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Metabolism of reactive oxygen species and reactive nitrogen species in pepper (*Capsicum annuum* L.) plants under low temperature stress

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ABSTRACT

Low temperature is an environmental stress that affects crop production and quality and regulates the expression of many genes, and the level of a number of proteins and metabolites. Using leaves from pepper (*Capsicum annum* L.) plants exposed to low temperature (8 °C) for different time periods (1 to 3 d), several key components of the metabolism of reactive nitrogen and oxygen species (RNS and ROS, respectively) were analysed. After 24 h of exposure at 8 °C, pepper plants exhibited visible symptoms characterized by flaccidity of stems and leaves. This was accompanied by significant changes in the metabolism of RNS and ROS with an increase of both protein tyrosine nitration (NO_2-Tyr) and lipid peroxidation, indicating that low temperature induces nitrosative and oxidative stress. During the second and third days at low temperature, pepper plants underwent cold acclimation by adjusting their antioxidant metabolism and reverting the observed nitrosative and oxidative stress. In this process, the levels of the soluble non-enzymatic antioxidants ascorbate and glutathione, and the activity of the main NADPH-generating dehydrogenases were significantly induced. This suggests that ascorbate, glutathione and the NADPH-generating dehydrogenases have a role in the process of cold acclimation through their effect on the redox state of the cell.

Key-words: antioxidants; NADP-dehydrogenases; nitric oxide; nitrotyrosine; RNS; ROS.

Abbreviations: APX, ascorbate peroxidase; G6PDH, glucose-6-phosphate dehydrogenase; GR, glutathione reductase; GSH, glutathione; ICDH, isocitrate dehydrogenase; MDAR, monodehydroascorbate reductase; ME, malic enzyme; NO, nitric oxide; $ONOO^-$, peroxynitrite; PVPP, polyvinylpolypyrrolidone; RNS, reactive nitrogen species; ROS, reactive oxygen species; SNOs, *S*-nitrosothiols; SOD, superoxide dismutase.

INTRODUCTION

The production of ROS, such as hydrogen peroxide (H_2O_2) and superoxide radicals ($O_2^{\cdot-}$), in different cell compartments in response to environmental stress is well established (Apel & Hirt 2004; Foyer & Noctor, 2005a; Gechev *et al*. 2006; Suzuki & Mittler, 2006; del Río *et al* 1996, 2009; Miller *et al*. 2010; McCarthy *et al*. 2011). More recently, the discovery that plant cells can generate the free radical NO has opened new ways of research since NO and other related molecules like *S*-nitrosoglutatione (GSNO) or $ONOO^-$, collectively designated as RNS, are also involved in the mechanism of response against environmental stress (Valderrama *et al*. 2007; Corpas *et al*. 2008; Chaki *et al*. 2009a, 2011). Additionally, the involvement of enzymatic components that regulate the production of essential antioxidant molecules such as GSH and NADPH indicates that the redox state of the cell is a cornerstone in the mechanism of regulation (Noctor 2006).

Low temperature (LT) is an environmental factor that has a significant influence in plant growth affecting photosynthesis, uptake of water and nutrients, among others. Many economically significant crops, such as cotton, maize, pepper, rice, soybean, tomato, some tropical fruits (e.g. bananas, papayas and mangoes) and subtropical fruits (e.g. grapes, oranges) are LT sensitive, which affects their production and quality (Sharma, Sharma & Deswal 2005). The influence of this type of stress has been studied at different levels from whole plants to single molecules. However, depending either on the type of plants (annual, biannual, shrubs or trees) or the intensity and duration of the exposure of plants to LT, the strategies used by plants can change. Thus, it has been shown that LT regulates the expression of many genes (Shinozaki, Yamaguchi-Shinozaki & Seki 2003), and there are biochemical changes that affect the level of a number of proteins, lipids and metabolites. These include the

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accumulation of cryoprotective peptides, synthesis of low molecular cryoprotective sugars (praline and raffinose), antifreeze proteins, dehydrins, ROS scavenging enzymes and soluble antioxidants (Thomashow 1999; Hannah, Heyer & Hincha 2005; Sharma *et al*. 2005; Renaut, Hausman & Wisniewski 2006; Lütz 2010).

Pepper (*Capsicum annuum* L.), a member of the Solanaceae family, is a very important crop, its fruits being the second worldwide consumable vegetables and excellent sources of many essential nutrients for humans, especially vitamin C, β-carotene and calcium. Additionally, some pepper cultivars contain significant quantities of capsaicinoids, a group of pungent phenolic-derived compounds with strong physiological and pharmacological properties (Topuz & Ozdemir, 2007). Thus, the growing global demand of pepper fruits implies several strategies to increase crop production and fruit quality through specific agricultural fertilization practices (Pascual *et al*. 2010) or promoting the investigation to improve the plant resistance to environmental stresses. Pepper plants are originally from tropic regions and require high temperature conditions for their development. Consequently, the optimum growth temperature is between 25 and 30 °C, in such a way that temperature changes affect a variety of physiological functions and morphological development. When temperature decreases below 15 °C, pepper growth is reduced, and bloom and fruit production stop (Mercado *et al*. 1997). LT affects pepper vegetative development and reproduction by disturbing the function of the flower female organs and the number of viable pollen grains per flower (Polowick & Sawhney 1985; Pressman *et al*. 1998, 2006; Shaked, Rosenfeld & Pressman 2004). Thus, fruits from plants that have been set under low night temperatures (14 °C or less) usually are deformed and seedless causing significant economical losses. Taking into account the important agronomical relevance of pepper (Mateos 2006), the main goal of this work was to study the antioxidant metabolism and homeostasis of ROS and RNS in this plant species under LT conditions, since this environmental stress considerably affects pepper growth. The results obtained showed that in pepper plants LT causes nitrosative and oxidative stress during the first 24 h but after this period, plants seem to recover by an acclimation of their metabolism, which involves important changes in their cellular antioxidant and redox state.

MATERIALS AND METHODS

Plant materials and growth conditions

Pepper (*Capsicun annuum* L) seeds, California type, were obtained from Syngenta Seeds S.A. (El Ejido, Almería, Spain). Seeds were germinated in Petri dishes containing Murashige and Skoog medium for 5 d at 30 °C in the dark. Then, the healthiest seedlings were transferred individually into 10-cm-diameter pots containing peat soil and grown in a growth chamber at 22/18 °C and a 16 h photoperiod for 30 d. Afterwards, plants were subjected to LT treatment (8 °C) for 1 to 3 d.

Crude extracts of plant tissues

Pepper leaves were frozen in liquid N_2 and ground in a mortar. The powder was suspended in a homogenizing medium containing 50 mM Tris-HCl, pH 7.8, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 5 mM dithiothreitol (DTT), 0.2% (v/v) Triton X-100, 10% (v/v) glycerol and 2% (w/v) PVPP. Homogenates were centrifuged at 27 000 *g* for 25 min at 4 °C and supernatants were immediately used for the assays.

Histochemical assay for lipid peroxidation

Histochemical detection of lipid peroxidation was performed with the Schiff's reagent, which detects aldehydes that originate from lipid peroxides (Yamamoto, Kobayashi & Matsumoto 2001). Leaves were incubated in the Schiff's reagent for 60 min and then were bleached by immersing in boiling ethanol until appearance of red/purple colour, which indicates the presence of lipid peroxidation.

Determination of H_2O_2, ascorbate and GSH

The concentration of H_2O_2 in pepper leaf extracts was determined spectrophotometrically by a peroxidase coupled assay using 4-amininoantipyrine and phenol as donor substrates (Frew, Jones & Scholes 1983). Soluble fractions (300–500 μL) were added to a reaction mixture containing 25 mM phenol, 5 mM 4-aminoantipyrine, 0.1 M potassium phosphate buffer (pH 6.9), 0.02 μM peroxidase and 2.5 μM H_2O_2. Quinone-imine formation was measured at 505 nm.

