Differences in Blossom-end Rot Resistance in Tomato Cultivars is Associated with Total Ascorbate rather than Calcium Concentration in the Distal End Part of Fruits *per se*

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Calcium is widely accepted as the main factor responsible for blossom-end rot (BER) appearance in tomato (*Solanum lycopersicum* L.) fruit. However, reactive oxygen species (ROS), which can damage plant tissues have also been proposed to initiate BER appearance in tomatoes and other fruit-bearing vegetables. Ascorbate, the major antioxidant in tomato fruit, is generally lower during green fruit development, which corresponds to the stage of BER appearance. Accordingly, one hypothesis is that tomato cultivars with a lower susceptibility to BER under salt stress have higher ascorbate contents and thus better control of ROS levels. In this study, to clarify the relationship between BER incidence and oxidative stress, two BER resistant cultivars, ‘Managua RZ’ and ‘House Momotaro’ and one BER-susceptible cultivar ‘Reiyoh’, were cultivated under salinity or standard nutrient solution (control) conditions. Calcium, potassium, magnesium, total hydro-soluble antioxidants, and ascorbate concentrations were measured in the distal pericarp 1 to 2 days prior to symptom appearance and during symptom appearance in healthy and affected fruits. When salt stress was applied, only BER-resistant cultivars showed a significant increase in ascorbate contents prior to BER appearance as compared with their levels under the control condition. In contrast, pre-BER Ca^{2+} concentrations did not associate with the BER susceptibility of each cultivar. Interestingly, ‘Reiyoh’ showed much higher $\text{K}^+/\text{Ca}^{2+}$ and $(\text{K}^+ + \text{Mg}^{2+})/\text{Ca}^{2+}$ ratios than the two other cultivars in healthy fruits due to a strong tendency towards lowered Ca^{2+} concentrations. A similar tendency was also observed in apple “bitter pit”. The ability to increase the fruit antioxidant capacity and maintain mineral balance under salt stress conditions may explain the resistance to BER development in highly resistant cultivars, probably by the avoidance of oxidative-induced cell necrosis and stabilization of the cell membranes, respectively.

Key Words: ascorbate, BER, cultivar, oxidative stress, ROS.

Introduction

Blossom-end rot (BER) is a physiological disorder in tomatoes and other fruit bearing vegetables. In tomatoes, it appears as a water-soaked area at the distal part (style-end) of green fruits aged 12 to 15 day after anthesis. It rapidly develops into black necrotic lesions and can cause severe yield losses (Geraldson, 1955; Spurr, 1959; Taylor and Locascio, 2004). Calcium is widely

accepted to be the main factor causing BER (Taylor and Locascio, 2004). In fact, BER appears when calcium is lacking in the distal part of tomato fruit (Bradfield and Guttridge, 1984). This calcium deficiency can be triggered by several factors including low calcium in the nutrient solution (Van Goor, 1968), nutrient imbalances (Hao and Papadopoulos, 2004; Raleigh and Chucka, 1944; Van Der Boon, 1973), salinity (Aktas et al., 2003; Bradfield and Guttridge, 1984; Robbins, 1937; Tabatabaie et al., 2004), drought (Pill and Lambeth, 1980), and low atmospheric humidity (Bradfield and Guttridge, 1984); all factors that can limit plant calcium uptake and translocation to the fruit (Taylor and

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Locascio, 2004).

At the cellular level, BER appears with low levels of Ca^{2+} in plasma membranes (Suzuki et al., 2003). The development of BER is described as an increase in cell membrane leakage, followed by plasmolysis and membrane breakdown that lead to the water-soaked symptoms and subsequent necrosis of tissues (Simon, 1978; Suzuki et al., 2003; Van Goor, 1968). These facts can be explained by the need for Ca^{2+} for cell membrane stability and semi-permeability (Marschner, 1995). Several authors tried to determine the critical calcium concentration under which BER is triggered in the fruit distal pericarp, but no consensus was established (Saure, 2001). In some cases, total Ca^{2+} concentration was even higher in BER fruits than healthy ones (Nonami et al., 1995). Based on these facts, Saure (2001) concluded that stress factors may be involved in BER appearance rather than Ca^{2+} *per se*.

Reactive oxygen species (ROS) were proposed to be involved in the causal mechanism of BER development (Aktas et al., 2003). ROS are a major player in stress-related mechanisms. ROS are well known to be involved in triggering cell damage and death by membrane lipid peroxidation, leading to increased membrane leakage and cell lysis (Van Breusegem and Dat, 2006), a very similar mechanism to what happens during BER development. Interestingly, the appearance of BER was found to correspond to the stages of fruit development at which the production of ROS was maximal while scavenging was limited in pepper fruits (Aktas et al., 2003). ROS levels can be controlled by several enzymatic and non-enzymatic mechanisms. For enzymatic control, enzymes like superoxide dismutase (SOD), peroxidase, and catalase can be deployed to control ROS levels, while for non-enzymatic control, two main metabolites, ascorbate and glutathione, are produced and recycled to protect the plant from oxidative damage (Mittler, 2002).

