University of São Paulo
"Luiz de Queiroz" College of Agriculture

Physiological and morphological mechanisms regulating blossom-end rot in tomato fruits

Lucas Baiochi Riboldi

Thesis presented to obtain the degree of Doctor in Science. Area: Plant Physiology and Biochemistry

Piracicaba
2018

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versão revisada de acordo com a resolução CoPGr 6018 de 2011

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With love,

To my family.

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One should not pursue goals that are easily achieved.

One must develop an instinct for what one can just barely

achieve through one's greatest efforts."

Albert Einstein

SUMMARY

RESUMO

Mecanismos fisiológicos e morfológicos que regulam a podridão apical em frutos de tomate

O Ca^{2+} é um nutriente para o desenvolvimento de plantas, e sua deficiência causa mal desenvolvimento celular em frutos de tomate, resultando em desordem fisiológica conhecida como podridão apical ou *blossom-end rot* (BER). Estudos sobre esta desordem fisiológica não são recentes e são focados principalmente na deficiência de Ca^{2+} nos tecidos e na interação entre planta e meio ambiente. No entanto, novas pesquisas têm se baseado na interação hormonal e nos mecanismos oxidativos como reguladores desta disordem fisiológica nos frutos. Hormônios como giberelinas, ácido abscísico e brassinosteróides têm sido implicados tanto na ativação quanto na inibição dos sintomas de BER. A aplicação de ácido abscísico e epibrassinolideo diminuiu a incidência de BER, reduzindo a concentração de Ca^{2+} nas folhas e aumentando-as nos frutos. A aplicação de ácido abscísico provocou diminuição na transpiração, o que explica a mudança no fluxo de seiva e cálcio no xilema que conduz da folha para fruto. O epibrassinolideo, por outro lado, aumentou a resposta antioxidante, diminuindo as concentrações de peróxido de hidrogênio e aumentando as de ácido ascórbico, ascorbato peroxidase, catalase e superóxido dismutase nos frutos. A seleção de genótipos mais tolerantes ao aparecimento de BER e a identificação de fatores que os tornam resistentes, são ferramentas importantes no processo de seleção de novas variedades. Neste trabalho, foram estudados muitos genótipos para compreender a relação entre a forma do fruto e o ambiente de crescimento sobre a incidência de BER. De acordo com os resultados obtidos, genótipos alongados são mais susceptíveis a podridão apical.

Palavras-chave: Podridão apical; Ácido Abscisico; Brassinoesteroides; Formato de frutos; Frutos alongados

ABSTRACT

Physiological and morphological mechanisms regulating blossom-end rot in tomato fruits

Ca^{2+} is a nutrient for plant development, and its deficiency causes poor cellular development in tomato fruits, resulting in a physiological disorder known as blossom-end rot (BER). Studies on this physiological disorder are not recent and mainly focused on Ca^{2+} deficiency in tissues and on the interaction between plant and the environment. However, new research has been based on hormonal interaction and oxidative mechanisms in fruits. Hormones such as gibberellins, abscisic acid, and brassinosteroids have been implicated in both activation and inhibition of BER symptoms. The application of abscisic acid and epibrasinolide decreased fruit susceitibility to BER. Abscisic acid reduces leaf transpiration, inhibiting xylem sap and calcium flow into the leaves and increasing into the fruit. Epibrassinolide increased fruit antioxidant responses, decreasing hydrogen peroxide and increasing ascorbic acid content, as well as increasing ascorbate peroxidase, catalase, and superoxide dismutase activities. In addition, the selection of varieties more tolerant to BER and the identification of factors that make new varieties resistant are important tools in the selection of new varieties. In this study, many varieties were evaluated in order to understand the relationship between fruit shape and growing environment on BER incidence in the fruit. According to the results, elongated fruit varieties showed higher susceptibility to BER.

Keywords: Blossom-end rot; Abscisic acid; Brassinoesteroids; Fruit shape; Elongated fruits

1. INTRODUCTION

Ca^{2+} is a plant nutrient, which has structural and metabolic roles requered for proper growth and development processes. In the soil, this nutrient has low mobility, competing with other cations, such as Al^{3+}, H^{+}, K^{+}, Mg^{2+}, Mn^{2+}, Na^{+}, NH_4^{+}, for root uptake (Taylor; Locascio, 2004).

Transport of Ca^{2+} by plants occur via transpiratory current; dissolved in water, this nutrient is mobilized from the roots to the aerial part of the plant, mainly high-growth and intense transpirations zones as meristems, leaves, and fruits (Ho et al., 1993). Nonetheless, there are several barriers for Ca^{2+} and other íons to reach all of these tissues. Its translocation occurs exclusively via xylem and is determined by transpiration and growth rates of different plant organs (De Freitas et al., 2011). Thus, transpiration occurring on the leaf surface indirectly help water and nutrients such as Ca^{2+} rise to growing tissues (Guichard et al., 2005). Once Ca^{2+} reaches these tissues, however, it becomes less mobile.

