h (Bartoli et al., 2000).

2.5. H₂O₂ production

Oxidative metabolism was characterized measuring *in vivo* H₂O₂ production and catalase activity. *In vivo* hydrogen peroxide

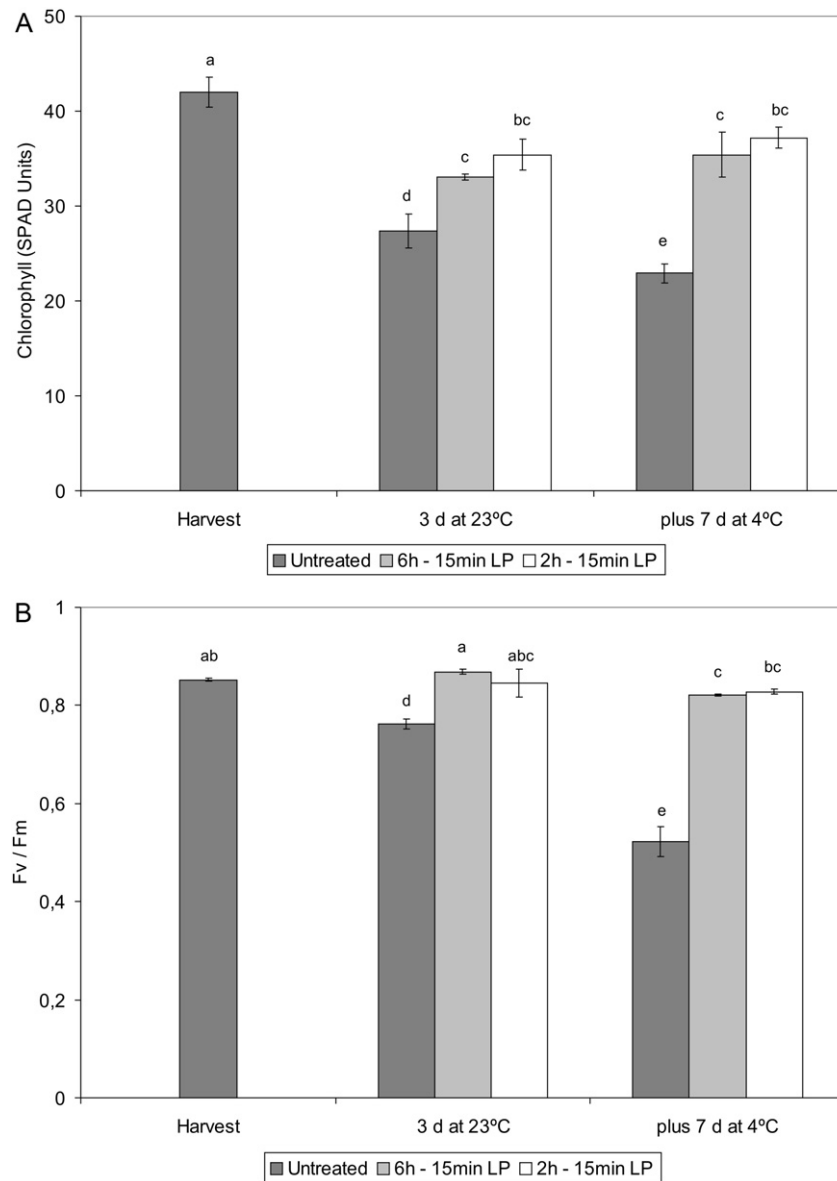


Fig. 1. Leaf senescence parameters during dark-induced senescence of spinach leaves untreated or treated with LP: chlorophyll content (A) and Fv/Fm (B). Treatments are detailed in Section 2. Values are the means of five independent experiments with three replicates each. Data with the same letters represent a statistically homogenous group (ANOVA $P \leq 0.05$).

production displayed by spinach leaves was determined as previously reported in Gómez et al. (2008) using the Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes®).

2.6. Statistical analysis

Data are expressed as the mean \pm SEM from 5 independent experiments after single-factor analysis of variance (ANOVA, $P \leq 0.05$) using Systat Inc. v.10 software package.

