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Spatial and temporal regulation of the metabolism of reactive oxygen and nitrogen species during the early development of pepper (*Capsicum annuum*) seedlings

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• **Background and Aims** The development of seedlings involves many morphological, physiological and biochemical processes, which are controlled by many factors. Some reactive oxygen and nitrogen species (ROS and RNS, respectively) are implicated as signal molecules in physiological and phytopathological processes. Pepper (*Capsicum annuum*) is a very important crop and the goal of this work was to provide a framework of the behaviour of the key elements in the metabolism of ROS and RNS in the main organs of pepper during its development.
• **Methods** The main seedling organs (roots, hypocotyls and green cotyledons) of pepper seedlings were analysed 7, 10 and 14 d after germination. Activity and gene expression of the main enzymatic antioxidants (catalase, ascorbate–glutathione cycle enzymes), NADP-generating dehydrogenases and *S*-nitrosoglutathione reductase were determined. Cellular distribution of nitric oxide ($^{\cdot}NO$), superoxide radical ($O_2^{\cdot-}$) and peroxynitrite ($ONOO^-$) was investigated using confocal laser scanning microscopy.
• **Key Results** The metabolism of ROS and RNS during pepper seedling development was highly regulated and showed significant plasticity, which was co-ordinated among the main seedling organs, resulting in correct development. Catalase showed higher activity in the aerial parts of the seedling (hypocotyls and green cotyledons) whereas roots of 7-d-old seedlings contained higher activity of the enzymatic components of the ascorbate glutathione cycle, NADP-isocitrate dehydrogenase and NADP-malic enzyme.
• **Conclusions** There is differential regulation of the metabolism of ROS, nitric oxide and NADP dehydrogenases in the different plant organs during seedling development in pepper in the absence of stress. The metabolism of ROS and RNS seems to contribute significantly to plant development since their components are involved directly or indirectly in many metabolic pathways. Thus, specific molecules such as H_2O_2 and NO have implications for signalling, and their temporal and spatial regulation contributes to the success of seedling establishment.

Key words: Antioxidant, *Capsicum annuum*, development, NADP dehydrogenases, nitric oxide, pepper, reactive oxygen species, RNS, ROS.

INTRODUCTION

Plant growth and development constitute a highly regulated process in which many internal and external factors are involved, including availability of nutrients, hormones, pattern formation, cell polarity, cell cycle control and auxin transport. Bearing in mind that the final goal of a plant is to reproduce and be perpetuated in time, the early development steps are crucial in establishing the new seedling. In this sense, numerous aspects have been studied from different points of view, such as seed germination, lateral root initiation, cell differentiation, orientation, division, development, architecture of roots and leaves (Ten Tusscher and Scheres, 2011; De Smet *et al*., 2012; Niu *et al*., 2013; Axelrod and Bergmann, 2014; Kalve *et al*., 2014).

Reactive oxygen and nitrogen species (ROS and RNS, respectively) constitute two families of molecules that have been generally associated with the mechanism of responses against adverse stress conditions (Foyer and Noctor, 2005*a*; Gechev *et al*., 2006; Miller *et al*., 2008; Corpas *et al*., 2007, 2011; Airaki *et al*., 2012). However, this concept started to change when some key molecules such as hydrogen peroxide (H_2O_2) and nitric oxide (NO) came to be recognized as second messengers implicated in a variety of developmental processes, including germination, root growth, senescence, flower development and fruit ripening (Leshem and Pinchasov, 2000; Foyer and Noctor, 2005*b*; Bethke *et al*., 2006; Carol and Dolan, 2006; Corpas *et al*., 2006, 2013*a*; Pitzschke *et al*., 2006; Diaz-Vivancos *et al*., 2010; Zafra *et al*., 2010; Leterrier *et al*., 2011; Kocsy *et al*., 2013; Ishibashi *et al*., 2013; Chaki *et al*., 2015). Therefore, the relevance of ROS and RNS metabolism in the physiology of higher plants under optimal conditions has begun to be well documented. A good example is the progress made in the understanding of the mechanism of NO and interactions

with other molecules (H_2O_2, auxin and gibberellins) in regulating primary and lateral root growth, which finally determines root architecture (Pagnussat *et al*., 2002; Correa-Aragunde *et al*., 2004; Fernández-Marcos *et al*. 2011; Duan *et al*., 2014). Moreover, the contributions of some of the enzymes involved in the regulation of ROS and RNS have been also studied in some specific physiological processes, such as germination, root formation, and hypocotyl development (Foreman *et al*., 2003; Mori and Schroeder, 2004; Carol and Dolan, 2006; Corpas *et al*., 2006; Bailly *et al*., 2008; Swanson and Gilroy, 2010; Šírová *et al*., 2011; Schippers *et al*., 2012).

In previous work we have studied the redox status and ROS and RNS levels in adult pepper plants at low temperature (Airaki *et al*., 2011, 2012) and pepper fruits during ripening (Mateos *et al*., 2009, 2013; Chaki *et al*., 2015). However, to our knowledge there is no report that has provided a wide framework for the behaviour of the key elements in the metabolism of ROS and RNS in the main organs during early plant development. Therefore, the main goal of this study was to provide a detailed analysis, using biochemical and cellular approaches, of the spatial and temporal regulation of ROS and RNS metabolism during the early development of pepper seedlings. Pepper is a plant with relevant agronomical interest since it is the second-most consumed vegetable worldwide according to its distribution and production (www.fao.org/publications; http://www.usaid.gov/sites/default/files/documents/1862/frutas_y_hortalizas.pdf). For this reason, key RNS and ROS parameters were selected, including the activity of enzymes involved in NO metabolism, such as *S*-nitrosoglutathione reductase (GSNOR), and different antioxidant enzymes (catalase and enzymes of the ascorbate–glutathione cycle). The cellular distributions of NO, the superoxide radical ($O_2^{\cdot-}$) and peroxynitrite ($ONOO^-$) were also studied.

MATERIALS AND METHODS

Plant materials and growth conditions

Pepper (*Capsicum annuum* L.) seeds, California type, were obtained from Syngenta Seeds S.A. (El Ejido, Almería, Spain). Seeds were surface-sterilized for 5 min in 50 % (v/v) commercial bleach, washed three times in sterile water and germinated in 100 × 100 mm plastic Petri dishes (six seeds per dish) containing Murashige and Skoog medium (Sigma) with a pH of 5.5 under sterile conditions. Then, they were sealed with Parafilm and placed in a vertical position for 5 d at 30 °C in the dark. The Petri dishes were then transferred to a growth chamber at 22/18 °C with a 16 h photoperiod. The seedlings were collected at different intervals between 7 and 14 d post-germination (Fig. 1).

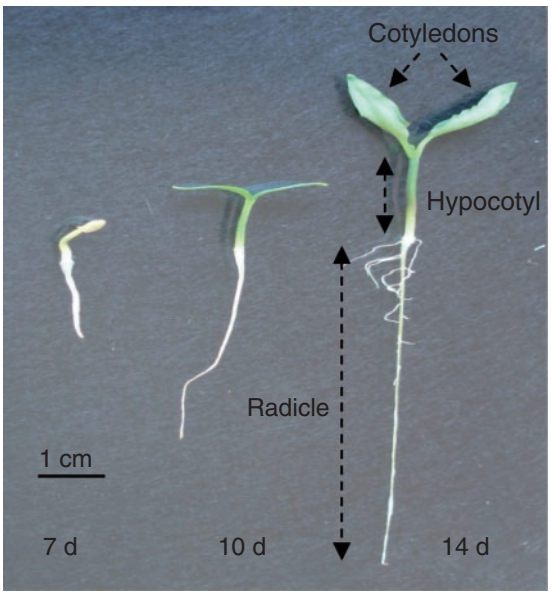

FIG. 1. Appearance of a pepper seedling at different stages of development (7, 10 and 14 d old). Three main organs are distinguishable after 10 d: radicle (embryonic root), hypocotyl (embryonic shoot) and green cotyledons.

Crude extracts of plant organs

To measure enzyme activities, for each individual experiment around 20–25 pepper seedlings were collected, and the roots, hypocotyls and green cotyledons were separated, pooled and frozen in liquid N_2 and ground in a mortar with a pestle. The powder was suspended in a homogenizing medium containing 50 mM Tris–HCl, pH 7.8, 0·1 mM EDTA, 5 mM dithiothreitol (DTT), 0·2 % (v/v) Triton X-100, 10 % (v/v) glycerol and 2 % (w/v) polyvinyl polypyrrolidone (PVPP). For the ascorbate peroxidase (APX) activity assay, 2 mM ascorbate was added to the homogenizing medium to preserve this activity (Miyake and Asada, 1996). Homogenates were centrifuged at 27 000 *g* for 25 min at 4 °C and supernatants were immediately used for the assays. For the GSNOR activity assay, the supernatants were passed through Sephadex G-25 gel filtration columns (NAP-10; GE Healthcare), which were equilibrated and eluted with the same buffer before the enzymatic assays (Barroso *et al*., 2006).