This mobility is directly linked to the role it plays in plant. Most Ca^{2+} in tissues is attached to the cell wall. Its structural role is related to the stabilization of pectin chains. Thus, Ca^{2+} is bound to de-esterified pectin molecules that act as reinforcement to the cellulosic chains in various plant tissues (De Freitas et al., 2012a).

In addition, Ca^{2+} plays an important role in the stabilization of membranes. It has been shown that, in tomato fruit, during the rapid expansion phase, there is an increasing amount of Ca^{2+} bound to the plasma membrane in healthy fruits and a decreasing amount in membranes of plasmolized cells from fruit tissues with visible blossom-end rot (BER) symptoms (Suzuki et al., 2003).

Citosolic Ca^{2+} has the function of signaling regulator, in which several proteins, such as calmodulins, nutrient pumps and exchangers are involved. Thus, Ca^{2+} concentrations within the cytoplasm should be maintained at very low levels, since any oscillation can trigger metabolic alteration, leading to cellular responses and even to cell death. In this case, most Ca^{2+} is stored within the vacuoles and other cellular organelles, where it also plays an important role in charge balance (Ho; White, 2005).

Symptoms of Ca^{2+} deficiency may be easily observed in plants. In leaves, the most classic symptoms are the burning of the edges of new leaves, disorder also

known as tipburn in lettuce (Uno et al., 2016). However, these damages are better known in fruits, whose classic symptom is the softening of the distal region of the fruits and posterior necrosis, better known as BER in tomato. In apples, the disorder caused by Ca^{2+} deficiency is known as bitter pit (De Freitas et al., 2010).

Damages resulting from this physiological disorder are enormous, especially in fruit, since the final product ends up being commercially depreciated. Many studies have attempted to understand the main mechanisms regulating BER in plants. Initially, the idea was that Ca^{2+} concentration in soil and tissues, as well as the balance between Ca^{2+} and other nutrients would trigger the BER symptoms (Castro, 1980; Ho, White, 2005). However, many studies show that, even under high Ca^{2+} conditions in both soil and fruit, there may be a high incidence of BER (De Freitas et al., 2011, De Freitas et al., 2014).

Environmental factors, especially water stress, low relative humidity, and high temperatures, very common in summer and in greenhouse conditions, are other factors that lead to a high incidence of BER. Thus, excess transpiration may displace water flow with higher intensity mainly to the leaves, in detriment of the fruits, mainly by modifying the water potential of the whole plant, depriving fruits, which do not transpire as intensely as leaves, from Ca^{2+} accumulation.

However, in recent years, studies have focused on other factors that could potentially regulate BER (De Freitas et al., 2014, De Freitas et al., 2016, De Freitas et al., 2017). Endogenous factors, such as hormones, fruit types and formats, morphological differences between proximal and distal portions, and antioxidant activity in fruits have led researchers to suggest new crop management approaches to control or minimized BER incidence.

There are several hormones that cause changes in plant growth and in this case the hormones gibberellin and abscisic acid have been quite studied when it comes to BER. The use of gibberellin was shown to promote plant growth (Serrani et al., 2007), however it was noted that there was an increase in the incidence of BER (De Freitas et al., 2012b). The mechanism by which gibberellins increase the incidence of BER are linked to accelerated growth and absence of Ca^{2+}, during fruit growth (Castro, 1980).

In addition, there may be an imbalance of some enzymes activity responsible for maintaining cell wall integrity, such as pectin methylesterase (De Freitas et al., 2012a). Pectin methylesterase activity is directly related to an increase in the

incidence of BER, since these enzymes increase de-esterification of pectins, increasing the amount of cell wall-bound Ca^{2+}, and decreasing the pool of free and soluble Ca^{2+} responsible for membrane stabilization (De Freitas et al., 2012a).

The abscisic acid (ABA) has been gaining ground in research lately, since it promotes a better response of the plant to adverse cultivation conditions, reducing transpiration and promoting activation of the plant's antioxidant system. In addition, ABA is able to promote a better redistribution of Ca^{2+} in fruits, mainly by promoting better functionality of the xylem vessels, which carry water and nutrients to the distal portion of fruits. This region typically presents the lowest levels of Ca^{2+} and the first symptoms of BER (De Freitas et al., 2011).

There are several pools of Ca^{2+} in fruit tissue, including: proximal, distal, wall-attached, organelles-attached, cytoplasmic, and apoplastic Ca^{2+}, and an adequate balance among different pools is necessary to avoid BER incidence. More precisely, apoplastic Ca^{2+} concentration is considered the main regulator of BER incidence, since it is always available and arrives rapidly in regions where growth is more active and there is a greater need for Ca^{2+} (De Freitas et al., 2014).

Brassinosteroids, still poorly studied, have similar effects as ABA, being able to suppress the negative effects of Ca^{2+} deficiency in tissues. Their mode of action is still not well understood, but it is speculated that they activate antioxidant defenses in plants, inhibiting membrane lipid peroxidation (Schnabel et al., 2001, Liu et al., 2009).