3. Results

3.1. Effect of LP on spinach senescence

Both 2 h- and 6 h-15 min LP treatments applied to dark storage spinach leaves delayed the progress of chlorophyll degradation and Fv/Fm declination during the first 3 d. This effect persisted after

conserving leaves under continuous dark at 4 °C for one week more (Fig. 1A and B).

3.2. Effect of LP on antioxidant content

While AA accumulation decreased in untreated leaves after storage for 3 d, it was kept high in LP treated leaves with similar levels of those observed at harvest. However, only 2 h-15 min LP treatment conserved higher AA content (about 70% of the content at harvest) after another week under dark-refrigerated storage (Fig. 2A). The redox state of AA was kept low (i.e. more reduced) only in leaves with 2 h-15 min LP treatment (Fig. 2B).

GSH content was increased around 20 or 50% by 6 h- and 2 h-15 min LP treatments 3 d after harvest, respectively. However, this high antioxidant content was only observed for 2 h-15 min LP treatment at the end of the experiments (3 d at 23 °C plus 7 d at 4 °C, Fig. 2C). GSH redox state was similar in all samples during the experiments (Fig. 2D).

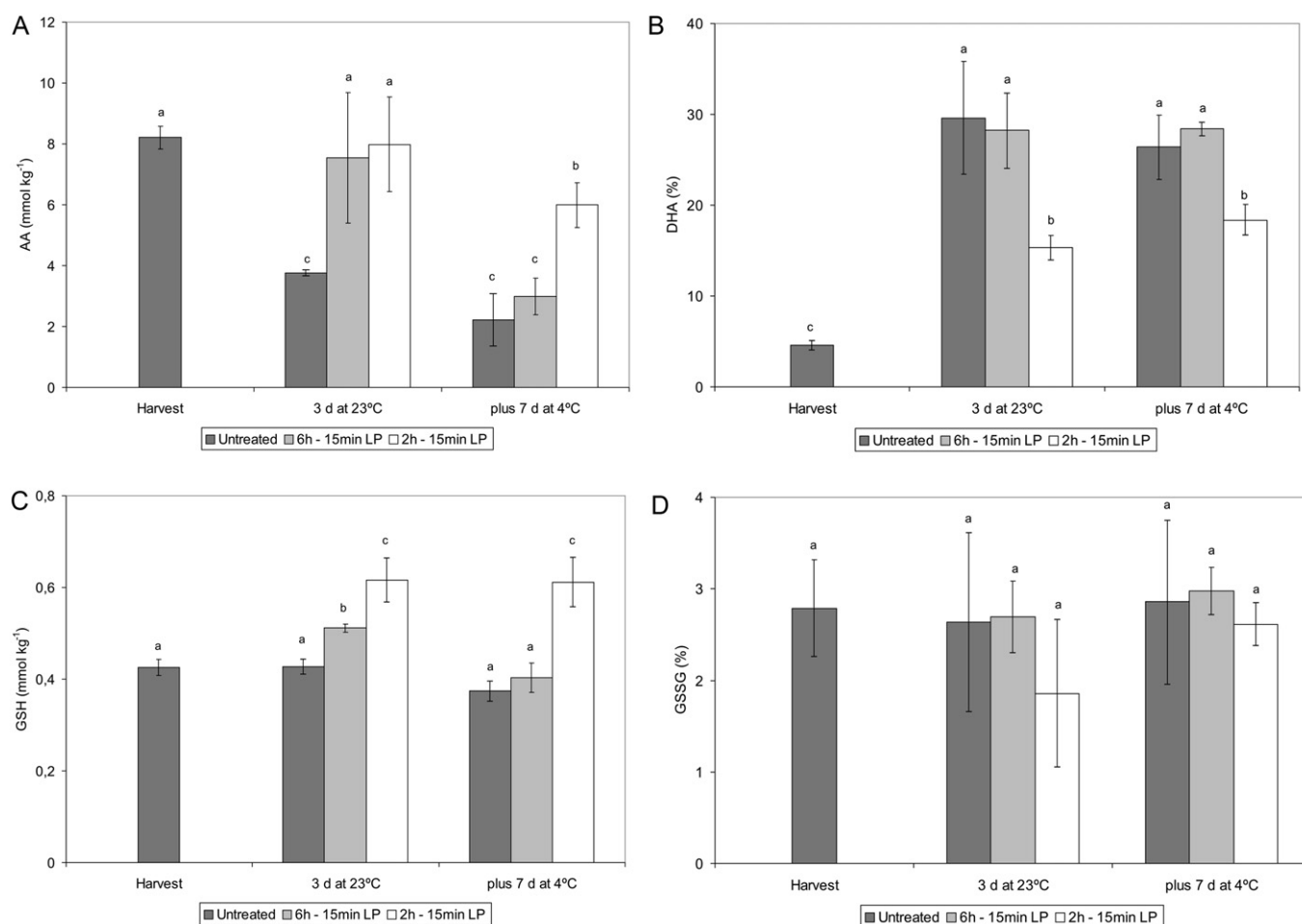


Fig. 2. Contents and redox state of antioxidants during dark-induced senescence of spinach leaves untreated or treated with LP: AA (A), DHA (B), GSH (C) and GSSG (D). Treatments are detailed in Material and Methods section. Values are the means of five independent experiments with three replicates each. Data with the same letters represent a statistically homogenous group (ANOVA $P \leq 0.05$).

Tocopherol contents were not modified by LP treatments at any time of the experiments (data not shown). Consequently, this lipid soluble antioxidant was not measured in the following assays.

3.3. Effects of combined 2 h-7 min LP and 1-MCP treatments on spinach senescence

Since the frequency of LP may be more important than their length, further studies of spinach postharvest characteristics were performed using treatments consisting of 7 min of light each 2 h in combination or not with 1-MCP.