Enzymatic activity assays

Catalase (EC 1.11.1.6) activity was determined by measuring the disappearance of H_2O_2, as described by Aebi (1984). Ascorbate peroxidase (EC 1.11.1.11) was determined by monitoring the initial ascorbate oxidation by H_2O_2 at 290 nm (Hossain and Asada, 1984). To test the feasibility of the assay, 20 mM *p*-chloromercuriphenylsulphonic acid, a specific inhibitor of APX, was used (Mittler and Zilinskas, 1991). Monodehydroascorbate reductase (MDAR; 1.6.5.4) was assayed by measuring monodehydroascorbate-dependent NADH oxidation, and monodehydroascorbate was generated by the ascorbate/ascorbate oxidase system (Leterrier *et al*., 2005). The rate of monodehydroascorbate-independent NADH oxidation (without ascorbate and ascorbate oxidase) was subtracted from the monodehydroascorbate-dependent reaction. Glutathione reductase (GR; EC 1.6.4.2) was assayed by monitoring the NADPH oxidation coupled to the reduction of glutathione disulphide (GSSG) (Edwards *et al*., 1990). The reaction rate was corrected for the small, non-enzymatic oxidation of NADPH by GSSG. Activity of GSNOR was assayed spectrophotometrically at 25 °C by monitoring the oxidation of NADH at 340 nm (Barroso *et al*., 2006). Glucose-6-phosphate

TABLE 1. *Oligonucleotides used for the cloning and semiquantitative RT–PCR analysis of enzymes involved in the metabolism of ROS and RNS. Letters 'F' and 'R' correspond to forward and reverse oligonucleotides, respectively*

Name	Oligonucleotide sequence (5–3)	Product size (bp)	Accession number
cDNA cloning			
F1-GSNOR	CTTGACAAAGTATGTGTCC	588	–
F2-GSNOR	AAGTCATTCGTTGCAAAGC		
R-GSNOR	GTGAGTGTAGAACTTCTCC	1121	EU652335
Semiquantitative RT–PCR			
F-CAT	GATTTCTTCTCTTTCCTCC		
R-CAT	CGATGTTCCTATTCAATACC	418	AF227952
F-APX	TGTGCTCCTCTTATGCTCC		
R-APX	CTCAAAACCAGAACGCTCC	485	X81376
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

dehydrogenase (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 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 (G6P). 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 (6PG) (Corpas *et al*., 1995, 1998). NADP isocitrate dehydrogenase (NADP-ICDH; EC 1.1.1.42) activity was measured by following NADP reduction according to Corpas *et al* (1999). 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 2*R*,3*S*-isocitrate. NADP-malic enzyme (NADP-ME; EC 1.1.1.40) activity was determined spectrophotometrically by recording the reduction of NADP at 340 nm using the same reaction mixture (1 ml), but in this case the reaction was initiated by the addition of 1 mM L-malate (Valderrama *et al*., 2006).

Protein concentration was determined with the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA) using BSA as standard.

Partial cloning of GSNOR cDNA

Using plant glutathione-dependent formaldehyde dehydrogenase sequences from the data bank, degenerate oligonucleotides were designed in conserved domains of these sequences (Table 1). Total RNA was isolated from 10-d-old seedlings with Trizol Reagent (Gibco BRL, Paisley, UK) as described in the manufacturer's manual, and RNA was quantified spectrophotometrically. Two micrograms of total RNA was used as a template for the reverse transcriptase reaction and added to a mixture containing 1·5 mM dNTPs, 1·6 µg polydT23 primer, 1 × reverse transcriptase buffer (25 mM Tris–HCl, pH 8.3, 5 mM $MgCl_2$, 50 mM KCl and 2 mM DTT), 0·9 U RNasin ribonuclease inhibitor and 20 U avian myeloblastosis virus reverse transcriptase (Finnzymes, Espoo, Finland). The reaction was carried out at 42 °C for 40 min, followed by a 5-min step at 98 °C and cooling to 4 °C for 10 min. Then, PCR was carried out as follows: 1 µl of the cDNA was added to 250 µM dNTPs, 1·5 mM $MgCl_2$, 1 × PCR buffer, 2·5 U of AmpliTaq Gold (Roche, Mannheim, Germany) and 0·5 µM of each primer (Table 1) in a final volume of 20 µl. Reactions were carried out in a Hybaid thermocycler (Ashford, UK). A first step of 2 min at 94 °C was followed by 30 cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min at 65 °C, with a final extension of 10 min at 65 °C. Amplified PCR products were detected after electrophoresis in 1 % (w/v) agarose gels stained with ethidium bromide, and the visualized bands were cut and extracted from the gel (Qiaex II gel extraction kit; Qiagen, Madrid, Spain). The purified fragments were cloned into the pGEM-T Easy Vector (Promega, Madrid, Spain). A partial cDNA of glutathione-dependent formaldehyde dehydrogenase was obtained, confirmed by sequencing and deposited in the data bank with accession number EU652335.

Semiquantitative reverse transcription–PCR

Total RNA was extracted from whole seedlings with Trizol as described by the manufacturer (Gibco BRL, Life Technologies). Two micrograms of total RNA was used to produce cDNA by reverse transcription (RT)–PCR (Mateos *et al*., 2009). Semiquantitative RT–PCR amplification of actin cDNA from pepper was used as control. Catalase, GR, MDAR, APX, GSNOR, G6PDH, 6PGDH, NADP-ICDH, NADP-ME and actin cDNAs were amplified by the PCR as follows: 1 µl of each cDNA (30 ng) was added to 0·250 mM dNTPs, 2·5 mM $MgCl_2$, 1 × PCR buffer, 0·5 U of HotMaster Taq™ DNA polymerase

(Eppendorf, Madrid, Spain) and 0·5 µM of each primer (Table 1) in a final volume of 20 µl. Reactions were carried out in the Hybaid thermocycler. A first step of 10 min at 95 °C was followed by 28–33 cycles (depending on the gene) of 30 s at 95 °C, 30 s at 55 °C and 45 s 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 camera. Band intensity was expressed as relative absorbance units. The ratio between each specific gene and actin amplification was calculated to normalize for initial variations in sample concentration (Marone *et al*. 2001). Means and standard deviations were calculated after normalization to actin.

TABLE 2. *Growth parameters of roots, hypocotyls and green cotyledons of pepper seedlings at different stages (7–14 d) of development. Data are means ± s.e. for at least six independent seedlings*

Day	Root		Hypocotyl length (cm)	Hypocotyl + cotyledon fresh weight (mg)
	Length (cm)	Fresh weight (mg)		
7	2·1 ± 0·1	12·0 ± 2·0	0·40 ± 0·05	11·8 ± 1·7
8	2·5 ± 0·1	14·7 ± 0·1	0·70 ± 0·05	15·3 ± 3·0
9	3·0 ± 0·3	16·3 ± 0·8	0·80 ± 0·03	19·5 ± 1·6
10	3·7 ± 0·2	18·6 ± 1·9	0·90 ± 0·04	29·5 ± 2·9
13	6·0 ± 0·1	29·3 ± 3·0	1·10 ± 0·10	50·9 ± 5·4
14	6·0 ± 0·1	32·0 ± 2·0	1·10 ± 0·10	62·7 ± 7·1

SDS–PAGE and immunoblot analysis

This was carried out in 10 % acrylamide slab gels. For western blot analysis, proteins were transferred to polyvinylidene difluoride (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 immunodetection, an affinity-purified goat anti-(rabbit IgG)–horseradish peroxidase conjugate (Bio-Rad) and an enhanced chemiluminescence kit (ECL Plus; Amersham, Piscataway, NJ, USA) were used. As positive control, commercial nitrated BSA (Sigma, St Louis, MO, USA) was used.

