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24-Epibrassinolide Mechanisms Regulating Blossom-End Rot Development in Tomato Fruit

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Abstract

Blossom-end rot (BER) is a physiological disorder believed to be triggered by low Ca^{2+} content in the distal fruit tissue. However, many other factors can also determine fruit susceptibility to BER. It is possible that during fruit growth, Ca^{2+} imbalance can increase membrane leakiness, which may trigger the accumulation of reactive oxygen species, leading to cell death. Brassinosteroids are a class of plant hormones involved in stress defenses, specially increasing the activity of antioxidant enzymes and the accumulation of antioxidant compounds, such as ascorbic acid. The objective of this study was to understand the mechanisms by which 24-epibrassinolide (EBL) reduces fruit susceptibility to BER. Tomato plants 'BRS Montese' were cultivated in a greenhouse and were weekly sprayed with water (control) or EBL (0.01 μM) after full bloom. Plants and fruits were evaluated at 15 days after pollination (DAP). According to the results, EBL treatment inhibited BER development, increased fruit diameter, length, and fresh weight. EBL-treated fruit showed higher concentrations of soluble Ca^{2+} and lower concentrations of cell wall-bound Ca^{2+}. EBL-treated fruit also had higher concentrations of ascorbic acid and lower concentrations of hydrogen peroxide, compared to water-treated fruit. EBL treatment increased the activity of the three main antioxidant enzymes known as ascorbate peroxidase, catalase, and superoxide dismutase. According to the results, EBL treatment maintained higher soluble Ca^{2+} and antioxidant capacity, reducing fruit susceptibility to BER.

Keywords Antioxidant capacity · Blossom-end rot · Brassinosteroids · Calcium deficiency · 24-Epibrassinolide · Oxidative stress

Introduction

Blossom-end rot (BER) is a physiological disorder that develops at the distal fruit tissues, leading to softening and subsequent necrosis. Although calcium (Ca^{2+}) deficiency has been considered for a long time the main cause of the disorder, recent studies have shown more complex mechanisms regulating BER incidence in tomato fruit, like oxidative stress, loss of xylem functionality, and expression of some genes related to Ca^{2+} transport in fruit cells (De Freitas et al. 2017; Ikeda et al. 2017).

Calcium deficiency is highly affected by environment factors such as high temperatures, low air relative humidity, and soil salinity, as well as by genetic predisposition of the genotypes (Ho and White 2005). In this case, a few studied factors such as fruit shape (Riboldi et al. 2018b) and loss of xylem functionality in the distal portion (Riboldi et al. 2018a) have emerged as key factors determining fruit susceptibility to BER.

Brassinosteroids (BRs) have been shown to induce stress tolerance in plants (Maia et al. 2018), and to increase cell viability under stress conditions by increasing the cellular capacity to scavenge reactive oxygen species (ROS) (Liu et al. 2009). Studies using exogenous applications of BRs have also shown that it can promote a better

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adaptation of plants to stresses (Wu et al. 2017; Maia et al. 2018; Riboldi et al. 2018c).

In some studies, it is possible to verify that the mechanism of BRs action is different from abscisic acid action, a traditional hormone studied in plants under stress. The use of BRs has been reported to activate plant antioxidant defenses such as a generation of compounds such as ascorbic acid or even to activate enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase (CAT) that inactivate ROS (Yadav et al. 2012; Maia et al. 2018). Thus, stress tolerance conferred through the exogenous application of this hormone is different from all the mechanisms already analyzed in studies on Ca^{2+} disorders (Saure 2001, 2014).

One of the main effects of Ca^{2+} in plants is maintenance of membrane function and stability (Hepler and Winship 2010). Thus, Ca^{2+} deficiency results in membrane degradation, which can be evidenced by the peroxidation of lipids and generation of reactive oxygen species (ROS). Indeed, previous studies have shown high levels of ROS, such as superoxide radicals, hydroxyl radicals, and singlet oxygen (O^{2-}), in fruit tissue with BER (Aktas et al. 2005; Turhan et al. 2006; Mestre et al. 2012). In this context, BR could increase fruit tissue capacity to scavenge ROS, which could prevent or reduce fruit susceptibility to Ca^{2+} deficiency (Turhan et al. 2006). However, an extensive study focusing on the relationship between oxidative metabolism and BER development revealed that reducing fruit Ca^{2+} concentration also reduced the activity of the main enzymes responsible for ROS detoxification, leading to H_2O_2 accumulation, lipid peroxidation, and BER symptom development in the fruit (Mestre et al. 2012).

Production of ROS can also be increased under exposure to many abiotic stresses (Wu et al. 2017). Under stress conditions, such as exposure to salt or water deficit, increasing H_2O_2 production is the major contributor to cell and tissue damage in plants (Choudhury et al. 2017). Plant cells have a large number of protective mechanisms to eliminate or reduce ROS (Cuypers et al. 2016). The activation of the enzymatic antioxidant system, which operates by the sequential and simultaneous actions of enzymes including SOD, CAT, and APX, is one of them (Gratão et al. 2015; Alves et al. 2017). The accumulation of H_2O_2 is prevented in plant cells either by CAT and peroxidases or by the ascorbate–glutathione cycle where APX reduces it to H_2O (Gratão et al. 2005). 24-Epibrassinolide (EBL) was shown to increase the activity of enzymes such as APX, CAT, SOD in plant cells, as well as enhance the synthesis of antioxidant substances such as ascorbic acid, resulting in lower contents of ROS, and lipid peroxidation in the cells (Maia et al. 2018). Ascorbic acid (AsA) can also directly scavenge ROS produced during aerobic metabolic processes such as photosynthesis or respiration; however, the extent to which the direct

reduction of ROS occurs in plants remains to be determined (Gallie 2013).

According to the previous studies, it is possible that neither total Ca^{2+} nor ROS alone can fully explain BER development in tomato fruit, but rather the interaction between Ca^{2+} and ROS levels in the tissue. Therefore, the objective of this study was to understand the mechanisms through which EBL inhibits BER development in tomato fruit.