Finally, a factor that is practically non-existent in studies is the influence of fruit format on the incidence of BER. There is popular knowledge that elongated fruits, represented mainly by the ‘San Marzano’, have high rates of BER incidence (Elmer, Ferrandino, 1991) and that BER is smaller in rounded and flattened shape, or even cherry (Ho; White, 2005). Nevertheless, the reason why these varieties are highly susceptible is still not well understood. Fruit shape may cause difficulties for the transport of Ca^{2+} along the fruit to the distal portion. As the distance from the proximal to the distal portion is greater in elongate fruits, the susceptibility of these fruits to BER is also generally greater. In addition, functionalities of xylem vessels in these varieties as well as xylem branching along the fruit tissue may also influence the appearance of BER symptoms.

Studies suggest that Ca^{2+} transport along the fruit may be limited by the loss of xylem vessels functionality during growth and development (De Freitas et al., 2014), possibly due to the accumulation of substances, embolism, and loss of

hydrostatic gradient (Bondada et al., 2005). As Ca^{2+} transport is coordinated by transpiratory current and carried to the fruits via xylem, it is necessary that its xylem vessels are functional, mainly in the distal portion. Consequently, Ca^{2+} may reach growing tissues, inhibiting the appearance of BER.

Therefore, it is clear that BER is not exclusively related to the concentration of Ca^{2+} in tissues, but to a combination of whole plant and fruit specific factors that cause this disorder. Morphological factors, together with physiological ones, determine the movement and redistribution of Ca^{2+} in plant tissues, promoting accumulation in fruits and inhibiting the appearance of BER. The main objectives of this study are to better understand the main factors regulating the susceptibility of tomatoes to BER, as well as to propose possible crop management approaches to reduce BER incidence and severity.

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2. ABSCISIC ACID, GIBBERELLIN AND 24-EPIBRASSINOLIDE MECHANISMS REGULATING BLOSSOM-END ROT DEVELOPMENT IN TOMATO FRUIT

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Abstract

Calcium (Ca^{2+}) is a macronutrient in plants, and low concentrations of this nutrient may result in the development of a physiological disorder known as blossom-end rot (BER) in tomatoes. Hormones can regulate the accumulation of Ca^{2+} and, consequently, fruit susceptibilities to BER. The objective of this study was to evaluate the effect of gibberellin (GA), abscisic acid (ABA), and 24-epibrassinolide (EBL) on Ca^{2+} accumulation and BER incidence in tomatoes. 'Tyna' tomato plants were sprayed biweekly, during anthesis, with water (control), GA (28.9 µM), ABA1 (90.8 µM), ABA2 (136.2 µM), EBL1 (0.01 µM) and EBL2 (0.1 µM). Treatments were applied until the physiological maturity of fruits of the first cluster, when evaluations were performed. ABA and EBL treatments reduced BER incidence. The bioregulators used had no effect on plant growth, fruit diameter, length, or color. However, application of GA and EBL reduced titratable acidity and, the first also reduced soluble solids content in the fruit. All treatments, except GA treatment, increased Ca^{2+} contents in the fruits, when compared with water treated fruit. The highest fruit Ca^{2+} content was observed in plants treated with 0.01 µM of EBL. According to our results, ABA and EBL increased Ca^{2+} concentrations and decreased BER incidence in tomato fruit.

Keywords: Bioregulators; Calcium balance; Stomatal conductance; Transpiration; Titratable acidity

2.1. Introduction

Calcium (Ca^{2+}) is an essential macronutrient in plants, actively playing a role in growth and development (White and Broadley 2003). This nutrient plays an important role in the cell wall structure and also in the maintenance of the integrity and functioning of cell membranes (Hepler 2005).

Translocation of Ca^{2+} occurs almost entirely via xylem, moving from roots to aerial parts of plants. Therefore, absorption is highly dependent on transpiration rate and plant growth (Ho et al. 1993). Uptake through the roots occurs by apoplastic and simplastic pathways (Karley and White 2009); however, as Ca^{2+} is maintained at low

concentrations in the cytoplasm, the apoplastic pathway is believed to be predominant (White and Broadley 2003). After entering the root, Ca^{2+} movement upward is regulated by means of sap flow in the xylem in response to the water potential gradient between the roots and the aerial parts of the plant (leaves and fruits).

Ca^{2+} is not commonly limiting under field conditions, but there are a number of disorders generally associated with low levels of this ion in plant tissue such as small root development, blossom-end rot (BER), leaf necrosis, bitter pit, aqueous spots, among others (White and Broadley 2003). Visual symptoms of Ca^{2+} deficiency in fruit tissue begin with cellular plasmolysis and soaked appearance of tissues, which progress to cell death and necrosis, resulting in dark-brown tissues (De Freitas et al. 2011). In tomato, watermelon, melon, and pepper, Ca^{2+} deficiency in distal parts of fruits is considered the main cause of the onset of symptoms of a physiological disorder known as BER (Ho and White 2005).

