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Biochemical responses of the ethylene-insensitive *Never ripe* tomato mutant subjected to cadmium and sodium stresses

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ABSTRACT

In order to further address the known interaction between ethylene and components of the oxidative system, we have used the ethylene-insensitive *Never ripe* (*Nr*) tomato (*Solanum lycopersicum* L.) mutant, which blocks ethylene responses. The mutant was compared to the control Micro-Tom (MT) cultivar subjected to two stressful situations: 100 mM NaCl and 0.5 mM $CdCl_2$. Leaf chlorophyll, lipid peroxidation and antioxidant enzyme activities in roots, leaves and fruits, and Na and Cd accumulation in tissues were determined. Although we verified a similar growth pattern and Na and Cd accumulation for MT and *Nr*, the mutant exhibited reduced leaf chlorophyll degradation following stress. In roots and leaves, the patterns of catalase (CAT), glutathione reductase (GR), ascorbate peroxidase (APX), guaiacol peroxidase (GPOX), superoxide dismutase (SOD) enzyme activity as well as malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) production under the stressful conditions tested were very similar between MT and *Nr* mutant. However, *Nr* fruits showed increased H_2O_2 production, reduced and enhanced APX activity in NaCl and $CdCl_2$, respectively, and enhanced GPOX in NaCl. Moreover, through non-denaturing PAGE, a similar reduction of SOD I band intensity in both, control MT and *Nr* mutant, treated with NaCl was observed. In leaves and fruits, a similar SOD activity pattern was observed for all periods, genotypes and treatments. Overall the results indicate that the ethylene signaling associated with NR receptor can modulate the biochemical pathways of oxidative stress in a tissue dependent manner, and that this signaling may be different following Na and Cd exposure.

1. Introduction

Salinity and metal stresses are two severe detrimental factors among the abiotic stresses which affect crop production (Gomes-Junior et al., 2006a; Teklic et al., 2008; Chen et al., 2010). A number of toxic effects of both stress types have been reported to affect plant development. For example, salinity causes lower soil water potential and an imbalance in the uptake of mineral nutrients and their accumulation within plants (Munns and Tester, 2008). Similarly, metal excess can alter the uptake of minerals by plants by reducing the availability of minerals, or by lowering the population of soil microbes in the soil (Moreno et al., 1999). Transpiration and photosynthesis are examples of processes that have been reported to be affected by metal excess (Pietrini et al., 2003; Guoa et al., 2007; Hattab et al., 2009).

The adverse effects induced by salinity and metal stress on plant growth and development involve biochemical disturbance mainly through oxidative damage. An exaggerated increase in the production of reactive oxygen species (ROS), such as the superoxide radical (O^{-}_2) and hydrogen peroxide (H_2O_2), triggers a complex detoxification mechanism which may involve non-enzymatic and enzymatic systems capable of preventing the cascades of uncontrolled oxidation (Gratão et al., 2005; Hafsi et al., 2010). Plant ROS-scavenging mechanisms include the action of some key enzymes, such as superoxide dismutase (SOD, EC 1.15.1.1), which dismutates O^{-}_2 to H_2O_2. Subsequently, H_2O_2 may be detoxified to H_2O by ascorbate peroxidase (EC 1.11.1.11), catalase (CAT, EC 1.11.1.6) and glutathione peroxidase (EC 1.11.1.9), among other peroxidases (Gratão et al., 2005; Passardi et al., 2007). In addition, for the detoxification of H_2O_2, phenolics can act as antioxidants by donating electrons to guaiacol-type peroxidases [guaiacol peroxidase (GPOX), EC 1.11.1.7] (Sakihama et al., 2002). The ascorbate–glutathione cycle is closed by regeneration of reduced glutathione (GSH) from oxidized glutathione (GSSG) by glutathione reductase (GR, EC 1.6.4.2) using NAD(P)H as a reducing agent (Foyer and Noctor, 2009).

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The literature has numerous reports published over the last fifty years which tend to support the involvement of ethylene in the response of plants to several stresses, including sodium (Na) and cadmium (Cd) (Iakimova et al., 2005; Cao et al., 2007; Liu et al., 2008). Although ethylene is generally believed to be as a stress-hormone (Kende, 1993) and ethylene signaling regulates multiple stress responses (Cao et al., 2009), it is not clear what specific roles it plays in signaling of stress responses (Cao et al., 2008). This raises questions as to whether the ethylene produced during stress is a causal agent or a consequence (Liu et al., 2008). For example, exogenous ethylene greatly stimulated Cd-induced stress and Cd treatment enhanced endogenous ethylene production in tomato (Iakimova et al., 2005), while sodium (Na) stress induced ethylene production in some species, whereas for others, ethylene levels were reduced (Zapata et al., 2007). Thus, these issues fall into the difficulty of understanding how ethylene interacts with ROS during stress. External addition of ethylene during cell death in Arabidopsis increased ROS production and caused increased cell death (Overmyer et al., 2000). On the other hand, exogenous application of H_2O_2 increased ethylene production in pine needles in a concentration-dependent manner (Ivenish and Tillberg, 1995), whereas Ke and Sun (2004) verified that it was the superoxide radical, but not H_2O_2, which was involved directly in osmotic stress-inducible ethylene biosynthesis. Despite all these aspects, it is now becoming clear that various ethylene-regulated stress responses are essential for stress tolerance and plant survival (Jung et al., 2009), but it is not clear which of the ethylene-dependent changes that occur in the oxidative system contributes to this tolerance.

Ethylene mutants have been powerful tools in genetic and biochemical research to elucidate ethylene modulation during stress. Analyses of these mutants have led to an understanding of the cell changes induced by ethylene as well as the identification of components of the ethylene signaling pathway involved in the response to a series of different stresses (Vahala et al., 2003; He et al., 2005; Wang et al., 2008; Kim et al., 2008; Chen et al., 2009). However, many major questions about the biochemical processes still remain to be answered (Tieman et al., 2000; Barry et al., 2005).

In this study, we have examined some aspects of the biochemical basis of oxidative stress in the dominant *Never-ripe* (*Nr*) tomato mutant (Wilkinson et al., 1995), which is blocked in ethylene perception (Lanahan et al., 1994), when it is exposed to sodium (Na) and Cd treatments. Although several researchers have used *Nr* as a tool to assess the role of ethylene in a range of environmental stresses (Lund et al., 1998; Ciardi et al., 2000; O'Donnell et al., 2001; Castagna et al., 2007), here we have indicated that the ethylene signaling associated with the NR receptor can modulate the biochemical pathways of oxidative stress in a tissue dependent manner, and that this signaling may be different for Na and Cd stresses.

2. Materials and methods

2.1. Plant material and growth conditions

Seeds of the Micro-Tom (MT) cultivar of tomato (*Solanum lycopersicum* L.) and its introgressed ethylene-insensitive *Never ripe* mutant (Lima et al., 2009; Carvalho et al., 2010) were sown in trays containing a 1:1 mixture of commercial substrate (Plantmax HT, Eucatex, Brazil) and expanded vermiculite, supplemented with 1 g l^{-1} 10:10:10 NPK and 4 g l^{-1} lime ($MgCO_3 + CaCO_3$) and maintained in a greenhouse with an average mean temperature of 28 °C, 11.5 h/13 h (winter/summer) photoperiod, and 250–350 μmol m^{-2} s^{-1} PAR irradiance (natural radiation reduced with a reflecting mesh (Aluminet–Polysack Industrias Ltda., Itápolis, Brazil). After the first true leaves appeared, seedlings were transplanted to 0.350 l Leonard pots (Vincent, 1975) (1 seedling per pot) filled with sand and polystyrene (1:1). Two days after transplanting, Hoagland's nutrient solution (Hoagland and Arnon, 1950) (0.250 mL) was added and renewed weekly. Up to the stage of anthesis, Leonard pots were maintained at zero NaCl and $CdCl_2$, by receiving Hoagland's solution only. At anthesis, 108 Leonard pots received Hoagland's solution containing 100 mM NaCl and 108 Leonard pots received Hoagland's solution containing 0.5 mM $CdCl_2$. At zero, 7, 20 and 36 days after anthesis, leaves, roots and fruits were collected, washed in distilled-deionized water and stored at −80 °C for further biochemical analysis.

2.2. Lipid peroxidation

Lipid peroxidation was determined by estimating the content of thiobarbituric acid reactive substances (TBARS) following the method of Heath and Packer (1968). The concentration of malondialdehyde (MDA) equivalents was calculated using an extinction coefficient of 1.55×10^{-5} mol^{-1} cm^{-1}.

2.3. Extraction and analysis of antioxidant enzymes

The following steps were carried out at 4 °C unless stated otherwise. Roots, leaves and fruits were homogenized (2:1, buffer volume:fresh weight) in a mortar with a pestle in 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM ethylenediaminetetraacetic acid (EDTA), 3 mM DL-dithiothreitol and 5% (w/v) insoluble polyvinylpolypyrrolidone (Azevedo et al., 1998). The homogenate was centrifuged at 10,000 × *g* for 30 min, and the supernatant was stored in separate aliquots at −80 °C prior to enzymatic analysis.