In tomato fruit, ascorbate is known to be the major antioxidant (Francesco et al., 2005). Ascorbate concentrations were reported to be limited during green fruit development (Torres and Andrews, 2006), which is also the stage of BER appearance (Spurr, 1959). Ascorbate concentration in tomato fruit was also found to be reduced under high salinity (D'Amico et al., 2003), heat (Massot et al., 2013) and light stress conditions (Ioannidi et al., 2009; Torres et al., 2006), all conditions favorable for BER appearance (Olle and Bender, 2009). Interestingly, fruit calcium concentration, the major known factor in BER initiation, has also been proposed to be positively correlated with ascorbate levels in tomato fruit (Premuzic et al., 1998) and the conditions favorable to BER, like salinity, drought and heat stress, were also found to increase ROS levels in tomato fruit (Leyva et al., 2013; Murshed et al., 2013, 2014). Taking all these facts into account, there seems to be a connection between conditions that lower ascorbate concentra-

tions and increase ROS levels to the susceptibility toward BER in tomatoes.

Therefore, we hypothesize that ascorbate is a major player in the control of BER appearance by protecting distal fruit cell membranes from oxidative damage. In our preliminary experiment, we could show the differences in BER susceptibility among some different tomato cultivars under saline conditions. Indeed, one cultivar showed high tolerance to BER under saline conditions regardless of its low Ca^{2+} concentrations. From these results, we expect that cultivars with less susceptibility to BER show higher ascorbate concentrations and antioxidant capacity prior to, and during the stages of BER appearance.

The objective of this study was to show the possible role of ascorbate as a fruit antioxidant in the protection from BER-inducing conditions. For that, three cultivars with different susceptibilities to BER were cultivated with a standard nutrient solution and a BER-inducing one, with high salinity and enriched with SO_4^{2-} , K^+ , and Mg^{2+} . The total hydrosoluble antioxidant, ascorbate, glutathione, and mineral concentrations were measured in fruit samples taken 1 to 2 days prior to symptom appearance and during symptom appearance in healthy and affected fruits. The status of each variable measured at each sampling stage, helped to clarify their degree of involvement in the resistance to BER appearance.

Materials and Methods

Plant materials and growing conditions

The experiment was conducted in a greenhouse of the Agricultural and Forestry Research Center in the University of Tsukuba, from April 29 until July 31 2016.

Three indeterminate type tomato (*Solanum lycopersicum* L.) cultivars with different susceptibilities to BER were used. These cultivars were selected after screening for BER susceptibility was performed on 8 cultivars from Japanese and European origins. The screening was performed by growing plants under salt stress conditions and three cultivars, in which Ca^{2+} concentrations in the distal pericarp and BER were not associated, were selected: 'Managa RZ' (RijkZwaan, De Lier, Netherlands) and 'House Momotaro', shown as 'H. Momotaro' in figures, (Takii & Co., Ltd., Kyoto, Japan) are BER-resistant cultivars, and 'Reiyoh' (Sakata Seed Co., Kanagawa, Japan) is a sensitive one. Seedlings were grown in compressed peat Jiffy pots (Jiffy International AS, Kristiansand, Norway) filled with coir substrate. Pots were set into a deep flow technique (DFT) system with Otsuka-A recirculating nutrient solution (Otsuka-A; Otsuka Chemical Co., Ltd., Osaka, Japan) adjusted to an electrical conductivity (EC) of $1.2 \text{ dS} \cdot \text{m}^{-1}$ and pH of 5.8–6.5 by adding a 1 M sulfuric acid solution. When 5 to 6 true leaves were fully expanded, twelve plants from each cultivar were