It is important to mention that some passive atmosphere modifications may happen during 1-MCP treatment and following storage in polyethylene bags, consequently, their effects on leaf senescence development must not be discarded. The oxygen concentration in the 40L containers decreased reaching $16.4 \pm 0.82\%$ and $16.7 \pm 0.26\%$ for treated or not with 1-MCP, respectively, after incubation of spinach leaves for 6 h. On the other hand, Fig. S1 shows that 1-MCP treatment alone produces a higher drop in oxygen concentration in the polyethylene bags after dark storage at 23°C for 3 d compared with the other treatments.

Both ethylene inhibitor and 2 h-7 min LP treatments alone delayed the progress of leaf senescence after storage for 3 d (Fig. 3A and B). However, either 2 h-7 min LP alone or combined with 1-MCP delayed spinach senescence at the end of the experiments, keeping

similar chlorophyll content and Fv/Fm values measured in leaves at the harvest day.

3.4. Effects of combined LP and 1-MCP treatments on spinach antioxidants and oxidative metabolism

The content of AA was kept higher in leaves receiving either 1-MCP or 2 h-7 min LP compared with untreated leaves but was not further improved when 1-MCP and 2 h-7 min LP were given together (Fig. 4A). AA redox state was conserved low by either 1-MCP or 2 h-7 min LP treatments applied alone or in combination after 3 d at 23°C. However, only the combination of both treatments kept leaf AA at the more reduced state compared with all other treatments after one week at dark-refrigerated conditions (Fig. 4B).

GSH accumulation that increased with 2 h-7 min LP treatment was even higher in combination with 1-MCP, reaching about two-fold concentration comparing with untreated leaves after either 3 d at 23°C or 3 d at 23°C plus 7 d at 4°C (Fig. 4C). GSH redox state was not modified by any treatment and sample time with the exception of a slightly reduction produced by 1-MCP after 3 d from harvest (Fig. 4D).