Materials and Methods

Plant Material, Growth Conditions, and Application of Treatments

This study was carried out using the 'BRS Montese' (EMBRAPA) tomato, which has long-shaped fruit. The seeds used in the study were donated by Agrocinco Seeds of Value (Monte Mor, São Paulo, Brazil). The experiment was performed in a greenhouse with average solar radiation at midday of 1000 µmol m^{-2} s^{-1}, average temperature of 18.3 °C, and relative humidity of 77% at night and 28.2 °C and 52% during daytime, respectively. Seeds were seeded separately in trays with a 1:1 (v/v) peat-based substrate mixture (Plantmax HT, Eucatex Brazil) and expanded vermiculite, and supplemented with 1 g L^{-1} of NPK (10:10:10) fertilizer and 4 g L^{-1} of dolomitic limestone, supplementing calcium and magnesium.

Thirty days after planting, the seedlings were transplanted into individual 30 L pots containing substrate mixture. Plants were fertilized every 20 days, during growing and fructification time, with 10 g of slow release fertilizer containing N (43 kg ha^{-1}), P_2O_5 (21.2 kg ha^{-1}), K_2O (32 kg ha^{-1}), MgO (5.3 kg ha^{-1}), S (13.3 kg ha^{-1}), Fe (1.1 kg ha^{-1}), Cu (0.13 kg ha^{-1}), Mn (0.16 kg ha^{-1}), Zn (0.05 kg ha^{-1}), B (0.05 kg ha^{-1}), Mo (0.04 ka ha^{-1}), but without Ca (Basacote Plus, Agricultural Soil Fertilizer; Compo Expert) to stimulate BER incidence.

When plants started blooming, they were sprayed weekly with a 125-mL solution per plant containing water (control) or 24-epibrassinolide—EBL (Sigma-Aldrich, Saint Louis, MO, USA) (0.01 µM). The EBL concentration was determined based on the level that has been shown to trigger plant responses in other studies (Ogweno et al. 2008; Jiang et al. 2012; Yadav et al. 2012; Zheng et al. 2016; Riboldi et al. 2018c). Fruit samples were harvested on the first cluster at 15 days after pollination (DAP).

BER Incidence, Growth Parameters, and Xylem Functionality

The incidence of BER was calculated by multiplying the number of fruits with BER symptoms by 100 and dividing

by the total number of fruit in the first cluster. The plant dry weight was determined by drying the samples (65 °C) until constant weight. Leaf area was determined through a leaf area meter (LI-3100C Area Meter, LI-COR, Lincoln, USA), using all the leaves. Both plant dry weight and leaf area were determined at full bloom. Fruit length and diameter were determined using a caliper. Fruit weight was determined using the average of fruits.

Xylem function was measured in developing fruit as previously described by Ho et al. (1993) and De Freitas et al. (2011b). Fruits were harvested 15 days after pollination and held in sealed plastic bags for 20 min with 100 mL of free water to reduce transpiration until the peduncle of each fruit was immersed in a solution of 1% Safranin-O at 20 °C under ≤20% relative humidity. After 24 h, fruit were cut into three equal sections at a 90° angle to the peduncle axis. The number of stained vascular bundles (xylem vessels) was counted in the placenta and pericarp tissues at the cut surfaces at the blossom and peduncle end regions of each fruit.

Determination of Leaf Stomatal Conductance, Leaf Transpiration Rate, and Leaf Water Potential

An infrared gas analyzer (IRGA) model LCpro+ (ADC Bio-Scientific LTD., Hertfordshire, UK) was used to determine stomatal conductance (g_s, mol H_2O m^{-2} s^{-1}) and transpiration rate (E, mmol H_2O m^{-2} s^{-1}). The evaluations were accomplished between 9 and 11 am in fully expanded leaves close to the first cluster at 15 DAP. For the determination of leaf water potential, we used the equilibrium vapor pressure method by means of a psychrometric technique using a microvoltmeter model HR-33T (Wescor, Logan, UT, USA) coupled to Wescor C-52 chambers (Wescor, Logan, UT, USA).

Total Tissue Ca²⁺, Mg²⁺, and K⁺ Contents in Leaf and Fruit and Ca²⁺ Bound to the Cell Wall

Nutrient analysis was accomplished in proximal and distal fruit tissues, as well as in fully expanded leaves close to the first fruit cluster. Samples were oven dried at 65 °C until constant weight. About 500 mg of dry material was added to 6 mL of nitroperchloric acid (2:1). The digestion was performed in a plaster block at 240 °C with 15 g of distilled water. Nutrient quantification was performed by atomic absorption, according to Malavolta et al. (1997). The results were expressed as g of Ca^{2+}, Mg^{2+}, and K^+ per kg of tissue dry weight.

Calcium bound to the cell wall was determined in fruit distal tissue after extracting cell-wall material following the protocol described by Campbell et al. (1990). The quantification of Ca^{2+} was carried out using the same method described above.

Apoplastic and Cytoplasmic Electrolytic Leakage and Soluble Ca²⁺ Content in Fruit Tissue

Fruit electrolyte leakage was performed according to the protocol described by De Freitas et al. (2011a). Three fruit pericarp discs with 1 cm in diameter and 0.7 cm thickness were collected in each replication. The discs were then added to 50 mL tubes containing a 0.4 M mannitol solution, which were placed on a rotary shaker (CT-165, Cientec). The conductivity in the mannitol solution was recorded for 6 h at 1 h intervals. Subsequently, the samples were frozen and thawed three times to determine the total conductivity (Saltveit 2002).

Apoplastic electrolyte leakage was considered to be the leakage of ions during the first 3 h of increasing solution conductivity, representing the ions leaking from the apoplast space in the tissue (Saltveit 2002). Cytoplasmic electrolyte leakage was considered to be the last 3 h of increasing solution conductivity, representing the ions leaking through the membranes in the tissue (Saltveit 2002). The results were expressed as the percentage increase of electrolyte leakage per gram of tissue per hour relative to total tissue conductivity.