selected and transplanted into each treatment area located in one of two different gutters of a nutrient film technique (NFT) cultivation system in a glasshouse. An Enshi nutrient solution containing $16 \text{ me}\cdot\text{L}^{-1}\text{NO}_3^-$, $4 \text{ me}\cdot\text{L}^{-1}\text{SO}_4^{2-}$, $4 \text{ me}\cdot\text{L}^{-1}\text{PO}_4^{3-}$, $8 \text{ me}\cdot\text{L}^{-1}\text{K}^+$, $4 \text{ me}\cdot\text{L}^{-1}\text{Mg}^{2+}$, $8 \text{ me}\cdot\text{L}^{-1}\text{Ca}^{2+}$, and $4 \text{ me}\cdot\text{L}^{-1}\text{NH}_4^+$ having an EC of $1.6\text{--}1.7 \text{ dS}\cdot\text{m}^{-1}$ was used and pH was maintained at $5.8\text{--}6.5$ in the same way described above. Planting density was $2.35 \text{ plants}\cdot\text{m}^{-2}$ ($26.5 \text{ cm} \times 160 \text{ cm}$) and plants were trained to a single stem. All lateral shoots were removed as they appeared and plants were pinched at three leaves above the fourth truss. Fully opened flowers were manually pollinated and their fruit age was monitored in terms of days after anthesis (DAA). The number of fruits set in each truss was limited to five starting from flower pollination to keep fruit growth rates close to their potentials in order to better express the BER susceptibility of each cultivar. Irrigation was applied for 15 min every hour during daytime and was done five times during the night. When flowering began, the nutrient solution treatment was started. The same Enshi nutrient solution was maintained for the control treatment while the BER-inductive treatment received a similar nutrient solution with $6.5 \text{ me}\cdot\text{L}^{-1}\text{SO}_4^{2-}$, $4 \text{ me}\cdot\text{L}^{-1}\text{K}^+$, and $2.5 \text{ me}\cdot\text{L}^{-1}\text{Mg}^{2+}$ added. The BER-inductive (BERi) nutrient solution's EC value was gradually raised to $8 \text{ dS}\cdot\text{m}^{-1}$ by using NaCl to avoid osmotic shock. This target EC was reached in 3 days, prior to full bloom. It was technically impossible to maintain the nutrient composition as it was set when first injected in the NFT tanks, so to insure that the composition was maintained as close as possible to the target ion values, the nutrient solution was exchanged once per week prior to flowering and twice per week from the start of flowering as plants grew bigger and consumed more water. Preliminary experiments showed that this nutrient solution induced higher incidences of BER than other ones with equivalent EC using only NaCl, or solutions enriched only with K^+ and Mg^{2+} . Also, Ca^{2+} concentration was maintained similar to the control in this nutrient solution to avoid Ca^{2+} deficiency symptoms in the plant itself. A shadowing net was placed above the culture from 9 a.m. to 3 p.m. to limit high solar radiation in summer conditions. Also, the top and side ventilation windows were automatically opened to prevent the air temperature from increasing.

BER appearance, averaged fruit fresh weight, and BER incidence

The average age of BER appearance was calculated for every plant in terms of DAA. Then, BER appearance was calculated by summing the obtained values, and dividing them by the corresponding number of plants. Averaged fruit fresh weight was calculated by summing the fresh weight of mature fruits in the first truss for each plant, and dividing them by the corresponding number of fruits. BER incidence was esti-

mated from six tomato plants for each combination of cultivar/nutrient solution treatment. It was expressed in terms of percentage of affected fruits from the total number of fruits in the first truss.

Fruit sample preparation

Sampling was performed on "BER-affected" fruits on the day of symptom appearance. The age of the affected fruit was noted and "healthy fruits" of the same age were collected from both the control and BER-inductive treatments. Fruits aged 1 to 2 days younger than the BER affected fruits were also collected from both nutrient solution treatments. These fruit pericarp samples were sliced from the proximal (peduncle side) and distal (style side) part of the fruit, frozen in liquid nitrogen, ground into a fine powder, and stored at -80°C .

Fruit mineral analysis

To determine the mineral composition in tomato pericarp, samples were freeze-dried for 48 h. After measuring dry weight, samples were placed into a muffle at 550°C for 4 h for ashing. The obtained ash was dissolved with $200 \mu\text{L}$ of water before adding $200 \mu\text{L}$ of concentrated HCl. The dissolved ash was then further diluted to determine calcium, potassium, and magnesium concentrations. Each element's concentration was measured by a polarized Zeeman atomic absorption spectrophotometer (ZA3300; Hitachi High-Technologies Co., Tokyo, Japan).

Total hydrosoluble antioxidant assay

The azino-bis(3-ethylbenzothiazolin-6-sulfonic) acid (ABTS) assay was used to determine the total hydrosoluble antioxidant concentration. The assay was performed following the method described by Marc et al. (2016) which was adapted to microplates. Briefly, an aqueous ABTS⁺ solution was prepared by the oxidation reaction of ABTS (Sigma Aldrich, Missouri, USA) with MnO_2 . 50 to 100 mg of frozen powder from each sample was dispatched into micro tubes (Micronic, Lelystad, Netherlands). $500 \mu\text{L}$ of 50% ethanol solution was added to the frozen powder for hydrosoluble antioxidant extraction. After mixing, tubes were centrifuged for 5 min at 4°C . $5 \mu\text{L}$ of the supernatant was added to $300 \mu\text{L}$ of the ABTS⁺ solution. The presence of antioxidants in the samples resulted in the discoloration of the mixture that was measured at 734 nm. This discoloration magnitude was proportional to the total hydrosoluble antioxidant capacity of the sample and was calculated using an ascorbate standard curve.