2.3.1. Catalase assay

Catalase (CAT) activity was assayed spectrophotometrically at 25 °C in a reaction mixture containing 1 mL of 100 mM potassium phosphate buffer (pH 7.5), which contains 2.5 μl of H_2O_2 (30% solution) prepared immediately before use. The reaction was initiated by the addition of 15 μl of plant extract, and the activity was determined by monitoring the removal of H_2O_2 at 240 nm over 1 min against a plant extract-free blank (Gomes-Junior et al., 2007). CAT activity is expressed as μmol min^{-1} mg^{-1} protein.

2.3.2. Glutathione reductase assay

Glutathione reductase (GR) activity was assayed spectrophotometrically at 30 °C in a mixture containing 3 mL of 100 mM potassium phosphate buffer (pH 7.5), which contains 1 mM 5,5″-dithiobis (2-nitrobenzoic acid), 1 mM oxidized glutathione (GSSG) and 0.1 mM NADPH. The reaction was started by the addition of 50 μl of enzyme extract. The rate of reduction of GSSG was followed by monitoring the increase in absorbance at 412 nm over 2 min (Gomes-Junior et al., 2006b). GR activity is expressed as μmol min^{-1} mg^{-1} protein.

2.3.3. Guaiacol peroxidase assay

Guaiacol peroxidase (GPOX) activity was determined as described by Gomes-Junior et al. (2006b). One enzyme activity unit (U) of GPOX corresponds to an increase of 0.001 in absorbance per min per mg protein. The reaction medium contained 250 μl phosphate–citrate buffer (sodium phosphate dibasic 0.2 M:citric acid 0.1 M) pH 5.0, 150 μl enzyme extract and 25 μl 0.5% guaiacol, which was vortex shaken and incubated at 30 °C for 15 min. The reaction was stopped by quickly cooling in an ice water bath, followed by the addition of 25 μl of 2% sodium metabisulphide

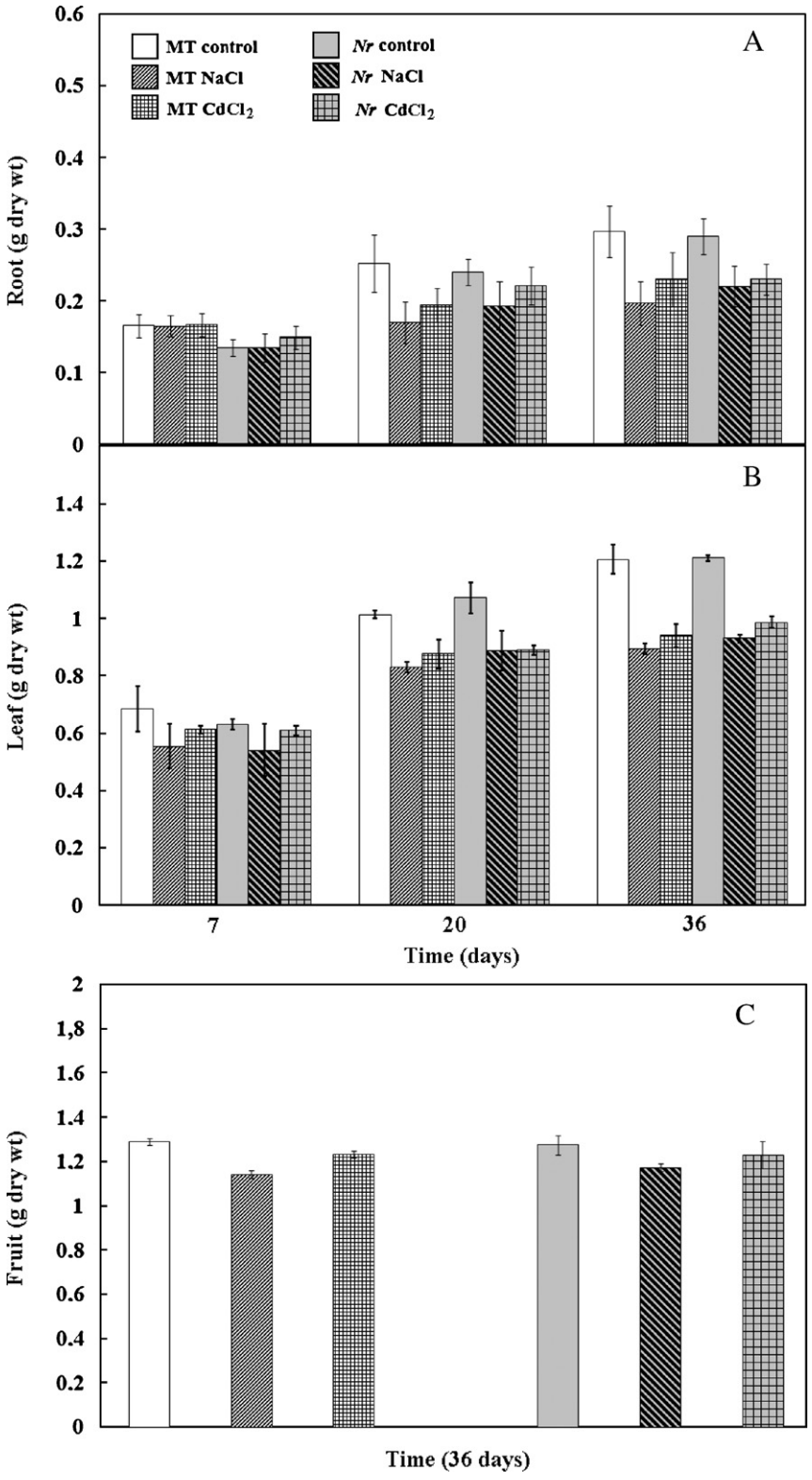

Fig. 1. Dry mass (g dry wt) in roots (A), leaves (B) and fruit (C) of *Nr* and MT grown, from anthesis, in the presence of NaCl (100 mM) and $CdCl_2$ (0.5 mM). The control plants were untreated. Values are the means of three replicates ± SEM.

solution. The reaction mixture was held for 10 min, and the GPOX activity was evaluated by monitoring the absorbance at 450 nm.

2.3.4. Ascorbate peroxidase

Ascorbate peroxidase (APX) activity was determined by monitoring the rate of ascorbate oxidation at 290 nm at 30 °C. The reaction was initiated by the addition of 40 μl plant extract to 1 mL of a medium containing 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbate, 0.1 mM EDTA and 0.1 mM H_2O_2. APX activity, expressed as nmol ascorbate min^{-1} mg^{-1} protein was calculated using the extinction coefficient 2.8 mM^{-1} cm^{-1} for ascorbate (Nakano and Asada, 1981).

Table 1
Sodium (Na) and cadmium (Cd) accumulation (mmol g^{-1} dry weight) during control Micro-Tom (MT) and *Never ripe* (*Nr*) mutant development. The plants were subjected to NaCl and $CdCl_2$ treatment and samples analyzed after 7, 20 and 36 days. The values represent mean ± SEM.

Organ	Genotype	Time (days)					
		NaCl			$CdCl_2$		
		7	20	36	7	20	36
Root	MT	48.66 ± 2.93[a]	70.43 ± 6.17[a]	71.07 ± 10.16[a]	*	*	*
		438.64 ± 22.18[b]	672.36 ± 0.82[b]	640.35 ± 7.46[b]	1.40 ± 0.18[c]	8.70 ± 0.82[c]	24.55 ± 0.88[c]
	Nr	62.41 ± 7.70[a]	79.40 ± 19.24[a]	110.14 ± 10.58[a]	*	*	*
		387.41 ± 111.3[b]	710.7 ± 19.21[b]	768.42 ± 66.54[b]	3.42 ± 0.68[c]	10.24 ± 0.42[c]	27.22 ± 2.45[c]
Leaf	MT	37.78 ± 1.10[a]	46.10 ± 6.92[a]	72.36 ± 17.43[a]	*	*	*
		342.58 ± 30.87[b]	845.26 ± 57.63[b]	1082.19 ± 48.34[b]	0.59 ± 0.02[c]	2.13 ± 0.40[c]	4.65 ± 0.22[c]
	Nr	37.78 ± 3.99[a]	60.19 ± 26.98[a]	64.03 ± 25.07[a]	*	*	*
		326.57 ± 34.63[b]	755.61 ± 79.98[b]	979.73 ± 38.42[b]	0.63 ± 0.08[c]	2.17 ± 0.23[c]	5.16 ± 1.03[c]
Fruit[d]	MT	–	–	23.69 ± 9.07[a]	–	–	*
		–	–	150.48 ± 14.54[b]	–	–	0.20 ± 0.06[c]
	Nr	–	–	23.69 ± 9.85[a]	–	–	*
		–	–	144.71 ± 9.85[b]	–	–	0.23 ± 0.00[c]

[*] The same values obtained with NaCl treatment.
[a] 0 mM NaCl.
[b] 100 mM NaCl.
[c] 0.5 mM $CdCl_2$.
[d] Values obtained only at 36 days; data not available for 0 day; (–) data not available.