At the end of the 6 h, 1 mL solution of the samples was collected to determine soluble Ca^{2+}, Mg^{2+}, and K^+ in fruit tissue according to the approach described above. The results were expressed as g of Ca^{2+}, Mg^{2+}, and K^+ per kg of tissue dry weight.

Ascorbic Acid, MDA, H₂O₂, and Enzymatic Assays

The determination of ascorbic acid (AsA) was based on the method described by Carvalho et al. (1990), of reducing 2,6-dichlorophenol indophenol sodium (DCIP) by ascorbic acid, which has a strong reducing action. Distal fruit tissue was used, obtained from fruits after 15 DAP.

The measurements of the malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) contents were performed in the same extraction, using distal fruits tissue, collected 15 days after pollination and leaves, collected next to the clusters. The concentration of MDA was calculated from the absorbance at 535 nm by using the absorbance coefficient 155 mM^{-1} cm^{-1}, following a correction for unspecific turbidity determined by the absorbance at 600 nm (Heath and Packer 1968).

H_2O_2 was measured spectrophotometrically after reaction with KI as described by Alexieva et al. (2001), using the same tissue used for MDA analysis. The reaction was developed for 1 h in darkness at room temperature and absorbance measured at 390 nm. The amount of H_2O_2 was calculated using a standard curve prepared with known concentrations of H_2O_2.

The total protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as a standard (Bio-Rad Protein Dye Reagent), using distal fruits tissue, collected 15 days after pollination and leaves, collected next to the clusters. Superoxide dismutase (SOD, E.C. 1.15.1.1) activity was carried out as reported by Constantine and Ries (1977) by the inhibition of NBT chloride photoreduction. One unit of SOD activity was defined as the amount of the enzyme required to inhibit the reduction of NBT by 50% under the specified conditions. SOD activity of the extracts was expressed as $U \, mg^{-1}$ protein.

Catalase (CAT, E.C. 1.11.1.6) activity was determined as described by Kraus (1995) with modifications as described by Azevedo et al. (1998). CAT activity was calculated using an extinction coefficient for H_2O_2 of $39.4 \, mM^{-1} \, cm^{-1}$ and results were expressed as $\mu mol^{-1} \, min^{-1} \, mg$ protein. Ascorbate peroxidase (APX, E.C. 1.11.1.11) activity was determined by monitoring the rate of ascorbate oxidation at 290 nm at 30 °C. APX activity was expressed as $\mu mol^{-1} \, min^{-1} \, mg$ protein (Nakano and Asada 1981).

Experimental Design

The experiment followed a randomized blocks design with five blocks per treatment and four plants per block. Therefore, each treatment was composed by a total of 20 plants. The data were subjected to analysis of variance (ANOVA) and the averages were compared by the t test with statistical significance at 5%, using SAS statistical software (Cary, North Carolina, USA). The data without normal distribution were analyzed using Friedman's non-parametric test with statistical significance at 5%. Pearson's correlation was also performed with statistical significance at 5%. Soluble Ca^{2+}+APX, Soluble Ca^{2+}+AsA, Soluble Ca^{2+}+SOD, Soluble Ca^{2+}+CAT, represent the sum of each respective average, whereas Soluble Ca^{2+}-H_2O_2 represents the subtraction of each respective average. In this case, the data were centered on the respective average so that, the magnitude of the values of different parameters had the same weight at the time of adding or subtracting. Total correlation refers to all values added or subtracted, depending on its correlation, inhibiting or triggering BER.

Results

BER Incidence, Biometric, and Physiological Parameters

The incidence of BER was markedly different between water- and EBL-treated plants. BER levels in the control were higher than in EBL-treated plants, with 44.2% of BER incidence reduction (Fig. 1).
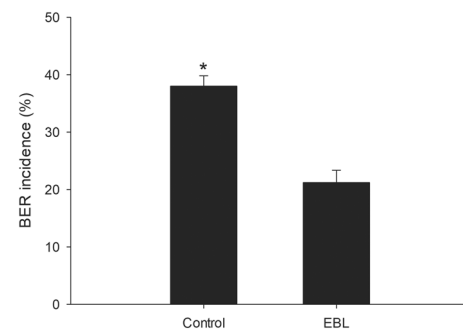

Fig. 1 Blossom-end rot (BER) incidence in tomato subject to EBL treatment. Fruit were harvested at 15 days after pollination. *Averages are statistically different according to the t test (5%). Data shown as mean ± standard deviation

There were also no differences between leaf area and dry weight between treatments (Table 1). Fruit length, diameter, and weight were higher in EBL-treated plants (Table 1).

There were no differences in leaf stomatal conductance, leaf transpiration, and leaf water potential between treatments (Table 1). Xylem functionality was similar between treatments (Table 2).

Nutrient Concentration in Leaf and Fruit Tissues and Membrane Permeability

The Ca^{2+} partitioning between leaves and fruits varied with the treatments (Table 3). In leaves, Ca^{2+} concentration was higher in control plants, compared to EBL-treated plants. In fruits, the highest concentration of soluble Ca^{2+} was observed in EBL-treated plants. In proximal and distal fruit tissue, the highest Ca^{2+} concentration was observed in control plants. The highest ratio between proximal and distal part (P/D) was observed in EBL-treated plants. Ca^{2+} bound to the cell wall in distal fruit tissue was higher in the control treatment. The ratio between the wall-bound Ca^{2+} and the soluble Ca^{2+}, both in the distal tissue (CW/D), was higher in control plants (Table 3).

In another way, there were no differences in Mg^{2+} concentration between treatments in leaves and fruit proximal and distal tissue (Table 4). EBL-treated plants had higher concentrations of soluble Mg^{2+} in comparison to control plants. K^+ concentration in leaves, fruit soluble, proximal, distal tissue (Table 4), and ratio P/D showed no differences between treatments.

There were no differences between treatments in cytoplasmic and apoplastic electrolyte leakage in proximal fruit tissue (Table 5). However, in the distal fruit tissue, the cytoplasmic electrolyte leakage was higher in control plants, whereas the apoplastic leakage was higher in EBL-treated plants.