Studies suggest that a genetic selection process aiming to increase fruit size and weight may have favored the selection of mechanisms that inhibit accumulation of Ca^{2+} and/or encourage storage of this ion in cellular organelles, reducing Ca^{2+} bound to the cell wall and allowing fruit growth (De Freitas et al. 2012b). These metabolic modifications may have led to increased fruit susceptibility to BER as large and elongated tomatoes are more susceptible to BER than small, round tomatoes (Ho and White 2005).

Studies have shown that BER does not occur exclusively due to insufficient availability of Ca^{2+}, but rather due to poor supply of Ca^{2+} caused by reduced transport of Ca^{2+} to the distal tissue of the fast growing fruit and also by an increased demand of the distal tissue for Ca^{2+} by accelerating fruit expansion (Ho, 1998; Saure 2005). In addition, a number of substances such as gibberellins (GA) (De Freitas et al. 2012a), abscisic acid (ABA) (De Freitas et al. 2014), ammoniacal fertilizers (Bar-Tal et al. 2001; Castro and Malavolta 1976), together with environmental conditions such as water stress (Guichard et al. 2005), can modify this translocation, preventing Ca^{2+} from reaching the fruit. Other factors affect the partition of Ca^{2+} in plants, such as excessive rate of transpiration or even the lack of it, induced by high temperatures and/or low relative humidity. Leaves have higher transpiration rates than fruits, thus resulting in a higher Ca^{2+} concentration in leaves (De Freitas et al. 2011).

Plant hormone ABA is known to reduce stomatal opening and to decrease transpiration of leaves, which may restrict the flow of sap and Ca^{2+} from the xylem to leaves and increase to fruits (De Freitas et al. 2014; Guichard et al. 2005). Gibberellins are plant hormones responsible for the growth of plant tissue, being a trigger for cellular expansion, together with auxins (Ho and White 2005). Studies have shown that, they inhibit tissue differentiation (Aloni 2001) and may affect development and functionality of xylem vessels responsible for Ca^{2+} transport in the plant, from root to fruit (Saure 2005). Studies in which ABA was applied in tomato plants showed increased Ca^{2+} in the pericarp tissue at the end of the peduncle. This occurred due to an increased xylem flow and decreased phloem flow towards fruits. In addition, a higher apoplastic Ca^{2+} concentration was observed in fruits, decreasing the permeability of membranes and reducing the incidence of BER (De Freitas et al. 2013).

Effects of brassinosteroids (BR) on the development of BER are being studied, but researches are not conclusive about the possible mechanisms involved. Some studies showed that BRs induce stress tolerance (Schnabl et al. 2001) and increase cell viability, strengthening fruit tissue capacity to eliminate reactive oxygen species (ROS) (Liu et al. 2009).

Studies using 24-epibrassinolide (EBL) have shown an increased activity of antioxidant enzymes, as well as a synthesis of antioxidant substances such as ascorbic acid (Liu et al. 2009). In addition, BR is known for its role in the development of xylem vessels, and in its absence, there is a predominance of phloem vessel formation (Nagata et al. 2001). If that is the case, BRs could stimulate the accumulation of Ca^{2+} and reduce the levels of reactive oxygen species (ROS) in tissues, reducing fruit susceptibility to BER. In addition, if BRs cause modifications in plants water relations, plant and fruit development, adaptation to the environment and nutrients partition, it is possible that BRs can play and important role on inhibiting BER.

The objective of this study was to evaluate the effect of bioregulators gibberellin, abscisic acid and 24-epibrassinolide, on Ca^{2+} partition and fruit susceptibility to BER.

2.2. Material and Methods

2.2.1. Plant material, growth conditions, and application of treatments

Elongated genotype of tomato, cultivar ‘Tyna’ was cultivated in a greenhouse. Plants were seeded in trays with a ratio of commercial product (Plantmax HT, Eucatex Brazil) to expanded vermiculite, supplemented with 1 g NPK 10:10:10 L^{-1} and 4 g L^{-1} limestone, indicated for seed germination. Thirty days after planting, seedlings were transplanted into individual 30 L pots containing oxysol and organic substrate in the ratio 2:1.

The plants were fertilized every 20 days, during de growing and fructification time, with 10 g of slow realease fertilizer containing N (16%), P_2O_5 (8%), K_2O (12%), MgO (2%), S (5%), Fe (0.4%), Cu (0.05%), Mn (0.06%), Zn (0.02%), B (0.02%), Mo (0.015%), but without Ca (Basacote Plus; Compo Expert; Soil fertilizer, Agricultural), to stimulate the incidence of BER, as well as soil with a low level of calcium (9 mmolc dm^{-3}) was used; added to pot for each plant. The plants were irrigated every other day until saturation of the substrate. Conditions of air temperature and relative humidity were monitored throughout the cycle, from planting to harvest, with a mean of 20°C and 71%, respectively.

From pollination to physiological maturation of fruits of the first cluster (60 days after pollination), plants were sprayed biweekly with a 125-mL solution per plant containing water (control), GA (GA_3, PROGIBB, Sumitomo Chemical do Brasil, Sao Paulo, SP) (28.9 µM), ABA1 (Valent Biosciences, Libertyville, IL, USA) (90.8 µM), ABA2 (Valent Biosciences) (136.2 µM), EBL1 (Sigma-Aldrich, Saint Louis, MO, USA) (0.01 µM) and EBL2 (Sigma-Aldrich) (0.1 µM). At the moment of the red ripe stage of fruits from the first cluster, evaluations of plants and fruits were carried out.