For the determination of ascorbate and GSH contents in whole leaves, crude extracts were prepared in 5% (w/v) meta-phosphoric acid and the methods of the bipyridyl and the reduction of 5,5′-dithiobis-(2-nitrobenzole) acid (DTNB), were used for ascorbate and GSH, respectively (Griffith 1980; Okamura 1980).

Enzymatic activity assays

Catalase activity (EC 1.11.1.6) was determined by measuring the disappearance of H_2O_2, as described by Aebi (1984). APX (EC 1.11.1.11) was determined by monitoring the initial ascorbate oxidation by H_2O_2 at 290 nm (Hossain & Asada, 1984). MDAR (1.6.5.4) was assayed by measuring the monodehydroascorbate-dependent NADH oxidation, with monodehydroascorbate being generated by the ascorbate/ascorbate oxidase system (Hossain, Nakano & Asada 1984). The rate of monodehydroascorbate-independent NADH oxidation (without ascorbate and ascorbate oxidase) was subtracted from the monodehydroascorbate-dependent reaction. GR (EC 1.6.4.2) was assayed by monitoring the NADPH oxidation coupled to the reduction of GSH (Edwards, Rawsthone & Mullineaux 1990). The reaction rate was corrected for the small, non-enzymatic oxidation of NADPH by glutathione disulfide (GSSG). GSNOR activity was assayed spectrophotometrically at 25 °C by monitoring the oxidation of NADH at 340 nm (Barroso *et al.* 2006).

G6PDH (EC 1.1.1.49) activity was determined spectrophotometrically by recording the reduction of NADP at 340 nm. Assays were performed at 25 °C in a reaction medium (1 mL) containing 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.6, 2 mM $MgCl_2$ and 0.8 mM NADP, and the reaction was initiated by the addition of 5 mM glucose-6-phosphate. For the determination of 6-phosphogluconate dehydrogenase (6PGDH; EC 1.1.1.44) activity, the reaction mixture was similar to that described for G6PDH, but the substrate was 5 mM 6-phosphogluconate (Corpas *et al.* 1998). NADP-isocitrate dehydrogenase (NADP-ICDH; EC 1.1.1.42) activity was also measured by following the NADP reduction according to Corpas *et al.* (1999). Thus, the assay was performed at 25 °C in a reaction medium (1 mL) containing 50 mM HEPES, pH 7.6, 2 mM $MgCl_2$ and 0.8 mM NADP, and the reaction was initiated by the addition of 10 mM 2R,3S-isocitrate. NADP-ME (EC 1.1.1.40) activity was also determined spectrophotometrically by recording the reduction of NADP at 340 nm using the same reaction mixture (1 mL) indicated earlier for other dehydrogenases, but in this case, the reaction was initiated by the addition of 1 mM L-malate (Valderrama *et al.* 2006).

SOD and NADPH oxidase isozymes

Native polyacrylamide gel electrophoresis was performed using acrylamide gels as described by Davis (1964).

SOD (EC 1.15.1.1) isozymes were separated by non-denaturing polyacrylamide gel electrophoresis (PAGE) on 10% acrylamide gels and visualized by a photochemical NBT (nitroblue tetrazolium) reduction method (Beauchamp & Fridovich, 1971). To identify the type of SOD isozymes, gels were incubated separately at 25 °C for 30–45 min in 50 mM K-phosphate, pH 7.8, in the presence or absence of either 2 mM KCN or 5 mM H_2O_2. CuZn-SOD is inhibited by CN^- and H_2O_2, Fe-SOD is inhibited by H_2O_2 but not by CN^-, whereas Mn-SOD is not inhibited by either CN^- or H_2O_2 (Corpas *et al.* 1998).

NADPH oxidase (NOX; EC 1.6.3.1. 1) isozyme activity was assayed in gels by the NBT reduction method of López-Huertas *et al.* (1999), as modified by Sagi & Fluhr (2001). Gels were incubated in the dark for 20 min in a reaction mixture solution containing 50 mM Tris-HCl buffer (pH 7.4), 0.2 mM NBT, 0.1 mM $MgCl_2$ and 1 mM $CaCl_2$. NADPH (0.2 mM) was added and the appearance of blue formazan bands was monitored. The reaction was stopped by immersion of the gels in distilled water. As control, 50 μM diphenyleneiodonium (DPI) was added as specific inhibitor of superoxide radical generation.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting

SDS–PAGE was carried out according to the method of Laemmli (1970) in 10% acrylamide slab gels. For Western blot analysis, proteins were transferred to PVDF membranes with a semi-dry Trans-Blot cell (Bio-Rad, Hercules, CA, USA). After transfer, membranes were used for cross-reactivity assays with a rabbit polyclonal antibody against 3-nitrotyrosine (Corpas *et al.* 2008) diluted 1:8000. For immuno-detection, an affinity-purified goat anti-(rabbit IgG)-horseradish peroxidase conjugate (Bio-Rad) and an enhanced chemiluminescence kit (ECLPLUS, Amersham, Piscataway, NJ, USA) were used. As positive control, commercial nitrated BSA (Sigma, St Louis, MO, USA) was used.

RNA isolation and semi-quantitative RT-PCR

Total RNA was extracted with Trizol according to Gibco BRL, Life Technologies (Rockville, MD, USA). Two micrograms of total RNA were used to produce cDNA by RT-PCR (Mateos *et al.* 2009). Semiquantitative reverse transcription–PCR amplification of actin cDNA from pepper was chosen as control. Catalase, SOD, GR, MDAR, APX and actin cDNAs were amplified by the PCR as follows: 1 μL of each cDNA (30 ng) was added to 250 mM dNTPs, 1.5 mM $MgCl_2$, 1 × PCR buffer, 0.5 U of Hot Master TaqTM DNA polymerase (Eppendorf, Hauppauge, NY, USA) and 0.5 mM of each primer (Table 1) in a final volume of 20 μL. Reactions were carried out in a Hybaid thermocycler. A first step of 2 min at 95 °C was followed by 28–33 cycles (depending on the gene) of 20 s at 94 °C, 20 s at 55 °C and 30 s at 65 °C plus a final step of 10 min at 65 °C. Then, PCR products were detected by electrophoresis in 1% (w/v) agarose gels and staining with ethidium bromide. Quantification of the bands was performed using a Gel Doc system (Bio-Rad Laboratories) coupled with a high-sensitive charge-coupled device (CCD) camera.

Spectrofluorometric detection of NO

To freshly prepared crude extracts of pepper leaves 4,5-diaminofluorescein (DAF-2) was added at a 10 μM final concentration. Then, reaction mixtures were incubated at 37 °C in the dark for 2 h, and the fluorescence was measured in a QuantaMaster™ QM-4 fluorescent spectrophotomer (PTI® Photon Technology International, Lawrenceville, NJ, USA) at excitation and emission wavelengths of 485 and 515 nm, respectively (Nakatsubo *et al.*, 1998). As control reaction mixtures leaf samples were pre-incubated for 30 min with: (1) 1 mM 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), a NO scavenger; (2) 1 mM N^G-nitro-L-Arg methyl ester (L-NAME), an arginine analog that inhibits mammalian NO synthase activity; (3) 2 mM aminoguanidine (AG) another inhibitor of mammalian NO synthase activity

Table 1. Oligonucleotides used for the semiquantitative reverse-transcription PCR (SQ-RT-PCR) analysis of enzymes involved in the metabolism of ROS