Table 1 Leaf area per plant, plant dry weight at maximum growth (60 days after planting), fruit length, fruit diameter, and fruit weight subjected to EBL treatment

Treatments	Leaf area (cm^2)	Dry weight (g)	Fruit length (cm)	Fruit diameter (cm)	Fruit fresh weight (g)
Control	7639.5 ± 787.7*	76.2 ± 17.9	85.9 ± 3.1	54.0 ± 2.3	140.8 ± 12.4
EBL	7626.6 ± 1597.9	68.1 ± 9.6	92.1 ± 3.5*	57.3 ± 1.28*	165.5 ± 14.4*
CV %	11.82	16.52	3.43	2.6	7.02

CV coefficient of variation

*Averages are statistically different according to the *t* test (5%). Data shown as mean ± standard deviation

Table 2 Leaf stomatal conductance (g_s), leaf water potential (Ψw), leaf transpiration (*E*), and xylem functionality (XF) of tomato subjected to EBL treatment

Treatments	g_s (mol H$_2$O m^{-2} s^{-1})	Ψw (MPa)	*E* (mmol H$_2$O m^{-2} s^{-1})	XF proximal	XF distal
Control	0.17 ± 0.01	−0.80 ± 0.14*	2.51 ± 0.14*	17.72 ± 3.95	3.95 ± 4.56
EBL	0.15 ± 0.02	−0.68 ± 0.60	2.32 ± 0.16	17.62 ± 3.92	3.92 ± 5.37
CV %	13.69	28.43	6.7	12.3	14.4

CV coefficient of variation

*Averages are statistically different according to the *t* test (5%). Data shown as mean ± standard deviation

Table 3 Ca^{2+} concentration in leaves (g kg^{-1} DW), soluble, proximal, and distal fruit tissue (g kg^{-1} FW), cell wall-bound Ca^{2+} (g kg^{-1} DW) in tomato subjected to EBL treatment

Treatments	Leaf	Soluble[a]	Proximal	Distal Ca^{2+}	*P/D*[b]	Cell wall[c]	CW/*D*[d]
Control	39.5 ± 1.7*	0.16 ± 0.01	1.6 ± 0.21*	1.1 ± 0.18*	1.45	0.9 ± 0.19*	0.8
EBL	35.9 ± 1.0	0.20 ± 0.02*	1.4 ± 0.15	0.8 ± 0.12	1.75	0.6 ± 0.11	0.7
CV %	3.35	6.28	5.4	16.7	–	17.1	–

CV coefficient of variation

*Averages are statistically different according to the *t* test (5%). Data shown as mean ± standard deviation

[a]*Soluble* soluble concentration in distal end

[b]*P/D* proximal/distal

[c]*Cell wall* Ca^{2+} bound to the cell wall of the distal end

[d]*CW/D* cell wall/distal

Table 4 Mg^{2+} and K$^+$ concentration (g kg^{-1} DW) in leaves, soluble, proximal, and distal fruit tissue from tomatoes subjected to EBL treatment

Treatments	Leaf	Soluble[a]	Proximal	Distal	*P/D*[b]
Mg^{2+}					
Control	5.7 ± 0.7	0.6 ± 0.12	1.9 ± 0.3	1.7 ± 0.12	1.12
EBL	5.8 ± 0.7	0.8 ± 0.13*	1.9 ± 0.2	1.9 ± 0.15	1.0
CV %	8.11	7.59	8.92	9.45	-
K$^+$					
Control	29.4 ± 5.9	35.7 ± 2.1	35.9 ± 7.1	35.0 ± 3.3	1.03
EBL	26.3 ± 6.0	37.0 ± 3.2	35.9 ± 4.7	34.5 ± 7.0	1.04
CV %	9.82	7.79	9.87	9.16	-

CV coefficient of variation

*Averages are statistically different according to the *t* test (5%). Data shown as mean ± standard deviation

Table 5 Electrolytic leakage from tomato fruits (% h^{-1}) in tomato fruits subjected to EBL treatment

Treatments	CP[a]	AP[a]	CD[b]	AD[b]
Control	1.6 ± 0.31	3.0 ± 0.48	1.3 ± 0.05*	2.7 ± 0.43
EBL	1.5 ± 0.43	3.0 ± 0.59	1.1 ± 0.08	3.2 ± 0.46*
CV %	10.11	11.73	11.61	10.11

CV coefficient of variation

*Averages are statistically different according to the *t* test (5%). Data shown as mean ± standard deviation

[a]CP and AP: cytoplasmic and apoplastic leakage from proximal part, respectively

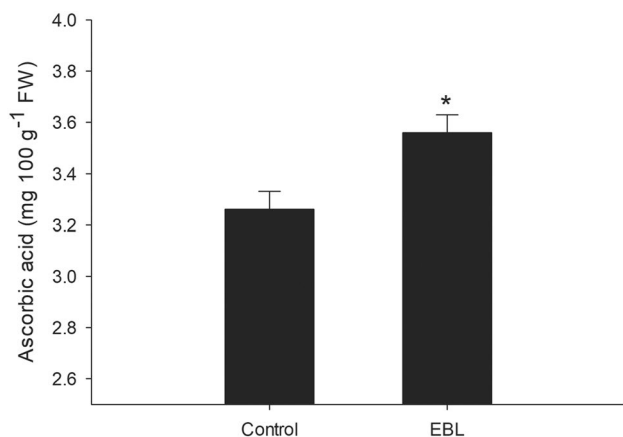[b]CD and AD: cytoplasmic and apoplastic leakage from distal part, respectively

Fig. 2 Ascorbic acid concentration in tomato fruits subjected to EBL treatment. Fruit were harvested at 15 days after pollination. *Averages are statistically different according to the *t* test (5%). Data shown as mean ± standard deviation

Antioxidant Concentration and Activity in Leaf and Fruit Tissues

The concentration of AsA was 8% higher in EBL-treated plants (Fig. 2). There was no significant difference in malondialdehyde (MDA) content in both leaves and fruits, using EBL treatment, compared to control (Fig. 3). Hydrogen peroxide (H_2O_2) content decreased in response to EBL treatment in leaves and fruits, being higher in leaves in comparison to fruits (Fig. 3).