2.4. Polyacrylamide gel electrophoresis (PAGE) and superoxide dismutase (SOD) activity staining

Electrophoretic analysis was carried out under non-denaturing condition in 10% polyacrylamide gels, followed by superoxide dismutase (SOD) activity staining as described by Vitória et al. (2001), with equal amounts of protein (60 μg) being loaded onto each gel lane. Electrophoresis buffers and gels were prepared as described by Gratão et al. (2008), except that SDS was excluded.

2.5. Hydrogen peroxide content

The content of H_2O_2 was determined according to Alexieva et al. (2001). Plant tissues were homogenized in 0.1% (m/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 12,100 × *g*, 15 min, 4 °C, and 200 μl of the supernatant was added to 200 μl of 100 mM potassium phosphate buffer (pH 7.0) and 800 μl of 1 M KI. The absorbance was read at 390 nm. H_2O_2 content for all samples was determined using H_2O_2 as a standard.

2.6. Determination of protein concentration

The protein content of all samples was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

2.7. Cadmium and sodium content

Assays for the determination of the Na and Cd contents of the plant tissues, were performed following digestion with a mixture of nitric and perchloric acids according to Malavolta et al. (1997). Na and Cd concentrations were measured using a flame photometer (Micronal – B462) and flame atomic absorption spectroscopy with a Perkin Elmer spectrometer model 310, respectively. Na and Cd concentrations are expressed as mmol per gram of dry tissue.

2.8. Chlorophyll determination

A Minolta SPAD-502 meter, which measures leaf transmittance at two wavelengths: red (approximately 660 nm) and near infrared (approximately 940 nm), was used to determine the leaf chlorophyll content. SPAD readings were taken weekly on the terminal leaflet of the forth leaf from the base of the shoot. The SPAD sensor was placed randomly on leaf mesophyll tissue only, with veins avoided.

2.9. Statistical analysis

The experimental design was randomized with twelve plants from three replicate pots, and the results are expressed as mean and standard error of mean (±SEM) of three independent replicate enzyme assays of each extract for TBARS and H_2O_2 contents, CAT, GR, GPOX, APX and SOD activities, and Cd and NaCl accumulation measurements.

3. Results

3.1. Plant growth

Over the 36 days of treatment, plants cultivated in NaCl and $CdCl_2$ exhibited growth reduction when compared to the untreated plants. This reduction was similar for MT and *Nr* in roots (Fig. 1A), leaves (Fig. 1B) and, to a lesser extent, in fruits (Fig. 1C).

3.2. Sodium and cadmium content

Table 1 shows the Na and Cd contents of the root, leaf and fruit tissues of the control MT and *Nr* genotypes after treatment with NaCl and $CdCl_2$. Although we did not determine the salt and metal accumulation at the first day when the treatments were initiated (day zero), after 7, 20 and 36 days, a gradual increase in Na and Cd occurred for both genotypes in roots and leaves. The fruit tissue also exhibited an accumulation of Cd and Na in a similar manner for the control MT and the *Nr* mutant. The similar behavior of both genotypes can also be observed in the untreated plants.

3.3. Lipid peroxidation

Lipid peroxidation (expressed by MDA content) was induced by the NaCl and $CdCl_2$ treatments over the three time-periods sampled (7, 20 and 36 days) in both the MT and *Nr* genotypes. However, a lower induction in the *Nr* roots (Fig. 2A) and leaves (Fig. 2A) was observed at 36 days in the NaCl treatment when compared to MT.

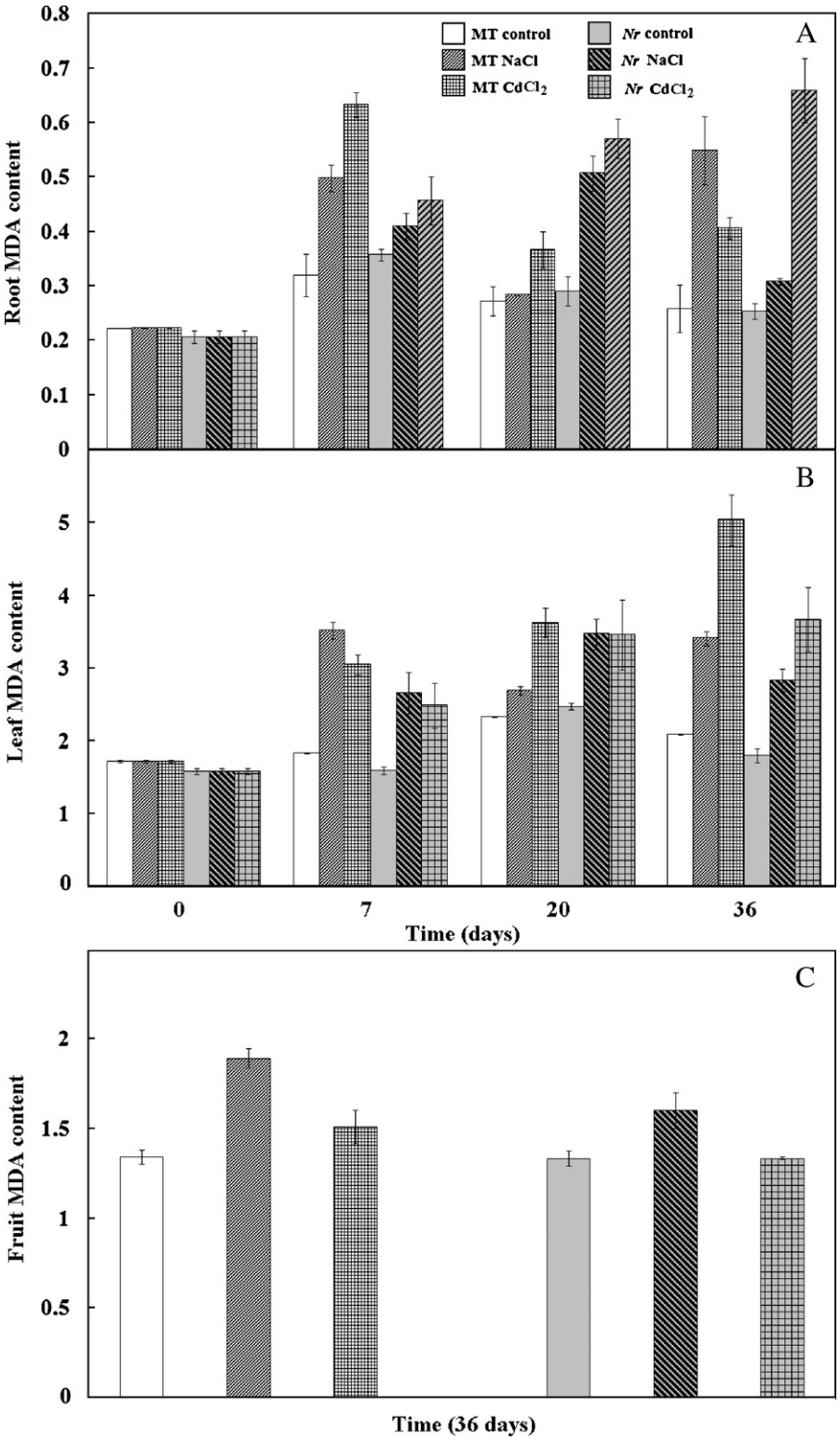

Fig. 2. Lipid peroxidation measured as malondialdehyde (MDA) content (nmol g^{-1} fresh weight) in roots (A), leaves (B) and fruit (C) of *Nr* and MT grown, from anthesis, in the presence of NaCl (100 mM) and $CdCl_2$ (0.5 mM). The control plants were untreated. Values are the means of three replicates ± SEM.

In *Nr* fruits, the NaCl and $CdCl_2$-induced MDA concentration was lower when compared to MT (Fig. 2C).

3.4. Hydrogen peroxide measurements

Following treatment with NaCl and $CdCl_2$, the MT and *Nr* roots (Fig. 3A) and leaves (Fig. 3B) exhibited an increase in H_2O_2 content when compared to the zero treatments at the three time points. Although a higher H_2O_2 induction in *Nr* roots and leaves prior to the beginning of the NaCl and $CdCl_2$ treatments was observed (Fig. 3A and B), during the time length of the treatments the wild type MT and *Nr* exhibited a similar H_2O_2 content. However, a greater increase in H_2O_2 was observed in the *Nr* mutant fruits compared to MT, when subjected to both stresses (Fig. 3C).