Ascorbate and glutathione assays

Total and reduced ascorbates were measured according to the procedure described by Stevens et al. (2006) with slight modifications. Briefly, ascorbate was extracted from 50 to 100 mg of frozen pericarp powder in

500 μ L of cold 6% phosphoric acid. After vigorous mixing, the homogenate was centrifuged at maximum speed (4000 rpm; $2271 \times g$) and 4°C for 25 min. For total ascorbate measurement, dehydroascorbate was reduced by incubating with 5 mM dithiothreitol (DTT) followed by a reaction with 40 mM N-ethylmaleimide (NEM) to eliminate excess DTT. For the reduced ascorbate, the same procedure was followed but DTT and NEM were replaced by the same volume of 0.4 M phosphate buffer, pH 7.4. A chromogenic reagent was prepared by mixing A and B solutions, for which the compositions were as follows: solution A: 31% orthophosphoric acid, 4.6% w/v trichloroacetic acid (TCA), and 0.6% w/v iron chloride; solution B: 4% 2,2-dipyridyl (w/v made up in 70% ethanol); and solutions A and B were mixed 2.75 parts (A) to 1 part (B). After incubation, the absorbance was read at 520 nm and ascorbate concentration was calculated using an ascorbate standard curve. A glutathione assay was performed with the same supernatant prepared for the ascorbate assay, according to the protocol described by Griffith (1980). The production of 5-thio-2-nitrobenzoic acid (TNB) was used to measure the total glutathione concentration present in the extract. TNB was produced by the reaction of reduced glutathione (GSH) with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB). The reaction was performed in the presence of glutathione reductase and started by the addition of 2 mM NADPH. The production rate of TNB, absorbing at 405 nm, was proportional to the concentration of GSH in the extract. Total glutathione concentration was calculated using a GSH standard curve. For the glutathione disulphide assay (Oxidized glutathione or GSSG), 50 μ L of the extract was incubated in the presence of 1 μ L of 0.5 M 4-vinylpyridine (4-vpd) to mask the presence of reduced glutathione. The same reaction described above was then performed, and GSSG concentration was calculated against a GSSG standard.

Statistical analysis

Statistical analysis was performed using IBM SPSS

Statistics for Windows, Version 24.0 (IBM Co., NY, USA). A two-way ANOVA ($P < 0.05$) with “Nutrient solution” and “Cultivars” as independent variables, then a (post-hoc) Tukey-HSD test was conducted.

Results

Average fruit fresh weight, BER incidence, and BER appearance

In the control treatment, average fruit fresh weight was 131.4 g, 112.8 g, and 101.6 g for ‘Managua RZ’, ‘Reiyoh’, and ‘House Momotaro’, respectively (Fig. 1A). In the BERi treatment, fruit fresh weight was significantly decreased to around 50 g per fruit for all three cultivars.

No BER was observed under the control condition (data not shown), while symptom incidence appeared differently between cultivars under the BERi condition (Fig. 1B). Namely, ‘Reiyoh’ showed the highest BER incidence of 63.9%. In contrast, ‘Managua RZ’ and ‘House Momotaro’ had significantly lower BER incidences of 2.8% and 11.7%, respectively. Those symptoms appeared 11 to 13 DAA for all three cultivars (Fig. 1C).

Distal pericarp mineral concentrations

Mineral concentrations were measured in the distal pericarp 1 to 2 days prior to symptom appearance (pre-BER) and also in healthy and affected fruits on the day symptoms appeared (Table 1). No significant differences were observed for K^+ concentration at each observation stage between cultivars, and treatments. For Mg^{2+} concentration, at the pre-BER stage, only ‘Reiyoh’ had a significant increase of 22% compared to the control, which made its concentration higher than the two other cultivars under the same treatment. In healthy fruits, ‘Reiyoh’ also tended to have more Mg^{2+} than the other cultivars. In the affected fruits, both Japanese cultivars ‘Reiyoh’ and ‘House Momotaro’ had more Mg^{2+} than ‘Managua RZ’.

In the pre-BER stage, Ca^{2+} concentrations were kept

Fig. 1. (A) Fruit fresh weight in tested cultivars ($n = 5-6$) and (B) BER incidence under the BER-inductive condition ($n = 6$). (C) BER appearance estimated as the averaged fruits age where BER symptoms appeared in tested cultivars under the BER-inductive condition ($n = 3-5$). Different letters between cultivars represent a statistical difference in BER incidence ($P < 0.05$). NS: ANOVA was not significant ($P < 0.05$). Data are means $\pm$ standard error (SE).