Detection of NO, $O_2^{\cdot-}$ and $ONOO^-$ by confocal laser scanning microscopy

Segments of pepper organs (roots, hypocotyls and green cotyledons) of ∼5 mm were taken from the elongation zone of roots and the middle area of hypocotyls and cotyledons. The segments were incubated in darkness with specific fluorescent probes. To detect NO, the organ segments were incubated at 25 °C for 1 h in darkness with 10 µM diaminofluorescein-FM diacetate (DAF-FM DA; Calbiochem) prepared in 10 mM Tris–HCl (pH 7.4) according to Corpas *et al*. (2006). For $O_2^{\cdot-}$, segments were incubated at 37 °C for 30 min with 10 µM dihydroethidium (DHE; Fluka) as described by Valderrama *et al*. (2007). For $ONOO^-$, the segments were incubated with 10 µM 3′-(*p*-aminophenyl) fluorescein (APF; Invitrogen) prepared in 10 mM Tris–HCl (pH 7.4) according to Chaki *et al*. (2009). The corresponding segments were then washed twice in the same buffer for 15 min each. After washing, sections were embedded in a mixture of 15 % acrylamide–bisacrylamide stock solution in 0·01 M PBS including 0·3 % tetramethylethylenediamine (TEMED) for 4 h and then polymerized in inclusion containers (1·5 × 0·9 × 0·5 mm; Sorvall Instruments) using 0·5 ml of a fresh 15 % acrylamide stock solution and 50 µl of 2 % persulphate ammonium. Then, 100-µm-thick sections, as indicated by the vibratome scale, were cut under 10 mM PBS. Sections were then soaked in glycerol : PBS (containing azide) (1 : 1; v : v) and mounted in the same medium for examination with a confocal laser scanning microscope (CLSM) system (Leica TCS SL), using standard filters and collection modalities for the different fluorescence probes (excitation at 495 nm, emission at 515 nm) (Corpas and Barroso, 2014).

Statistical analysis

Data are presented as the mean ± s.e.m. of at least three independent experiments. Pairwise analysis of variance (ANOVA) was used to detect differences among stage of development and organs. Values of $P < 0·05$ were considered statistically significant.

RESULTS

Pepper plants have a relative long period of growth. However, post-germination development is a key step in the establishment of the seedlings, a process involving various morphological, physiological, biochemical and molecular adaptations. Figure 1 shows a representative image of the appearance of pepper seedlings at the ages of development selected to carry out this work, i.e. 7, 10 and 14 d. These stages were selected because the major organs (roots, hypocotyls and green cotyledons) undergo significant changes in size, and hypocotyls and cotyledons become green, forming the first photosynthetic organs of the young plant. This step involves drastic biochemical changes. Table 2 summarizes length and biomass data for pepper seedlings between days 7 and 14 of development.

Activity of antioxidant enzymes and NADPH-generating dehydrogenases in the main organs during the development of pepper seedlings

The results of this are analysis shown in Fig. 2. Catalase activity increased during seedling development in hypocotyls and green cotyledons; however, in roots the activity did not show significant differences during the analysed period (Fig. 2A). Among all organs, the highest catalase activity was found in hypocotyls of 14-d-old seedlings. The APX activity was highest in roots of 7-d-old seedlings and was ∼25 % lower in roots of 10- and 14-d-old seedlings (Fig. 2B). Activity of MDAR had a different distribution; it was higher in roots than in other organs, the highest activity being found in the roots of 10-d-old seedlings (Fig. 2C). In hypocotyls and cotyledons MDAR activity increased with the age of the seedlings. Figure 2D shows that

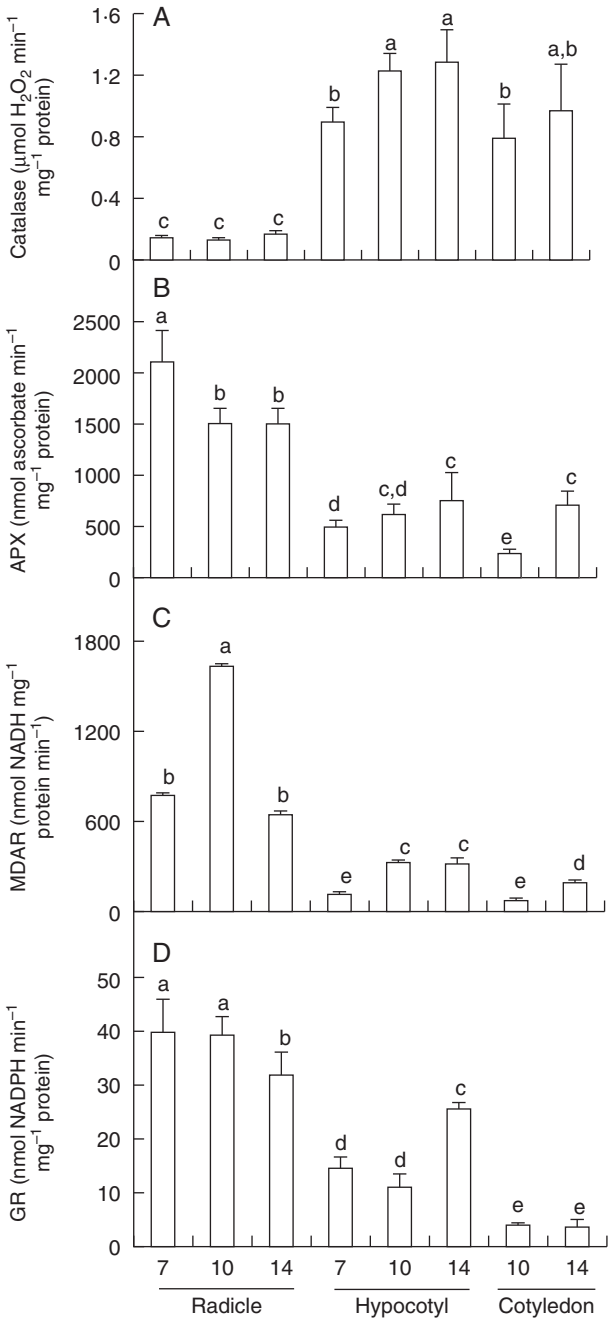

FIG. 2. Activity of catalase and ascorbate–glutathione cycle enzymes in radicles, hypocotyls and green cotyledons of pepper seedlings at different stages of development (7, 10 and 14 d old). (A) Catalase. (B) Ascorbate peroxidase (APX). (C) Monodehydroascorbate reductase (MDAR). (D) Glutathione reductase (GR). Data are mean ± s.e.m. of at least three experiments. Different letters indicate significant differences between stages and organs ($P \leq 0{\cdot}05$).

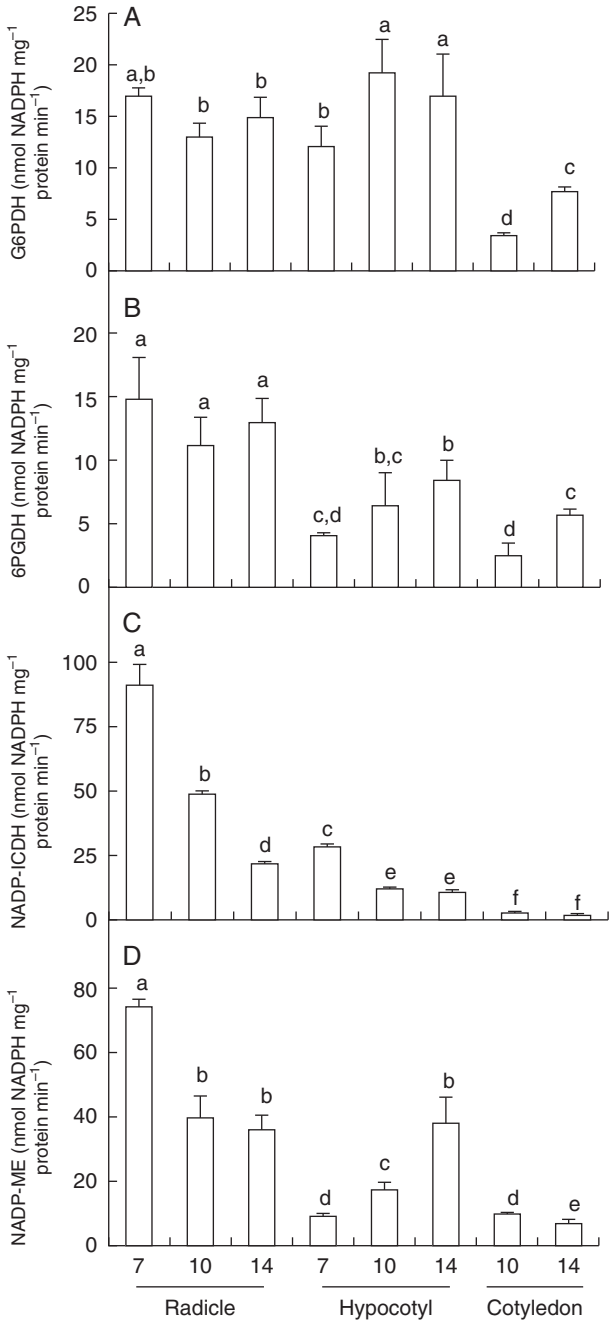

FIG. 3. Activity of NADP dehydrogenases in radicles, hypocotyls and green cotyledons of pepper seedlings at different stages of development (7, 10 and 14 d old). (A) Glucose-6-phosphate dehydrogenase (G6PDH). (B) 6-Phosphogluconate dehydrogenase (6PGDH). (C) NADP isocitrate dehydrogenase (NADP-ICDH). (D) NADP-malic enzyme (NADP-ME). Data are mean ± s.e.m. of at least three experiments. Different letters indicate significant differences between stages and organs ($P \leq 0{\cdot}05$).

