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Impact of Ripening and Salinity on Tomato Fruit Ascorbate Content and Enzymatic Activities Related to Ascorbate Recycling

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ABSTRACT

Our objectives were i) to determine the impact of ripening and salinity on fruit ascorbate content in a cherry tomato 'Cervil', a medium sized tomato 'Levovil' and a larger sized tomato 'Marmara', ii) to compare the impact of increasing salinity by adding NaCl alone (Na) or NaCl plus $CaCl_2$ (Ca+Na) as calcium may limit the salinity stress and iii) to determine how increased salinity and ripening modified the activity of antioxidant enzymes related to ascorbate recycling. Tomato fruits were harvested at three ripening stages: mature green (MG), pink (P) and red ripe (RR), on plants irrigated with a control solution (3 dS m^{-1}) or with increased salinity solutions (Na or Ca+Na at 7.6 dS m^{-1}). During ripening, ascorbate content and oxidative parameters increased whereas antioxidant activities of enzymes did not show any obvious trend. 'Cervil' had higher ascorbate and oxidative parameters but lower ascorbate peroxydase (APX) activity compared to 'Marmara' and 'Levovil'. Salinity increased the reduced ascorbate content (except for RR fruits of 'Cervil'), and the dehydroascorbate content in 'Cervil' and 'Marmara'. Despite qualitatively similar impacts on the ascorbate pools, adding Na had a stronger impact on the oxidative parameters (hydrogen peroxide and lipid peroxidation) compared to adding Ca+Na. Increasing salinity did not increase the activity of antioxidant enzymes in Marmara, but it significantly enhanced them in 'Levovil' or 'Cervil'. The stronger antioxidant enzyme activities observed in 'Cervil' when adding Ca+Na could be linked to a lower accumulation of Na^+ compared to other genotypes. We discuss a possible link between the oxidative parameters, the ascorbate content and the activity of ascorbate recycling enzymes in genotypes harvested at different ripening stages and receiving different salinity solutions.

Keywords: antioxidant enzyme, ascorbate glutathione cycle, fruit quality, oxidative stress, *Solanum lycopersicon*
Abbreviations: APX, ascorbate peroxidase; **AsA**, ascorbate; **Ca+Na**, increased salinity treatment by addition of NaCl and $CaCl_2$; **DHA**, dehydroascorbate; **DHAR**, dehydroascorbate reductase; **DPA**, day(s) post anthesis; **DW**, dry weight; **FW**, fresh weight; **MDA**, malonyldialdehyde; **MDHAR**, monodehydroascorbate reductase; **MG**, mature green; **Na**, increased salinity treatment by addition of NaCl; **P**, pink; **ROS**, reactive oxygen species; **RR**, red ripe; **SOD**, superoxide dismutase

INTRODUCTION

To face the decrease in water resource in the countries around the Mediterranean, irrigation of tomato crop with salinized water could provide a convenient solution (D'Amico *et al.* 2003; Incerti *et al.* 2007). Several studies have indeed shown that irrigation with a moderate concentration in salts improved tomato fruit quality with a low impact on the commercial yield (Ehret and Ho 1986; Adams 1991; De Pascale *et al.* 2001; Dorais *et al.* 2001). This improvement is mainly due to increased concentrations in dry matter, sugars, titratable acidity (Petersen *et al.* 1998; De Pascale *et al.* 2001) but not in flavour volatiles (Lin and Glass 1999). Salinity was also reported to increase the content in health promoting molecules such as antioxidants, for example, ascorbate and lycopene (De Pascale *et al.* 2001; Dorais 2001; Dumas *et al.* 2003; Krauss *et al.* 2006; Dorais *et al.* 2008).

The impact of increasing salinity on fruit ascorbate content is not so clear compared to the impact on fruit dry matter. Depending on the experiments, fruit ascorbate content either did increase (Adams 1991; Petersen *et al.* 1998; Krauss *et al.* 2006) or did not increase at all (Fanasca *et al.* 2007). This might be linked to the key role of ascorbate (Smirnoff and Pallanca 1996) as a non enzymatic system complementary to enzymatic systems that remove reactive oxygen species (ROS) produced in excess by an oxidative stress (Mittler 2002). Consequently, any factor that will affect oxidative stress may affect ascorbate pool and the activities of ascorbate recycling enzymes (Conklin 2001). Thus the differences reported in ascorbate content following an increased salinity treatment might be due

i) to differences in the intensity of the salinity stress applied to the plants (composition and concentration of the nutrient solution, plant developmental stage at the onset of the salinity treatment, the duration of the salinity treatment),
ii) to interactions with other factors such as the climate (Dumas *et al.* 2003) or the ripening stage (Jiménez *et al.* 2002) that can affect oxidative stress,
iii) to differences in cultivars or species response to increased salinity (Cuartero *et al.* 2006; Incerti *et al.* 2007; Zushi and Matsukoe 2009).

Plants have developed two antioxidant defence systems, enzymatic and non enzymatic scavenging systems, to minimize the concentrations of ROS and protect plant cells from oxidative damages (Allen 1995). The key enzyme involved in the first steps of the ROS scavenging system is the

Received: 12 March, 2009. Accepted: 15 July, 2009.

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superoxide dismutase (SOD) (Gomez *et al.* 2004). It catalyzes the dismutation of the superoxide anion ($O_2^{\bullet-}$) to oxygen and hydrogen peroxide (H_2O_2) (Fridovich 1986). Catalase, guaiacol peroxidase or ascorbate peroxidase (APX) convert hydrogen peroxide (H_2O_2) into non-toxic water (Asada 2006). H_2O_2 dismutation by APX enzyme is coupled with ascorbate (AsA) oxidation to monodehydroascorbate (MDHA), which can either dismutate back to AsA, or to dehydroascorbate (DHA). DHA is then rapidly reduced to AsA (Mano *et al*. 1997; Asada 2006). Different enzymes are involved in the regeneration of AsA from MDHA (monodehydroascorbate reductase enzyme, MDHAR) or from DHA (dehydroascorbate reductase enzyme, DHAR). The active ascorbate–glutathione cycle is responsible for efficient removal of excess ROS (Noctor and Foyer 1998; Asada 2006). In the non-enzymatic scavenging system, antioxidant compounds such as AsA, glutathione (GSH), α-tocopherol, and carotenoids (lycopene, carotene) also play important roles (Foyer *et al*. 2005; Munné-Bosch 2005). Following an excessive production of ROS, the plant may increase accumulation of AsA and upregulate the antioxidant enzymes to limit oxidative stress (Davey *et al.* 2000). The protective role of AsA towards salt oxidative stress was previously reported in *Arabidopsis thaliana* leaves: the VTC mutants (which accumulated less ascorbate) were more sensitive to salt oxidative stress (Smirnoff 2000). Similarly, it was previously shown in roots and leaves of wild salt-tolerant tomato that salt induced changes in enzymatic and non enzymatic antioxidants (Shalata and Tal 1998; Shalata *et al.* 2001; Mittova *et al.* 2003), but there are few studies on the impact of increasing salinity on fruit antioxidant systems (Zushi and Matsukoe 2009).