Name	Oligonucleotide sequence (5′–3′)	Product size (bp)	Accession number
SQ-RT-PCR			
F-CAT	GATTTCTTCTCTTTCCTCC		
R-CAT	CGATGTTCCTATTCAATACC	418	AF227952
F-APX	TGTGCTCCTCTTATGCTCC		
R-APX	CTCAAAACCAGAACGCTCC	485	X81376
F-MnSOD	CATGCAGCTTCATCACCAGA		
R-MnSOD	ATAACAAGGCGCTTCAGCTC	314	AF036936
F-FeSOD	CATCACAGGACCTATGTCG		
R-FeSOD	GGTGTTTTCACAACTACAAGC	352	AY173123
F-CuZnSOD	TGTTAGTGGCACCATCCTCT		
R-CuZnSOD	GGCCGATAATACCACAAGCA	459	AF009734
F-GR	TTTGGTTTATGGAGCTGCC		
R-GR	CAGTGGGAGTTGCTTTCTG	509	AY547351
F-MDAR	ATGGAGAGGGTGAAGTCCG		
R-MDAR	GCCTTGACAGCCTGCTCAG	279	AY652702
F-G6PDH	ATTTGTTGGTGCTGCGTT		
R-G6PDH	CATTGATTGAAGGACCT	255	AY652703
F-6PGDH	TGTAGTTATGCTCAGGGGATG		
R-6PGDH	CTCTCATATGTGTGAGCCCC	374	AY532646
F-ICDH	TTGTGCCAGAAGGTACAGAC		
R-ICDH	CAGATTCCAGCCTCCTCGTA	418	AY572426
F-ACT	ACTCTTAATCAATCCCTCC		
R-ACT	GCACTGTATGACTGACACC	573	AY572427

CAT, catalase; MnSOD, manganese-containing SOD; FeSOD, iron-containing SOD; CuZnSOD, copper/zinc-containing SOD; 6PGDH, 6-phosphogluconate dehydrogenase; ICDH, isocitrate dehydrogenase; ACT, actin.

(Corpas *et al.* 2009c); (4) 2 mM Na-tungstate, a nitrate reductase (NR) inhibitor (Cantrel *et al.* 2011); (5) 1 μM rotenone, inhibitor of mitochondrial electron transport since it prevents reduction of complex I (Møller 2001); and (6) 2 mM α-difluoromethylornithine (DFMO; DL-α-difluoromethylornithine hydrochloride hydrate), inhibits polyamine biosynthesis by the selective, irreversible inhibition of ornithine decarboxylase (Yoda, Hiroi & Sano 2006).

Detection of NO, $ONOO^-$ and SNOs by confocal laser scanning microscopy (CLSM)

NO was detected in pepper leaf transversal sections with 10 μM 4-aminomethyl-2′,7′-difluorofluorescein diacetate (DAF-FM DA, Calbiochem, San Diego, CA, USA) prepared in 10 mM Tris-HCl (pH 7.4) as described elsewhere (Corpas *et al.* 2008). $ONOO^-$ was detected with 10 μM 3'-(*p*-aminophenyl)-fluorescein (APF, Invitrogen) prepared in 10 mM Tris-HCl (pH 7.4) according to Chaki *et al.* 2009b. SNOs were detected using the fluorescent reagent Alexa fluor 488 Hg-link phenylmercury (Valderrama *et al.* 2007). In all cases, leaf transversal sections were examined with a confocal laser scanning microscope (Leica TCS SL, Leica Microsystems, Heidelberg GmbH, Wetzlar, Germany).

Other assays

Protein concentration was determined with the Bio-Rad Protein Assay, using bovine serum albumin as standard. To estimate the statistical significance between means, the data were analysed by Student's *t*-test.

RESULTS

The phenotype of the 30-day-old pepper plants exposed to LT for several days (1–3 d) is shown in Fig. 1. LT caused an increase of flaccidity in both stems and leaves from the first to the third days, the most evident symptoms being observed at the first day. After 24 h shoots started to recover their original appearance.

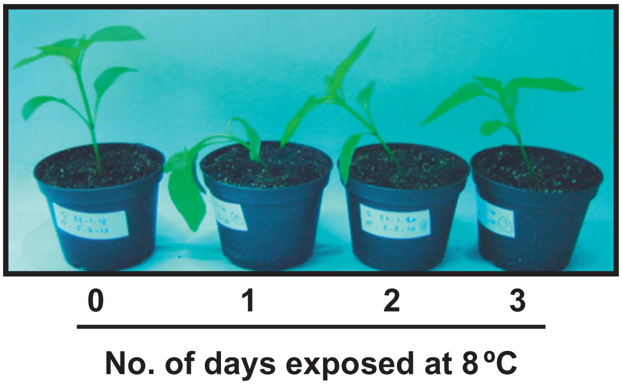

Figure 1. Phenotype of 30-day-old pepper plants exposed to low temperature (8 °C) for 0 to 3 d.

Table 2. Total ascorbate and GSH (reduced and oxidized) content in leaf extracts from pepper plants exposed to low temperature (8 °C) for 1 to 3 d. Data are the mean ± standard error of the mean (SEM) of at least three different experiments

No. of days	Ascorbate ($\mu g \cdot g^{-1}$ FW)	GSH ($\mu g \cdot g^{-1}$ FW)	GSSG ($\mu g \cdot g^{-1}$ FW)	GSH/GSSG ratio
0	77.28 ± 4.13	91.9 ± 5.6	7.20 ± 0.56	13
1	104.04 ± 1.43*	172.5 ± 7.6*	3.59 ± 0.34*	48
2	112.30 ± 1.45*	141.9 ± 6.5*	8.09 ± 0.26	18
3	101.26 ± 2.45*	142.9 ± 1.8*	6.53 ± 0.55	22

*Differences from control values were significant at $P < 0.05$. [Correction added after online publication (15 September 2011): Values in columns 3 and 5 were amended.]

Effect of LT on the metabolism of ROS

To know how LT affects the status of non-enzymatic antioxidants, the content of GSH and ascorbate in pepper leaves was studied (Table 2). Thus, the total ascorbate (reduced + oxidized) and GSH increased about 35 and 88%, respectively, in leaves exposed 1 d to LT compared with control plants, and afterwards similar levels were maintained during the second and third days of LT treatment. The content of oxidized GSH (GSSG) was reduced about 50% after 1 d exposure to LT and then no significant changes were observed in comparison with control plants. The ratio GSH/GSSG underwent an increase of 3.7-fold after 1 d of LT treatment.

The activity of catalase, a characteristic antioxidant enzyme, increased 34% to 36% during the first and second days, and then it was not significantly affected by LT (Fig. 2a). The analysis of SOD activity by native PAGE showed in all treatments the presence of four SOD isozymes that were identified as a Mn–SOD, a Fe–SOD and two CuZn–SODs, which were named as isozymes CuZn-SOD I and CuZn–SOD II according to their increasing electrophoretic mobility (Fig. 2b). No significant changes were observed in the SOD isoenzymatic pattern of plants after 3 d under LT.

On the other hand, the activity of the antioxidative ascorbate–GSH cycle enzymes, including APX, MDAR and GR was also analysed (Fig. 3). Thus, APX and MDAR activities were induced 40 and 31%, respectively, after 1 d LT treatment (Fig. 3a,b, respectively), but then no significant differences were observed in the MDAR activity. On the contrary, the GR activity was reduced 49 and 73% during the second and third days of LT stress, respectively (Fig. 3c).