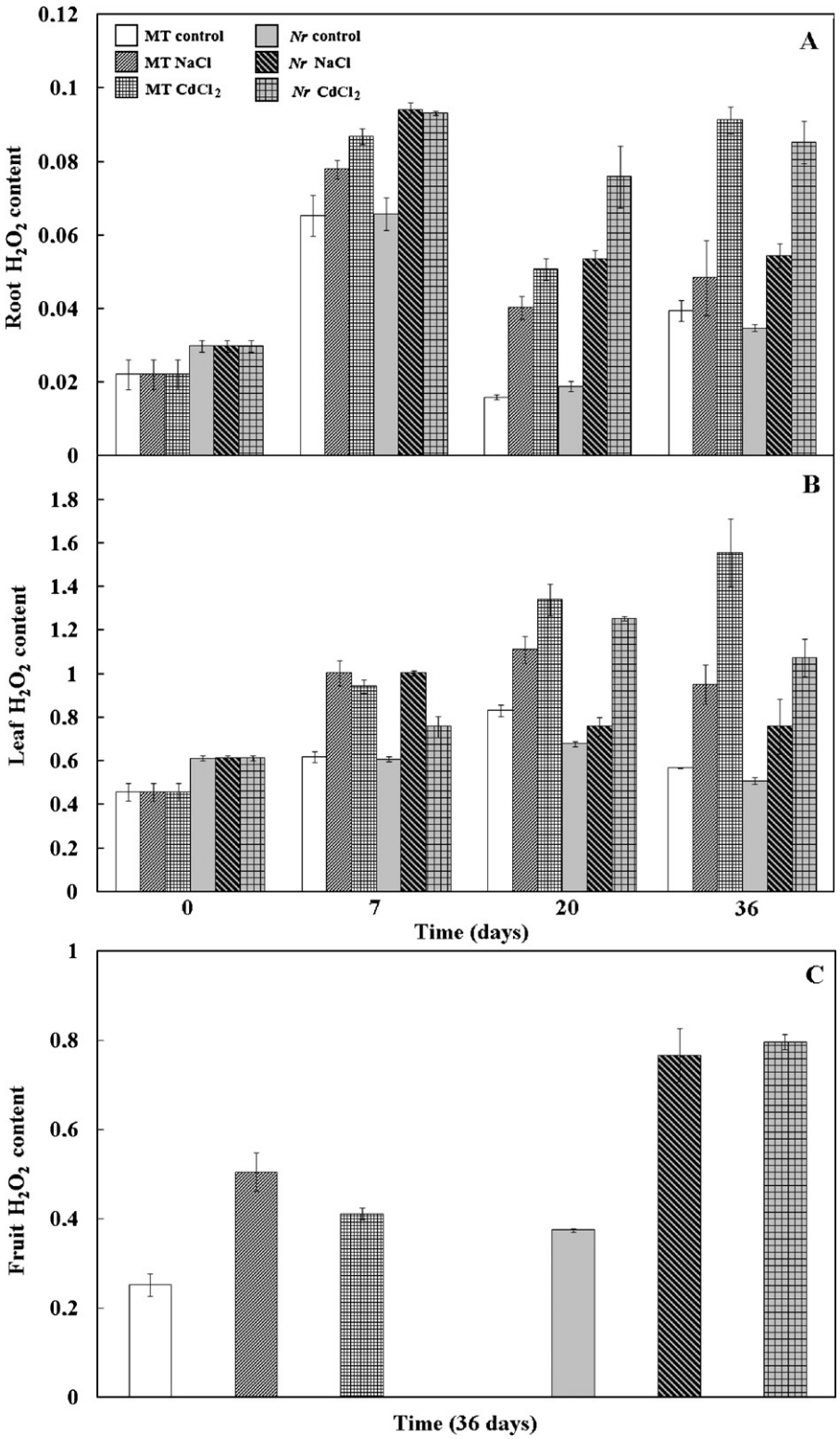

Fig. 3. Hydrogen peroxide (H_2O_2) content ($\mu mol\, g^{-1}$ fresh weight) in the roots (A), leaves (B) and fruits (C) of *Nr* and MT grown, from anthesis, in the presence of NaCl (100 mM) and $CdCl_2$ (0.5 mM). The control plants were untreated. Values are the means of three replicates ± SEM.

3.5. Chlorophyll content

The chlorophyll content of the leaves reduced during the time length of the experiment under both zero and treatment conditions. However, after 28 and 35 days of Cd treatment, the reduction was significantly higher in the MT, although it was also observed in the *Nr* mutant (Fig. 4).

3.6. Antioxidant enzymes

SOD activity staining using non-denaturing PAGE is presented in Figs. 5–7 showing SOD isoforms activity pattern and distribution in roots, leaves and fruits, respectively, after NaCl and $CdCl_2$ exposure. Five distinct SOD isoforms were detected and characterized as Mn/SOD (SOD I), present in all three tissues; Mn/SOD (SOD II),

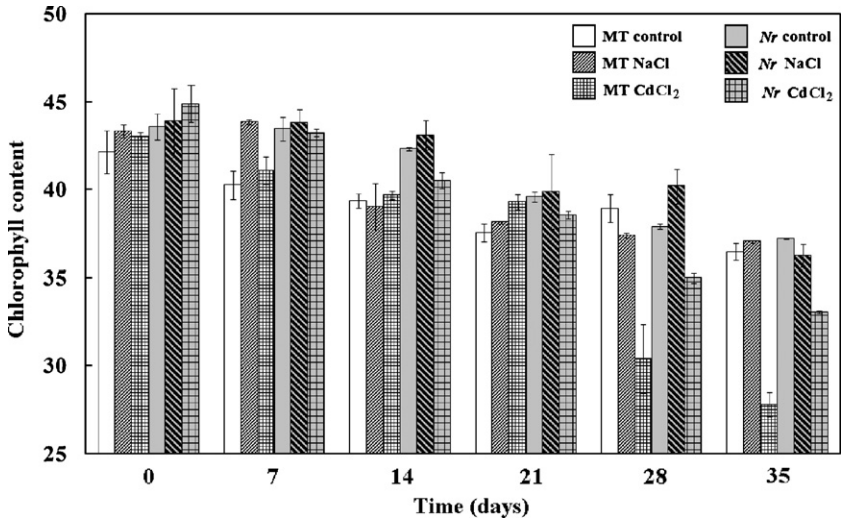

Fig. 4. Chlorophyll content measured in leaves of *Nr* and MT grown, from anthesis, in the presence of NaCl (100 mM) and $CdCl_2$ (0.5 mM). The control plants were untreated. A Minolta SPAD-502 chlorophyll meter was used to take readings on forth leaves. Values are the means of four replicates ± SEM.

only present at very low level in roots; Fe/SOD (SOD III), present in roots and leaves, depending on the stage of plant development; and Cu-Zn/SODs (SOD IV and V) highly active in all three tissues (classification of gels not shown). Considering that SOD isoforms I, IV and V are the predominant isoforms in terms of total SOD activity, the most obvious alteration encountered was observed in roots at 36 days, at which period a similar reduction of SOD I band intensity in both, MT and the *Nr* mutant, treated with NaCl (Fig. 5C), was detected. In leaves and fruit, a similar SOD activity pattern was observed for all periods, genotypes and treatments (Figs. 6 and 7). It is also possible to mention that SOD III appeared to be stage dependent since it only appeared in the leaves of 36-day treated plants, but was independent of genotype and stress.

Analysis of CAT clearly showed Cd and to a lesser extent NaCl induced increases in the total activity in the roots and leaves of both, MT and *Nr* (Fig. 8A and B) during the time length of the experiment. On the other hand, the CAT activity patterns in the fruit tissue of MT and *Nr* were strikingly similar with the NaCl treatment leading to a small reduction in CAT activity, an effect that was not observed for Cd (Fig. 8C).

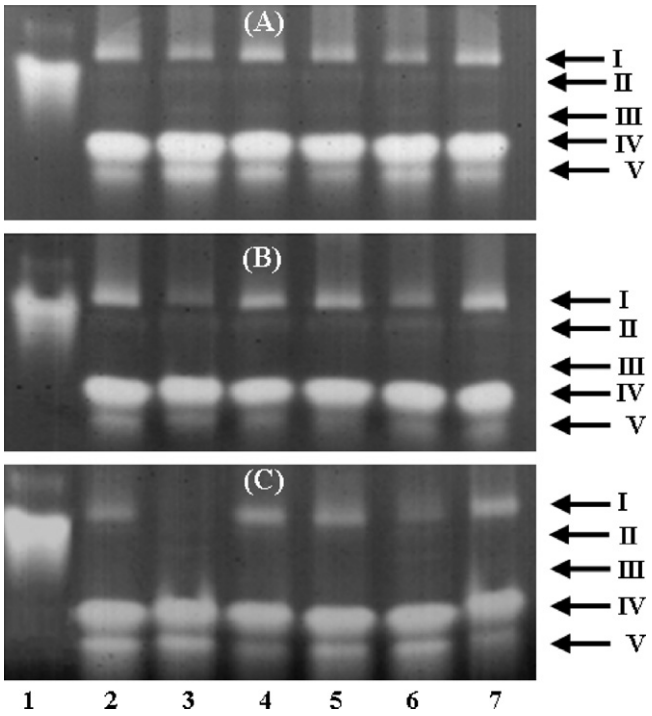

Fig. 5. Activity staining for superoxide dismutase (SOD) following non-denaturing polyacrylamide gel electrophoresis of extracts of roots isolated from MT and *Nr* plants grown over a 7 (A), 20 (B) and 36 (C)-day period in the presence of NaCl and $CdCl_2$. The lanes listed are: (1) bovine SOD standard; (2) MT, zero; (3) MT, 100 mM NaCl; (4) MT, 0.5 mM $CdCl_2$; (5) *Nr*, zero; (6) *Nr*, 100 mM NaCl and (7) *Nr*, 0.5 mM $CdCl_2$. The SOD isoforms are: (I) Mn-SOD; (II) Mn-SOD; (III) Fe-SOD; (IV) Cu/Zn-SOD and (V) Cu/Zn-SOD.