As previously outlined, the contradictory results reported on the impact of salinity on ascorbate content in tomato fruit might be related to genetic differences in the sensitivity to salinity stress and to the possibility for a plant to limit or not salt accumulation within its tissue (Rajasekaran *et al.* 2000) triggering differences in the intensity of the salinity stress perceived by the plant. This might be modulated also through the ionic composition of the nutrient solution. Increasing sodium in the nutrient solution may dramatically increase sodium storage in the plant. Increasing salinity by adding calcium or a mixture of sodium and calcium has been reported to trigger a lower stress than adding sodium alone as it reduces sodium accumulation within the plant tissues (Cramer 2002) and consequently salinity stress. In the present study, we estimated the tolerance or sensitivity of a genotype to salinity stress by measuring the accumulation of sodium, hydrogen peroxide and malonyldialdehyde (MDA) (as an indicator of membrane lipid peroxidation). During tomato fruit ripening, under optimal conditions there is an increase in ROS production and their removal by antioxidant systems. It was previously reported that ripening stage may interact with an environmental factor to strongly change AsA content (Gautier *et al.* 2009). Therefore, we decided to take into account three fruit ripening stages to get an overall view of the oxidative stress triggered both by salinity and ripening. The aim of the present study was thus to look for correlations between ascorbate content, oxidative parameters and the activity of ascorbate related antioxidant enzymes in tomato fruits harvested at three developmental stages on three contrasted genotypes receiving different nutrient solutions with increased salinity.

MATERIALS AND METHODS

Plant material, salinity treatments and growth conditions

The impact of increased salinity was studied on three contrasted sized genotypes of tomato: a cherry tomato (*Solanum lycopersicum* L., cv. 'Cervil', Vilmorin®, France), a medium-sized tomato (*S. lycopersicum* L., cv. 'Levovil', Vilmorin®, France) and a larger sized tomato (*S. lycopersicum* L., cv. 'Marmara', Seminis®). Tomato plants were grown in three adjacent compartments (240 m^2) of a greenhouse at the Ctifl Research Station (Bellegarde, Southern France, 43° 45′ N). On November 23, 2006, seeds were sown on rockwool rolls covered with vermiculite (20 × 27 mm, Grodan BV, Roermond, The Netherlands). After eight days, seedlings were transferred to larger rockwool cubes (65 × 75 × 75 mm) and, finally, on December 19, 2006, plants with three or four true leaves were transplanted onto rockwool blocks (two plants per block, 100 × 15 × 25, Grodan BV, Roermond, The Netherlands). Planting took place in greenhouse compartments, with a planting density of 2.4 plants m^{-2}.

From sowing until January 22 (which corresponded to the flowering of the second truss in 'Marmara' and 'Levovil' and the third truss in 'Cervil'), plants received the same nutrient solution with an electro-conductivity of 3 dS m^{-1} (**Fig. 1, Table 1**). After that, control plants were irrigated with the 3 dS m^{-1} solution. For other plants, the solution conductivity was increased to 7.6 dS m^{-1} either by adding a combination of NaCl plus $CaCl_2$ (16.4 meq L^{-1} Na, 17.7 meq L^{-1} Ca; Ca+Na treatment) or by NaCl only (40.8 meq L^{-1} Na; Na treatment). Nutrient solutions were prepared with de-ionized water and a commercial mixture to obtain the desired mineral composition (**Table 1**). The solutions were supplied using a drip irrigation system in order to maintain at least 30% drainage.

All plant side shoots were removed as they appeared and the

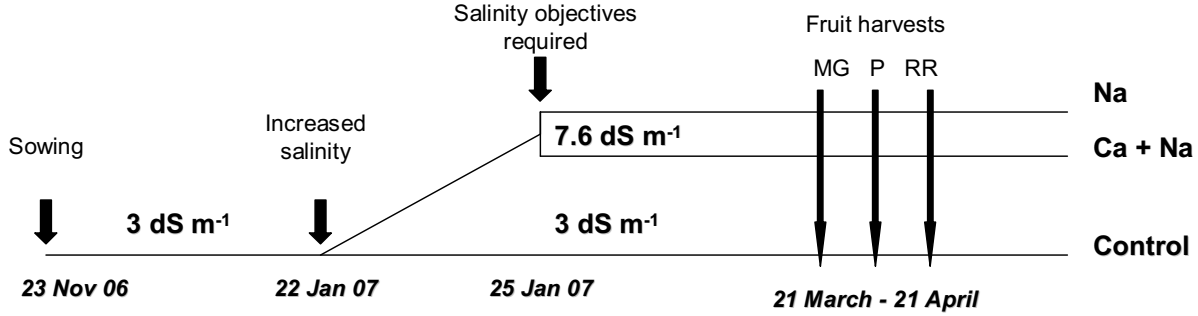

Fig. 1 Electroconductivity of the nutrient solution applied to the control and the two salinity treatments (Na, Ca+Na) during the assay. Salinity was progressively increased from 3 (on January 22) to 7.6 dS m^{-1} (on January 25), which corresponded to the second truss flowering in 'Marmara' and 'Levovil' and to the 3rd truss flowering in 'Cervil'. Na: salinity increased by addition of NaCl; Ca + Na: salinity increased by addition of NaCl and $CaCl_2$.

Table 1 Content in macro-elements (meq L^{-1}) of the three different nutrient solutions delivered to the plant.

Electrical conductivity dS m^{-1}		**[N]**	**[P]**	**[K]**	**[S]**	**[Cl]**	**[Ca]**	**[Mg]**	**[Na]**	**[Ca]/[Na]**
Control	3	13.8	1.6	9.7	5.7	4.1	11.1	4.6	0.7	15.9
Ca + Na	7.6	24.3	1.6	14.1	14.5	25.2	28.8	5.9	17.1	1.7
Na	7.6	13.8	1.6	9.7	5.7	45	11.1	4.6	41.5	0.3

old leaves were removed every 15 days. Fruit load was set at four, five and 20 fruits truss^{-1} in 'Marmara', 'Levovil' and 'Cervil', respectively. The mean air temperature varied from 18.7°C in January to 21.7°C in June with a relative humidity varying from 83 to 72%.

Fruit and plant sampling, physical trait measurement and sample preparation

Flowers were tagged at anthesis and tomato fruits were harvested from the beginning of April until the beginning of May to obtain for each genotype and treatment the three following ripening stages: mature green (MG), pink (P) and red ripe (RR). Fruit ripening stages were defined from fruit external coloration in agreement with Jiménez *et al*. (2002): the mature green stage (with white distal end, fully developed green fruits, usually 2-4 days before breaker stage), the pink stage (2-3 days after breaker stage) and the red ripe stage defined as the pink stage plus 5 days. For each genotype, three sub-samples of 10 fruits were harvested per treatment and per developmental stage. After harvest, fruits were weighed and pericarp tissue was frozen in liquid nitrogen and maintained at -80°C until it was ground in liquid nitrogen. Means (± standard error) corresponded to the average of three independent samplings, extractions and analyses. Fruit dry matter content was determined on a sub sample after lyophilisation.