The capacity to synthesize AA was increased with 2 h-7 min LP application during the storage and was not modified by 1-MCP (Table 1). MDHAR and DHAR activities were higher in either 1-MCP or 2 h-7 min LP applied alone or in combination than untreated

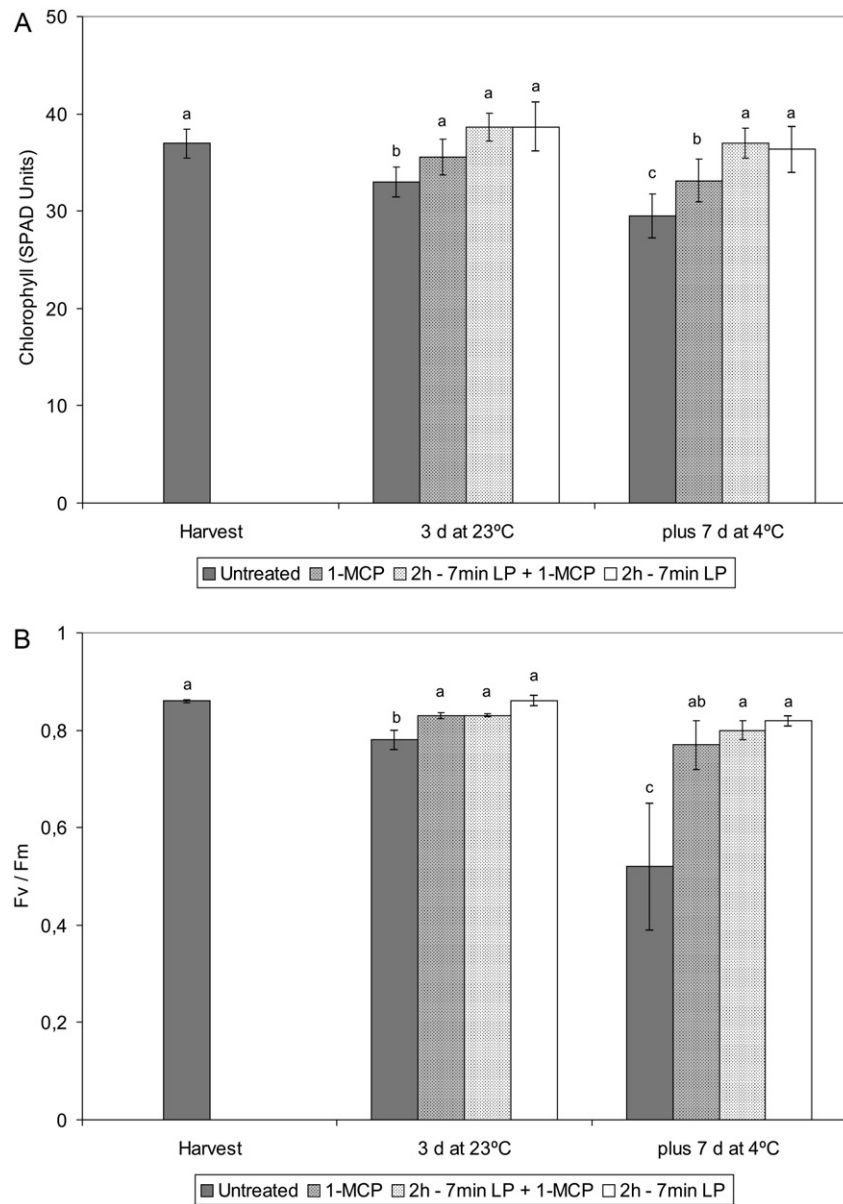


Fig. 3. Leaf senescence parameters during dark-induced senescence of spinach leaves untreated or treated with LP in combination or not with 1-MCP: chlorophyll content (A) and Fv/Fm (B). Treatments are detailed in Material and Methods section. Values are the means of five independent experiments with three replicates each. Data with the same letters represent a statistically homogenous group (ANOVA $P \leq 0.05$).

Table 1

AA synthesis, recovery from oxidized forms and catalase activity during dark-induced senescence of spinach leaves untreated or treated with LP in combination or not with 1-MCP. Treatments are detailed in Section 2.