On the other hand, the activity of SOD, CAT, and APX was higher in leaves and fruits of EBL-treated plants (Fig. 4). Focusing on the effects of their activity on fruits, SOD, CAT, and APX had 16, 12.5, and 18.4% increases after EBL treatment, respectively (Fig. 5).

Correlation Among Parameters Related to BER

The correlation analysis between the variables evaluated in the study revealed that some of the physiological variables correlated positively with BER (Table 6). The variables that
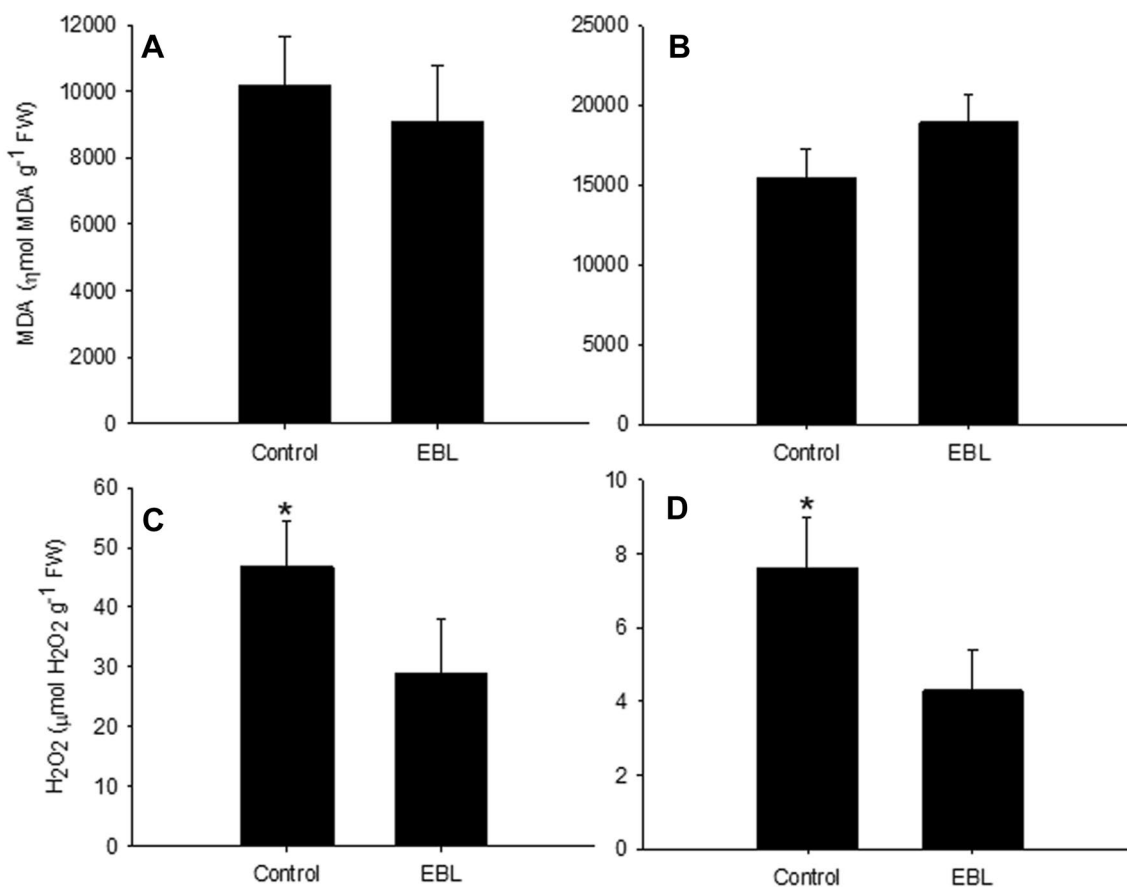