The semi-quantitative reverse-transcription PCR analysis of the mRNA expression of seven antioxidant enzymes, including catalase, Mn-SOD, FeSOD, CuZnSOD, APX, MDAR and GR in pepper plants exposed to LT during several days is shown in Fig. 4. In general, none of the analysed genes showed significant changes during this period of time. Likewise, analysis of the H_2O_2 content in pepper leaves did not show significant differences during the period of treatment (Fig. 5)

The effect of LT on the activity of the main NADP-dehydrogenases is shown in Fig. 6. The activity of G6PDH increased from 32 to 44% during the three days of exposure to LT (Fig. 6a). The 6PGDH activity also increased between 30 and 33% after the first day and this increase was maintained till the end of the treatment (Fig. 6b). The NADP-ICDH activity increased 26% in the first and second day of treatment, but no significant changes were observed in the third day compared with control plants (Fig. 6c). The ME activity increased 23% during the first day, and 40 and 31% during the second and third days under LT, respectively (Fig. 6d).

Peroxidation of unsaturated lipids in biological membranes is recognized as a marker of oxidative damage by ROS. To verify the potential ROS damage in pepper plants during the LT treatment, a histochemical method with the Schiff's reagent was used to detect aldehydes that originate from lipid peroxides (Yamamoto *et al.* 2001). Pepper leaves

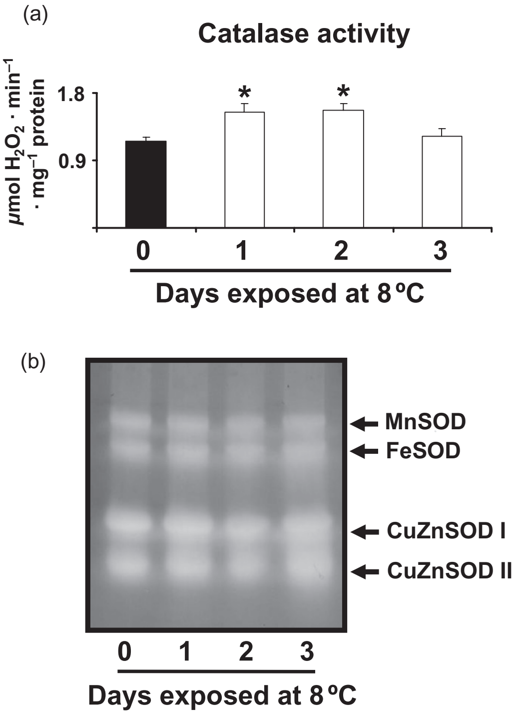

Figure 2. Catalase and SOD activities in leaf extracts of 30-day-old pepper plants exposed to low temperature (8 °C) for 0 to 3 d. (a) Catalase activity. Values are means of at least three independent experiments. *Differences from control values were significant at $P > 0.05$. (b) SOD isozymes were separated by native-PAGE (10% acrylamide) and stained by a photochemical method (20 μg protein per lane).

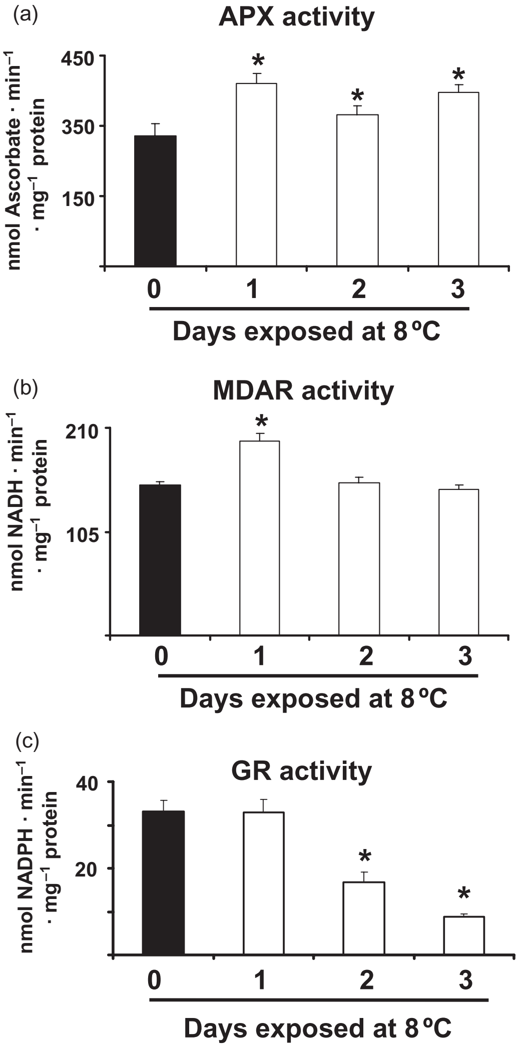

Figure 3. Activity of the ascorbate-GSH cycle enzymes in leaf extracts of 30-day-old pepper plants exposed to low temperature (8 °C) for 0 to 3 d. (a) APX activity. (b) MDAR activity. (c) GR activity. Values are means of at least three independent experiments. *Differences from control values were significant at $P > 0.05$.

from plants exposed to LT for several days and stained for lipid peroxidation are shown in Fig. 7. The red/purple colour corresponds to the presence of lipid oxidation and a clear intensification of this colour was observed throughout the main and secondary veins in leaves of plants exposed for 1 and 2 d to LT. On the contrary, in leaves from plants treated for 3 d there were no differences compared with controls.

NADPH oxidase (NOX) activity is considered an important source of superoxide radicals in plants (Sagi & Fluhr 2001; Fluhr 2009; Miller *et al*. 2009). The detection of NOX activity in pepper leaves using non-denaturing gels showed the presence of three isozymes, designated I to III in order to their increasing mobility in gels (Fig. 8). It was found that isozyme NOX III was induced in pepper leaves after 1 d of plant exposure to LT, whereas the activity of NOX I diminished after 24 h at LT.

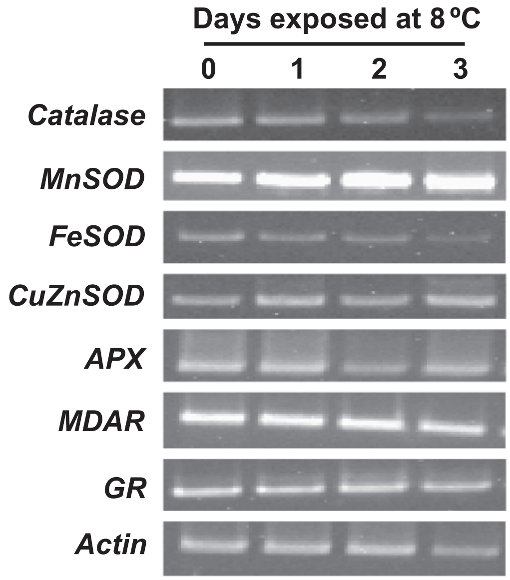

Figure 4. Analysis of the mRNA expression of the antioxidative enzymes catalase, SOD isozymes (MnSOD, FeSOD, CuZnSOD), APX, MDAR and GR in pepper leaves of plants exposed to low temperature (8 °C) for 0 to 3 d. Semiquantitative reverse transcription–PCR was performed on total RNA isolated from pepper leaves. Representative agarose electrophoresis gels of the amplification products visualized by ethidium bromide staining under UV light.

Effect of LT on the metabolism of RNS

In plants, NO biosynthesis can be mediated by different pathways including L-arginine-NO synthase activity, NR activity, mitochondrial electron transport or mediated by polyamines. To examine the relative contributions of these potential sources in pepper leaves a pharmacological approach was used. Accordingly, the effects of specific inhibitors of each pathway were studied in the NO release (Fig. 9a). As a result, leaf samples were preincubated with 1 mM L-NAME (an arginine analog that inhibits mammalian NO synthase activity), 1 mM aminoguanidine (an animal NOS inhibitors), 1 mM Na-tungstate (a NR inhibitor), 1 μM rotenone (inhibitors of mitochondrial electron

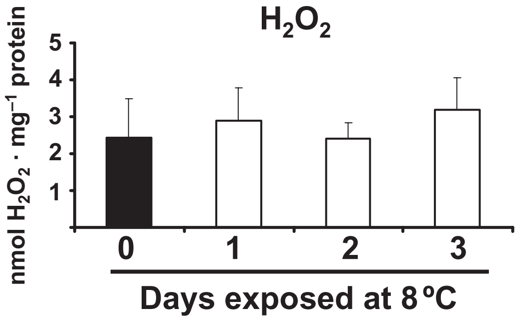

Figure 5. Hydrogen peroxide content in pepper leaves of plants exposed to low temperature (8 °C) for 0 to 3 d. Values are means of at least three independent experiments.