2.2.2. Growth evaluations

Evaluations were divided into plant and fruit analyses. The former consisted in measuring plants’ final height. The latter evaluated the number of fruits, diameter and average length, and percentage of fruits with visual symptoms of BER. Only fruits of the first cluster were used for evaluations. Incidence of BER was also evaluated in subsequent clusters.

2.2.3. Determination of total leaf and fruit Ca^{2+} contents

Whole fruits and leaves, oven-dried at 65ºC until constant weight, were used. Samples of mature leaves were removed from the region close to the fruits of the first cluster. Five-hundred milligrams of dry material were taken, 6 mL of nitroperchloric acid (2:1) was added and the material digested in gypsum block at 240°C and completed to 15 g with distilled water. Total Ca^{2+} was determined by atomic absorption, according to Malavolta et al. (1997). Results were expressed in g of Ca^{2+} per Kg of fruit dry matter.

2.2.4. Determination of physical-chemical parameters in fruits

Soluble solids (SS) were determined in fruit juice after trituration in a domestic centrifuge. A drop of juice was used, and readings were performed in an Atago digital refractometer, Palete 101 model (Atago, Ribeirao Preto, SP, Brazil), with two readings per repetition. Results were expressed as percentage. Titratable acidity (TA) was determined in fruit juice after crushing, where 10 g of juice was weighed and filled with distilled water to make 100 mL. Subsequently, it was titrated with NaOH (0.1 N) to the pH 8.1, under constant agitation, according to the methodology described by Carvalho et al. (1990). Results were expressed as % citric acid in the juice. Ratio: was expressed as a ratio between soluble solids/titratable acidity.

The color of fruit skin was determined using a Minolta colorimeter, CR-300 model (Konica Minolta Holdings Inc., Tokyo, Japan), with the following configuration: CIELab color system, D65 illuminator and standard observer 2º. Two readings per fruit were performed on opposite sides of its equatorial region, and the results were expressed in color angle (h).

2.2.5. Determination of stomatal conductance and transpiration

A LI-COR-1600 (LI-COR Biosciences, Lincoln, NE, USA) model was used. The analyses were performed between 9 and 11 am at 10, 25, 40, and 55 days after pollination of the flowers of the first cluster. Mature leaves close to first cluster were used. The values obtained for transpiration and stomatal conductance correspond to

the average of the four evaluations performed on leaves during the period of experiment.

2.2.6. Experimental design

Experimental design was a completely randomized design with five replicates and two plants per replicate. Results were submitted to analysis of variance and, when significant, were submitted to the Tukey test at the 5% probability. Data were also submitted to principal component analysis (PCA).

For selection of factors to be analyzed, the Kaiser criterion (Kaiser 1960) was used for factors with their own value greater than 1. The PCA results were submitted to cluster analysis using the HCPC command (Hierarchical Clustering on Principle Components). Analysis was performed using the FactoMineR bookstore (Husson 2014) in the R project.

2.3. Results

ABA (1 and 2) and EBL1 treatments showed a 6.6 - 9.0% reduction in BER incidence compared with control treatment (Table 1). GA and EBL2 treated plants had similar BER incidence to control plants. The BER incidence was higher in the first clusters and lower in the second, third and fourth clusters (Table 1).

Table 1. Average percentage of blossom-end rot incidence in fruits by cluster, in the first four clusters

Treatments[a]	1st cluster (%)	2nd cluster (%)	3rd cluster (%)	4th cluster (%)
C	20.62±1.09 ab*	4±8.94 ns**	0	0
GA	21.6± 3.45 a	0	0	0
ABA1	14.04±5.47 c	2.22±4.96 ns	0	0
ABA2	13.34±6.03 c	6.81±10.89 ns	0	0
EBL1	11.66±2.19 c	0	0	0
EBL2	14.72±0.85 bc	0	3.33±7.45 ns	4±8.94 ns

[a] C = control; GA = 28.9 µM; ABA 1= 90.8 µM; ABA 2 = 136.2 µM; EBL 1 = 0.01 µM; EBL 2 = 0.1 µM. *The average followed by same letter were not statistically different (Tukey 5%). **ns= the values were not statistically different (Tukey 5%)

Application of bioregulators had no effect on plant growth (Table 2). The average height of all plants was 154.7 cm. No significant differences were observed between diameter and length of fruits among treatments (Table 2). Color of epidermis was statistically the same for all treatments, which presented a mean value for the 30° color angle (°h) at the red ripe stage (López-Camelo and Gómez 2004).