Treatments	0 d	3 d at 23°C				Plus 7 d at 4°C			
	Harvest	Un-treated	1-MCP	1-MCP + 2 h-7 min LP	2 h-7 min LP	Un-treated	1-MCP	1-MCP + 2 h-7 min LP	2 h-7 min LP
AA synthesis capacity (mol kg ⁻¹ s ⁻¹)	1.86 a	1.03 b	1.00 b	1.53 a	1.72 a	0.69 c	0.86 bc	1.08 b	1.11 b
MDHAR (mol kg ⁻¹ s ⁻¹)	0.068 d	0.032 a	0.085 e	0.082 e	0.087 e	0.06 cd	0.052 bc	0.045 b	0.030 a
DHAR (mol kg ⁻¹ s ⁻¹)	0.237 ab	0.222 a	0.307 cd	0.295 cd	0.278 bc	0.203 a	0.340 de	0.363 e	0.212 a
Catalase (mol kg ⁻¹ s ⁻¹)	0.009 a	0.015 b	0.019 c	0.012 b	0.011 b	0.017 c	0.020 c	0.020 c	0.008 a

Values are the means of five independent experiments. Data with the same letters represent a statistically homogenous group (ANOVA $P \leq 0.05$).

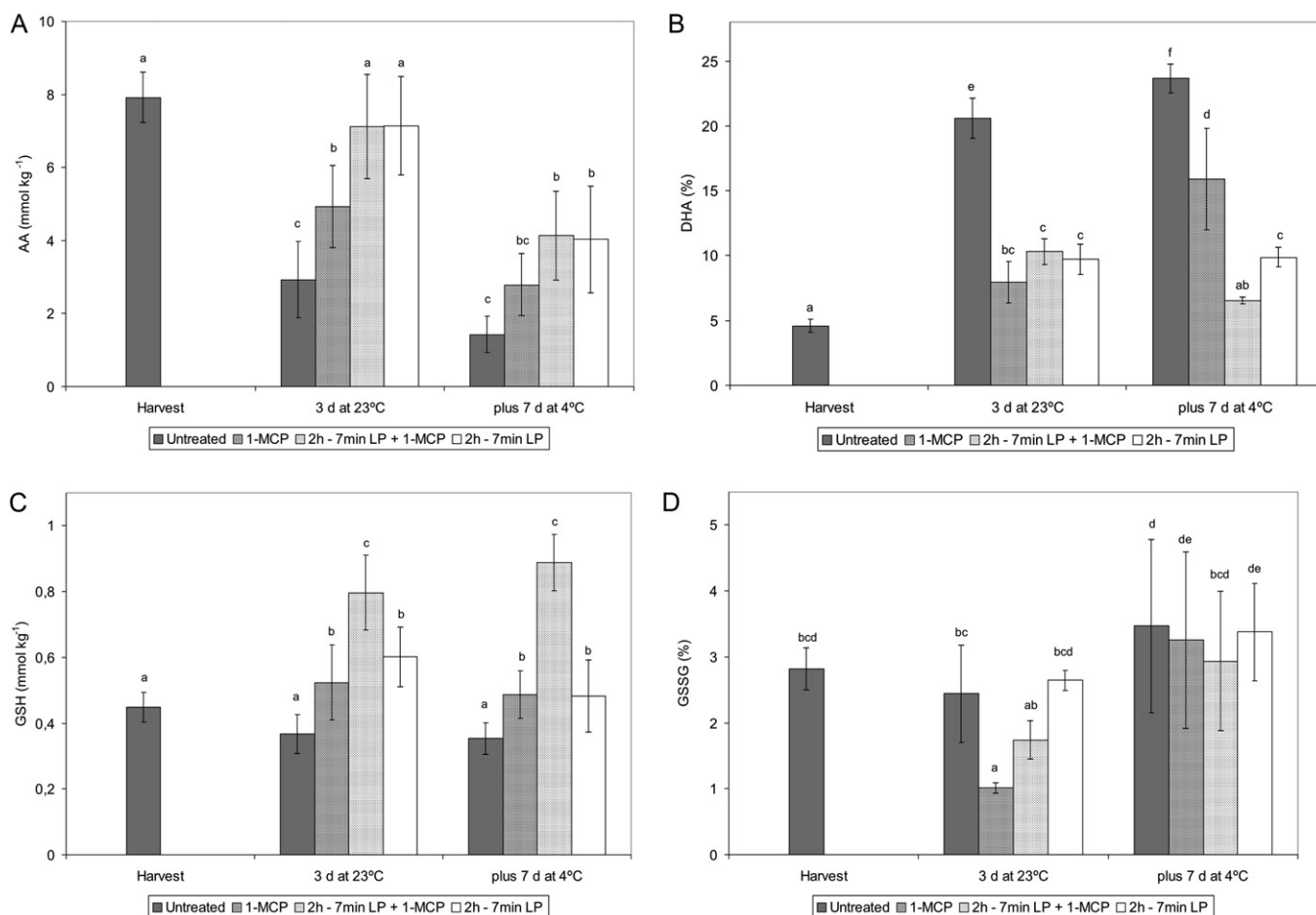


Fig. 4. Contents and redox state of antioxidants during dark-induced senescence of spinach leaves untreated or treated with LP in combination or not with 1-MCP: AA (A), DHA (B), GSH (C) and GSSG (D). Treatments are detailed in Material and Methods section. Values are the means of five independent experiments with three replicates each. Data with the same letters represent a statistically homogenous group (ANOVA $P \leq 0.05$).

leaves after 3 d (Table 1). MDHAR activity was similar or lower than untreated leaves for 1-MCP or 2 h-7 min LP treatments, respectively, at the end of the dark storage. At this time, DHAR activity was only higher in leaves treated with 1-MCP compared with those receiving other treatments.

The H_2O_2 *in vivo* production increased during dark storage of spinach leaves (Fig. 5). However, it was lower for 2 h-7 min LP or 1-MCP treatments and was further decreased when both treatments were applied together (Fig. 5). Catalase activity also increased during spinach storage and with the 1-MCP application. However, this modification was abolished by the 2 h-7 min LP treatment.

4. Discussion