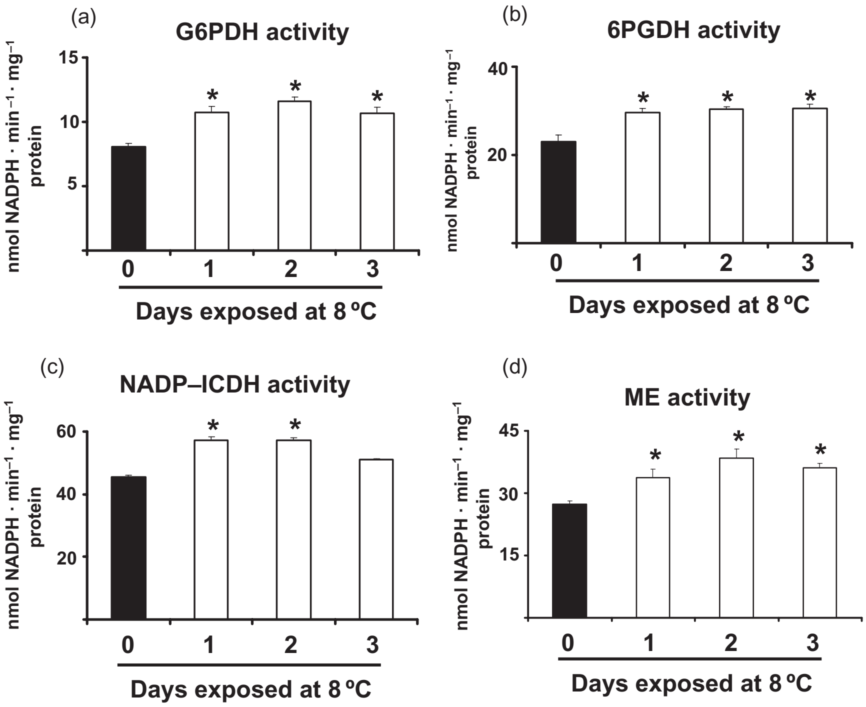

Figure 6. Activity analysis of NADP-dehydrogenases in leaves from pepper plants exposed to low temperature (8 °C) for 0 to 3 d. (a) G6PDH activity. (b) 6PGDH activity. (c) NADP-ICDH activity. (d) ME activity. Data are the mean ± SEM of at least three different experiments. Asterisks indicate that differences from control values were statistically significant at $P < 0.05$.

transport since prevents reduction of complex I) and 2 mM DFMO (inhibits polyamine biosynthesis by the selective, irreversible inhibition of ornithine decarboxylase). Additionally, the NO scavenger cPTIO was used as negative control because it efficiently impaired the fluorescence detected with DAF-2 in comparison with control samples by 68%. Thus, NO production was strongly reduced with the tungstate (by 70%) a NR activity inhibitor, followed for the NOS inhibitors aminoguanidine (by 53%) and L-NAME (by 49%). The rotenone reduced the NO production by 19% and DFMO (inhibits polyamine biosynthesis) by 9%. Therefore, the results suggest that NR and L-arginine-dependent NO synthase activities are the major contributors of NO production in pepper leaves.

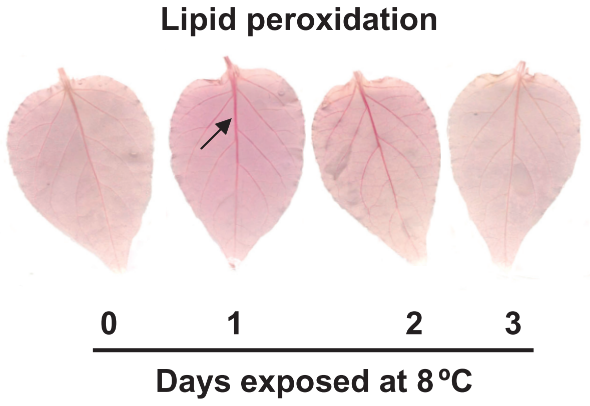

Figure 7. Histochemical detection of lipid peroxidation in leaves of 30-day-old pepper plants exposed to low temperature (8 °C) for 0 to 3 d. The red/purple colour indicates the presence of lipid peroxidation detected with the Schiff's reagent.

When pepper plants were exposure at 8 °C, the NO content in leaves was reduced between 52 and 64% with respect to control plants during the three days at 8 °C (Fig. 9b). The GSNO reductase activity showed a rise of 32% during the first and third days of treatment but no significant effect was observed after 2 d (Fig. 9c). The immunoblot analysis of the protein profile of tyrosine nitration in pepper leaves during the three days of exposure to LT, using an antibody against nitrotyrosine (NO_2-Tyr), is shown in

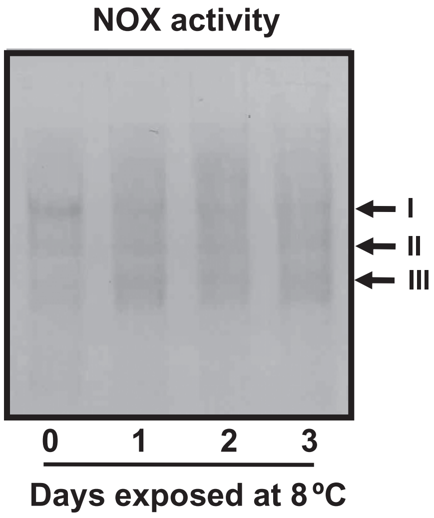

Figure 8. Detection of NOX isozymes fom leaves of pepper plants exposed to low temperature (8 °C) for 0 to 3 d. Protein samples (20 μg) were separated by native PAGE (6% acrylamide), then gels were incubated with NBT and NADPH until the appearance of blue formazan bands was observed.