Table 2. Plant height, diameter, length, and average weight of fruits, number of fruits by cluster and epidermic color of fruits from 'Tyna' tomato

Treatments[a]	Height (cm)[ns**]	Diameter (mm)[ns]	Length (mm)[ns]	Weight (g)	Fruit number[ns]	Color index[ns]
C	151±14.8	44.6±4.0	54.8±5.4	556.5 ab*	5.2±4.1	32.5± 1.7
GA	165±9.9	44.5±3.5	56.6±5.7	700.5 ab	6.9±3.2	32.1±1.2
ABA1	154±10.0	44.1±3.0	55.3±4.6	673.8 ab	5.8±2.9	33.3±1.1
ABA2	154±13.4	46.7±3.8	56.7±4.1	714.8 a	4.1±7.9	32.5±0.6
EBL1	152±8.3	48.4±4.7	56.8±3.3	858.8 a	6.6±3.8	32.9±1.5
EBL2	152±6.1	43.8±3.6	52.9±4.5	346.6 b	4.3±2.7	31.2±1.7
CV %	7.0	7.52	7.8	28.8	25.73	ns

[a] C = control; GA = 28.9 µM; ABA 1=90.8 µM; ABA 2 = 136.2 µM; EBL 1 = 0.01 µM; EBL 2 = 0.1 µM. . *The average followed by same letter were not statistically different (Tukey 5%). **ns =The values were not statistically different (Tukey 5%)

The titratable acidity (TA) was lower in fruits treated with GA, EBL1 and EBL2 than in control fruit (Table 3). There was no significant variation of fruit pH among treatments, with a mean of 3.7. Only GA treated fruit showed lower soluble solids content, compared with control fruit (Table 3). The observed SS/AT ratio (SS, Soluble solids) was around 6 and treatments using EBL promoted a higher SS/AT ratio, compared with control and ABA treatments (Table 3).

Table 3. Tritatable acidity (TA), Soluble solids (SS), pH, and Ratio of 'Tyna' tomato fruits

Treatments[a]	TA (% citric acid)	SS (°Brix)	pH[ns**]	Ratio
C	0.84 a*	5.04 a	3.62	6.0 c
GA	0.52 c	3.78 b	3.59	7.27 bc
ABA1	0.85 a	5.58 a	3.69	6.56 bc
ABA2	0.78 ab	4.62 ab	3.65	5.92 c
EBL1	0.52 c	5.19 a	3.77	9.98 a
EBL2	0.67 b	5.57 a	3.88	8.31 b
CV%	10.93	9.94	5.39	10.45

[a] C = control; GA = 28.9 µM; ABA 1= 90.8 µM; ABA 2 = 136.2 µM; EBL 1 = 0.01 µM; EBL 2 = 0.1 µM. *The average followed by same letter were not statistically different (Tukey 5%). **ns =The values were not statistically different (Tukey 5%)

In leaves, a reduced Ca^{2+} content was observed in treatments with ABA1 and EBL2, when compared with control (Table 4). This reduction was more pronounced in ABA1 treatments. In fruits, the lowest Ca^{2+} levels were observed in the control and GA treatments (Table 4). The highest levels of Ca^{2+} were observed in treatments with EBL1 (Table 4).

Table 4. Leaf stomatal conductance (g_s), leaf transpiration, and Ca^{2+} content in leaf and fruit tissue of 'Tyna' tomato

Treatments[a]	g_s (cm s^{-1})	Transpiration (µg cm^{-2} s^{-1})	Leaf Ca^{2+} (g kg^{-1} DM)	Fruit Ca^{2+} (g kg^{-1} DM)
C	0.99 ab*	7.82 a	24.68 ab	0.52 e
GA	1.02 a	8.28 a	25.87 a	0.62 d
ABA1	0.83 d	7.21 b	17.75 d	0.82 b
ABA2	0.74 e	6.64 c	23.80 ab	0.87 b
EBL1	0.93 bc	8.33 a	23.24 b	0.95 a
EBL2	0.90 cd	8.15 a	20.03 c	0.72 c
CV%	4.01	3.55	4.73	4.3

[a] C = control; GA = 28.9 µM; ABA 1= 90.8 µM; ABA 2 = 136.2 µM; EBL 1 = 0.01 µM; EBL 2 = 0.1 µM. *The average followed by same letter were not statistically different (Tukey 5%). DM= dry mass

The lowest transpiration rates were observed in treatments with ABA1 and ABA2 (Table 4). Stomatal conductance was lower in ABA1-, ABA2- and EBL2-treated plants, when compared with control (Table 4).

There was a significant positive correlation among several factors; however, a highly significant correlation (R = 0.9) between BER and Ca^{2+} concentration in fruits was verified, being the highest among all variables observed (Table 5). Likewise, BER is positively correlated with leaf Ca^{2+} concentration, stomatal conductance and leaf transpiration (Table 5), as well as negatively correlated with Ca^{2+} concentration in fruits. Soluble solids content is negatively correlated with incidence of BER in fruits (Table 5).