Chemical analyses

1. Determination of mineral composition, soluble sugars and starch content

Nutrient solutions were analysed for total Ca, Mg, K, Na, P, S on an Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES, Vista PRO, VARIAN, Palo Alto, USA) using certified calibrated standards. Ionic interference was avoided using a solution of CsCl (10 g L^{-1}), which was added to all samples, including the standards. Nitrogen and chlorine contents were determined by a continuous flow colorimetric analysis (Evolution II, Alliance Instrument, Méry sur Oise, France) using certified calibrated standards. The mineral composition of RR fruits was assayed according to the method of Kjeldahl for N quantification and the method described by Pinta (1973) for other minerals. Soluble sugars and starch were assayed as previously described (Gomez *et al.* 2002, 2007). Briefly, the soluble sugars were extracted at 4°C from 5 mg of freeze dried fruit powder. First, 1 ml of a methanol/water solution (1: 1, v/v) was added, then 0.3 ml of chloroform. Samples were shaken for 30 min at 4°C and centrifuged (5 min at 16,000 × *g* at 4°C). 0.8 ml of the methanol/water supernatant was recovered, evaporated under vacuum (Speed-Vac) and stored at -20°C until measurement of soluble sugars. Sugars were estimated using the micro-method described in Gomez *et al*. (2007). Glucose, fructose and sucrose concentrations were successively quantified by enzymatic assays measuring the production of NADH directly in each well at 340 nm using the multiskan Ascent MP reader (Labsystels, Thermo Fisher Scientific, Courtaboeuf, France). The contents in glucose, fructose and sucrose were pooled to obtain the content in soluble sugars. For starch measurements, 1ml of methanol was added to the tube containing chloroform and fruit powder and the tube was shaken for 20 min before centrifugation (5 min at 16,000 × *g* at 4°C). The supernatant was discarded and the pellet was used for starch assay. Starch was dispersed by autoclaving for 2 h (120°C) and then hydrolysed for 1.5 h at 56°C by addition of amyloglucosidase solution. The glucose released by starch hydrolysis was measured as described previously (Gomez *et al*. 2007) using 150 µL of diluted extract, 100 µL of a solution containing ATP, NAD and 20 µL of a solution containing glucose-6-phosphate dehydrogenase and hexokinase.

2. Determination of ascorbate content

Assays of total and reduced AsA content were carried out as previously described (Stevens *et al.* 2006) on material stored at -80°C. Briefly, tomato tissue was ground in liquid nitrogen, and 0.5 to 1 g of powder was homogenised with 600 µl of ice cold 6% trichloroacetic acid (TCA). Samples were centrifuged for 15 min at 16,000 × *g* at 4°C. 20 µL of the supernatant were used in each assay. Two assays were carried out on each sample, one to measure the total AsA (including addition of 5 mM dithiothreitol (DTT)) and one to quantify the reduced AsA content (omission of DTT from the assay). 20 µL of each sample or standard were distributed into at least two wells (for two repetitions) of a 96-well microplate and mixed with 20 µl of 5 mM DTT (total AsA assay) or 0.4 M phosphate buffer pH = 7.4 (reduced AsA assay). The plate was incubated at 37°C for 20 min. 10 µL of *N*-ethyl maleimide (total AsA assay) or 0.4 M phosphate buffer pH = 7.4 (reduced AsA assay) were added and mixed followed by the addition of 80 µl of colour reagent (Stevens *et al.* 2006). After incubation at 37°C for 50 min, the absorbance was read at 550 nm using the multiskan Ascent MP reader. The standard curve obtained from the standard solution values allowed calculation of the AsA concentration of the samples after correction for the quantity of water introduced by the tomato fruit sample.

3. Determination of hydrogen peroxide (H_2O_2) content

Hydrogen peroxide levels were determined as described by Murshed *et al*. (2008a). Frozen fruit powder was homogenized in an ice bath with 1 mL 0.1% (w/v) TCA. The homogenate was centrifuged at 12,000 × *g* for 15 min at 4°C. Aliquots of 100 µL from each tube were placed in 96-well plates and 50 µL of 10 mM potassium phosphate buffer (pH 7.0) and 100 µL of 1 M KI were added in each well. Each plate also contained commercial H_2O_2 to generate a standard curve. Plate was briefly vortexed and the absorbance readings were taken at 390 nm in a micro-plate reader. The content of H_2O_2 was given on a standard curve.

4. Determination of the malonyldialdehyde content (MDA)

For the measurement of lipid peroxidation in fruits, the thiobarbituric acid (TBA) test, which determines malonyldialdehyde (MDA) as an end product of lipid peroxidation (Murshed *et al.* 2008a), was used. Frozen fruit powder (0.25 g) was homogenized in 1 mL 0.1% (w/v) TCA solution. The homogenate was centrifuged at 12,000 × *g* for 15 min and 0.5 mL of the supernatant was added to 1 mL 0.5% (w/v) TBA in 20% TCA. The mixture was incubated in boiling water for 30 min, and the reaction stopped by placing the reaction tubes in an ice bath. Tubes were briefly vortexed and triplicate, 200 µL aliquots from each tube were placed in 96-well plates, and the absorbance of supernatant was read at 532 nm in a micro-plate reader. The value for non-specific absorption at 600 nm was subtracted. The amount of MDA–TBA complex (red pigment) was calculated from the extinction coefficient 155 mM^{-1} cm^{-1}.

5. Antioxidant enzyme assays

Extraction of enzymes: Protein extraction was performed according to Murshed *et al*. (2008b). Frozen fruit powder (0.20 to 0.4 g) was homogenized in 1 ml of 50 mM MES/KOH buffer (pH 6.0), containing: 40 mM KCl, 2 mM $CaCl_2$, and 1 mM AsA. Extracts were centrifuged at 4°C for 15 min at 16,000 × *g*, and the supernatants were analysed immediately for enzyme activities. Protein was quantified by Bradford's method (Bradford 1976), but due to the strong impact of fruit developmental stage on protein content (a 117% increase between green to red stage, $P<0.0001$, data not shown), enzyme activities were expressed per fresh weight instead of mg proteins.

Enzyme assays: All enzyme activities were determined in 200 µL volume kinetic reactions at 25°C, using a micro-plate reader. APX, DHAR and MDHAR activities were measured by the method of Murshed *et al*. (2008b). APX activity was measured in reaction mixtures consisting of 50 mM potassium phosphate buffer (pH 7.0), 0.25 mM AsA, 10 µL extract and 5 mM H_2O_2. Activity was determined by measuring the decrease in reaction rate at A290 and calculated from the 2.8 mM^{-1} extinction coefficient. DHAR activity was assayed in a reaction mixture consisting of 50 mM HEPES buffer (pH 7.0), 0.1 mM EDTA, 2.5 mM GSH, 0.2 mM DHA, and 10 µL extract. Activity was determined by measuring the increase in reaction rate at A265 and calculated from the 14.0 mM^{-1} extinction coefficient. MDHAR activity was determined in

Table 2 Impact of salinity treatments on fruit fresh weight, dry matter and sugar content. Data are means ± se. Fruit traits (fresh weight, %dry matter, soluble sugar and starch content) were subjected to a two way analysis of variance considering the ripening stage, the salinity treatments and the interaction between them. Mean comparison was performed with a Fisher test; significant differences (α=5%) between salinity treatments and ripening stages within a genotype were indicated by different letters in a column.