Table 1. Potassium (K^+), magnesium (Mg^{2+}), and calcium (Ca^{2+}) concentrations and ratios of K/Ca and $(K+Mg)/Ca$ in the distal pericarp of Pre-BER, healthy and affected tomato fruits under standard nutrient solution treatment (Control) and BER inductive nutrient solution treatment (BERi) with a summary of the Two-way ANOVA analysis. Data is expressed as percentage (%) of dry weight.

	Sampling	Control			BERi			Source ^z		
		'Managua RZ'	'Reiyoh'	'H. Momotaro'	'Managua RZ'	'Reiyoh'	'H. Momotaro'	Cv ^x	T	Cv×T
K^+	Pre-BER	3.16±0.10 NS	3.09±0.10 NS	2.69±0.10 NS	3.12±0.06 NS	3.31±0.25 NS	3.08±0.2 NS	NS	NS	NS
	Healthy	3.60±0.10 NS	3.37±0.31 NS	3.15±0.20 NS	2.99±0.15 NS	2.81±0.30 NS	3.20±0.08 NS	NS	NS	NS
	Affected				2.82±0.186 a	3.33±0.06 a	3.35±0.1 a	*	—	—
Mg^{2+}	Pre-BER	0.174±0.002 b ^y	0.189±0.007 b	0.171±0.006 b	0.179±0.002 b	0.228±0.01 a	0.199±0.003 b	**	**	*
	Healthy	0.177±0.008 ab	0.221±0.010 a	0.197±0.006 ab	0.175±0.008 b	0.210±0.01 ab	0.189±0.009 ab	**	NS	NS
	Affected				0.157±0.006 b	0.205±0.009 a	0.191±0.002 a	**	—	—
Ca^{2+}	Pre-BER	0.054±0.003 a	0.045±0.005 ab	0.042±0.003 ab	0.029±0.006 bc	0.012±0.003 c	0.014±0.003 c	**	**	NS
	Healthy	0.088±0.005 a	0.055±0.013 ab	0.038±0.005 bc	0.021±0.003 c	0.003±0.0004 c	0.031±0.007 bc	**	**	**
	Affected				0.020±0.003 a	0.009±0.002 b	0.009±0.002 ab	*	—	—
K^+/Ca^{2+}	Pre-BER	59.0±3.5 c	71.3±8 bc	66.4±8.7 bc	122.5±22.4 b	221.9±10.6 a	204.7±18.5 a	NS	*	NS
	Healthy	41.6±4.0 b	79.1±28.3 b	92.4±17.5 b	152.1±14.8 b	922.7±209.1 a	157.9±62.7 b	**	**	**
	Affected				159.8±29.2 NS	433.8±124.8 NS	381.0±84.4 NS	NS	—	—
$(K^++Mg^{2+})/Ca^{2+}$	Pre-BER	62.3±3.7 c	75.7±8.5 bc	70.6±9.1 bc	129.5±23.6 b	237.8±11.9 a	217.4±20 a	NS	*	NS
	Healthy	43.6±4.0 b	84.4±30.2 b	98.1±18.4 b	161.1±15.9 b	993.9±219.7 a	167.4±66.6 b	**	**	**
	Affected				168.5±30.5 NS	459.3±132 NS	402.7±88.9 NS	NS	—	—

^z NS, *, or **: The effect was not significant ($P>0.05$), significant ($0.01<P<0.05$), or highly significant ($P<0.01$).

^y Different letters represent a statistical difference with Tukey-HSD test ($P<0.05$). Data are means±standard error (SE). (n=3–5)

^x Cv: "cultivar"; T: "nutrient solution treatment".

above 0.04% for all cultivars under the control condition and were significantly decreased by BERi condition treatment. It was 0.029% for 'Managua RZ' and tended to be even lower, around 0.01%, for the two other cultivars. In the healthy fruits, control treatment fruits of 'Managua RZ' and 'Reiyoh' had more Ca^{2+} than in the previous stage, reaching 0.088% and 0.055% respectively. However, 'House Momotaro' maintained similar levels. The decrease in Ca^{2+} concentration under the effect of BERi treatment was even more drastic at this stage for 'Managua RZ' and 'Reiyoh', with fruits showing concentrations of 0.021% and 0.003%, respectively; there was a strong interaction between cultivars and nutrient solution treatment too ($P<0.01$). Interestingly, 'House Momotaro' maintained comparable concentrations to the control in the BERi treatment. Affected fruits showed Ca^{2+} concentrations of 0.02% for 'Managua RZ' and lower levels, around 0.009%, for both remaining cultivars.