Table 5. Correlation analysis between physiological analysis and BER incidence to determine parameters potentially inhibiting ($-R^2$) or triggering ($+R^2$) BER in 'Tyna' tomato

Inhibiting BER	**R^{2*}**	**Triggering BER**	**R^{2*}**
Fruit Ca^{2+}	-0.93	Stomatal conductance	+0.71
pH	-0.61	Height	+0.55
Solide solubles	-0.59	Leaf Ca^{2+}	+0.55
Fruit diameter	-0.55	Transpiration	+0.30
Ratio	-0.43	Fruit number	+0.26
Color index	-0.26		
Fruit total weight	-0.24		
Fruit length	-0.06		
Tritratable acidity	-0.01		

* Positive correlations mean a proportional correlation between two variables and negative correlations mean inversely proportional correlation between the variables

Principal component analysis was used to reduce dimensionality of database (Figure 1), and a total of four dimensions explained 88.51% of total database variability. First component explained 34.10% of database, and the main variables that contributed to this component were soluble solids, pH, height, fruit number leaf Ca^{2+} and leaf stomatal conductance. Second component explained 28.62%, and the main variables were % BER, fruit Ca^{2+} fruit diameter, fruit length and color index. Third component explained 22.55%, and variables were titratable acidity, ratio and

transpiration. Fourth component explained 8.73% of database, and its main variable was weight and finally the fifth component explained 5.99% of database and no variable was explained in this component.

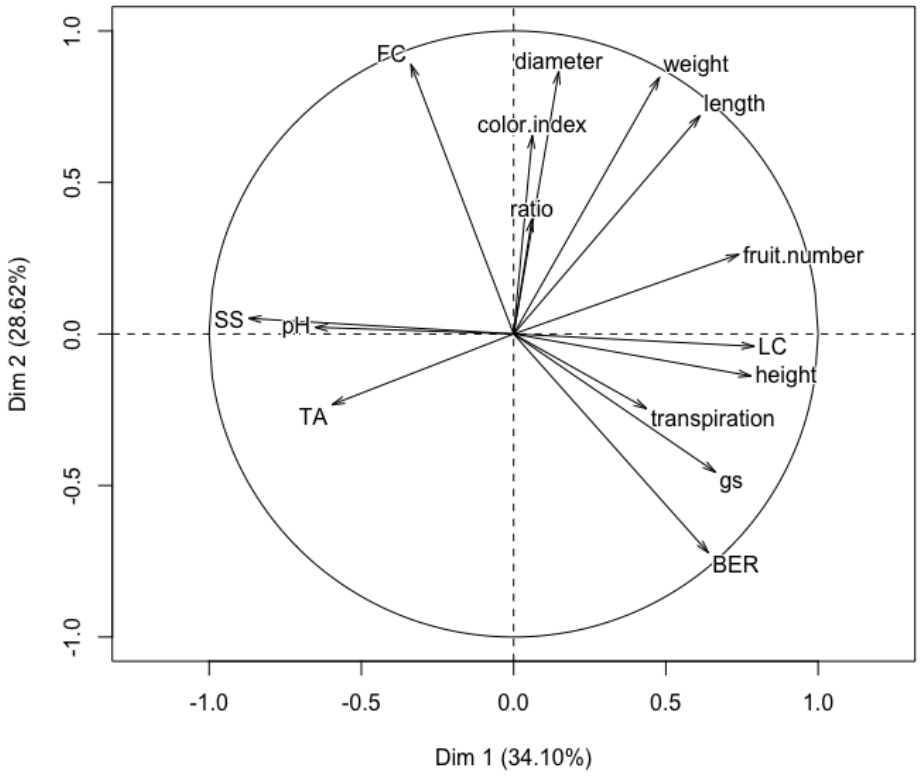

Figure 1. Relationship among variables (PCA) shown by scatter plot of the first two principal components based on traits. The variables are: SS- solide solubles, TA- tritatable acidity, FC- fruit Ca^{2+}, LC- leaf Ca^{2+}, gs- leaf stomatal conductante, BER- blossom-end rot

2.4. Discussion

In the present study, the incidence of BER was higher in the first clusters, possibly due to rapid growth of the distal part of the fruits, which increases the demand for nutrients, compared with fruits of other clusters (Ho 1998). During development of the first clusters, the plant presents a high vegetative growth, mainly in cultivars with indeterminate cycle, which have a continuous vegetative and reproductive growth, as is the case of 'Tyna'. Therefore, there is a greater

competition for nutrients between leaves and fruits in the first clusters. Calcium uptake into the leaves strongly limits fruit Ca^{2+} uptake, considering that leaf Ca^{2+} is not mobile in the phloem and does not move into the fruit (Dražeta et al. 2004).

After the appearance of the first cluster, vegetative growth rates of the plant tend to fall and, consequently, the demand for Ca^{2+} in leaves decreases. Therefore, a greater amount is translocated from plant to fruits via xylem, taking into account their growth and transpiration demands. However, rates of transpiration and growth are much higher in leaves than in fruits (De Freitas et al. 2011; Ho and White 2005). Factors such as these leading to a reduced transport of Ca^{2+} to the distal part of fruit may promote the onset of the disorder (Ho 1998).