		Fruit fresh weight g	Dry matter g / 100 g FW	Soluble sugars g / 100 g DM	Starch g / 100 g DM
Cervil	Control	5.9 ± 0.1 c	11.1 ± 0.1 b	26.2 ± 0.4 c	14.3 ± 0.5 b
MG : Mature green	Ca + Na	6.0 ± 0.0 bc	13.1 ± 0.2 a	23.1 ± 0.7 d	19.4 ± 0.5 a
	Na	5.8 ± 0.1 c	12.7 ± 0.3 a	23.0 ± 0.5 d	20.7 ± 1.0 a
	Control	6.2 ± 0.1 b	11.2 ± 0.1 b	36.0 ± 1.0 b	7.8 ± 0.3 c
P : Pink	Ca + Na	6.0 ± 0.0 bc	12.9 ± 0.2 a	34.9 ± 0.4 b	12.9 ± 0.4 b
	Na	6.1 ± 0.0 b	13.1 ± 0.2 a	34.6 ± 0.7 b	13.6 ± 0.4 b
	Control	6.9 ± 0.0 a	11.1 ± 0.1 b	48.1 ± 0.6 a	2.2 ± 0.1 d
RR : Red ripe	Ca + Na	6.1 ± 0.0 b	13.0 ± 0.1 a	47.9 ± 1.8 a	3.7 ± 0.1 d
	Na	6.1 ± 0.1 b	13.2 ± 0.1 a	46.6 ± 0.6 a	3.5 ± 0.7 d
Levovil	Control	139 ± 4 a	4.0 ± 0.1 d	35.1 ± 0.7 de	9.1 ± 0.6 b
MG : Mature green	Ca + Na	110 ± 2 bc	6.9 ± 0.2 b	33.5 ± 0.1 e	9.9 ± 0.5 ab
	Na	103 ± 4 c	7.4 ± 0.2 a	33.6 ± 0.7 e	11.3 ± 0.4 a
	Control	133 ± 4 a	4.8 ± 0.1 c	41.3 ± 0.4 cd	0.5 ±0.0 e
P : Pink	Ca + Na	102 ± 4 c	7.5 ± 0.2 a	44.2 ± 0.7 bc	2.5 ± 0.1 d
	Na	103 ± 3 c	7.2 ± 0.2 ab	50.4 ± 6.6 ab	5.1 ± 0.8 c
	Control	141 ± 5 a	5.1 ± 0.2 c	41.8 ± 0.9 cd	0.4 ± 0.1 e
RR : Red ripe	Ca + Na	118 ± 3 b	6.9 ± 0.1 b	49.1 ± 0.9 ab	0.7 ± 0.2 de
	Na	105 ± 3 c	7.5 ± 0.1 a	51.0 ± 0.7 a	1.1 ± 0.1 de
Marmara	Control	120 ± 2 cd	4.6 ± 0.1 c	36.2 ± 0.4 de	0.8 ± 0.1 d
MG : Mature green	Ca + Na	100 ± 5 f	6.1 ± 0.1 b	35.0 ± 0.4 de	10.9 ± 0.2 b
	Na	103 ± 4 ef	6.9 ± 0.1 a	34.0 ± 1.4 e	14.1 ± 1.1 a
	Control	150 ± 8 ab	3.6 ± 0.1 d	37.3 ± 1.5 d	0.6 ± 0.1 d
P : Pink	Ca + Na	133 ± 2 bc	6.9 ± 0.1 ab	44.8 ± 0.7 bc	2.5 ± 0.3 c
	Na	115 ± 4 de	7.1 ± 0.4 a	44.6 ± 0.4 bc	3.5 ± 0.3 c
	Control	159 ± 6 a	4.7 ± 0.1 c	42.5 ± 0.2 c	0.4 ± 0.0 d
RR : Red ripe	Ca + Na	133 ± 4 c	6.7 ± 0.2 ab	46.9 ± 0.9 ab	0.8 ± 0.0 d
	Na	127 ± 5 c	6.9 ± 0.1 a	47.8 ± 0.8 a	0.8 ± 0.1 d

reaction mixtures consisting of 100 mM HEPES buffer (pH 7.6), 2.5 mM AsA, 0.25 mM NADH, 20 µL extract, and 0.4 unit ascorbate oxidase. Activity was determined by measuring the decrease in reaction rate at A340 and calculated from the 6.22 mM^{-1} extinction coefficient.

SOD activity was determined using modified method of Dhindsa *et al*. (1981). SOD activity was assayed in a 1.0 ml reaction mixture containing 50 mM potassium phosphate (pH 7.8) buffer, 13 mM methionine, 75 µM nitro blue tetrazolium (NBT), 0.1 mM EDTA, 10 µL of sample supernatant and 2 µM riboflavin. Tubes were briefly vortexes and triplicate, 200 µL aliquots from each tube, were placed in plastic 96-well plates. Each plate also contained commercial SOD in amounts of 0.6, 1.2, 1.8, 2.4 and 3 units to generate a standard curve. The plates were then placed above white light provided by a commercial overhead-transparency projector (Horizon, Model Apollo, Lincolnshire, IL, USA) for 5 min. After the light treatment, absorbance readings were taken at 560 nm in a plate reader. SOD in the extract quenched the photochemical reduction of NBT to blue formazan. Activity was expressed in units of SOD from the standard curve of activity units versus absorbance.

Statistical analyses

Fruit traits (fresh weight, dry matter soluble sugars and starch content), fruit ascorbate content, oxidative parameters and enzyme activities were subjected to a two-way analysis of variance considering the "salinity" factor, fruit ripening stage and their interaction using XLSTAT software (Addinsoft, France). When the salinity treatment was significant (α=5%), mean comparison was performed with a Fisher test; significant differences were indicated by different letters in the tables. Correlations among indicators of oxidative stress (H_2O_2, MDA), ascorbate and enzymatic activities related to ascorbate recycling were studied on the whole set of data (3 genotypes, 3 developmental stages, 3 salinity treatments and 3 repetitions).

RESULTS

Impact of salinity on fresh weight and dry matter and sugars contents

In control conditions, the fresh weight (FW) of fruits increased during ripening in 'Cervil' and 'Marmara', but not in 'Levovil' (**Table 2**). The Na treatment decreased fruit FW at MG, P and RR stages in 'Levovil', at P and RR stages in 'Marmara' and at RR stage in 'Cervil'. Adding Na or of a combination of Ca+Na had similar effect on the FW of 'Cervil'. But the Na treatment decreased more fruit FW in 'Levovil' and 'Marmara' compared to the Ca+Na treatment ($P<0.001$). Salinity increased fruit dry matter (DM) content at the different ripening stages but the Ca+Na treatment triggered a lower DM increase compared to the Na treatment. During ripening, soluble sugars increased whatever the genotype. Salinity had no effect on soluble sugars content in 'Cervil' (except a small decrease at MG stage). In contrast, salinity increased soluble sugars content in 'Levovil' and 'Marmara' at P and RR stages. Salinity also increased starch content at MG and P stages in 'Cervil' and 'Levovil' and at MG stage in 'Marmara'. The Ca+Na treatment limited starch accumulation in 'Levovil' and 'Marmara' compared to the Na treatment.

Impact of salinity on fruit ionic composition

Fruit ionic content in N, P and K expressed per g FW was about twice in 'Cervil' that in larger fruits ('Levovil' and 'Marmara', **Table 3**). This difference may be due to the higher fruit dry matter content in 'Cervil' (**Table 2**). In contrast, 'Cervil' did not accumulate much Ca and Na compared to larger sized genotypes. Increasing salinity favoured N accumulation in the three genotypes tested, but P and K were either not or only slightly affected.

As attempted, raising the Na supply in the nutrient solutions significantly induced an increase of Na^+ content in the fruit. This increase was more important in fruits harvested

Table 3 Ionic composition of red ripe fruits. Data expressed as µg g^{-1} FW are means ± se. Data were subjected to a two way analysis of variance considering the genotype, the salinity treatments and the interaction between them. Mean comparison was performed with Fisher's test; significant differences (α=5%) between salinity treatments and genotypes were indicated by different letters in a column.