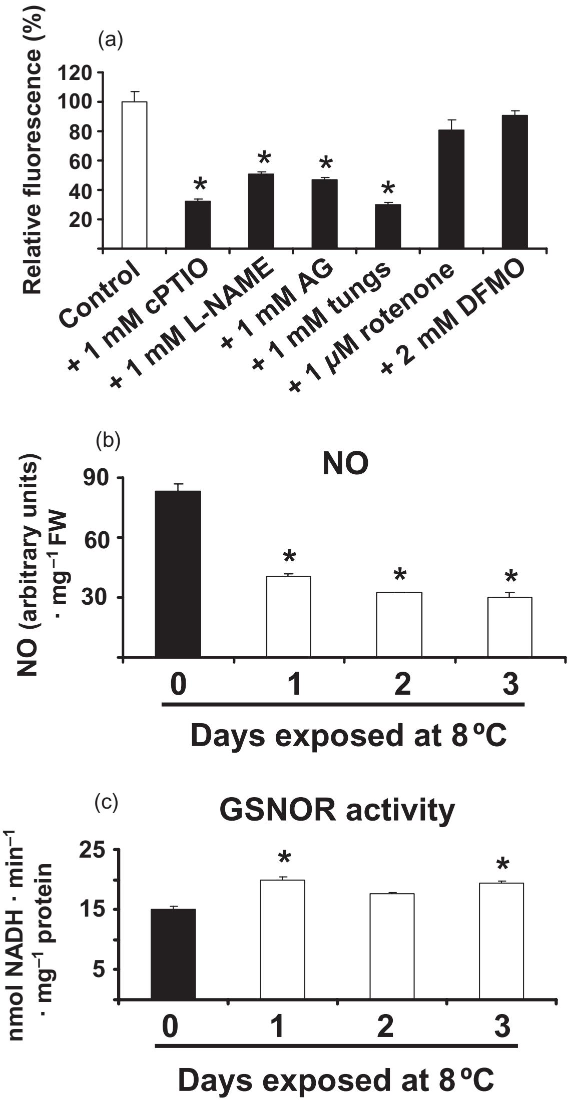

Figure 9. (a) Pharmacological characterization of NO source(s) in pepper leaves samples by spectrofluorometric assay using DAF-2 (see Material and Methods). In all cases pepper leaves samples were pre-incubated for 30 min with different inhibitors including 1 mM L-NAME (an arginine analog that inhibits mammalian NO synthase activity), 1 mM aminoguanidine (AG, an animal NOS inhibitors), 1 mM Na-tungstate (a NR inhibitor), 1 μM rotenone (inhibitor of mitochondrial electron transport which prevents reduction of complex I) and 2 mM α-difluoromethylornithine (DFMO; inhibits polyamine biosynthesis by the selective, irreversible inhibition of ornithine decarboxylase). As control, leaf samples were preincubated with cPTIO as NO scavenger. (b) Spectrofluorometric detection of NO with DAF-2 in pepper leaves of plants exposed to low temperature (8 °C) for 0 to 3 d. The fluorescence produced was expressed as arbitrary units per milligram of fresh weight (FW). (b) Spectrophotometric assay of *S*-nitrosoglutathione reductase (GSNOR) activity in pepper leaves of plants exposed to low temperature (8 °C) for 0 to 3 d. Results are means ± SEM of samples from at least three different experiments. Asterisks indicate that differences from control values were statistically significant at $P < 0.05$.

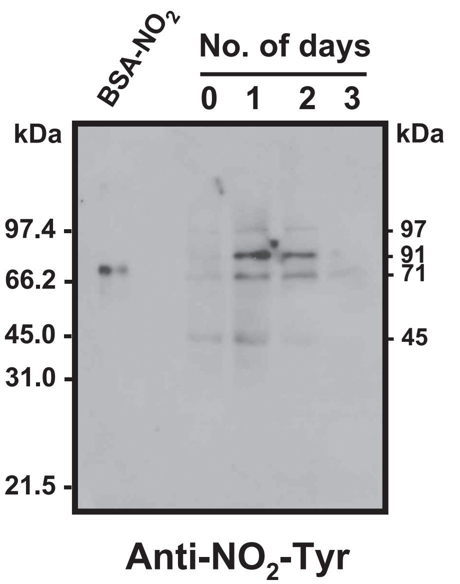

Figure 10. Representative immunoblot showing the protein tyrosine nitration in leaves from pepper plants exposed to low temperature (8 °C) for 0 to 3 d. Leaf samples (50 μg of protein per lane) were subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting analysis using an antibody against 3-nitrotyrosine (NO_2-Tyr) (dilution 1: 8000). Commercial nitrated BSA (NO_2-BSA) (2 μg of protein) was used as positive control. The numbers on the left side of the immunoblot indicate the relative molecular masses of the protein markers.

Fig. 10. In control leaves four weak immnunoreactive bands were observed, with molecular masses of approximately 45, 71, 91 and 97 kDa, the most prominent bands being those of 71 and 91 kDa. After 1 d exposure to LT an intensification of these four nitrated-protein bands took place, but after 2 d the intensity of these bands decayed, being almost undetectable at the third day.

Considering that many of the analysed parameters seem to undergo a significant modification after the first day of plant exposure to LT, this time was selected for the cellular analysis of the RNS metabolism of pepper leaves by CLSM, using specific fluorescent probes for NO, $ONOO^-$ and SNOs (Fig. 11). NO was analysed using the fluorescent probe DAF-FM DA where the green fluorescence is attributable to NO. In control pepper leaves, the localization of endogenous NO showed an intense green fluorescence in palisade mesophyll, vascular tissues (xylem and phloem), and a lower intensity in the upper and lower epidermal cells (Fig. 11a). In plants exposed to LT for 1 d the green fluorescence was slightly reduced in all cell types (Fig. 11b). On the other hand, the cellular production of $ONOO^-$ was analysed using the fluorescent probe 3'-(*p*-aminophenyl) fluorescein (APF). In control leaves, the green fluorescence attributable to $ONOO^-$ was scarcely observed in vascular tissue (Fig. 11c). However, in plants exposed for one day to LT, green fluorescence was more intense in vascular tissue and started to be observed in palisade mesophyll (Fig. 11d). SNOs were detected using the fluorescent probe, Alexa

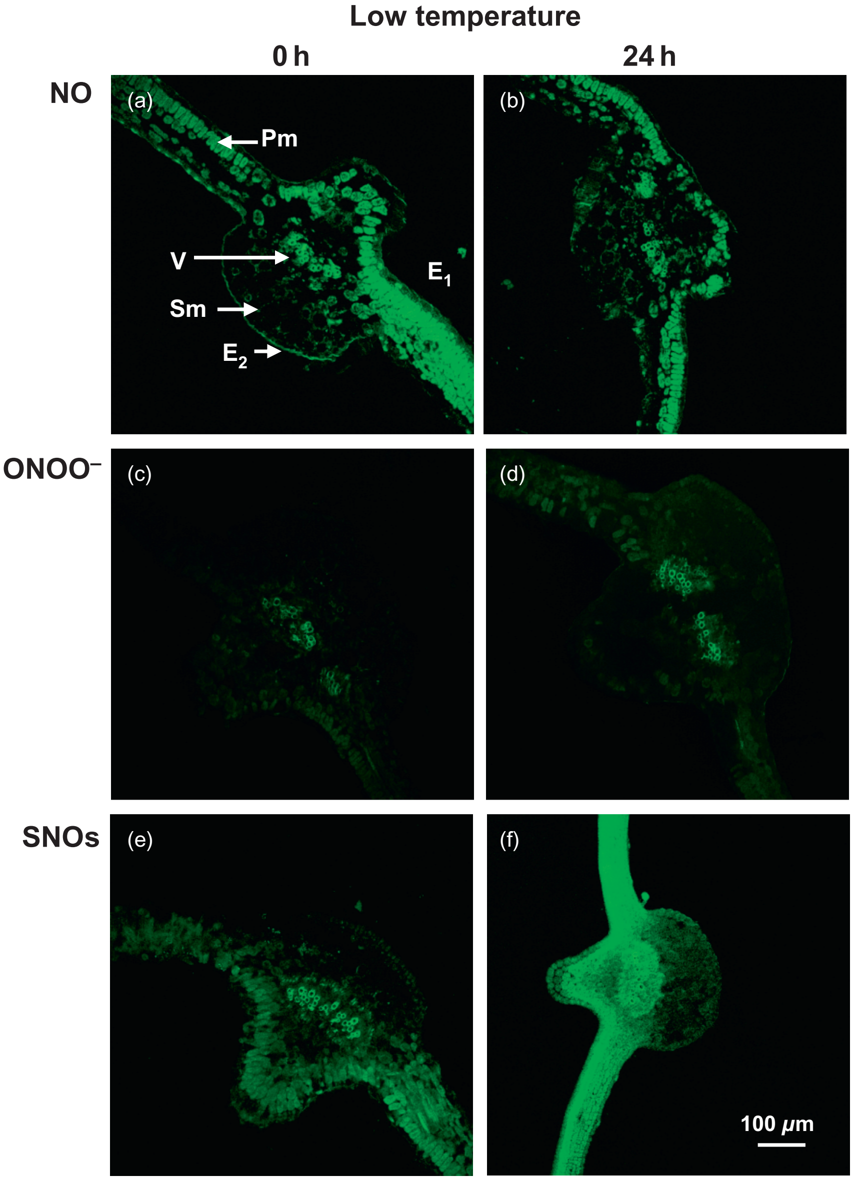

Figure 11. Representative images illustrating the CLSM detection and visualization of NO (a and b), ONOO$^-$ (c and d) and SNOs (e and f) in transversal cross-sections of pepper leaves of plants exposed to low temperature (8 °C) for 0 (control) and 24 h. The bright green fluorescence corresponded to the detection of NO, ONOO$^-$ and SNOs in the corresponding panels. E$_1$, adaxial epidermis. E$_2$, abaxial epidermis. Pm, palisade mesophyll. Sm, spongy mesophyll. V, main vein.