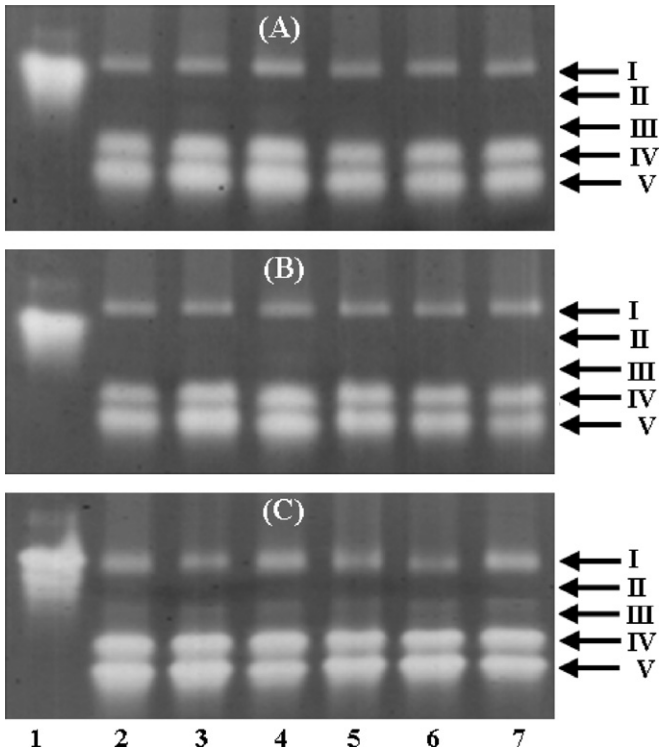

Fig. 6. Activity staining for superoxide dismutase (SOD) following non-denaturing polyacrylamide gel electrophoresis of extracts of leaves isolated from MT and *Nr* plants grown over a 7 (A), 20 (B) and 36 (C)-day period in the presence of NaCl and $CdCl_2$. The lanes listed are: (1) bovine SOD standard; (2) MT, zero; (3) MT, 100 mM NaCl; (4) MT, 0.5 mM $CdCl_2$; (5) *Nr*, zero; (6) *Nr*, 100 mM NaCl and (7) *Nr*, 0.5 mM $CdCl_2$. The SOD isoforms are: (I) Mn-SOD; (II) Mn-SOD; (III) Fe-SOD; (IV) Cu/Zn-SOD and (V) Cu/Zn-SOD.

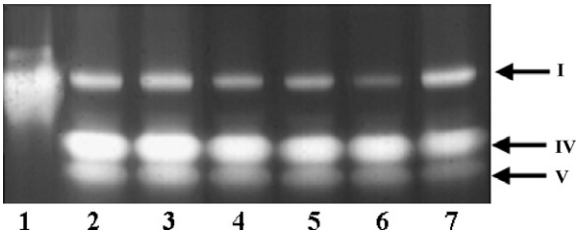

Fig. 7. Activity staining for superoxide dismutase (SOD) following non-denaturing polyacrylamide gel electrophoresis of extracts of fruits isolated from MT and *Nr* plants grown over a 36-day period in the presence of NaCl and $CdCl_2$. The lanes listed are: (1) bovine SOD standard; (2) MT, zero; (3) MT, 100 mM NaCl; (4) MT, 0.5 mM $CdCl_2$; (5) *Nr*, zero; (6) *Nr*, 100 mM NaCl and (7) *Nr*, 0.5 mM $CdCl_2$. The SOD isoforms are: (I) Mn-SOD; (IV) Cu/Zn-SOD and (V) Cu/Zn-SOD.

GR activity was also determined (Fig. 9) and the results revealed a very similar behavior to that observed for CAT (Fig. 8). For instance, NaCl and $CdCl_2$ clearly induced GR activity increases with time and in both, MT and *Nr* roots (Fig. 9A) and leaves (Fig. 9B). However, in fruits GR activity was not altered in MT whereas in *Nr*, a small increase in activity was observed in response to Cd (Fig. 9C).

The clear patterns of CAT and GR enzyme activity stimulation induced by NaCl and Cd were not observed for APX (Fig. 10), although significant changes were observed, they were not as dramatic as for the other two enzymes. On the other hand, APX at day zero followed the pattern exhibited for CAT (Fig. 8) and GR (Fig. 10), so that the *Nr* mutant showed a slightly higher CAT (Fig. 8A), GR (Fig. 9A) and APX (Fig. 10A) activity in roots when compared to the MT, which was not observed in the leaves. There was a slight APX activity change in the fruit tissue, the NaCl treated MT plants exhibited the highest activity, whereas for *Nr*, the $CdCl_2$ treated plants had the highest activity (Fig. 10C).

The activity patterns observed for GPOX revealed some more specific behavior (Fig. 11). Initially, MT and *Nr* exhibited similar levels of activity at day zero in roots and leaves. NaCl and $CdCl_2$ treatments also resulted in induced activity, but after 36 days the activities were not as high as observed at 20 days, particularly for the *Nr* mutant in roots and leaves (Fig. 11A and B). In leaves, GPOX activity was reduced in both the untreated MT and *Nr*.

4. Discussion

We used the ethylene insensitive *Nr* tomato mutant to study indirectly the potential of ethylene modulation on the biochemical changes induced by Na and Cd stresses. We have opted for the analysis of tomato plants that would reach the fruiting stage after at least 30 days of treatment with 100 mM NaCl and 0.5 mM $CdCl_2$. Such a strategy was based on our prior study in which we verified that after 75 days of exposure to $CdCl_2$, MT plants acquired tolerance to a concentration of 1 mM $CdCl_2$ (Gratão et al., 2008). Thus, we have chosen a short acute treatment prior to the tolerance acquisition stage. The same strategy was adopted for the NaCl treatment so both stresses could be directly compared.

NaCl and $CdCl_2$ have obvious detrimental effects on plants, such as inhibition of root and shoot growth, nutrient uptake, and photosynthesis (Shalat and Neumann, 2001; Madhaiyan et al., 2007; Ghanem et al., 2008; Gratão et al., 2008). The chlorophyll breakdown is one of the first visible symptoms and is closely linked to metal and salt stress induction (Demiral and Türkan, 2005). For example, the production of transgenic tobacco plants with delayed senescence resulted in outstanding stress tolerance (Rivero et al., 2007). Although this last report provides evidence for the important role of cytokinin in the senescence control, a large number of other reports showed that the stress-induced senescence is modulated by ethylene (Abeles et al., 1992; Morgan and Drew, 1997; Bleecker and Kende, 2000; Munné-Bosch et al., 2004). In this current work, we were expecting to find an increase in the chlorophyll content in untreated *Nr* plants due to the classic effect of ethylene on senescence (Noodén et al., 1997) and from previous observations of the delayed leaf senescence shown by this mutant (Lanahan et al., 1994). *Nr* appeared to be more resistant to the toxic effect of NaCl and $CdCl_2$ during leaf chlorophyll degradation, since its pigment content after 36 days of treatment was shown to be substantially higher that that observed in wild type MT leaves (Fig. 4). The potential higher resistance of the *Nr* mutant to Cd, when compared to MT, agrees with previous work with ethylene-insensitive mutants, including the *Nr* mutant, which demonstrated an increased tolerance to virulent strains of biotrophic pathogens infection, apart from reduced senescence (Bent et al., 1992; Lund et al., 1998; Hoffman et al., 1999; Ciardi et al., 2000). However, the alterations of the resistance of plants generated by ethylene insensitivity seem to be quite complex; for example, ethylene insensitive plants also have increased susceptibility to a number of diseases, particularly those caused by necrotrophic pathogens (Knoester et al., 1998; Thomma et al., 1999; Geraats et al., 2002; Klee and Clark, 2002). Furthermore, mutants that overproduce ethylene do not necessarily show premature leaf senescence (Grbic and Bleecker, 1995; Wang et al., 2005), indicating that the programmed senescence is regulated through multiple, interrelated but distinct signaling pathways (Jing et al., 2002; De Paepe et al., 2005). This is in agreement with the multiplicity of responses controlled by ethylene signaling, which may derive from differences in developmental stage, plant species and culture systems (Kim et al., 2008). This may explain, for example, the similar reduction between the wild type MT and *Nr* in root, leaf and fruit dry weight (Fig. 1) as well as similar accumulation of both, metal and salt, in the three tissues (Table 1).

The more obvious and perhaps expected biochemical response during stress is the enhanced ROS production. Rapid and non-specific reactions of ROS result in severe cell biochemical changes during oxidative stress, including lipid peroxidation and damage to proteins and DNA, which may lead to cell death (Dietz, 2005). Such changes have been reported to be strongly modulated by ethylene (Mayak et al., 1983; Hodges and Forney, 2000; Desikan et al., 2001; Munné-Bosch et al., 2004; Vranova et al., 2002; Overmyer et al., 2003; Ghanem et al., 2008; Liu et al., 2008). Ours results with the *Nr* mutant provide some extra insights into the role of ethylene sensitivity on oxidative stress triggered by NaCl and $CdCl_2$.