GR activity behaved differently among the three organs. Roots had the highest GR activity, but this activity decreased in 14-d-old seedlings. In hypocotyls, GR activity was 2-fold higher in 14-d-old seedlings compared with 7- and 10-d-old seedlings. Green cotyledons showed the lowest GR activity in comparison with roots and hypocotyls, and this activity was very similar in 10- and 14-d-old plants.

The activity of the main NADP dehydrogenases in the different pepper organs during plant development is represented in Fig. 3. Activity of G6PDH was highest in hypocotyls of 10-d-old seedlings (Fig. 3A). In roots, G6PDH activity did not show significant differences between growth stages. In hypocotyls, activity was up to 1.5-fold higher in 10- and 14-d-old

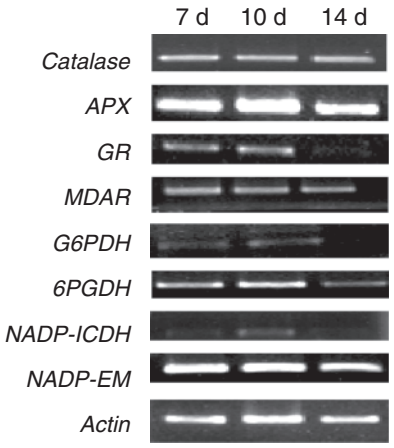

FIG. 4. mRNA expression of catalase, ascorbate peroxidase (*APX*), glutathione reductase (*GR*), monodehydroascorbate reductase (*MDAR*) and NADP dehydrogenases in whole pepper seedlings at different stages of development (7, 10 and 14 d old). Semiquantitative RT–PCR was performed on total RNA isolated from whole seedlings. Representative agarose electrophoresis gels of the amplification products visualized by ethidium bromide staining under UV light. Actin was used as internal control.

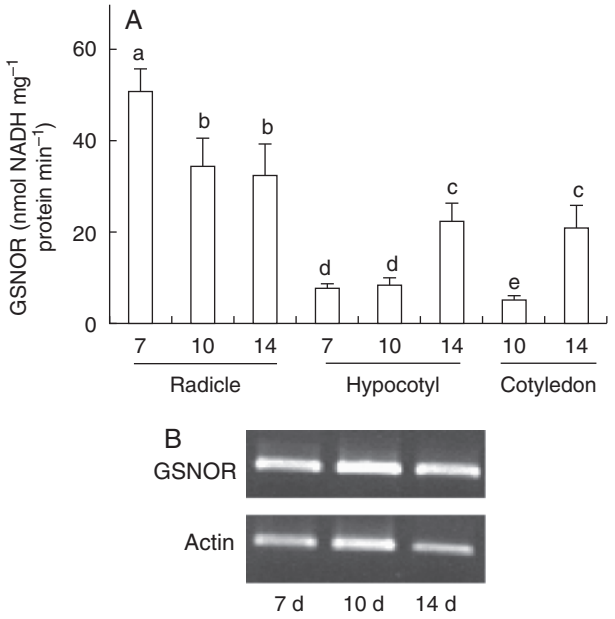

FIG. 5. Activity of *S*-nitrosoglutathione reductase (GSNOR) and *GSNOR* gene expression in radicles, hypocotyls and green cotyledons of pepper seedlings at different stages of development (7, 10 and 14 d old). (A) Spectrophotometric assay of GSNOR activity. Data are mean ± s.e.m. of at least three experiments. Different letters indicate significant differences between stages and organs ($P \leq 0{\cdot}05$). (B) mRNA expression of *GSNOR* in whole seedlings. Semiquantitative RT-PCR was performed on total RNA isolated from whole seedlings. Representative agarose electrophoresis gels of the amplification products visualized by ethidium bromide staining under UV light. *Actin* was used as internal control.

seedlings compared with 7-d-old seedlings. Green cotyledons had the lowest activity, but activity was 2·2-fold higher in cotyledons of 14-d-old seedlings compared with those of 10-d-old seedlings. The activity of 6PGDH is shown in Fig. 3B. The highest activity was observed in roots but there were no significant changes during the period analysed. In hypocotyls and green cotyledons, activity was 2·2-fold higher in 14-d-old seedlings than at younger ages. Activity of NADP-ICDH (Fig. 3C) showed different behaviour compared with both dehydrogenases of the pentose phosphate pathway (G6PDH and 6PGDH). Thus, in roots and hypocotyls NADP-ICDH activity significantly decreased from 7 to 14 d (4-fold and 2·4-fold, respectively). Among all organs and stages, the highest NADP-ICDH activity was found in roots of 7-d-old seedlings and the lowest activity in green cotyledons. The behaviour of NADP-ME differed among organs (Fig. 3D). In roots, activity decreased with age, being reduced by half in 14-d-old compared with 7-d-old seedlings. However, in hypocotyls activity increased 4-fold from 7 to 14 d. No difference in activity was observed in green cotyledons.

Transcript analysis of *catalase*, three enzymes of the antioxidative ascorbate-glutathione cycle (*APX*, *MDAR* and *GR*) and the family of NADP dehydrogenases, was performed by semiquantitative RT–PCR using whole seedlings of different ages (Fig. 4). No significant changes in antioxidant transcripts were observed, with the exception of *GR*, which was lower in 14-d-old seedlings. Expression of NADP dehydrogenase mRNAs was lower at 14 d than at younger ages, and in the case of *NADP-ICDH* there was maximum expression in 10-d-old seedlings.

Metabolism of RNS in the main organs during pepper seedlings development

As part of the analysis of RNS metabolism in pepper seedlings, we studied GSNOR activity and its mRNA expression (Fig. 5A, B), and the pattern of protein tyrosine nitration (NO_2-Tyr) in the different organs (Fig. 6).

The activity of GSNOR was highest in roots of 7-d-old seedlings, and decreased with age, being ~6-fold lower in roots of 14-d-old plants (Fig. 5A). However, its behaviour was different in hypocotyls and green cotyledons, as activity was 1·8-fold and 3·4-fold higher, respectively, in 14-d-old seedlings compared with 7- and 10-d-old seedlings. To analyse the mRNA expression of *GSNOR* by semiquantitative PCR, it was necessary to prepare a partial cDNA of this pepper gene. Thus, a cDNA of 1000 bp (accession number EU652335) that coded for a polypeptide of 333 amino acids was identified. This protein showed 96 % identity with alcohol dehydrogenase 3 of *Solanum tuberosum* (P14675), 91 % identity with *Lactuca sativa* (BAA07911) and 89 % identity with *Ricinus communis* (XP_002534157). Semiquantitative RT–PCR of mRNA expression of *GSNOR* is shown in Fig. 5B. The mRNA did not show any significant change at any of the stages of development analysed.

The tyrosine nitration pattern in the three organs of pepper seedlings analysed during natural development is presented in Fig. 6. The profile of immunoreactive bands differed among the three organs. In roots, the number of immunoreactive bands apparently did not change with the age of seedlings; however, the intensity of the immunoreactive bands of 91, 62, 56 and 38 kDa seemed to diminish (Fig. 6A). A similar result was observed in hypocotyls, with the immunoreactive bands of 91, 64, 42 and 34 kDa diminishing and only the 59-kDa band increased (Fig. 6B). In cotyledons the tyrosine nitration profile was different in comparison with the other organs (Fig. 6C).