BERi treatment increased K^+/Ca^{2+} ratios in pre-BER fruits by 2–3 fold for all cultivars compared with the control, and the resulting ratio was higher for Japanese cultivars than for 'Managua RZ'. More than a 10-fold increase was observed for 'Reiyoh' in the healthy fruits as compared with the control condition. The resulting ratio of 923 was 6 times higher than the values observed in the other cultivars in the same conditions. The $(K^++Mg^{2+})/Ca^{2+}$ ratio was also increased by BERi treatment at the pre-BER stage and the resulting ratio was more than 1.6 times higher for the Japanese cultivars

than 'Managua RZ'. In the healthy fruits, the same trends were found, with K^+/Ca^{2+} being much higher in 'Reiyoh' than in 'Managua RZ' and 'House Momotaro'. In the two Japanese cultivars, affected fruits also tended to show higher values for both ratios.

Total hydrosoluble antioxidants

In the control condition, pre-BER stage, there was no statistical difference between all cultivars (Fig. 2A). Under the BERi treatment, only fruits of 'Managua RZ' and 'House Momotaro' had significantly increased antioxidants, with increases of 56% and 45% respectively. In 'Reiyoh' the increase was of only 37% and was not significant. The resultant antioxidant concentration in the BERi condition was the highest for 'Managua RZ', reaching 1675 nmol·g⁻¹ FW. It was intermediate for 'House Momotaro' with 1390 nmol·g⁻¹ FW and the lowest for 'Reiyoh' with 1172.5 nmol·g⁻¹ FW. During BER appearance, there was a trend in healthy fruits to have more antioxidants in the BERi treatment than in the control (Fig. 2B). Also, 'Managua RZ' tended to have more antioxidants than the two Japanese cultivars. This increase was less important than in the pre-BER stage. In affected fruits (Fig. 2C), 'Managua RZ' tended to have more antioxidants than 'Reiyoh', which also tended to have more than 'House Momotaro'.

Ascorbate concentrations

Ascorbate redox ratios were always higher than 80%, so only total ascorbate concentrations were considered

Fig. 2. Total hydro-soluble antioxidant concentration in the distal pericarp of (A) pre-BER, (B) healthy, and (C) affected tomato fruits, expressed in equivalent L-sodium ascorbate ($n = 5$). NS: ANOVA was not significant ($P < 0.05$). Different letters represent a statistical difference in total hydro-soluble antioxidants ($P < 0.05$). Data are means $\pm$ standard error (SE).

Fig. 3. Total ascorbate (ASA) concentrations in the distal pericarp of (A) pre-BER, (B) healthy, and (C) affected tomato fruits ($n = 3-5$). NS: ANOVA was not significant ($P < 0.05$). Different letters represent a statistical difference in total ASA ($P < 0.05$). Data are means $\pm$ standard error (SE).

(data not shown). In the pre-BER stage control treatment, all cultivars had within $800 \text{ nmol} \cdot \text{g}^{-1} \text{ FW}$ ascorbate in their distal pericarp (Fig. 3A). These concentrations were only significantly higher in 'Managua RZ' and 'House Momotaro' in the BERi treatment. In comparison with the control, the increases were of 46% and 63%, respectively, making their concentrations higher than $1300 \text{ nmol} \cdot \text{g}^{-1} \text{ FW}$. For 'Reiyoh', the increase was only 10.8%. During symptom appearance in healthy fruits, the increase in ascorbate was not significant for any cultivars. In the BERi treatments, 'Managua RZ' and 'House Momotaro' fruits maintained their trend of having more ascorbate than 'Reiyoh', with concentrations above $1150 \text{ nmol} \cdot \text{g}^{-1} \text{ FW}$, while in 'Reiyoh' concentrations were below $900 \text{ nmol} \cdot \text{g}^{-1} \text{ FW}$. When comparing control and BERi treatments, 'House Momotaro' tended to have more ascorbate in the BERi condition, but the two other cultivars had comparable levels (Fig. 3B). In the affected fruits, all cultivars had comparable ascorbate concentrations within $1200-1300 \text{ nmol} \cdot \text{g}^{-1} \text{ FW}$ (Fig. 3C).

Glutathione concentrations

ANOVA analysis was not significant in terms of stages or fruit types (healthy and affected) for the GSSG concentration of the distal pericarp (Fig. 4, $P < 0.05$). 'House Momotaro' showed a tendency to have more glutathione (GSH) than the other cultivars in treatments prior to symptom appearance and in healthy fruits under control treatment (Fig. 5A, B). For the BERi treatment, healthy fruits of 'Managua RZ' showed significantly more glutathione than 'Reiyoh', while 'House Momotaro' had an intermediate level. In affected fruits, the effect on cultivars was not significant (data not shown) (Fig. 5C, $P < 0.05$). For the redox ratio of glutathione, the effect of treatment was significant (data not shown) only in healthy fruits during symptom appearance, with no substantial differences between cultivars (Fig. 6B).