A reduction in the MDA content, which is a product of lipid peroxidation, would be predicted in the *Nr* mutant since previous reports showed that the membrane peroxidation is induced by ethylene (Sylvestre and Paulin, 1987; Sylvestre et al., 1989; Hodges and Forney, 2000; Flors et al., 2007). However, the similar results obtained in the treated and untreated tissues of MT and *Nr* (Fig. 2A–C) indicates a limited role of ethylene signaling in lipid peroxidation. On the other hand, at least in fruits, *Nr* exhibited a slightly elevated H_2O_2 content under salt and metal stress when compared to MT, even though the untreated *Nr* (no Na or Cd) naturally exhibited a higher H_2O_2 production when compared to MT (Fig. 3). Thus, such results may also be related to ethylene production. For instance, Na and Cd-induced ethylene production occurred at the same time that H_2O_2 was increased in tomato (Iakimova et al., 2005) and chickpea (Nandwal et al., 2007), and also exogenous application of H_2O_2 increased ethylene production (Ivenish and Tillberg, 1995). Recently, Liu et al. (2008) speculated that ethylene and H_2O_2 act in a synergistic manner in tomato and the function of ethylene in relation to Cd-induced cell death is dependent on H_2O_2. On the other hand, Ke and Sun (2004) verified that H_2O_2 was not involved in osmotic stress induced ethylene production.

As a direct response to the stress and ROS production, the antioxidant defense systems are expected to respond. The enzymatic

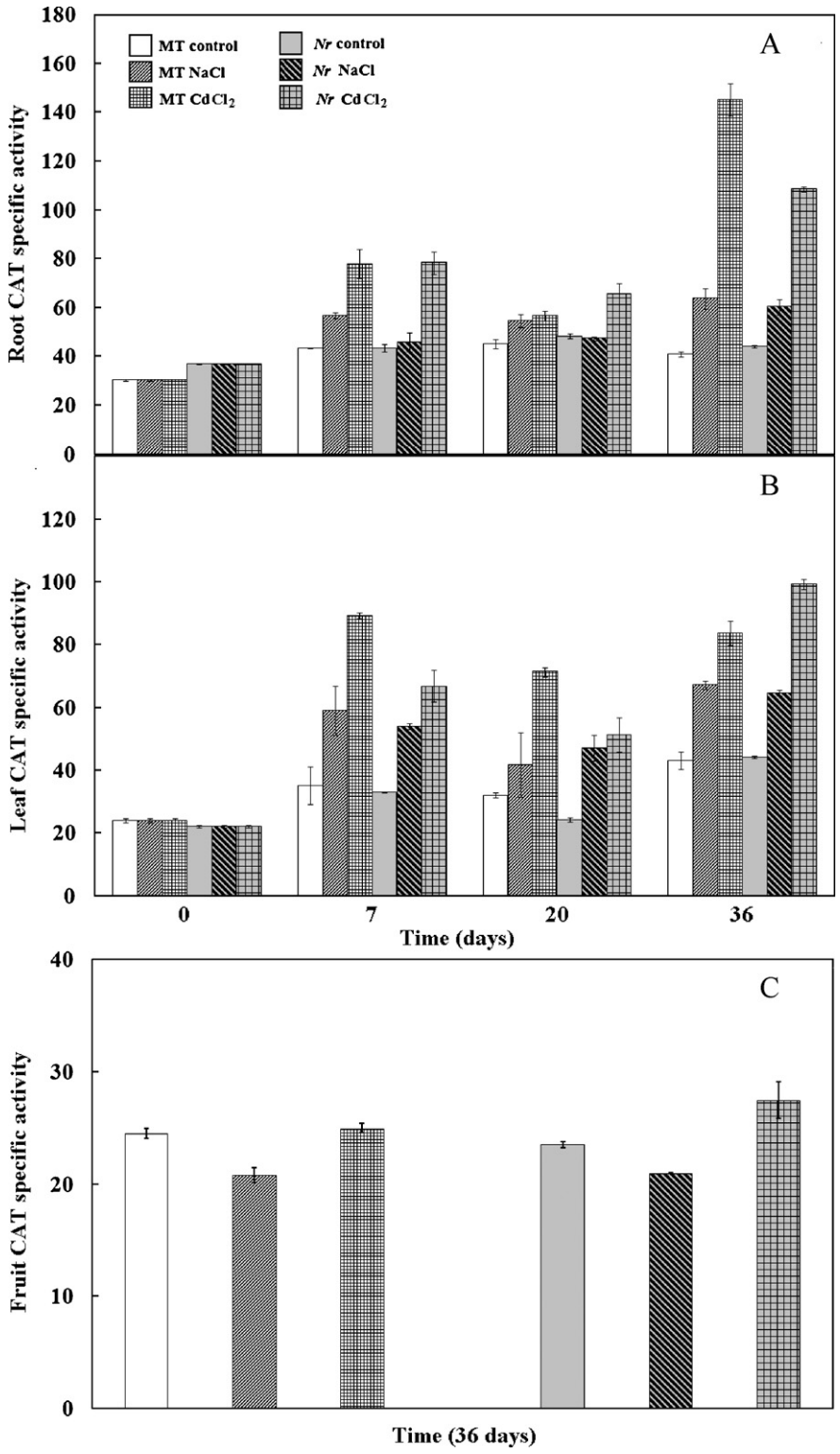

Fig. 8. Specific activity of catalase (CAT) (μmol min^{-1} mg^{-1}) in the roots (A), leaves (B) and fruits (C) of *Nr* and MT grown, from anthesis, in the presence of NaCl (100 mM) and $CdCl_2$ (0.5 mM). The control plants were untreated. Values are the means of three replicates ± SEM.

detoxification mechanisms of plants are capable of quenching ROS involving signaling from stressor to response (Gratão et al., 2005; Baycu et al., 2006; Pauly et al., 2006), probably also dependent on ethylene (Molassiotis et al., 2005; Yakimova et al., 2006; Castagna et al., 2007; Qin et al., 2008). Although non-enzymatic antioxidant systems are likely to be involved in the response, we have initially investigated some key antioxidant enzymes which have been shown previously to respond to the stress induced by NaCl and $CdCl_2$.

When we analyzed the antioxidant enzyme activities of *Nr* and MT, we verified that the interplay between ethylene signaling and the antioxidant system extends to mechanisms dependent on stressor, time, tissue and, additionally, cell compartmentalization type. For example, SOD isoforms are normally located in different