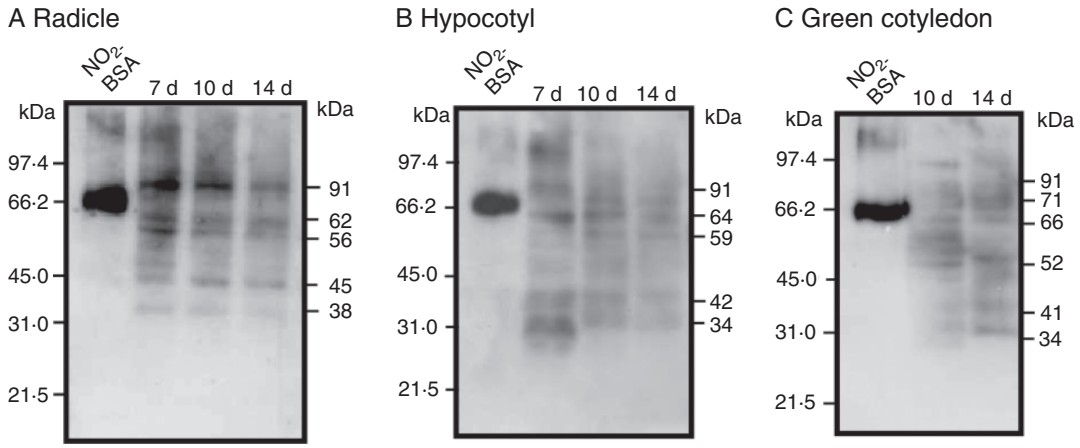

FIG. 6. Tyrosine nitration pattern in radicles, hypocotyls and green cotyledons of pepper seedlings at different stages of development (7, 10 and 14 d old). (A) Radicle samples (5 μg per lane). (B) Hypocotyl samples (10 μg per lane). (C) Cotyledon samples (20 μg per lane). Commercial nitrated BSA (NO_2-BSA; 2 μg of protein) was used as a positive control. Numbers to the right of the immunoblots indicate relative molecular masses of the detected immunoreactive bands, and relative molecular masses of the protein markers are shown to the left. Western blots were probed with a rabbit anti-nitrotyrosine polyclonal antibody at 1 : 8000 dilution.

Cellular detection of NO, $O_2^{\cdot-}$ and $ONOO^-$ by CLSM

Figure 7 illustrates the cellular localization of NO (panels A–C), $O_2^{\cdot-}$ (panels D–F) and $ONOO^-$ (panels G–I) in roots of 7-, 10- and 14-d-old pepper seedlings. Nitric oxide, observed as intense green fluorescence, was localized mainly in epidermal cells and root hairs, and was less prominent in vascular tissue of roots from 7-, 10- and 14-d-old seedlings (Fig. 7A–C). However in roots of 10-d-old seedlings, slight green fluorescence was also generally distributed in parenchyma cells of the cortex (Fig. 7B). The orange colour corresponds to autofluorescence, which was clearly observed in the parenchyma cells of the cortex (Fig. 7A, B). The superoxide radical, observed as green fluorescence in longitudinal root sections, showed a general distribution in the epidermal cells of roots, with a slight intensification in 14-d-old seedlings (Fig. 7F) in comparison with 7- and 10-d-old seedlings (Fig. 7D and E, respectively). Peroxynitrite, observed as intense green fluorescence, was localized in almost all cell types in the root cross-sections, including root hairs, epidermal cells, parenchyma cells of the cortex, endodermis, xylem and phloem of 7-d-old seedlings (Fig. 7G). Similar localization of $ONOO^-$ was observed in roots of 10- and 14-d-old seedlings, but with a lower intensity of green fluorescence in the parenchyma cells of the cortex (Fig. 7H, I). The orange colour corresponds to the autofluorescence distributed in almost all parenchyma cells of the cortex (Fig. 7I).

Figure 8 shows the cellular localization of NO (panels A–C), $O_2^{\cdot-}$ (panels D–F) and $ONOO^-$ (panels G–I) in transverse sections of hypocotyls of 7-, 10- and 14-d-old pepper seedlings. Nitric oxide, observed as intense green fluorescence, was localized mainly in epidermal cells and xylem, and was less prominent in cortex cells of hypocotyl sections of 7-d-old seedlings (Fig. 8A). In the case of 10- and 14-d-old seedlings, green fluorescence was distributed in all cell types, although it was less intense in epidermal cells (Fig. 8B and C, respectively). The orange colour corresponds to autofluorescence. Superoxide, observed as green fluorescence, was distributed in almost all cell types of the cortex in the hypocotyls of 7-d-old seedlings (Fig. 8D). In hypocotyls of 10-d-old seedlings, fluorescence was less intense (Fig. 8E) and in hypocotyls of 14-d-old seedlings it was again distributed in all cell types (Fig. 8F).

Figure 9 shows the cellular localization of NO (panels A and B), $O_2^{\cdot-}$ (panels C and D) and $ONOO^-$ (panels E and F) in transverse sections of green cotyledons of 10- and 14-d-old pepper seedlings. Nitric oxide, observed as intense green fluorescence, was localized in vascular tissue, epidermal and palisade mesophyll cells of green cotyledons of 10-d-old seedlings (Fig. 9A). In 14-d-old seedlings, green fluorescence intensity had diminished in all cell types (Fig. 9B). The orange colour corresponds to autofluorescence. Superoxide, observed as green fluorescence, showed a discrete distribution in spongy mesophyll cells and in vascular tissue in the cotyledons of 10- and 14-d-old seedlings (Fig. 9C, D). In the case of $ONOO^-$, green fluorescence was also present in spongy mesophyll cells and in vascular tissue in the cotyledons of 10-d-old seedlings (Fig. 9E) but was reduced in the green cotyledons of 14-d-old seedlings (Fig. 9F).

DISCUSSION

The pepper plant has enormous agronomical relevance since its fruits are the second most consumed vegetable worldwide and are also characterized for their high levels of vitamin C, provitamin A and calcium (Howard *et al.*, 1994). Thus, according to a Dietary Reference Intakes report, a medium-sized green bell pepper contains 177 % of the recommended dietary allowance of vitamin C (http://ods.od.nih.gov/factsheets/VitaminC-HealthProfessional/), which is necessary to maintain good cardiac and vascular health (Vanderslice and Higgs, 1991; Kirsh *et al.*, 2006). In previous studies we analysed some aspects of the metabolism of ROS and RNS in 30-d-old pepper plants exposed to low temperature during different periods of time that provoked nitro-oxidative stress (Airaki *et al.*, 2012) and in fruits during the ripening process (Mateos *et al.*, 2009, 2013). Considering that ROS and RNS are also involved in other physiological processes, including plant growth and development, in this work the key enzymatic elements involved in the