Discussion

In this experiment, the BERi treatment succeeded triggering the appearance of BER fruits at the same fruit age in all 3 cultivars. However, there was a large difference between 'Reiyoh', which was highly suscep-

Fig. 4. Oxidized glutathione (GSSG) concentration in the distal pericarp of (A) pre-BER, (B) healthy, and (C) affected tomato fruits ($n = 3-5$). NS: ANOVA was not significant ($P < 0.05$). Different letters represent a statistical difference in oxidized glutathione ($P < 0.05$). Data are means $\pm$ standard error (SE).

Fig. 5. Reduced glutathione (GSH) concentration in the distal pericarp of (A) pre-BER, (B) healthy, and (C) affected tomato fruits ($n = 3-5$). NS: ANOVA was not significant ($P < 0.05$). Different letters represent a statistical difference in reduced glutathione ($P < 0.05$). Data are means $\pm$ standard error (SE).

tible, and the two other cultivars, which showed very low incidence and could be considered as unsusceptible or resistant. These results are in accordance with a previous study in which BER susceptibility was found to be variable between a range of genotypes (Adams and Ho, 1992; Ho et al., 1995).

In comparison with the control nutrient solution, the BERi nutrient solution was enriched in K^+ , Mg^{2+} , and its EC level was increased by up to $8 \text{ dS} \cdot \text{m}^{-1}$ with the addition of NaCl, while Ca^{2+} concentration was maintained to avoid the adverse effects of Ca^{2+} deficiency on plants (Simon, 1978). The combination of these factors succeeded in increasing BER incidence in 'Reiyoh' to levels higher than described in the literature under comparable salt stress (Adams and Ho, 1992; Ho et al.,

1995), or by only changing the nutrient solution ratios of Ca^{2+} to K^+ (Van Der Boon, 1973) or Mg^{2+} (Hao and Papadopoulos, 2004). Salinity is thought to lower plant Ca^{2+} uptake and distribution into fruits, and the distal-end part of the fruit is thought to be particularly exposed to Ca^{2+} deficiency (Ho and Adams, 1989), which triggers BER (Bradfield and Guttridge, 1984). The drastic reduction in fruit fresh weight indicates the level of osmotic stress that was imposed on the plants. K^+ and Mg^{2+} enrichment were proposed to increase BER incidence by competition with Ca^{2+} absorption limiting its uptake, resulting in nutrient imbalances inside the fruit (Bar-Tal and Pressman, 1996; Hao and Papadopoulos, 2004; Raleigh and Chucka, 1944; Van Der Boon, 1973).

Fig. 6. Glutathione redox ratio in the distal pericarp of (A) Pre-BER, (B) healthy, and (C) affected tomato fruits ($n = 3-5$). NS: ANOVA was not significant ($P < 0.05$). Different letters represent a statistical difference in glutathione redox ratio ($P < 0.05$). Data are means $\pm$ standard error (SE).

Nutrient solution treatment, sampling time and cultivars did not show any significant differences in fruit K^+ concentrations, while Mg^{2+} was only significantly higher prior to symptom appearance in 'Reiyoh'. Importantly, we found no association between BER susceptibility and calcium content in pre-BER fruits, the stage which is thought to be critical for BER appearance. In fact, under the BERi condition, Ca^{2+} concentrations were decreased in the distal part of young pre-BER fruits. In addition, the reduction was more severe in 'Reiyoh' and 'House Momotaro' than in the Dutch cultivar. Moreover, Ca^{2+} concentrations measured in both Japanese cultivars were much more lower than the lowest levels found in the literature for BER-inductive conditions (Bradfield and Guttridge, 1984; De Freitas et al., 2012; Ward, 1973). BER incidence was much higher in 'Reiyoh' than in 'House Momotaro'. It was only in healthy fruits, during BER appearance, that Ca^{2+} levels tended to be particularly low in 'Reiyoh'. These results support the idea that Ca^{2+} is indeed an important factor involved in the development of BER, but that other factors are probably also involved (Saure, 2001). Interestingly, K^+/Ca^{2+} and $(K^+ + Mg^{2+})/Ca^{2+}$ ratios found in healthy fruits during symptom appearance were associated with BER incidence, as 'Reiyoh' showed much higher ratios than the other two cultivars. Indeed, De Freitas et al. (2015) showed that in apple fruit, bitter pit (BP), another fruit storage Ca-deficiency disorder, was associated with a high fruit $(K^+ + Mg^{2+})/Ca^{2+}$ ratio. In their study, this high ratio was due to higher K^+ and Mg^{2+} concentrations in BP affected fruits of a BP-susceptible cultivar, while calcium concentrations were similar in both BP-susceptible and resistant cultivar fruits. The authors of this study proposed that this ratio probably acts by reducing Ca^{2+} binding sites in the cell membrane (Schönherr and Bukovac, 1973; Yermiyahu et al., 1994) and/or by promoting fruit growth which in-