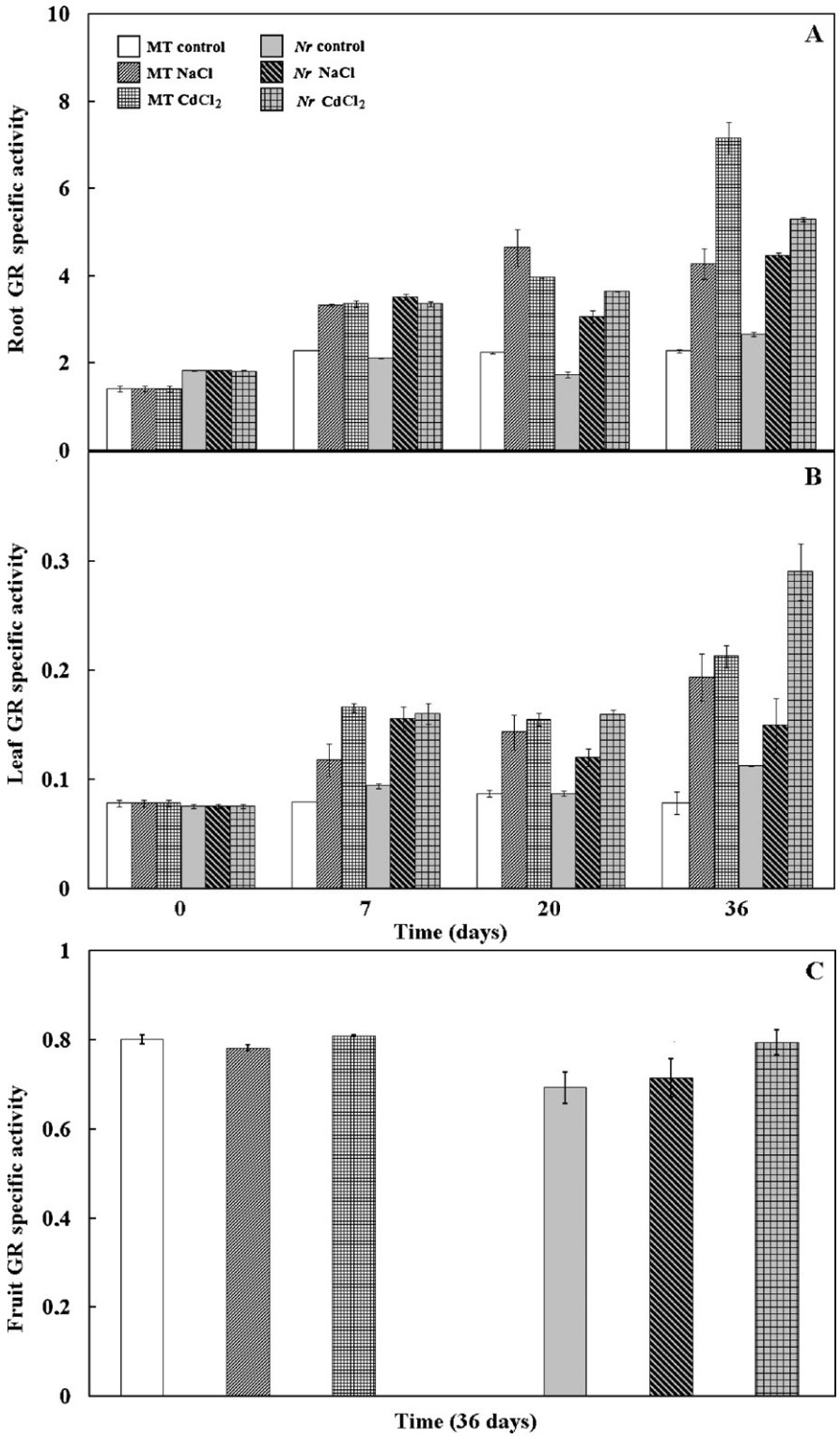

Fig. 9. Specific activity of gluthatione reductase (GR) (μmol min^{-1} mg^{-1}) in the roots (A), leaves (B) and fruits (C) of *Nr* and MT grown, from anthesis, in the presence of NaCl (100 mM) and $CdCl_2$ (0.5 mM). The control plants were untreated. Values are the means of three replicates ± SEM.

cell organelles: Mn-SOD is located in the mitochondria, Fe-SOD in the chloroplast, whereas Cu/Zn-SODs can be found in the chloroplast, the cytosol and, possibly the extracellular space (Alscher et al., 2002); and our results indicate that distinct strategies are likely to be involved when SOD activity response is concerned: (i) SOD isoform pattern distribution is not ethylene dependent since both genotypes exhibited the same isoform pattern in all three tissues tested (Figs. 5–7), but (ii) it is stress signaling dependent since there was an specific effect of NaCl (*i.e.* not genotype) on SOD I in roots of both genotypes, which was more accentuated at 36 days of exposure (Fig. 5C); (iii) compartmentalization dependent, according to differences observed from SOD isoforms activity staining (Figs. 5–7). From these observations, it is curious to note that although H_2O_2 was increased in *Nr* fruits during salt and metal stress (Fig. 3C), *Nr* SOD did not differ in this tissue, being not unreasonable to suggest that there is another H_2O_2 genera-

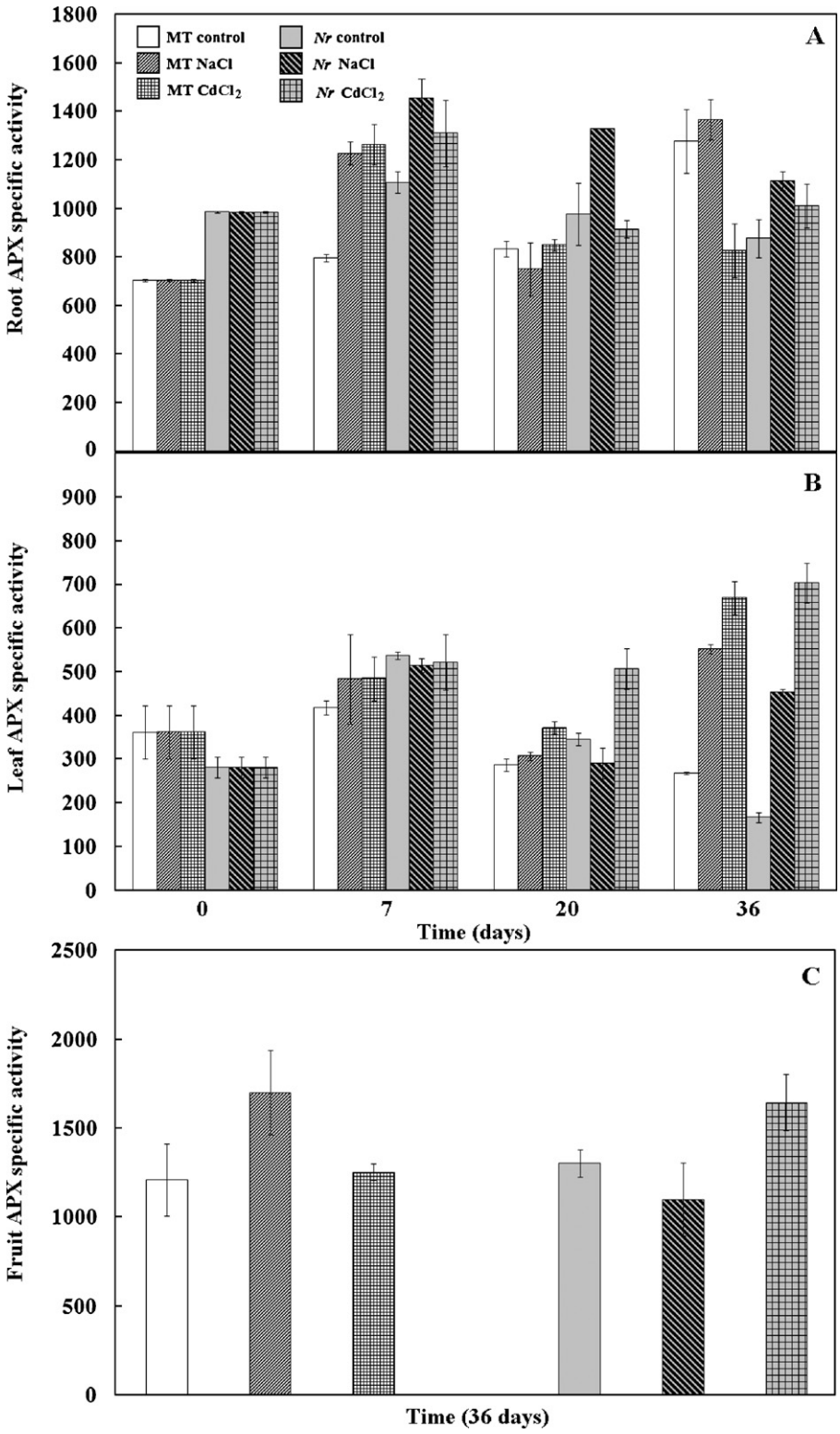

Fig. 10. Specific activity of ascorbate peroxidase (APX) (μmol min^{-1} mg^{-1}) in the roots (A), leaves (B) and fruits (C) of *Nr* and MT grown, from anthesis, in the presence of NaCl (100 mM) and $CdCl_2$ (0.5 mM). The control plants were untreated. Values are the means of three replicates ± SEM.

tion pathway, not from the dismutation of O_2^- to H_2O_2 by SOD, and that at least in fruit the H_2O_2 generation may be ethylene dependent.

As H_2O_2 can subsequently be reduced to H_2O by CAT and APX, a reduction in H_2O_2 in *Nr* roots could be expected, due to the higher CAT and APX activities, but this was not the case (Fig. 3A). H_2O_2 is also known to be produced through non-enzymatic processes in plants (Nandwal et al., 2007), thus making it difficult to assign to CAT and APX, a role in the reduction of H_2O_2 during salt and metal stress. Consistent with this complexity, Castagna et al. (2007) verified that although *Nr* produced H_2O_2 during an O_3-induced oxidative burst, *Nr* displayed reduced activities of APX, indicating the involvement of complex ROS-producing and scavenging systems.