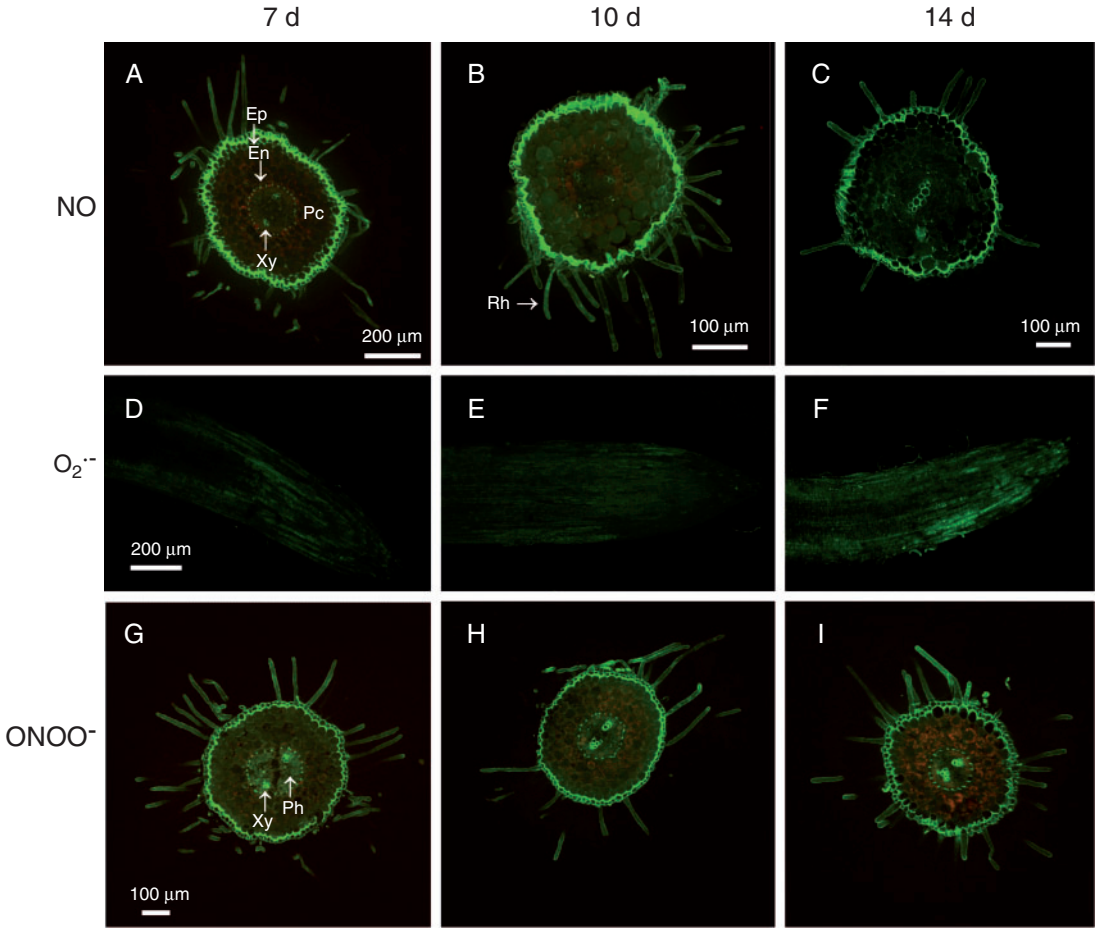

FIG. 7. Representative images of radicles of pepper seedlings illustrating confocal laser scanning microscopy (CLSM) detection and visualization of endogenous nitric oxide (NO), superoxide radical ($O_2^{\cdot-}$) and peroxynitrite ($ONOO^-$) at different stages of development (7, 10 and 14 d old). (A–C) Detection of endogenous NO using diaminofluorescein-FM diacetate as a fluorescent probe in cross-sections of pepper radicles. (D–F) Detection of endogenous $O_2^{\cdot-}$ using dihydroethidium as a fluorescent probe in root tips. (G–I) Detection and visualization of $ONOO^-$ using 3′-(*p*-aminophenyl) fluorescein as a fluorescent probe in transverse sections of pepper radicles. Strong and bright green fluorescence corresponds to each specific fluorescent probe in the corresponding panels. Each picture was prepared from 30 sections, which were analysed by CLSM. The orange–yellow colour corresponds to autofluorescence. En, endodermis; Ep, epidermis; Pc, parenchyma cells of the cortex; Ph, phloem; Rh, root hair; Xy, xylem.

metabolism of ROS and RNS during the early stages of pepper seedlings (7–14 d old) were analysed in the main organs (roots, hypocotyls and green cotyledons) of this plant. It must be pointed out that, due to the small size of the different organs during the developmental stage, of seedlings, it was decided to use individual organs for the analysis of biochemical parameters, whereas whole seedlings were used for the gene expression analyses. To our knowledge there are no reports that provide a wide overview of these components and its modulation in the establishment of higher plant seedlings, something which is critical for the future success of the plant and its fruit quality.

Catalase has the highest activity in the aerial part of pepper seedlings, whereas the enzymatic components of the ascorbate glutathione cycle show higher activity in roots

Reactive oxygen species are produced during normal cellular metabolism, being involved in many physiological aspects. For example in seed physiology, ROS are involved in the disruption of the seed dormancy (Bailly, 2004; Bailly *et al*., 2004; Oracz *et al*., 2007, 2009; Müller *et al*., 2009) since some ROS serve as messenger molecules in seedling growth and developmental processes (Rodríguez *et al*., 2002; Foreman *et al*., 2003; Carol and Dolan, 2006; Swanson and Gilroy, 2010).

The main function of catalase is the removal of H_2O_2, either generated inside peroxisomes due to their own metabolism or accumulated in this organelle through cell metabolism. Our results on catalase activity in the early days of development of pepper seedlings showed that there were significant differences among the analysed organs, roots having the lowest activity. Thus, catalase is probably the main antioxidant enzyme involved in the control of H_2O_2 in the aerial part of pepper seedlings during development. Similar behaviour of catalase activity has been reported in other plant species, such as maize (*Zea mays*) seedlings (Redinbaugh *et al*., 1990). In other species, the inhibition of catalase has been reported to contribute to a higher level of H_2O_2, which was necessary to break the seed

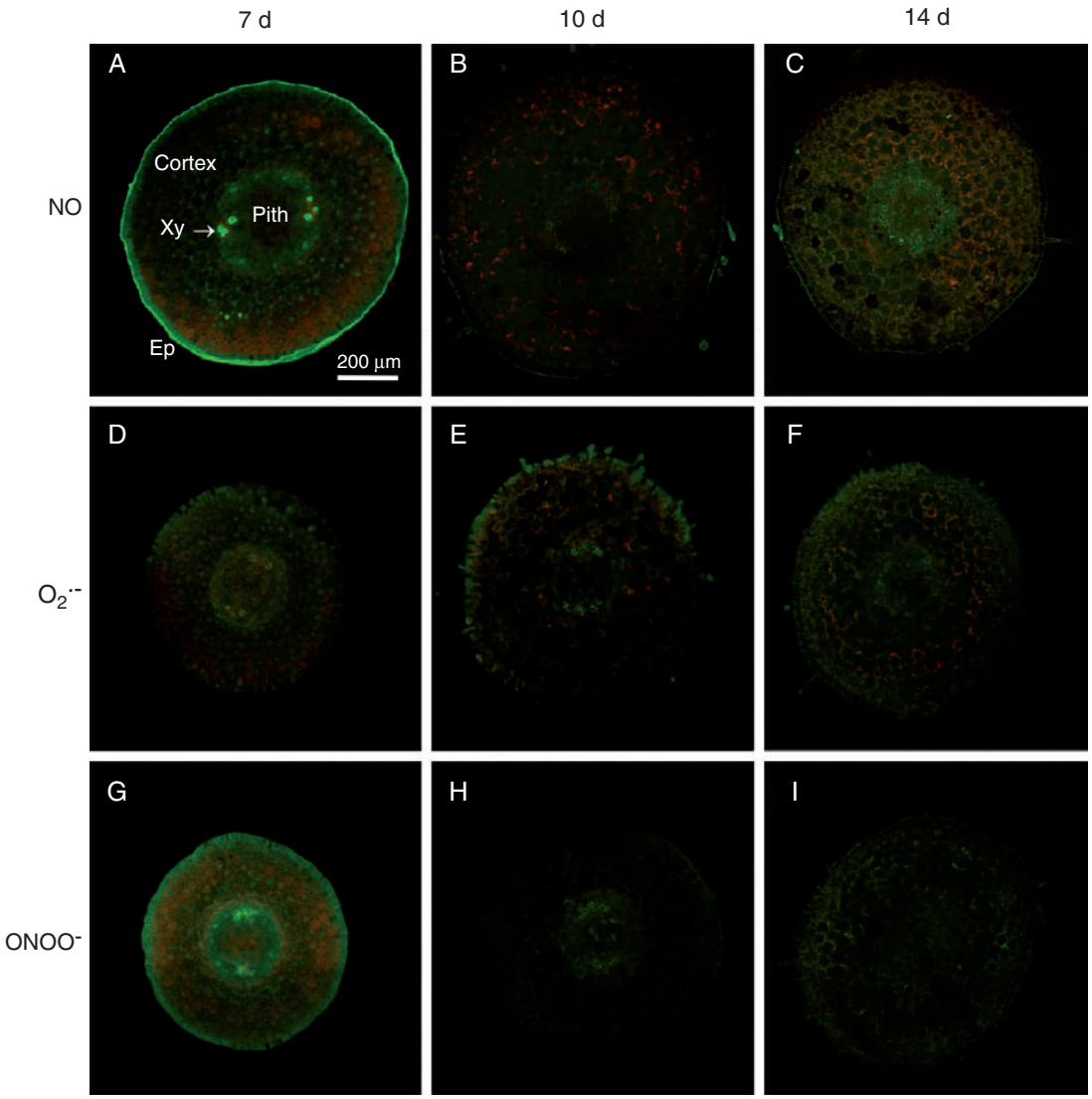

FIG. 8. Representative images of transverse sections of hypocotyl of pepper seedlings illustrating confocal laser scanning microscopy (CLSM) detection and visualization of endogenous nitric oxide (NO), superoxide radical ($O_2^{\cdot-}$) and peroxynitrite ($ONOO^-$) at different stages of development (7, 10 and 14 d old). (A–C) Detection of endogenous NO using diaminofluorescein-FM diacetate as a fluorescent probe. (D–F) Detection of endogenous $O_2^{\cdot-}$ using dihydroethidium as a fluorescent probe. (G–I) Detection of $ONOO^-$ using 3′-(*p*-aminophenyl) fluorescein as a fluorescent probe in transverse sections. Strong and bright green fluorescence correspond to each specific fluorescent probe in the corresponding panels. Each picture was prepared from 30 sections, which were analysed by CLSM. The orange–yellow colour corresponds to autofluorescence. En, endodermis; Ep, epidermis; Xy, xylem.