		[N]	[P]	[K]	[Ca]	[Na]	[Ca]/[Na]
Cervil	Control	3000 ± 40 c	524 ± 31 a	4083 ± 77 a	52 ± 1 d	19 ± 0 g	2.76 ± 0.01 b
	Ca + Na	3390 ± 90 a	458 ± 18 b	3255 ± 28 b	106 ± 2 a	37 ± 0 f	2.84 ± 0.05 b
	Na	3215 ± 115 b	484 ± 20 ab	3298 ± 34 b	96 ± 1 b	73 ± 0 d	1.32 ± 0.02 d
Levovil	Control	935 ± 5 f	235 ± 2 c	2217 ± 4 e	61 ± 0 c	15 ± 0 h	4.08 ± 0.04 a
	Ca + Na	1170 ± 20 de	250 ± 16 c	2685 ± 26 d	60 ± 2 c	70 ± 0 e	0.87 ± 0.03 e
	Na	1165 ± 15 de	262 ± 5 c	2672 ± 13 d	58 ± 2 c	296 ± 1 a	0.20 ± 0.01 g
Marmara	Control	1110 ± 30 e	270 ± 4 c	2712 ± 6 cd	45 ± 0 e	20 ± 1 g	2.26 ± 0.08 c
	Ca + Na	1335 ± 25 d	269 ± 15 c	2822 ± 47 c	53 ± 0 d	88 ± 1 c	0.60 ± 0.01 f
	Na	1245 ± 35 de	261 ± 9 c	2671 ± 34 d	50 ± 1 d	209 ± 1 b	0.24 ± 0.00 g

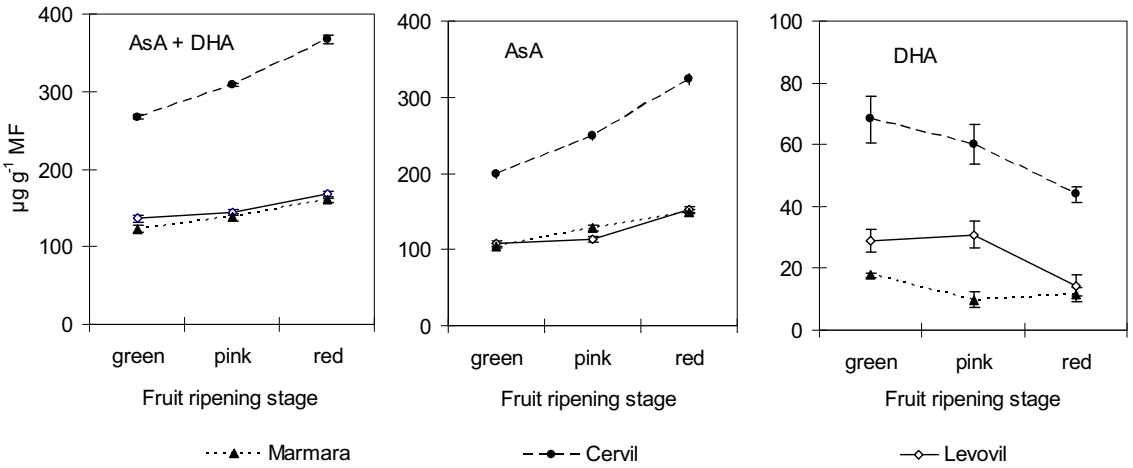

Fig. 2 Total ascorbate (AsA+DHA), reduced ascorbate (AsA) and oxidized ascorbate (DHA) contents during fruit ripening among genotypes grown under control conditions. Data are mean ± SE.

from plants irrigated with Na alone compared to Ca+Na treatment. Nevertheless, 'Cervil' accumulated much lower Na^+ (+95% with Ca+Na treatment and +280% with Na) compared to 'Levovil' (+370 and +1,870%, respectively) or 'Marmara' (+340 and +945%, respectively).

Fruits also accumulated more calcium under salinity treatments in 'Cervil' and 'Marmara' but not in 'Levovil'. Consequently, the [Ca]/[Na] ratio was strongly decreased (down to 20 times) in the larger fruit genotypes and the addition of calcium was not sufficient to maintain this ratio. In contrast, this ratio was not modified in 'Cervil' fruits under the Ca+Na treatment, or decreased by about 50% under the Na treatment.

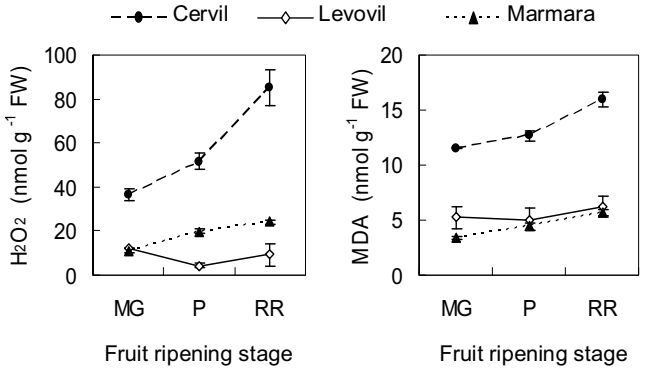

Fig. 3 H_2O_2 and MDA contents during fruit ripening among genotypes grown under control conditions. Data are mean ± se. MG: mature green, P: pink; RR: red ripe.

Comparison of ascorbate content, indicators of oxidative stress and activities of ascorbate recycling enzymes among genotypes grown under control conditions

In the three genotypes, total ascorbate and reduced ascorbate increased during fruit ripening while the oxidized form DHA decreased (**Fig. 2**). 'Cervil' (the cherry tomato) had the highest total ascorbate content more than twice that of 'Levovil' (or 'Marmara'). This was mainly due to higher content in the reduced AsA form, but also in DHA.

Fig. 3 shows H_2O_2 and MDA contents in fruits during ripening for the three genotypes. 'Cervil' had the highest contents in H_2O_2 and MDA which were about twice that found in fruits of the larger sized genotypes. During ripening, these molecules increased in 'Cervil' and 'Marmara' (**Fig. 3**). The activities of SOD, MDHAR and DHAR during ripening were similar among 'Cervil' and 'Levovil' but much higher in 'Marmara' (around 3 times more, **Fig. 4**). 'Cervil' had a very low APX activity compared to the larger sized genotypes. The MDHAR and DHAR activity decreased with ripening stage (except for MDHAR in 'Marmara').

Comparison of ascorbate content, indicators of oxidative stress and activities of ascorbate recycling enzymes among genotypes grown under increased salinity

Increasing salinity increased total ascorbate content by 18 to 70% ($P<0.0001$) depending on the genotype and fruit developmental stage except at RR stage in 'Cervil' (**Fig. 5**). This was mostly due to increased AsA content by Na addition which triggered the maximal increase compared to the Ca+Na addition. Increasing salinity increased DHA in

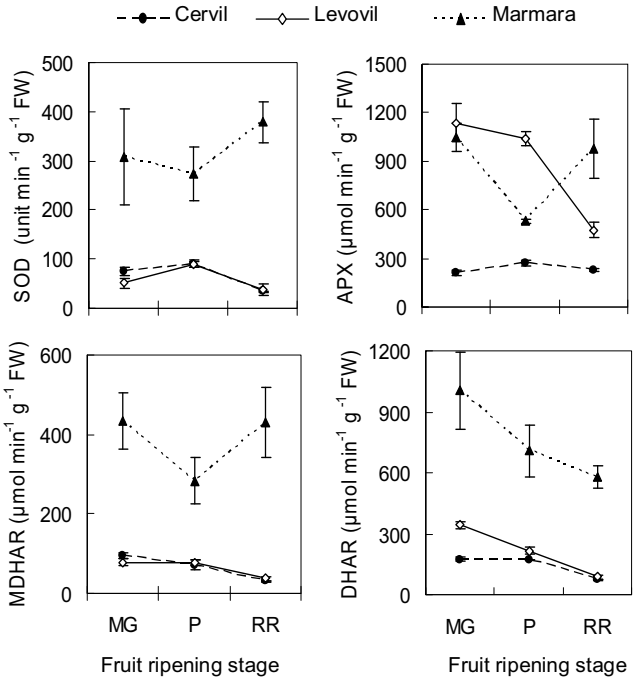

Fig. 4 Antioxidant enzyme activities (SOD, APX, DHAR, MDHAR) during fruit ripening among genotypes grown under control conditions. Data are mean ± se. MG: mature green, P: pink; RR: red ripe.