creases Ca^{2+} dilution under salt stress (Lopez and Satti, 1996). However, in our study this increased ratio was the result of lowered Ca^{2+} concentrations in 'Reiyoh' while the K^+ and Mg^{2+} levels were similar in all cultivars under the BERi condition. Therefore, our results support the possible involvement of increased K^+ and Mg^{2+} to Ca^{2+} ratios with the susceptibility to Ca^{2+} deficiency disorders, but without providing sufficiently strong evidence (De Freitas et al., 2015; Do Amarante et al., 2013).

In our study, under the BERi condition, pre-BER fruits tended to show more antioxidants than control fruits. Additionally, levels of antioxidants were different between cultivars. For the total hydrosoluble antioxidants, if compared to 'Reiyoh', only 'Managua RZ' had significantly more antioxidants, while 'House momotaro' tended to show more antioxidants but without significant differences. Interestingly, prior to symptom appearance, the resistant cultivars 'Managua RZ' and 'House Momotaro' showed more total ascorbate than the susceptible cultivar 'Reiyoh', while no difference was observed in the ascorbate redox status. A similar trend was maintained in healthy fruits during symptom appearance, but only for ascorbate. Also, there was no significant difference found between cultivars for the glutathione redox status. These results clearly indicate that there is a link between BER and the ROS scavenging capacity, 1 to 2 days prior to symptom appearance and the ascorbate pool appeared as a major contributor of this antioxidant capacity. This supports the proposed hypothesis that BER is the result of cell damage provoked by oxidative stress (Aktas et al., 2003). Thus, if ROS happen to be the major causal factor in BER appearance, it is possible that BER is an indirect result of a lack of ROS scavenging capacity represented by ascorbate during stress conditions, which are known to boost ROS levels. Therefore, the

fact that BER appears at 12–15 DAA could be a consequence of the regulation of ascorbate and ROS levels in tomato fruit during its development, most specifically prior and during BER appearance stages. Accordingly, the levels of ascorbate were already reported to be the lowest around at 7 and 15 DAA in tomato fruit (Carrari et al., 2006), which further supports our hypothesis.

To confirm the results of this study, it will be important to evaluate the ROS content in this experimental system. It will also be important to evaluate cell damage by lipid peroxidation (Van Breusegem and Dat, 2006), which indicates whether there was protection from oxidative damage in BER-resistant cultivars or not compared with 'Reiyoh'. Additionally, the capacities of enzymes involved in the production and scavenging of ROS could also be measured, as they may have a major role (Mittler, 2002). Also, it can be argued that fruits affected by BER in all cultivars displayed comparable antioxidant and ascorbate levels. The assay was performed on tissues surrounding the affected area, which tended to be close to the fruit mid-section in 'Reiyoh' due to the large size of the necrosis. Affected fruits of 'Reiyoh' had more ascorbate in the proximal pericarp than the distal one (data not shown), so it is possible that the samples from them showed comparable antioxidant and ascorbate concentrations to the other cultivars because of their proximity to the peduncle side.

In conclusion, our results support the involvement of ROS as a major protagonist in BER appearance. In our experiment, BER-resistant cultivars showed a larger increase in their ROS scavenging capacity, represented by ascorbate, in response to BER-inductive growth conditions. These differences in ascorbate antioxidant capacity among cultivars were observed right before the symptoms started to appear in the distal part of tomato fruits and tended to be maintained during symptom appearance. It is unclear whether these differences are due to a concentration effect by the limitation of fruit size or the activation of stress-resistance mechanisms. Also, it seems that the critical phase for BER initiation is around 10 DAA under salt stress, which suggests that there is a peak of ROS production during that stage. Thus, further studies should be performed to confirm the occurrence of actual redox stress. Higher K^+/Ca^{2+} and $(K^++Mg^{2+})/Ca^{2+}$ ratios and a tendency to have less Ca^{2+} were only observed in 'Reiyoh' during symptom appearance. These nutrient imbalances could be involved in increasing the instability of the cell membrane as mentioned above. We hypothesize that these cell nutrient imbalances may be a trigger for cell disintegration after damage by excessive ROS in the tissues affected.

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