dormancy in lettuce (*Lactuca sativa*) (Hendricks and Taylorson, 1975). In other cases, such as oil seedlings during the transition from dark to light conditions, catalase activity and/or isozyme number are differentially modulated. This modulation has been described in cotton (*Gossypium hirsutum*) (Ni *et al*., 1990; Ni and Trelease, 1991) and pumpkin (Yamaguchi *et al*., 1995). However, APX, which is part of the ascorbate–glutathione cycle, showed the highest activity in roots in the present study, suggesting that these two systems are complementary in regulating the level of H_2O_2 during seedling development. The activities of the other enzymes of the ascorbate–glutathione cycle, MDAR and GR, followed the same pattern as APX activity, being higher in the roots than in the other organs. Taking these results together, the highest antioxidant activity of these enzymes, which are differentially modulated in roots, hypocotyls and cotyledons, might allow speculation about the possible involvement of ROS in the processes of growth and development in pepper seedlings. The involvement of antioxidant enzymes has also been reported in other plants. During germination and the early stages of root development in *Ipomoea triloba*, the activities of catalase, superoxide dismutase and guaiacol peroxidase were probably the crucial enzymes involved in the neutralization of ROS, since they had higher levels of activity compared with other enzymes, such as APX and GR (Pergo and Ishii-Iwamoto, 2011). In germinating pea (*Pisum sativum*) seeds, increases in catalase and superoxide dismutase activities have also been reported, probably due to the increased oxygen uptake which is accompanied

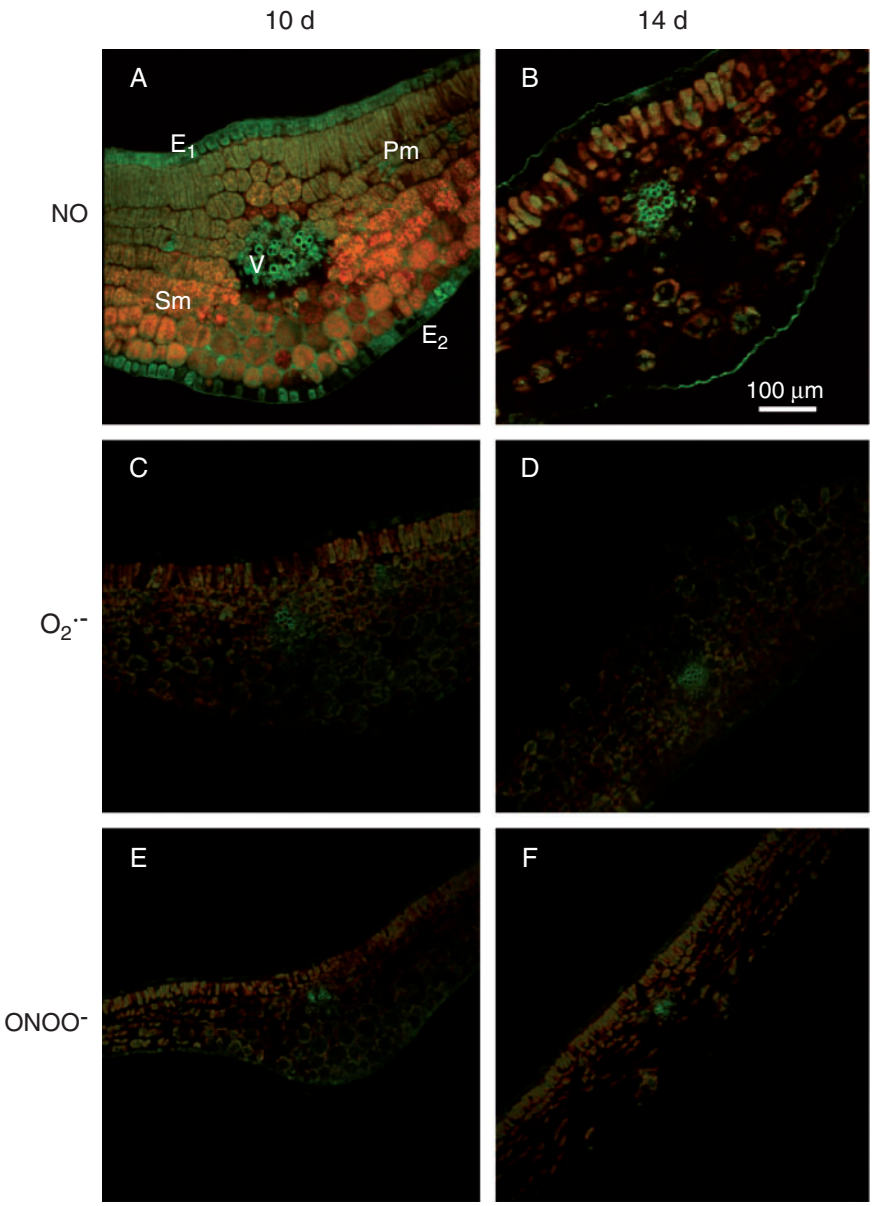

FIG. 9. Representative images of transverse sections of green cotyledons of pepper seedlings illustrating confocal laser scanning microscopy (CLSM) detection of endogenous nitric oxide (NO), superoxide radical ($O_2^{\cdot-}$) and peroxynitrite ($ONOO^-$) at different stages of development (10 and 14 d old). (A, B) Detection of endogenous NO using diaminofluorescein-FM diacetate as a fluorescent probe. (C, D) Detection of endogenous $O_2^{\cdot-}$ using dihydroethidium as a fluorescent probe in root tips. (E, F) Detection and visualization of $ONOO^-$ using 3′-(*p*-aminophenyl) fluorescein as a fluorescent probe. Strong and bright green fluorescence corresponds to each specific fluorescent probe in the corresponding panels. Each picture was prepared from 30 sections analysed by CLSM. The orange–yellow colour corresponds to autofluorescence. E_1, adaxial epidermis; E_2, abaxial epidermis; Pm, palisade mesophyll; Sm, spongy mesophyll; V, main vein.

by a set of events (mobilization of fatty acids, cell elongation and division in the embryo, radical protrusion, etc.) including the release of ROS (Wojtyla *et al*., 2006).

Roots have the highest capacity to regenerate NADPH via NADP dehydrogenases

NADPH is an essential cofactor in cell growth, proliferation and detoxification in eukaryotic cells. It is remarkable that, among the three pepper organs analysed, the activity of NADP-ICDH and NADP-ME was highest in roots and especially in 7-d-old seedlings, suggesting a higher demand of NADPH for growth in this period. NADP-ICDH catalyses the oxidative decarboxylation of isocitrate to 2-oxoglutarate, with the concomitant production of the reduced coenzyme NADPH and it has therefore been associated with the metabolism of amino acids and ammonia (Fieuw *et al*., 1995; Gálvez and Gadal, 1995; Palomo *et al*., 1998). In this context, the high NADP-ICDH activity found in roots of 7-d-old seedlings must be related to the relevance of this enzyme in the metabolism of nitrogen, which is critical for plant growth. These results are in a good

agreement with previous data in 3-week-old pea plants, in which NADP-ICDH activity was 3-fold higher in roots than in green leaves (Chen *et al*., 1988), and in 6-week old *Arabidopsis* plants, in which NADP-ICDH activity in roots was 3.2-fold higher than in leaves (Leterrier *et al*., 2012).

On the other hand, during the imbibition and germination process in soybean seeds there was an increase in the NADPH/NADP ratio, which was mainly due to NADP-ICDH activity and to a lesser extent to G6PDH (Duke *et al*., 1977). In pine, NADP-ICDH is also implicated in the primary development of seedlings (Palomo *et al*., 1998; Pascual *et al*., 2008). NADP-ME catalyses the reaction of malate with NADP to yield α-oxaloacetate and NADPH. Thus, this activity is involved in different processes, including pyruvate metabolism, carbon fixation and lignin biosynthesis (Schaaf *et al*., 1995). Therefore, the high NADP-ME activity in roots could also be correlated with the demand for both NADPH and α-oxalacetate by biosynthetic pathways such as gluconeogenesis and the amino acid and fatty acid synthesis necessary for root growth. Activities of G6PDH and 6PGDH were also higher in roots and hypocotyl seedlings in the early days of development.

The highest activity of the NADP dehydrogenases observed in roots of 7-d-old seedlings again suggests a higher demand for NADPH by biosynthetic pathways, such as sugar biosynthesis, fatty acid and carotenoid biosynthesis. Moreover, the presence of both dehydrogenases (G6PDH and 6PGDH) of the pentose phosphate pathway also supports the efficient recycling of NADPH for its subsequent use in the ascorbate–glutathione cycle, which would help to reduce a potential overproduction of H_2O_2 in the cell (Mateos *et al*., 2013). Furthermore, it is also well recognized that NADPH, together with glutathione and ascorbate, modulates the redox state of the cell and transmits the necessary information that regulates signalling pathways (Noctor, 2006; Gallie, 2013). Therefore, the generated NADPH may be used by the plasma membrane NADPH oxidase of plant cells to produce ROS, which are involved in cell expansion through the activation of Ca^{2+} channels (Foreman *et al*., 2003).