'Cervil' at MG stage but mostly in 'Marmara' when adding Na (compared to Ca+Na).

During the ripening period, under Na treatment, H_2O_2 content increased by 40% in 'Cervil' ($P<0.0001$), and in the larger fruits 'Levovil' (+250% at P stage) and 'Marmara' (+80% at MG and P stages) (**Fig. 6**). In response to Ca+Na treatment, H_2O_2 increased in 'Cervil' fruits at MG and P stages and in 'Marmara' at the MG stage. Considering fruits at RR stage under the two salinity treatment, a good correlation ($R^2 = 0.77$, data not shown) was found between increased fruit content in Na and increased H_2O_2 content; this confirms that genotypes which were not able to limit Na influx within the fruits had increased oxidative parameters.

Under Na treatment, lipid peroxidation estimated from MDA content was increased in 'Marmara' ($P<0.001$) and in 'Levovil', but not significantly in 'Cervil'. The Ca+Na treatment had no impact on MDA content in 'Cervil' and 'Levovil' while an increase in MDA content was observed in 'Marmara' at all stages.

For a given genotype, increasing salinity by adding Ca+Na (respectively by adding Na) globally triggered similar impact on the four enzymatic activities measured (**Fig. 7**). But these changes in activity were strongly dependent on the genotypes and on the salinity treatment. SOD activity increased in response to Ca+Na treatment (and to a lower extend to Na treatment) in 'Cervil' ($P<0.001$) and in 'Levovil' ($P<0.01$), but it was not significantly modified by Ca+Na or was slightly decreased by Na in 'Marmara' (**Fig. 7**). APX activity which removed excessive [H_2O_2] was significantly increased by salinity in 'Cervil' ($P<0.001$) and 'Levovil' ($P<0.06$) but not in 'Marmara'. The presence of calcium promoted the increase in APX activity in 'Cervil' only.

MDHAR activity increased with salinity in 'Cervil' ($P=0.03$) and 'Levovil' ($P=0.07$) mostly at the RR stage, but it decreased in 'Marmara' ($P<0.001$). DHAR activity also increased in 'Cervil' ($P<0.01$) and in 'Levovil' ($P<0.0001$) with salinity and mostly in RR fruits. In contrast DHAR activity was not modified in 'Marmara' by addition of Ca+Na or it was slightly decreased with Na alone ($P<0.1$). Similarly to what observed for other enzymes activity, Calcium promoted the increase in MDHAR and DHAR activity with salinity in 'Cervil' only.

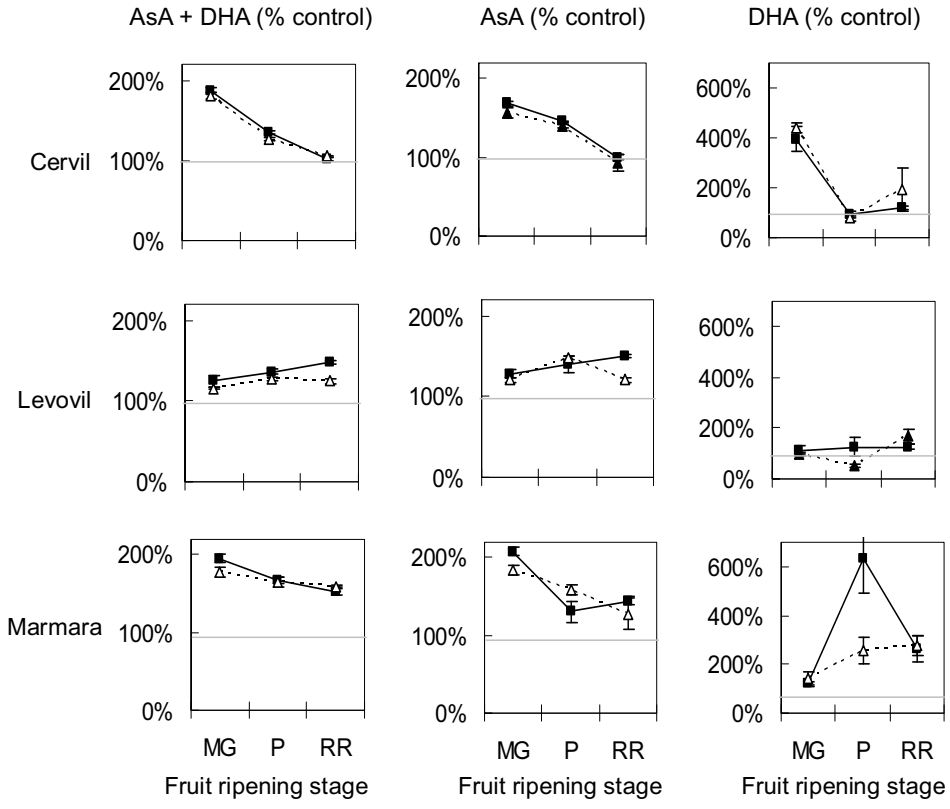

Fig. 5 Impact of salinity treatments on total ascorbate (AsA+DHA), reduced ascorbate (AsA) and oxidized ascorbate (DHA) contents in fruits harvested at mature green (MG), pink (P) and red ripe stage (RR). Each point represents the mean (±se) of three independent analyses (corresponding to three batches of 10 fruits) expressed as percent of the control (3 dS m^{-1}). The grey line at 100% represents the response observed in the controlled treatment (3 dS m^{-1}). Black squares and solid line: Na, salinity increased by addition of NaCl; white triangles and dashed line: Ca + Na, salinity increased by addition of NaCl and $CaCl_2$.

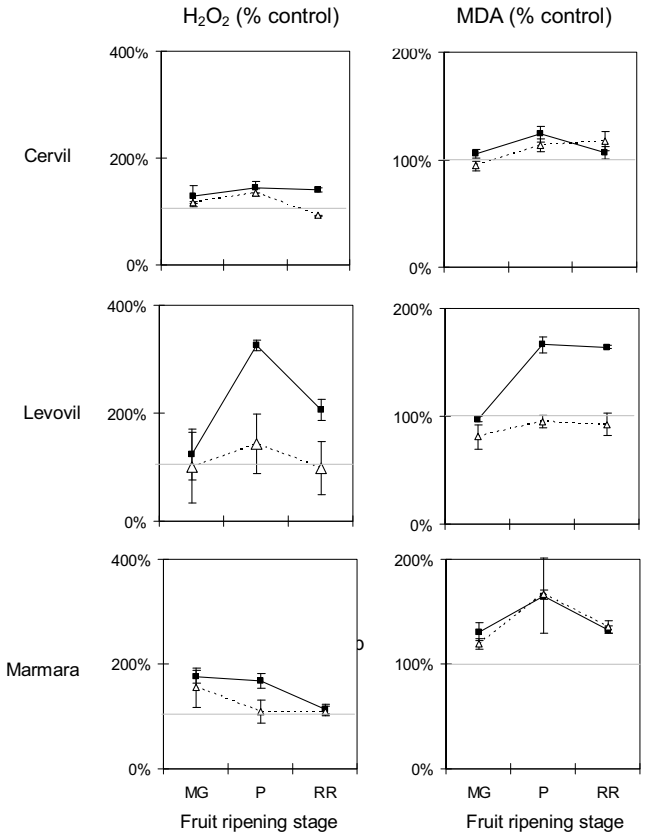

Fig. 6 Impact of salinity treatments on H_2O_2 and MDA contents. Legend is similar to **Fig. 5**.