Fig. 3 Oxidative damage induced by EBL treatment in tomato tissues expressed as MDA content (nmol g^{-1} fresh weight) and H_2O_2 µmol g^{-1} fresh weight. **a** MDA content in leaves; **b** MDA content in fruits; **c** H_2O_2 content in leaves; **d** H_2O_2 content in fruits. Fruit were harvested at 15 days after pollination. *Averages are statistically different according to the *t* test (5%). Data shown as mean ± standard deviation
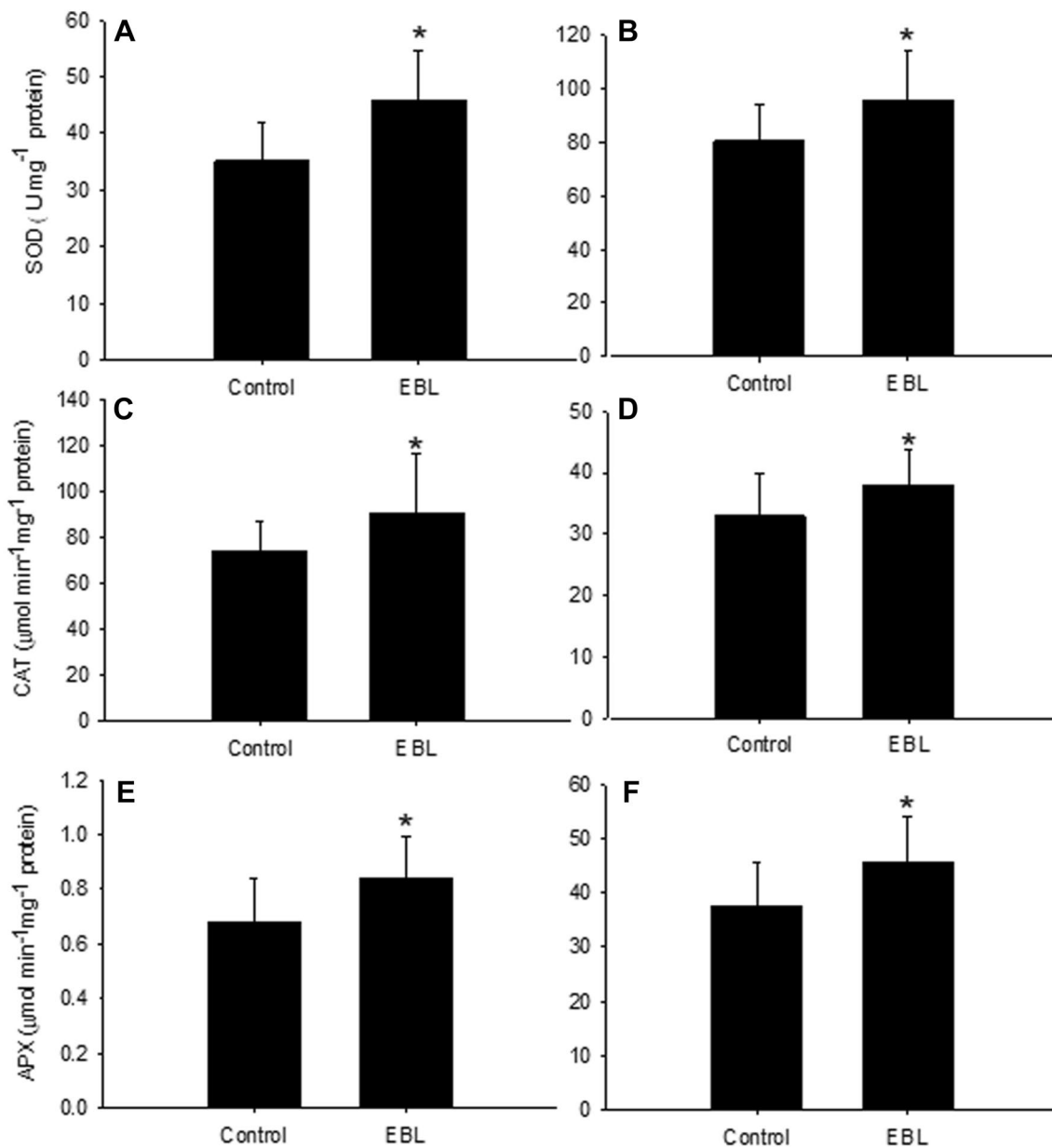
Fig. 4 Oxidative stress-related enzymes activity in tomato plants and fruits subjected to EBL treatments. **a** Superoxide dismutase activity in leaves; **b** Superoxide dismutase activity in fruits; **c** Catalase activity in leaves; **d** Catalase activity in fruits; **e** Ascorbate peroxidase activity in leaves; **f** Ascorbate peroxidase activity in fruits. Fruit were harvested at 15 days after pollination. *Averages are statistically different according to the t-test (5%). Data shown as mean ± standard deviation

had positive correlations with BER were cytoplasmic leakage, H_2O_2, soluble Ca^{2+}-H_2O_2, cell wall-bound Ca^{2+}, CAT, MDA, SOD, total variables related to triggering BER. The variables that had negative correlations with BER were AsA, apoplastic leakage, soluble Ca^{2+}+APX, soluble Ca^{2+}+AsA, APX, soluble Ca^{2+}, soluble Ca^{2+}+SOD, soluble Ca^{2+}+CAT, total variables related to inhibiting BER.
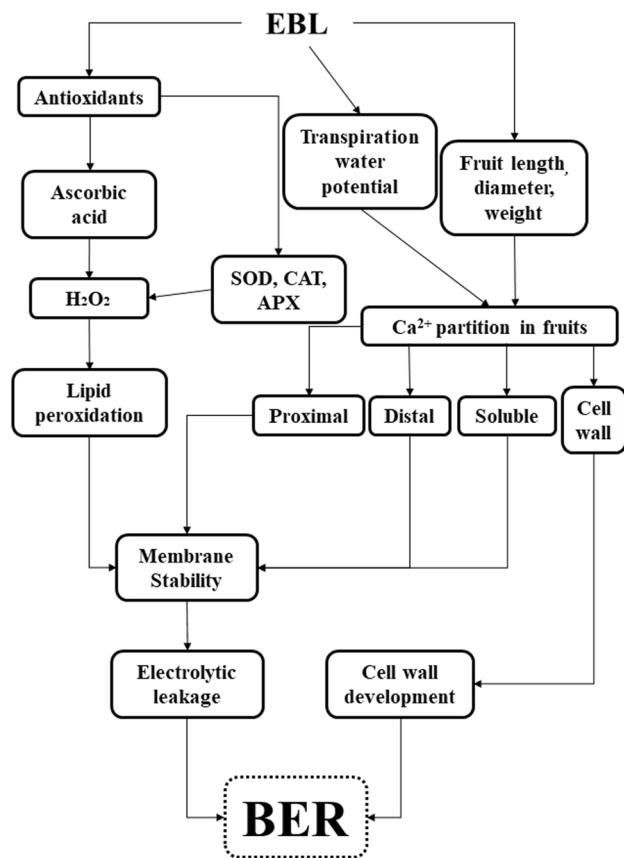
Fig. 5 Suggested physiological and morphological mechanisms regulating BER incidence in tomato varieties with elongated fruits

Discussion

Blossom-end rot (BER) is a disorder that affects growing fruits. It increases oxidative compounds in fruit distal tissue, leading to membrane peroxidation and cell-wall damages, reaching all tissue in a later stage (Aktas et al. 2003). Some hormones, such as ABA, are being used to reduce BER damage, but focusing on adapting the plants to a restrictive environment (Riboldi et al. 2018c).

Recently, some hormones started to be tested, with new approaches, such as brassinosteroids. They are a class of hormones with many responses in plants and main effects are related to stress responses (Soares et al. 2016; Shahzad et al. 2018). In this study, EBL had a great effect on reducing BER incidence in developing tomato fruit by about 44.2%. Due to some well-known evidence, it was noted how EBL may modulate the antioxidant activity in plants (Liu et al. 2009; Xia et al. 2009; Soares et al. 2016). It is possible that these plants responded by increasing stress-related defenses, like the antioxidant response.