We observed a discrepancy between the expression of genes encoding antioxidant enzymes and NADP dehydrogenases and their corresponding activities in the different seedling growth stages analysed (7, 10 and 14 d) since gene expression was not affected, with the exception of *GR*, *G6PDH* and *ICDH*, which showed slight modulation. This could suggest that the modulation of these enzymes during this period is due to post-translational regulations. This behaviour has previously been observed in pepper plants exposed to low temperature (Airaki *et al*., 2012) and in pepper fruits during the ripening process (Mateos *et al*., 2009, 2013). However, it will be possible to interpret these observations more precisely when the different isoenzymes of these enzymatic systems have been identified and localized.

The NO content of pepper seedlings is modulated differentially during development depending on the organ

Nitric oxide plays an important role in a wide range of physiological responses during growth and development of plants (Lamattina *et al*., 2003; Shapiro, 2005; Corpas *et al*., 2006; Wilson *et al*., 2008). This gas can regulate plant growth depending on its concentration, such that low concentrations stimulate growth but high concentrations may inhibit it (Anderson and Mansfield, 1979; Hufton *et al*., 1996; Leshem and Haramaty, 1996; Gouvea *et al*., 1997; Jin *et al*., 2009). The NO molecule seems to be synthesized mainly in tissues that are in the growth phase, such as the embryonic axis and cotyledons, whereas lower levels are produced in mature and senescent organs (Beligni and Lamattina, 2001). In roots the application of exogenous NO induces root elongation (Kopyra and Gwóźdź, 2003) and causes the formation of roots spontaneously (Pagnussat *et al*., 2002). Our experimental model showed the presence of NO in the root epidermis of 7- and 10-d-old seedlings and this may indicate the involvement of NO in a signalling cascade during development and the formation of new roots. Kopyra and Gwóźdź (2003) reported that NO donors counteract the inhibitory effects of heavy metals and salinity on the root growth, and this suggests that the NO detected in roots may have a protective role in abiotic stress conditions. Furthermore, production of NO in the root could be involved in the interaction of the plant with beneficial soil microorganisms, including mycorrhiza and rhizobacteria (Stöhr and Ullrich, 2002; Horchani *et al*., 2011) and as a mechanism of defence against pathogens (Murakami *et al*., 2011).

The analysis of tissue NO showed that each organ had a different content depending on the age of the seedling. The hypocotyl of 7-d-old seedling had a clearly higher NO content, which could participate in elongation, meristem development and communications between organs through the vascular system. Similar behaviour was reported during the early development of the main organs of pea seedlings at 5, 7, 9, 11, 13 and 15 d of growth, and stems of 11-d-old seedlings showed the highest NO production (Corpas *et al*., 2006). On the other hand, the cellular localization of NO showed that this free radical is mainly located in epidermal cells and vascular tissue, which makes sense considering that NO production has been reported to be involved in the formation of xylem (Gabaldón *et al*., 2005). In roots, NO clearly accumulated in the root hairs, which also correlates well with the involvement of NO in determining lateral root development (Correa-Aragunde *et al*., 2004), nitrate perception and root elongation (Manoli *et al*., 2014). The cellular localization of peroxynitrite and the superoxide radical follows a similar pattern, especially in roots, where they may be involved in defence mechanisms or lignification, considering that the superoxide radical, some peroxidases and NO participate in the lignification of vascular tissue (Puntarulo *et al*., 1991; Ogawa *et al*., 1997; Gabaldón *et al*., 2005; Gómez-Ros *et al*., 2012). In addition, it has been reported recently that one of the primary events in initially differentiating tracheary elements during root xylem development is a burst of NO in thin-walled cells, followed by synthesis of H_2O_2 (Bagniewska-Zadworna *et al*., 2014).

Currently, it is well established that *S*-nitrosoglutathione (GSNO) is the preferred physiological substrate of the glutathione-dependent enzyme formaldehyde dehydrogenase (Leterrier *et al*., 2011; Corpas *et al*., 2013*a*), which catalyses the NADH-dependent reduction of GSNO to GSSG and NH_3. For this reason, this enzyme has been renamed as *S*-nitrosoglutathione reductase (GSNOR). The presence of GSNOR activity in plants has been conclusively demonstrated in many species (Sakamoto *et al*., 2002; Feechan *et al*., 2005; Barroso *et al*.,

2006; Espunya *et al*., 2006; Leterrier *et al*., 2011); this GSNOR activity is necessary for normal plant development under optimal growth conditions (Lee *et al*., 2008; Kwon *et al*., 2012).

In *Arabidopsis*, analysis of the activity and expression of GSNOR by immunolocalization and histochemical methods showed that this protein was differentially expressed, being higher in roots and leaves from the earliest stages of development (Espunya *et al*., 2006). Our results are in good agreement with these data because roots of 7-d-old seedlings showed the highest activity, and activity diminished with seedling age. Conversely, GSNOR activity increased with seedling age in hypocotyls and cotyledons. Furthermore, over-expression and deletion of *GSNOR* in transgenic *Arabidopsis* plants produced a short root phenotype that correlated with a low level of intracellular glutathione and altered spatial distribution in the roots. This suggested that GSNOR and, consequently GSNO, could be involved in the regulation of the redox state of the root (Espunya *et al*., 2006). In this context, to our knowledge this is the first time that the *GSNOR* gene has been reported in pepper plants. Moreover, the analysis of this gene in other plant species, including *Arabidopsis*, tomato and tobacco, has shown that it is present in a single copy (Díaz *et al*., 2003; Lee *et al*. 2008; Xu *et al*., 2013; Kubienová *et al*., 2013).

Nitration of tyrosine is a covalent modification of proteins by the addition of a nitro group (-NO_2) forming 3-nitrotyrosine (Gow *et al*., 2004; Radi, 2004). In plants, this modification of proteins has been used as a reliable marker of nitro-oxidative stress situations (Corpas *et al*., 2007, 2013*b*; Valderrama *et al*., 2007; Airaki *et al*., 2012). However, the presence of this post-translation modification mediated by NO-derived molecules in plants under normal physiological conditions suggests that nitration could have an additional function. In pepper seedlings grown under optimal conditions, the pattern of protein nitration in roots, hypocotyls and green cotyledons during the different stages of development was quite different. Several reports have identified some protein targets of this post-translation modification mediated by NO-derived molecules such as peroxynitrite. Thus, nitrated proteins have been identified in 9-d-old sunflower hypocotyls (Chaki *et al*., 2009), 2-week old *Arabidopsis* seedlings (Lozano-Juste *et al*., 2011), bitter orange (*Citrus aurantium*) roots (Tanou *et al*., 2009) and 71-d-old pea roots (Begara-Morales *et al*., 2013). Interestingly, some antioxidant enzymes were found among the identified nitrated proteins, such as catalase, APX, MDAR, GR and NADP-ICDH. In some cases, it has been demonstrated that nitration provokes inactivation of the enzyme activity (Corpas *et al*., 2015), which reflects a clear interplay between ROS and RNS metabolism. Moreover, in rice seedlings grown under normal conditions it has been reported that the tyrosine nitration of α-tubulin inhibits cell division and, consequently, cell growth (Jovanović *et al*., 2010), which provides evidence for the interconnection between post-translational modifications mediated by NO during plant development.

In summary, the present results show that during the development of pepper seedlings there is differential regulation of the metabolism of ROS, NO and NADP dehydrogenases, depending on the plant organ. Under these circumstances, it can be concluded that the metabolism of ROS and RNS seems to contribute significantly to plant development since their components are involved directly or indirectly in many metabolic pathways (β-oxidation, carbon and nitrogen metabolism, etc.). Thus, specific molecules such as H_2O_2 and NO have signalling implications (regulating gene expression and mediating post-translational modifications, such as nitration and *S*-nitrosylation, which affect protein function) (Begara *et al*., 2013, 2014) and their temporal and spatial regulation must contribute to the success of seedling establishment. Additionally, considering the NO profile during seedling development, it seems that this gaseous radical must be involved in the signalling cascade that allows root formation and elongation of pepper plants, as has been proposed for tomato (Correa-Aragunde *et al*., 2004) and sunflower plants (Corti-Monzón *et al*., 2014).

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