Correlations between fruit content in ascorbate, oxidative parameters and the activities of antioxidant enzymes

The most significant correlations were found among total ascorbate and the indicators of oxidative stress [MDA] and [H_2O_2] (**Table 4, Fig. 8**). The correlation was not so good when considering DHA (**Table 4**). Strong correlations were also found among MDHAR or DHAR and the SOD activity: The latter initiates the removal of active oxygen producing [H_2O_2] followed by AsA consumption (by APX) to reduce H_2O_2 and the former (MDHAR and DHAR) were then involved in AsA regeneration.

DISCUSSION

The present study showed on three contrasted genotypes how ripening and salinity may affect fruit composition (dry matter, sugars, starch and ascorbate), oxidative parameters and antioxidant enzyme activity. Both ripening and salinity have been shown to trigger oxidative stress response in tomato fruit. Jiménez *et al*. (2002) reported changes in oxidative and anti-oxidative parameters during tomato fruit ripening. They found that [H_2O_2] increased at breaker stage and then decreased during ripening. Similarly, Mondal *et al*. (2004) reported on tomato that ripening was accompanied by a progressive increase in oxidative stress; at the beginning of ripening, fruit respond to the increase oxidative stress by increasing their ascorbate content as well as the activity of scavenging enzymes. Later on, ROS scavenging system was not sufficient to cope with ROS production leading to the accumulation of ROS. Ahn *et al*. (2002) also observed in cherry tomato a transient increase in the SOD from MG to orange stage followed by a decrease at the end of the ripening, which was in agreement with the present data on 'Cervil' and 'Levovil'.

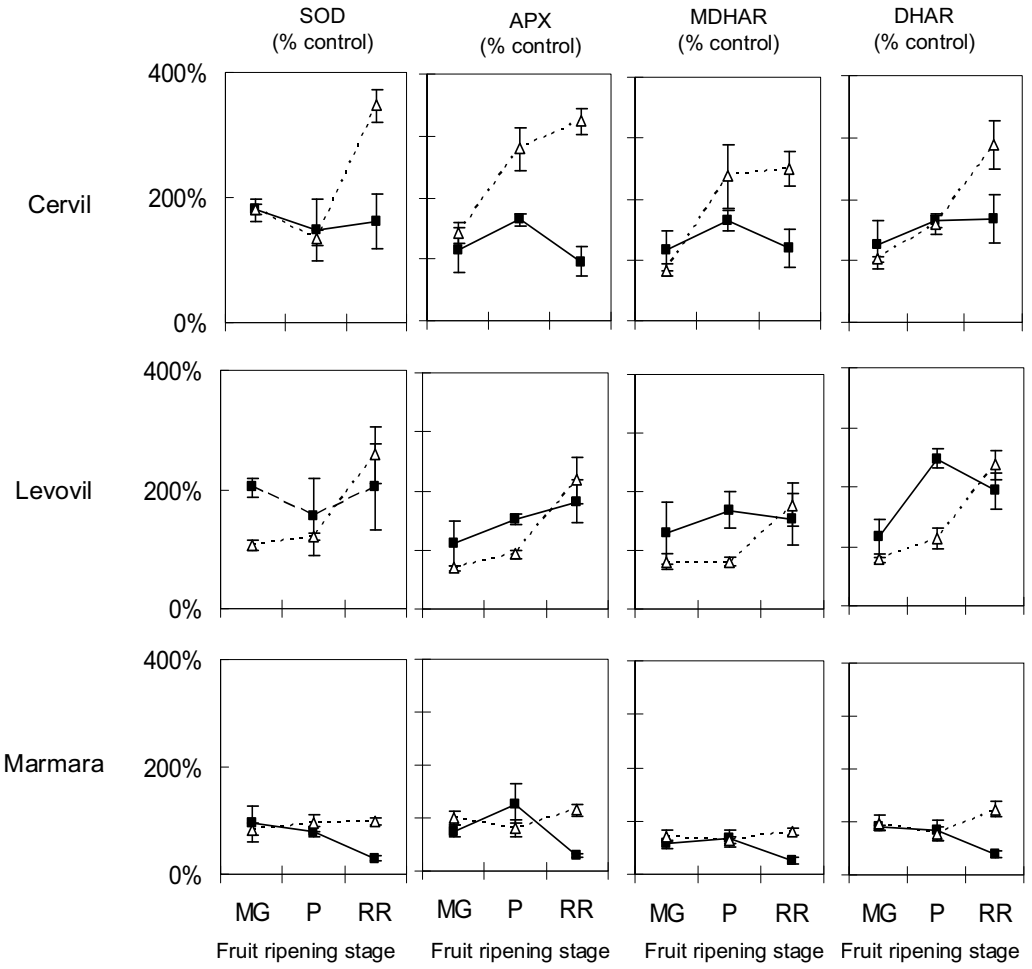

Fig. 7 Impact of salinity treatments on antioxidant enzyme activities (SOD, APX, DHAR, MDHAR). Legend is similar to **Fig. 5**.

Table 4 Pearson's correlation coefficients among variables considering the whole set of data (81 points corresponding to three genotypes, three developmental stages, three salinity treatments and three replicates per treatment). Bold values are significantly different from 0 ($P<0.05$). **Fig. 8** illustrates the highest correlations among total ascorbate and indicators of oxidative stress. t-AsA: total ascorbate, r-AsA: reduced ascorbate.

Variables	APX	DHAR	SOD	MDHAR	H_2O_2	MDA	t-AsA	r-AsA	DHA
APX	**1**								
DHAR	**0.48**	**1**							
SOD	**0.38**	**0.86**	**1**						
MDHAR	**0.36**	**0.87**	**0.91**	**1**					
H_2O_2	**-0.49**	**-0.34**	-0.19	**-0.25**	**1**				
MDA	**-0.50**	**-0.52**	**-0.33**	**-0.40**	**0.88**	**1**			
t-AsA	**-0.51**	**-0.40**	-0.22	**-0.32**	**0.85**	**0.90**	**1**		
r-AsA	**-0.49**	**-0.35**	-0.18	**-0.27**	**0.82**	**0.86**	**0.96**	**1**	
DHA	**-0.31**	**-0.32**	**-0.23**	**-0.32**	**0.50**	**0.55**	**0.59**	**0.36**	**1**