The improvement of storage conditions is an important task to extend postharvest life of plant edible organs. The use of healthy and environmentally safe technologies (such as 1-MCP or low light treatments) during manipulation of vegetables is very well appreciated by consumers. This work demonstrates that the application of brief LP at low irradiance can be used during storage of detached spinach leaves, improving their appearance and nutritional characteristics.

4.1. LP treatments delay spinach senescence

Dark storage accelerates the senescence of detached spinach leaves (Philosoph-Hadas et al., 1991). The exposition to

continuous low irradiance under refrigerated conditions was successfully used to improve the storage of spinach leaves (Lester et al., 2010) but in this work brief LP treatments were enough delaying the progress of spinach senescence. Low irradiance may trigger some CO_2 uptake contributing to maintain the sugar pool and delaying spinach senescence (Toledo et al., 2003). Here, it can be assumed that there is no effect of LP treatments on *de novo* synthesis and increased accumulation of sugars during postharvest storage of spinach leaves. In one hand, the irradiance selected for LP treatments corresponded to the light compensation point and in the other hand, LP are presumably shorter than the time needed to get a full activation of Calvin cycle. The activation of chloroplast metabolism needs some minutes after light is switched on to achieve a full photosynthetic activity (von Caemmerer and Quick, 2000).

4.2. LP treatments keep higher antioxidant contents

It was previously observed that continuous low light exposition ameliorates AA degradation during storage of spinach leaves under refrigerated conditions (Toledo et al., 2003; Lester et al., 2010). The synthesis of a highly abundant antioxidant like AA is derived from D-glucose (Smirnoff, 1996). However, the conservation of high levels of AA induced by LP treatments would not be linked to the accumulation of sugars produced by photosynthesis. Another signal originated from chloroplasts might trigger those physiological processes connected with antioxidant increments. Yabuta et al.