In addition to oxidative stress factors, other possible mechanisms related to morphological factors such as fruit size, weight, and number of xylem functionality, physiological factors such as stomatal conductance, water potential, and transpiration, as well as nutritional factors such as Ca^{2+}, Mg^{2+}, and K^+ may also be possibly involved in determining fruit susceptibility to BER.

Physiological/Morphological and Nutritional Factors Regulating BER Development

Although EBL is a hormone related to plant growth, its effect was only observed in the fruit and not on leaf area or plant dry weight. Ca^{2+} is transported through the transpiratory current, reaching the fruit and aerial parts of plants via the vascular system, especially through the xylem vessels. As suggested in other studies, functional xylem vessels are required to maintain proper fruit Ca^{2+} uptake to support the active growing cells at the fruit distal end (Bondada et al. 2005).

However, the EBL treatment had no effect on the number of functional xylem vessels in the fruit during growth and development. Some studies have shown that using BRs in tissue culture can lead to a greater differentiation of xylem vessels instead of phloem vessels (Nagata et al. 2001). It is

Table 6 Correlation analysis between physiological analysis and BER incidence to determine parameters potentially inhibiting ($-R^2$) or triggering ($+R^2$) BER in tomato fruits subjected to EBL treatments

Inhibiting BER	R^{2a}	p value	Triggering BER	R^{2a}	p value
AsA	−0.32	0.36	Cytoplasmic leakage	+0.50	0.14
Apoplastic leakage	−0.30	0.4	H_2O_2	+0.32	0.37
Soluble Ca^{2+}+APX	−0.28	0.43	Soluble Ca^{2+}-H_2O_2	+0.32	0.36
Soluble Ca^{2+}+AsA	−0.27	0.45	Cell wall Ca^{2+}	+0.29	0.41
APX	−0.24	0.49	CAT	+0.19	0.59
Soluble Ca^{2+}	−0.24	0.5	MDA	+0.12	0.74
Soluble Ca^{2+}+SOD	−0.06	0.85	SOD	+0.11	0.76
Soluble Ca^{2+}+CAT	−0.03	0.93	Total	+0.54	0.33
Total	−0.34	0.8			

[a]Positive correlations mean a proportional correlation between two variables and negative correlations mean inversely proportional correlation between the variables. Pearson at 5%

also important to consider that xylem and phloem vessel differentiation depends on other plant hormones and their final ratio in the tissue, which may explain the lack of EBL effect on functional xylem vessels.

The effects of EBL controlling plant susceptibility to environmental stresses are important and this relation is becoming clearer with new studies (Singh and Shono 2005; Dobrikova et al. 2014; Soares et al. 2016; Shazad et al. 2018). Nevertheless, it was possible to see that EBL treatment reduced plant transpiration, changing leaf water potential. The movement of water and solutes in the plant takes place in response to water potential gradients. Therefore, the EBL reduction of leaf transpiration and the increase in water potential observed resulted in lower leaf Ca^{2+} accumulation in EBL-treated plants.

The content of Ca^{2+} in fruits was also affected by EBL treatment. In proximal and distal fruit parts, water-treated fruit showed greater tissue Ca^{2+} content. However, recent studies have shown that the concentration of total Ca^{2+} in the tissue cannot fully explain BER incidence, because other pools of Ca^{2+} at the cellular level are also involved (De Freitas et al. 2017).

Despite higher total fruit tissue Ca^{2+} content in water-treated plants, the content of soluble Ca^{2+} in the distal tissue was higher in EBL-treated fruit and also negatively correlated with BER. Furthermore, the Ca^{2+} bound to the cell wall was higher in water-treated plants. In that case, higher Ca^{2+} bound to the cell wall reduces the Ca^{2+} available for other cellular functions such as membrane structure and stability, potentially increasing fruit susceptibility to BER. The observed lower Ca^{2+} bound to the cell wall may be the result of lower pectin methylesterase (PME) activity and/or lower synthesis of desterified pectin in the tissue in response to EBL treatment (Peaucelle et al. 2012). De Freitas et al. (2012) have shown that reducing PME expression and activity can reduce Ca^{2+} binding to the cell wall, increasing other pools of Ca^{2+} in the cell and inhibiting BER development in tomato fruit.

The results of electrolytic leakage confirm some evidence of maintenance of membrane integrity and soluble Ca^{2+} content. The electrolytic leakage in the apoplastic portion is directly related to the solutes present in the apoplast and that the analytical solution is mixed in the first hours. In this case, there is a higher apoplast leakage in EBL-treated plants. Complementing what has already been discussed above, for Ca^{2+} to protect the membranes it must be in the soluble form, it becomes immobile once bound to the cell wall. Thus, there was a lower incidence of BER in EBL-treated plants, and this can be explained by the higher concentration of these solutes in the apoplastic portion, mainly composed of soluble Ca^{2+}.

In the cytoplasmic leakage, which corresponds to the simplastic portion, there is greater leakage in the control plants

and at the same time, a higher concentration of Ca^{2+} bound to the wall and lower soluble Ca^{2+}. It is also the highest positive correlation, considering the main variables. This analysis corresponds directly to the stability of the membrane, since they represent the solutes that were mixed in the solution, passing through the membrane after several hours of analysis.

BER is believed to be triggered by a cell-localized Ca^{2+} deficiency that leads to plasma membrane damage, cell plasmolysis, and water-soaked tissue at the blossom-end region of the fruit that becomes dark-brown, as cells die (Ho and White 2005; Rached et al. 2018). Therefore, the loss of integrity of these membranes leads to higher cytoplasmic leakage.