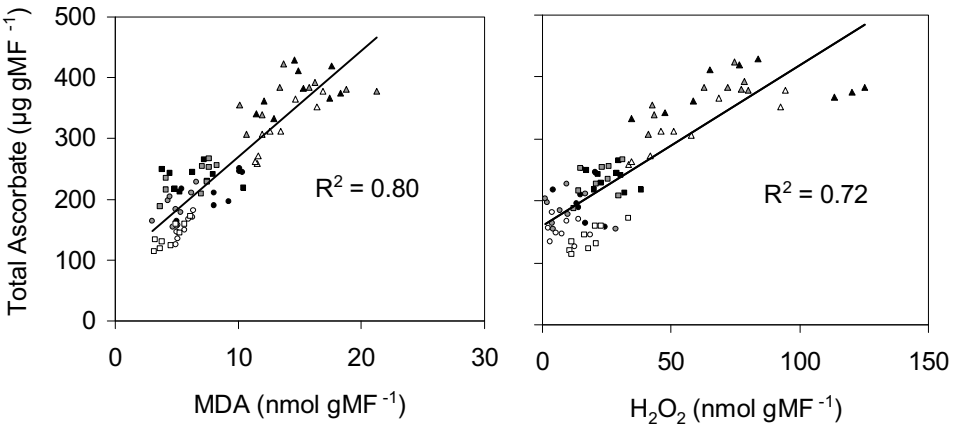

Fig. 8 Correlations among total ascorbate (AsA+DHA) and indicators of oxidative stress such as fruit content in MDA and H_2O_2. Correlations were established gathering data from the three genotypes, the three salinity treatments and the three developmental stages. Each point represents an analysis made on the mixed frozen powder issued from 10 fruits. 'Cervil': triangle, 'Levovil': circle, 'Marmara' square; white symbols: control; grey symbols: Ca+Na treatment, black symbols: Na treatment.

In the present study, we observed contrasted responses linked to the genotypes: 'Cervil' showed a strong increase in [H_2O_2] content from MG to P and even more from P to RR stage, whereas 'Marmara' showed a smaller increase in [H_2O_2] content mostly from MG to P stage, and in contrast [H_2O_2] content slightly decreased from MG to P stage in 'Levovil'. During ripening, lipid peroxidation also increased in the three genotypes but to a larger extent in 'Cervil'. This could be linked to the higher accumulation of H_2O_2 in 'Cervil' as we found a nice correlation between these two compounds (H_2O_2 and MDA, **Table 4**). In a previous study (Jiménez *et al.* 2002), the increased lipid peroxidation in tomato fruit coincided with the increased [H_2O_2] content during ripening, both indicating increased oxidative activity. Considering AsA accumulation, 'Cervil' was once again very different from the larger sized genotypes ('Levovil' and 'Marmara') having more than the double content in ascorbate may be linked to increased [H_2O_2] accumulation and lower APX activity.

This hypothesis, that higher AsA content coupled to higher ascorbate recycling activities may induce an higher tolerance to increased H_2O_2 and limit lipid peroxidation was then tested by comparing the control treatment to a moderate salinity stress (Ca+Na) or to a stronger salinity stress (Na). The strong correlation observed between ascorbate content and oxidative parameters confirmed the involvement of ascorbate as a strong antioxidant in response to oxidative stresses induced by ripening or salinity. Moreover, we were able to discriminate three types of response to increasing oxidative stress depending on the genotype:

'Marmara' had a low content in ascorbate and oxidative parameters that slightly increased during ripening. The activities of antioxidant enzymes were already very high under control conditions which may be related to low MDA accumulation during ripening. Increasing salinity strongly increased fruit content in Na^+, and both form of ascorbate (AsA and DHA), but it did not enhance antioxidant enzyme activity (**Fig. 7**), so that oxidative parameters (mostly MDA) increased with salinity (**Fig. 6**).

'Levovil' had a low content in ascorbate and oxidative parameters that did not vary a lot with ripening. The activities of antioxidant enzymes were low under controlled condition (except APX activity). Increasing salinity strongly increased fruit content in Na^+, and in reduced ascorbate. It also increased antioxidant enzyme activity specifically in the Na treatment compared to the Ca+Na treatment, but this increase was not sufficient to cope with increased oxidative stress in the Na treatment and consequently both indicators of oxidative stress strongly increased.

'Cervil' had a high content in ascorbate and oxidative parameters which increased a lot with ripening. The activities of antioxidant enzymes were low under control conditions, so that H_2O_2 accumulate a lot. Increasing salinity triggered a moderate increase in Na^+ content, in AsA and DHA (no more significant at RR stage). It also increased antioxidant enzyme activities to a larger extent in the Na treatment compared to the Ca+Na treatment. This increase was sufficient to limit the accumulation of oxidative parameters with salinity. This increase in APX activity might be related to the higher tolerance to salt oxidative stress in 'Cervil'. Indeed, Tsugane *et al*. (1999) previously observed in an Arabidopsis mutant (*pst1*) showing higher salt tolerance an increase in APX activity in response to salt photo-oxidative stress. Shalata and Tal (1998) also suggested that in a wild salt-tolerant tomato species (*Lycopersicon pennelli*) the tolerance to salt stress could be due to higher salt induced activities of SOD, APX, and MDHAR coupled to higher inherited activities of SOD and APX.

'Cervil' fruits content in Ca increased with salinity (both Ca+Na and Na treatments), in contrast to the larger sized genotypes for which Ca was not significantly modified. This might be due to the lower vegetative development of the cherry tomato compared to 'Levovil' and 'Marmara'. Consequently the transpiration flux from the leaves might be reduced in 'Cervil', allowing more Calcium to reach and accumulate in 'Cervil' fruits compared to larger sized geno-

types. Such a relation between calcium transport into the fruits and transpiration flux linked to humidity during the day was already underlined by Adams and Holder (1992). Lopez and Satti (1996) previously reported protective effect of Calcium that limited the impact of salinization of the nutrient solution. They observed that addition of NaCl reduced root volume and fresh weight and consequently fruit yield and that addition of calcium reversed these negative impacts. In the present study, Na treatment strongly increased oxidative stress parameters ([H_2O_2] or [MDA]) compared to the Ca+Na treatment, which triggered a lower or no increase in these parameters. These data confirmed that the combination of Ca+Na compared to Na treatment led to a lower stress that may be due to lower Na concentration within the fruit (**Table 3**), despite similar electroconductivity of the nutrient solution. These data are in agreement with previous results (Rajasekaran *et al.* 2000) in tomato leaves for which the salt tolerance of tomato species was correlated among others to their ability to exclude Na^+ from the leaves. Thus, increased Na content in fruit tissue may be responsible for increased oxidative parameters.

The present study underlines strong correlations among the different traits measured to characterize the oxidative stress. The reductase activities (MDHAR and DHAR) were correlated, and also correlated to the SOD activity. This indicates that the detoxification of superoxide radicals on one side (by SOD) and the regeneration of reduced AsA on the other side (by MDHAR or DHAR) were tightly correlated. But as the intensity of the response to salinity was genotype dependent: the increased activity of ascorbate recycling enzymes was only sufficient in the cherry tomato to avoid further increased in oxidative parameters. This apparent tolerance of 'Cervil' to salinity stress might be related to the fact that i) it already strongly responded to the oxidative stress induced by ripening by accumulating high amount of antioxidant such as ascorbate, ii) it did not accumulate much Na^+ compared to the other genotypes, and iii) it strongly increased its enzymatic activities related to ascorbate recycling in the Ca+Na treatment.

ACKNOWLEDGEMENTS

We would like to thank L. Rosso for plant management and control of salinity treatments, S. Gagnier for her help in fruit harvesting and blending and G. Heral-Llimous and K. Alary for ionic assays in the Cirad laboratory.

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