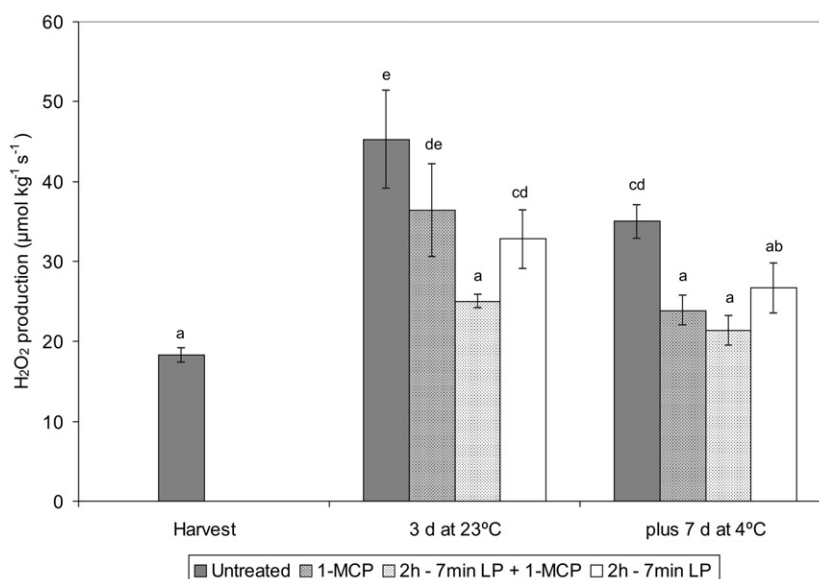


Fig. 5. *In vivo* hydrogen peroxide production during dark-induced senescence of spinach leaves untreated or treated with LP in combination or not with 1-MCP. Treatments are detailed in Material and Methods section. Values are the means of five independent experiments with three replicates each. Data with the same letters represent a statistically homogenous group (ANOVA $P \leq 0.05$).

(2007) demonstrated that AA accumulation in *Arabidopsis* leaves depends on the photosynthetic electron transport chain. In addition, the increment in AA pool depends on its synthesis through the activity of L-GalLDH activity and on the reduction of MDHA and DHA (Smirnoff et al., 2001). Both AA synthesis capacity and recovery from oxidized forms were augmented by LP treatments, keeping LP treated samples with increased AA content and highly reduced. Interestingly, GSH content increased in response to LP treatments demonstrating the close association between these antioxidants (Noctor et al., 2011). It was previously observed that light quality (*i.e.* Red/Far red ratio), in addition to irradiance, is an important factor regulating the content and redox state of both AA and GSH (Bartoli et al., 2009). Here, the source of light consisted of a Red/Far red ratio higher than 1.2 which stimulates the increments of antioxidants and NAD(P)H foliar contents (Bartoli et al., 2009).

The accumulation of antioxidants also depends on their degradation that may be accelerated by reactions with reactive oxygen species that increase during dark storage (Hodges et al., 2004). Here it was observed that LP treatments maintain lower hydrogen peroxide production of spinach leaves during storage. Higher biosynthesis and lower detoxification reactions may lead to keep high AA and GSH contents in LP treated leaves.

4.3. 1-MCP treatment improved the effect of LP on spinach leaves

The inhibition of ethylene action with 1-MCP was effective delaying senescence and improving quality of spinach leaves during storage under darkness at either 23 or 4°C (Gergoff et al., 2010a). The combination of LP with 1-MCP may improve other spinach attributes like antioxidant or vitamin contents. It was previously observed that 1-MCP delays the decrease of GSH content during the dark storage of spinach (Gergoff et al., 2010a). Surprisingly, 1-MCP duplicated the accumulation of GSH when was added in combination with LP compared with untreated samples or receiving separate treatments. Further experiments are needed to understand the mechanisms involved in this high GSH accumulation. In addition, AA redox state was kept highly reduced in samples receiving both treatments simultaneously (probably related with the increased GSH content). Antioxidant redox state is an important metabolic signal that modifies gene expression, regulating the

growth and development of plants (Foyer and Noctor, 2005; Potters et al., 2010). The combined effect of 1-MCP and LP treatments on GSH accumulation and AA redox state suggest that might be an interaction of hormone and light signaling pathways for the control of plant metabolism.

Taken as a whole these results demonstrate that low irradiance brief LP treatments are effective improving spinach postharvest quality. In addition, these data show that some desirable and not visible characteristics (*v.g.* vitamins or antioxidants) of spinach leaves can be further improved combining LP treatments with 1-MCP.

Finally, it is important to mention that leaves may not be completely exposed to light and this would affect the efficiency of LP treatments during spinach commercialization. Further studies must demonstrate that a partial surface exposition to LP treatments improves whole spinach leaves.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.postharvbio.2012.10.012>.

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