Fluor 488 Hg-link (AF) that reacts with *S*-nitrosylated thiols (SNOs) via the Saville reaction (Chaki *et al*. 2009a). In control leaves, the green fluorescence attributable to SNOs was present mainly in vascular tissue, palisade and spongy mesophyll (Fig. 11e) and after 1 d at 8 °C a general intensification of fluorescence in the same cell types was observed (Fig. 11f).

Considering that pepper plants seem to undergo a process of cold acclimation since some of the analysed parameters were recovered to normal level, it was analysed the behaviour of some of the above parameters including lipid peroxidation, NO content, catalase and NADP-ICDH activities in leaves of pepper plants exposed to several situations: (I) control pepper plants grown under optimal temperature; (II) pepper plants exposed to LT for 24 h; (III) pepper plants exposed to 8 °C for 24 h and then moved to optimal temperature for another 24 h; and (IV) pepper plants exposed to 8 °C for 24 h, moved to optimal temperature for 24 h and moved back again to 8 °C for another 24 h (Supporting Information Fig. 1). Thus, lipid peroxidation was reduced during the treatment III being the damage less intense after treatment IV (Supporting Information Fig. S1a). Similar behaviour was observed with catalase and NADP-ICDH activities (Supporting Information Fig. S1b,c). However, in the case of NO content seems to be still significantly affected even after a period of acclimation for 24 h (treatment III) where the cold stress is alleviated.

DISCUSSION

Among the different abiotic stresses that affect plants, LT can be considered one of the most harmful (Hannah *et al*. 2005; Sharma *et al*. 2005; Janská *et al*. 2010). Thus, many studies have shown that LT induces changes in expression of hundreds of genes followed by an increase in the levels of many metabolites, some of which are known to have protective effects against the damaging effects of cold stress (Seki *et al*. 2002; Shinozaki *et al*. 2003; Sharma *et al*. 2005; Chinnusamy, Zhu & Zhu 2007; Zhu, Dong & Zhu 2007; Winfield *et al*. 2010). Although there are some reports focused on the analysis of antioxidant response to LT, less information is available of the involvement of NO and NO-derived molecules in this process. Consequently, the main goal of this work was to study the antioxidant metabolism and homeostasis of ROS and RNS in this plant species under LT conditions, since this environmental stress considerably affects pepper growth.

LT induces antioxidative systems and disturbs the redox homeostasis in pepper leaves

During the period of time in which pepper plants were exposed to LT (3 d) an overall induction of enzymatic and non-enzymatic antioxidant systems was observed. Catalase and APX, involved in the direct removal of H_2O_2, were induced after 24 h even when the content of this molecule apparently did not change. A similar increase was observed for the MDAR activity as well as for the content of ascorbate and GSH. However, GR kept its activity during the first day with a significant reduction during the second and third days of exposure to LT. In other plant species, the response of the antioxidative systems to LT stress depends of how low the temperature and how long the treatment are. For example, in tobacco plants exposed to LT under normal light conditions, chloroplastic Fe-SOD and cytosolic CuZn-SOD are induced (Tsang *et al*. 1991). In rice, LT induces a rapid increase in APX activity and then gradual increases of SOD, MDAR and GR activities what suggests differential regulations of these enzymes; conversely, catalase activity was not significantly affected (Oidaira *et al*. 2000). In rice seedling exposed to heat (42 °C for 24 h) before LT (5 °C for 7 d) an interesting behaviour of APX was observed. Plants did not develop chilling injury and a higher level of APX activity and expression was found, whereas catalase activity was decreased and no significant difference in SOD activity was observed (Sato *et al*. 2001). In pea plants exposed to LT (8 °C) for 48 h, MDAR activity did not show any significant change but the transcripts of *MDAR* and *APX* increased 2.2- and 2.6-fold, respectively (Leterrier *et al*. 2005, 2007). In addition, GR activity was increased slightly but the transcription level of both chloroplastic and cytosolic *GR* genes was increased four- and threefold, respectively (Romero-Puertas *et al*. 2006). In haw (*Crataegus azarolus*) leaves exposed to LT (4 °C) for 24 h, the activities of both MDAR and DHAR increased and reached a maximum after 16 h of treatment. This was accompanied by an increase in the gene expression of both enzymes (Eltelib *et al*. 2011). In the *Coffea* genus, different degrees of cold tolerance have been observed, being the genotype Icatu the best documented because it has significant activity increases of chloroplastic CuZnSOD and APX (Fortunato *et al*. 2010). In embryonic axes of lotus (*Nelumbo nucifera*) exposed to LT (4 °C) for 48 h the MnSOD gene was induced eightfold (Naydenov *et al*. 2010).

GSH is one of the major soluble low molecular weight antioxidants, as well as the major non-protein thiol in plant cells (Foyer & Noctor 2005a,b; Noctor 2006; Szalai *et al*. 2009). It contributes to keep the cellular redox homeostasis and signalling (Meyer 2008). Thus GSH exerts a protective role based on the reducing capacity of GSH and the half-cell reduction potential of the GSH/GSSG couple. In leaves of pepper plants exposed to LT a significant increase of GSH from the first to third day of LT treatment and a significant increase in the ratio GSH/GSSG were observed. In maize plants the treatment with a combination of two herbicide safeners provoked a rise of total GSH, with a concomitant protection against LT (5 °C) (Kocsy *et al*. 2001a). A similar result has been found in mung bean plants where the pretreatment of leaves with H_2O_2 provoked a rise of the GSH level and, consequently, an increase of LT tolerance (Yu, Murphy & Lin 2003).

Ascorbate is a potent ROS scavenger that reacts with H_2O_2 in a reaction catalysed by APX, and non-enzymatically with $^1O_2$, $O_2^{\cdot -}$ and lipid hydroperoxides (Foyer 2001; Asada 2006). In pepper leaves, LT induced a rise in the content of ascorbate and similar results have been described in other plant species. In spinach leaves exposed to 10 °C, the content of ascorbate increased and after 7 d it was about 41% higher than in plants grown at 25 °C (Proietti *et al*. 2009). In the microscopic algae *Dunaliella salina*, growth under LT (13 °C for 24 h) increased the total ascorbate pool by 10–50%. This was accompanied by a rise of GSH and SOD activity (Haghjou, Shariati & Smirnoff 2009).

It must be kept in mind that both ROS production and antioxidative systems have multiple subcellular localizations including chloroplasts, cytosol, nuclei, mitochondria and peroxisomes, among others, and in our experimental conditions they might participate in a fine modulation of the ROS levels that could contribute either to control potential damage or be used for signalling purposes (Corpas, Barroso & del Río 2001; Mittler 2002; Møller, Jensen & Hansson 2007; Miller *et al*. 2010; Møller & Sweetlove 2010). In *Arabidopsis* subjected to LT, H_2O_2 was accumulated in the cells, and the APX enzyme activity increased (O'Kane *et al*. 1996). In maize leaves, LT induced changes in the distribution of H_2O_2 and antioxidants between the bundle sheath and mesophyll cells (Pastori, Foyer & Mullineaux 2000). In our case, the H_2O_2 content did not show any significant change by LT treatment, which could suggest a participation of this metabolite in signalling processes without inducing cell damages.