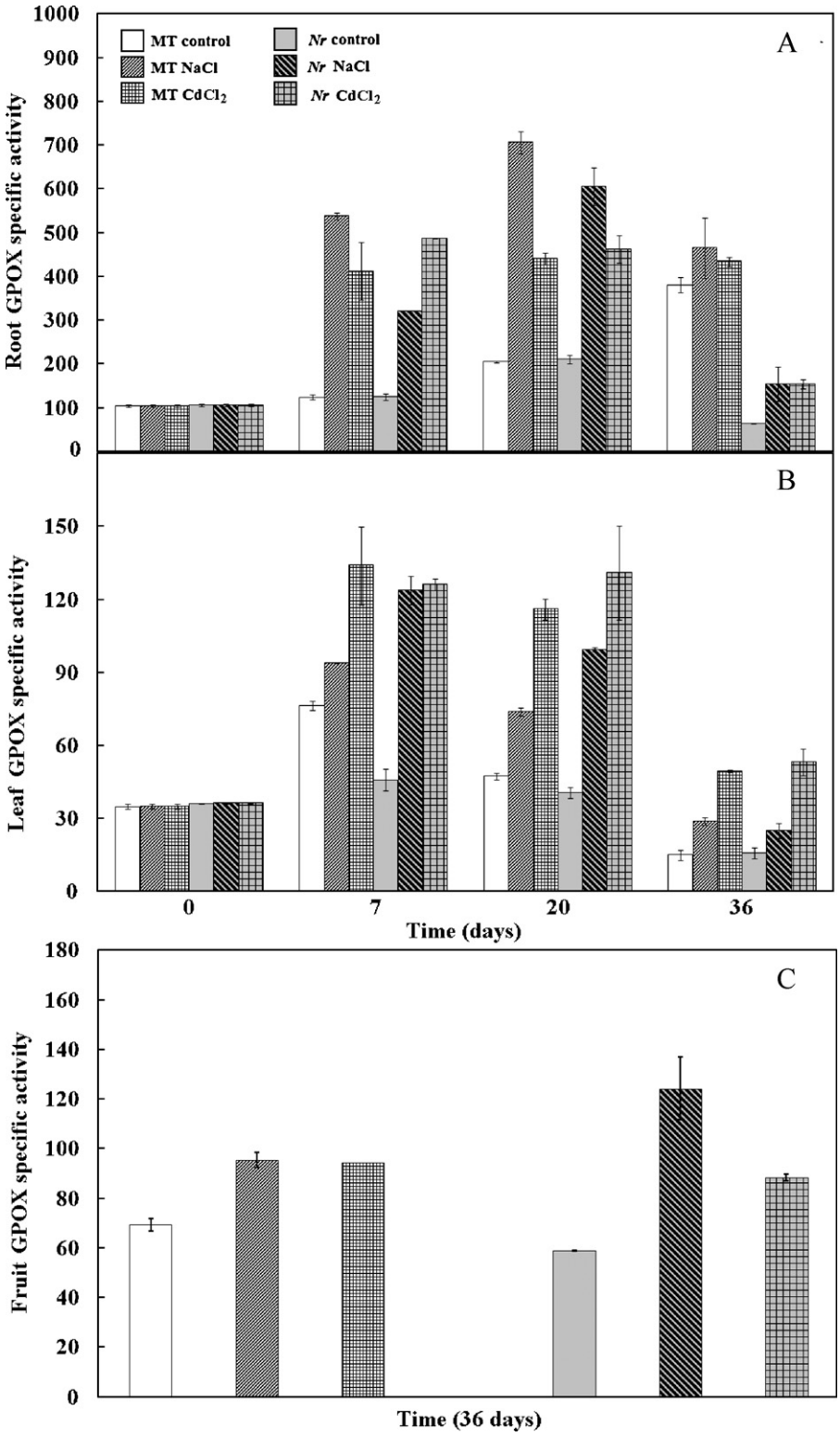

Fig. 11. Specific activity of guaiacol peroxidase (GPOX) (U) in the roots (A), leaves (B) and fruits (C) of *Nr* and MT grown, from anthesis, in the presence of NaCl (100 mM) and $CdCl_2$ (0.5 mM). The control plants were untreated. Values are the means of three replicates ± SEM.

It is noteworthy that *Nr* roots exhibited enhanced CAT, GR and APX activities at the beginning of the treatments, when compared to the wild type MT (Figs. 8–10A). However, when exposed to NaCl and $CdCl_2$, the enzymes did not exhibit different behaviors when compared to the control MT. On the other hand, at least for APX, the *Nr* fruits appeared to contain a different signaling mechanism in response to NaCl and $CdCl_2$ stress, since there were opposite responses showing a reduced and an enhanced APX activity in NaCl and $CdCl_2$, respectively (Fig. 10C). This indicates that if APX is dependent of ethylene signaling, only in fruits different mechanisms for salt and metal stress may be involved.

In *Nr* roots, there was a considerable reduction in GPOX after 36 days (Fig. 11A), and in fruits the increase of *Nr* APX in NaCl (Fig. 11C) are the most obvious enzymatic alterations expressed by

the mutant. These results provide strong evidences of a close GPOX and ethylene signaling interaction through mechanisms which appears to be stressor and tissue-dependent, since the GPOX activity patterns observed for MT and *Nr* leaves are similar (Fig. 11B). However, based on our data it is still difficult to draw any specific interaction between GPOX and ethylene and more research is needed, even though such an interaction between other peroxidases (*e.g.* APX and CAT) and ethylene have already been exploited (Nandwal et al., 2007).

Although signal transduction from ethylene receptors involves a subset of downstream components (Lin et al., 2008), receptors had been the target of active research, since ethylene receptor expression seems to be induced by multiple stresses (Cao et al., 2008). For instance, the expression of the ethylene receptor gene *ETR1* seems to be down-regulated by salt and osmotic stress at both the transcription and protein levels in *Arabidopsis thaliana* (Zhao and Schaller, 2004). The gain-of-function mutant *etr1-1*, with ethylene insensitivity, showed increased sensitivity to salt stress during germination and early seedling development (Zhou et al., 2006; Cao et al., 2007; Wang et al., 2008). On the contrary, the strong loss-of-function mutant *etr1-7* of *A. thaliana,* which displays enhanced sensitivity to ethylene, showed increased tolerance to salt stress (Wang et al., 2008). Additionally, transgenic *A. thaliana* and tobacco plants overexpressing the ethylene receptor gene *NTHK1* from tobacco showed sensitivity to salt stress compared to the wild-type plants (Zhou et al., 2006; Cao et al., 2006, 2007). These results indicate that ethylene receptors regulate salt-sensitive responses, whereas in this paper we have suggested that this regulation can occur at the level of oxidative stress. Although the cause of this differential response observed for *Nr* is still unknown, it is suggestive of a varying degree of penetrance of the dominant mutation in different tissues (Barry et al., 2005). In accordance with this, the receptors are expressed in various temporal and spatial patterns, depending on developmental stage and external stimuli (Lashbrook et al., 1998; Klee and Tieman, 2002; Lin et al., 2008). *LeETR1*, for example, is expressed constantly in all tissues examined and shows no induction by exogenous ethylene, whereas *NR* (*LeETR3*) expression increases during ripening, senescence and abscission (Lashbrook et al., 1998; Payton et al., 1996). The oxidative stress responses by the *Nr* mutant may also help to elucidate the role that this receptor plays during Na and Cd-induced stress as alteration in ethylene sensitivity may result in the unexpected or unrecognized modification of another signaling pathway (Alexander and Grierson, 2002), allowing the plant to initiate an ethylene response in one tissue while suppressing the response in others (Tieman et al., 2000). Consistent with this, Castagna et al. (2007) verified that the *Nr* mutant retains a partial sensitivity to ethylene, thus suggesting only a marginal role of NR receptor in mediating the complex response of tomato plants to O_3 and implying the involvement of other ethylene receptors. Moreover, a fascinating question in ethylene signaling and response is the involvement of other hormonal signaling pathways (Mittler, 2002; Neill et al., 2002). For example, *A. thaliana* mutant loci have been cloned and revealed to encode either downstream components of the ethylene signaling pathway or peripheral components that function in, or integrate multiple hormone signaling pathways, involving auxin and abscisic acid (Kwak et al., 2002; Larsen and Cancel, 2003; Li et al., 2004). These issues make it clear how much still is to come from future research.

In this paper we provided indirectly a background to ethylene signaling during oxidative stress trigged by NaCl and $CdCl_2$. From the data obtained with the *Nr* mutant we have suggested that in tomato this signaling can be tissue and stressor dependent. However, our approach relies on a fundamental role of NR receptors on modifications of the oxidative system. For a complete understanding of the ethylene signaling from NR many efforts are still necessary. Moreover, recent evidence indicated that the *Nr* mutant has leaves with light disorganization of the chloroplast, and when exposed to $CdCl_2$ treatment, the leaves of this mutant show an increase in the intercellular spaces and decrease in the size of the mesophyll. Whereas in the roots, there were alterations in diameter and disintegration of the epidermis and the external layers of the cortex (Gratão et al., 2009). Thus, these anomalies should also be taken into consideration when evaluating the biochemical responses of the *Nr* mutant during Cd stress. However, it is surprising that although *Nr* does show the evident disorganization of the chloroplast (Gratão et al., 2009), SOD did not differ between *Nr* and MT, indicating a complex antioxidative system dependent of compartmentalization which needs to be explored. Moreover, another question to be answered is whether such ultrastructural alterations also occur as a result of NaCl treatment, which has not yet been investigated. Finally, it would also be interesting in future studies to analyze whether the fruit ultrastructure is also affected by $CdCl_2$ and NaCl.

Considering a distinct approach, but still highlighting the ethylene signaling from NR, transgenic tomato plants overexpressing wild-type *NR*, which is suggested to reduce ethylene sensitivity due to the fact that NR receptor can be a negative regulator of ethylene response, displayed tolerance to virulent *X. campestris* pv. *vesicatoria*, as evidenced by reduced necrosis and greater membrane integrity (Ciardi et al., 2000). Is this pathogen tolerance, as well as reduced senescence during NaCl and $CdCl_2$ treatment observed here in *Nr*, triggered by an altered antioxidant system response? Further analysis, including new approaches such as plant omics (Arruda and Azevedo, 2009), in addition to biochemical experiments, are thus necessary to elucidate the roles played by the NR receptors and other ethylene receptors in mediating the plant response to salt and metal stress. These should help to unravel the relative importance of ethylene receptors in regulating the cell responses to different kind of stresses sharing similar signaling routes.

Acknowledgements

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