Then, for the control plants, which exhibited a higher BER incidence, soluble and cell wall-bound Ca^{2+} levels regulated the onset of BER. On the other hand, plants treated with EBL presented lower rates of BER precisely because they had more stable membranes and soluble Ca^{2+} available in the apoplastic solution. Therefore, using physiological and nutritional parameters, it is possible to consider that the increase of cell wall-bound Ca^{2+}, leaf Ca^{2+}, cytoplasmic distal leakage, could have triggered BER in the fruit and EBL could lower the electrolytic leakage and improve the integrity of the membranes, which represented lower BER incidence in this treatment.

Oxidative Stress-Related Factors Regulating BER Development

Brassinosteroids can stimulate growth, as well as enhance the plant's ability to overcome stresses, such as drought, high temperatures, or oxidative stress (Liu et al. 2009; Soares et al. 2016). In this way, EBL could improve the relationship between plants and the environment (Singh and Shono 2005).

In normal conditions, ROS are produced by cell metabolism, which is aggravated under stress conditions. Generally, in these conditions, there is an increase in the rate of lipid peroxidation, resulting from an increase in accumulation of ROS. In this way, some compounds such as singlet oxygen, hydroxyl radical, anion superoxide, or hydrogen peroxides attack the unsaturated lipids, especially fatty acids, and cause their peroxidation (Van Breusegem and Dat 2006), leading to the liberation of malondialdehyde (MDA) (Yamauchi et al. 2008).

Brassinosteroids are well known as acting to increase the antioxidant defense. In this study, EBL treatment increased ascorbic acid (AsA) content in the fruit, which could help explaining the reduction in BER incidence in EBL-treated fruit. Ascorbic acid is the most abundant, powerful, and water-soluble antioxidant that acts to prevent or minimize the damage caused by ROS in plants (Athar et al. 2008),

protecting and preventing membrane damage and leakage. It is also possible to observe that AsA had the highest negative correlation, showing the importance of this compound in inhibiting BER development.

Furthermore, our results show a great increase in the activity of the enzymes SOD, CAT, and APX, in both leaves and fruit. Our study reveals an important stimulation of SOD activity in EBL-treated leaves and fruits and a high increase of CAT activity in EBL-treated tomato leaves and fruits. APX is part of the ascorbate–glutathione cycle and is responsible for the elimination of hydrogen peroxide. The stimulation of the APX activity has been reported to be triggered as a plant response to stress conditions (Liu et al. 2009; Borges et al. 2018), such as ROS generation during BER development in fruits. Therefore, these enzymes act as scavengers, transforming ROS into less dangerous compounds, such as water.

Indeed, studies have shown that high expression and activity of these enzymes in response to brassinosteroids resulted in plants more tolerant to stresses such as drought, oxidative, salt, and some metals conditions (Liu et al. 2009). In this study, total enzyme activities were considered, but it is important to bear in mind that most of these enzymes are present as isoenzymes in a number of plants, including tomato (Gratão et al. 2005, 2015; Pompeu et al. 2017; Carvalho et al. 2018). Therefore, it is not possible to establish whether the changes observed in this study are due to an overall change by all isoenzymes of a particular enzyme, for instance SOD, or to only one or another isoenzyme. Ongoing research in our laboratories is considering these possibilities particularly in the case of SOD whose distinct isoenzymes reallocate in distinct cell compartments, and therefore respond to specific changes in superoxide in different organelles.

Our study was the first to show the effects of EBL on different pools of Ca^{2+} at the cellular level as well as on antioxidant mechanisms inhibiting BER development in fruit tissue. Therefore, the combined effect of specific pools of Ca^{2+} and enzymes involved in stress resistance resulted in fruits less susceptible to BER in response to EBL treatment.

Possible Mechanism Inhibiting BER in Response to EBL

For many years, researchers have considered BER as a disorder caused only by Ca^{2+} depletion in the tissues in the distal portion of the fruit. However, more recently, it has been concluded that localized Ca^{2+} deficiency may lead to membrane leakage, which results in BER symptom development (Saure 2014).

Under stress conditions, increasing tissue ROS production to levels greater than the cells can metabolize may result in lipid peroxidation and cell death. In this case, we can treat BER as a direct consequence of ROS accumulation in the tissue (Rached et al. 2018), because the main symptoms observed during the development of the disorder are the loss of membrane stability and tissue necrosis.

In this study, EBL increased tissue antioxidant capacity, minimizing the effects of ROS on tissue oxidation and BER incidence. Therefore, spraying the plants with EBL increased plant resistance to low Ca^{2+} availability and stress conditions. In addition, EBL reduced cell wall-bound and increased water-soluble Ca^{2+} contents, as well as increased AsA leaves, both showing a strong negative correlation with BER incidence.

Higher activity of oxidative stress-related enzymes such as APX, CAT, and SOD, possibly helped reducing H_2O_2 concentration and lipid peroxidation, which resulted in the observed lower distal fruit tissue membrane leakage and BER incidence. Accordingly, the correlation analysis showed that cytoplasmic leakage and H_2O_2 levels were the main factors triggering BER in the fruit. In that case, ROS possibly acted by disrupting membranes, increasing membrane leakage, and triggering BER in the fruit.

Conclusions

In this study, EBL inhibited BER development in 'BRS Montese' tomato fruit. EBL maintained higher soluble Ca^{2+} and lower cell wall-bound Ca^{2+} contents in fruit tissue, reducing fruit susceptibility to BER. EBL also increased ascorbic acid content and decreased hydrogen peroxide contents, as well as increased the activity of the three main antioxidant enzymes known as ascorbate peroxidase, catalase, and superoxide dismutase in fruit tissue.

The results show that not only Ca^{2+} content plays a role in determining fruit susceptibility to BER, but also the oxidative activity in distal fruit tissues.

Although brassinosteroids are still not commercially used in agriculture, there is a great potential use of these hormones to improve plant stress resistance and reduce tomato fruit susceptibility to BER.

Future studies should be carried out to optimize doses, timing of applications, and possible interactions between brassinosteroids and other hormones. Furthermore, studies are also required to better understand the mechanisms regulating the activity of the main antioxidant enzymes, as well as increasing soluble Ca^{2+} and decreasing cell wall-bound Ca^{2+}.

Compliance with Ethical Standards

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