NADPH-generating dehydrogenases are involved in the mechanism of response to LT

NADPH is essential for defense against oxidative stress, since it is the coenzyme required for the reduction of oxidized GSH by GR in the ascorbate–GSH cycle, and is also necessary for the NADPH oxidase and the thioredoxin reductase activity (Noctor, Queval & Gakière 2006). The general rise in the activity of the main NADPH-generating dehydrogenases, including G6PDH, 6PGDH, NADP-ICDH and ME, observed in pepper plants exposed to LT indicated the involvement of these enzymes in the mechanism of response to this stress. In the case of LT, an increase in G6PDH activity has been reported in alfalfa (Krasnuk, Jung & Witham 1976), ryegrass (Bredemeijer & Esselink 1995), soybean (van Heerden *et al*. 2003), banana and populus (Lin *et al*. 2005). In rice roots and pea leaves, the activity and protein expression of NADP-ICDH are also induced by LT (Saruyama & Tanida 1995; Lu *et al*. 2005; Leterrier *et al*. 2007; Lee *et al*. 2009). More recently, in leaves of maize seedlings it has been reported that *ME* transcripts are up-regulated in response to cold stress (Nguyen *et al*. 2009).

Taken together, these results suggest that under LT stress pepper plants seem to require increasing NADPH production since the rise of all the NADP-dehydrogenase activities is maintained throughout the 3 d treatment. This suggests that these enzymes must play an important role in the process of cold acclimation observed during the third day of LT exposure.

LT affects NO homeostasis

In pepper leaves, LT caused a decrease in the NO content and this could be correlated with the rise of GSH since both molecules can react nonenzymatically to form GSNO, which could be considered the most abundant *S*-nitrosothiol in plant cells and a long-distance signal molecule that can operate under certain stress conditions (Lindermayr, Saalbach & Durner 2005; Barroso *et al*. 2006; Chaki *et al*. 2009a, 2011). This behaviour is also well correlated with the increase of GSNOR activity observed during the first 24 h of exposure to LT. Additionally, the analysis by CLSM allowed to observe that the leaves of pepper plants exposed to LT showed a rise of $ONOO^-$, which is formed by a very quick reaction between NO and $O_2^{\cdot-}$ ($k = 1.9 \times 10^{10}$ $\mathrm{M}^{-1}\ \mathrm{s}^{-1}$) (Kissner *et al*. 1998). This action could explain the increase of protein tyrosine nitration found during the first and second days of exposition to LT stress, because $ONOO^-$ is a molecule that can mediate this post-translational modification (Radi 2004; Szabó, Ischiropoulos, Radi 2007). The origin of the NO necessary to form $ONOO^-$ could be the increased content of SNOs. It is known that SNOs in the presence of some metals and reductants, such as GSH and ascorbate, which are augmented by LT (Table 2), can be decomposed releasing NO (Holmes & Williams 2000; Smith & Dasgupta 2000). On the other hand, in other plant species it has been reported that LT can promote an opposite effect on the NO content. In leaves of pea plants exposed to LT for 48 h, a rise of the NO content was observed, which was accompanied by an increase of SNOs, GSNO reductase activity and tyrosine-nitrated proteins (Corpas *et al*. 2008). A similar behaviour of NO was reported in *Arabidopsis thaliana* exposed to 4 °C for 1–4 h (Cantrel *et al*. 2011) or during cold acclimation (Zhao *et al*. 2009) where the NO content increased. Consequently, all these results support the existence of a connection between NO metabolism and LT stress.

LT causes oxidative and nitrosative stress

In pepper plants, the phenotypic symptoms observed during the first and second days were accompanied by a rise of lipid peroxidation and protein tyrosine nitration (NO_2-Tyr), which are considered biochemical markers of both oxidative and nitrosative stress, respectively (Requena *et al*. 1996; Corpas, del Río & Barroso 2007). There are numerous examples where the rise of oxidized products such as lipids is a reliable parameter to evidence the damage of the cellular membranes being the chloroplast membranes the first target and also the most severely injured (Krarsch & Wise 2000). In our case, a histochemical procedure to detect lipid oxidation was used because it offers an advantage over the biochemical methods, since it allows detecting lipid peroxidation in single cells of a tissue composed of different cell populations. Thus, in leaves it was observed that LT produces lipid oxidation in vascular tissues, what is in good agreement with the leaf flaccidity observed during the first 48 h of exposure to LT. Similarly, the swelling recovery observed during the third day correlated with the slight lipid peroxidation detected in the leaves. Additionally, the rise determined in the membrane NADPH oxidase activity can also contribute to the damage produced. The oxidative stress observed by LT treatment in leaves of pepper plants during the first and second days is in agreement with previous studies reported in maize plants (Prasad *et al*. 1994; Hodges *et al*. 1997; Pinhero *et al*. 1997).

With regard to the rise of protein tyrosine nitration (NO_2-Tyr), less information is available in plant systems (Corpas *et al*. 2009a). However, an increased number of data start indicating that this parameter could be also a good marker to determine if a specific stress is accompanied by nitrosative stress. Several reports suggest that this correlation takes place because different environmental stresses, which provoke oxidative stress, also induce a rise of protein tyrosine nitration. Thus, this mechanism occurs in olive and *Arabidopsis* under salt stress conditions (Valderrama *et al*. 2007; Corpas *et al*. 2009b), in pea and sunflower seedlings by mechanical wounding (Corpas *et al*. 2008; Chaki *et al*. 2011), in tobacco BY-2 suspension cells in response to biotic stress (Saito *et al*. 2006), and in sunflower seedlings after infection by mildew (Chaki *et al*. 2009a).

Cold stress and cold acclimation involves redox state homeostasis

The results obtained in this work showed that in pepper plants exposed to LT during 24–48 h a general imbalance of the ROS and RNS metabolism was produced with the result of a rise in lipid peroxidation and protein tyrosine nitration, which strongly suggests the induction of oxidative and nitrosative stress. However, it was also observed that during the third day under LT pepper plants started to recover and this might indicate that a mechanism of cold acclimation was taking place, with the NADPH-generating dehydrogenases contributing to obtain the optimum redox state (high levels of GSH and ascorbate) that allow pepper plants to acclimate to LT. This is supported by the recovery of the turgence of leaves of a normal phenotype and the reduction of lipid oxidation and protein tyrosine nitration. This process was corroborated when the pepper plants were exposed to optimal temperature for a period of 24 h where the cold stress was alleviated and some of the analysed parameters showed less intense changes.

According with the data reported in other plants species (Kocsy, Galiba & Brunold 2001b), the observed high content of GSH in pepper leaves could be one of the factors contributing to the cold acclimation observed. Thus, this strategy would guarantee the supply of GSH for the ascorbate–GSH cycle and other processes such as the reaction with NO to form *S*-nitrosoglutathione. From an applied viewpoint, this process of cold acclimation could be used as an agronomical strategy to improve the resistance of pepper plants to LT and perhaps also other environmental stresses.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. (a) Histochemical detection of lipid peroxidation (b) catalase activity (c) NADP-ICDH activity, and (d) NO content in leaves of pepper plants maintained for different periods of times to low temperature (8 °C) and optimal conditions. (I) Control plants exposed to optimal temperature. (II) Plants exposed to LT for 24 h. (III) Plants exposed to LT for 24 h and then moved to optimal temperature for another 24 h. (IV) Plants exposed to LT for 24 h, moved to optimal temperature for 24 h and exposed again to LT for another 24 h.