A strategy that can be used to overcome this problem is to diminish Ca^{2+} transport to the leaves by reducing transpiration rates (Li et al. 2001). In the present study, high temperature peaks and low relative humidity, typical of the local winter, were observed, which may have contributed to the high transpiration rates observed (around 6 μg cm^{-2} s^{-1}) and, therefore, to the high content of Ca^{2+} in leaves. In addition, a decrease in air relative humidity, and consequently an increase in the vapor pressure deficit, contributes to a greater occurrence of BER (De Freitas et al. 2014)

Great variations in air relative humidity were observed during the period of fruit growth and development. Accordingly, it is possible that leaf transpiration had an important role in the development of BER in fruits. Through the correlation analysis, it was possible to identify negative correlation between leaf transpiration and Ca^{2+} content in fruits.

Although the total fruit Ca^{2+} content is important for determining BER incidence, metabolism of this nutrient at the cellular level may be an even more important factor. Calcium may bind to cell wall pectins, phosphate groups of cell membranes, or even to negative charged compounds within organelles (De Freitas et al. 2012). Cell-bound Ca^{2+} presents the major part of its content in fruits (De Freitas et al. 2010; Demarty et al. 1984) and has a great effect on the availability of soluble Ca^{2+} in apoplast, which is necessary to maintain plasma membrane functionality and integrity (De Freitas et al. 2014). Our results concerning total Ca^{2+} in fruits showed that, even under normal growing conditions and low availability of Ca^{2+} in the soil, BER is present when there is no agronomic intervention in the crop.

Therefore, it is not possible to minimize BER only by raising Ca^{2+} levels in soil, but by looking at factors that modify nutrient partitioning and allocation between different drains, such as transpiration and growth rates. In this regard, the use of bioregulators proves efficient as a way to alter such physiological responses, thus minimizing damages caused by a high incidence of BER in fruits. There are many studies showing that growth regulators may have the same (saturated responses) or opposite (feedback inhibition) responses depending on the dose and timing of application, which can explain the fact that there is no dose response in our study.

2.4.1. Role of GA in the incidence of BER

During fruit growth, the main hormones that promote this regulation are auxins and gibberellins (Ho and White 2005). Endogenous synthesis of GA is responsible for growth responses (Kumar et al. 2014), and exogenous applications can improve and homogenize such responses. However, a previous study reports an increased percentage of fruits with BER through exogenous applications of GAs, even without major changes in plant growth (De Freitas et al. 2011).

However, in the present study, we verified that application of GA did not increase the incidence of BER in tomato fruits when compared with the control, although, in both cases, the rates of BER incidence were high when compared with other treatments. It is possible that high endogenous concentrations of GAs in control plants resulted in saturation of physiological responses to this hormone. In this regard, application of exogenous GA had no additional effect when compared with control plants. The high BER index observed in control plants, which are under the effect of endogenous GAs, and in plants treated with exogenous GA may be related to its effects on leaf transpiration and leaf stomatal conductance.

As discussed earlier, BER develops not only because of a reduction in Ca^{2+} content in the plant, but also because of a change in the nutrient partitioning of the xylem sap for different drains. In cases of high transpiration rates, the leaves become preferential drains to the detriment of the fruits (De Freitas et al. 2013). Although there was no significant reduction in Ca^{2+} content in the fruits, the presence of both endogenously and exogenously applied GAs leads to an accelerated growth of the fruits, increasing their demand for Ca^{2+}. This fact, together with high transpiration

rates, favored the development of the disorder in fruits of control and GA-treated plants.

2.4.2. Role of ABA and EBL in the incidence of BER

Studies conducting ABA application directly on fruits showed that this yielded a significant reduction in the percentage of BER incidence (De Freitas et al. 2014). This efficiency in maintaining adequate Ca^{2+} levels occurs mainly through control of leaf transpiration, modifying the Ca^{2+} partition between leaves and fruits (De Freitas et al. 2011). In PCA (Figure 1), it was possible to observe a positive correlation between BER, leaf transpiration and leaf Ca^{2+} content.

Since ABA regulates leaf stomatal closure, its foliar application causes a greater part of Ca^{2+} that arrives at the aerial part of the plant to be directed to the fruits, when compared with plants not treated with ABA (De Freitas et al. 2014). In the present study, there was a significant reduction in the occurrence of BER in respose to ABA, with a reduction in transpiration rates and leaf stomatal conductance, as well as an increased in fruit Ca^{2+} content, compared with the control plants.

Additionally, studies suggest that ABA changes the antioxidant capacity of plants (Saure 2014). Plants submitted to regular foliar applications with ABA had high enzymatic activity of ascorbate peroxidase, catalase, and superoxide dismutase (Ibrahim and Jaafar, 2013). Reduction of ROS generation may be closely related to the reduction of susceptibility to BER, and this may be another mechanism through which ABA inhibited fruit susceptibility to BER (De Freitas et al. 2016).

Here, we observed a beneficial effect of EBL1 on the reduction of BER incidence, associated with a decrease in the Ca^{2+} content in leaves and its increase in fruits. As mentioned before, partition of Ca^{2+} between leaves and fruits has a great importance in developing Ca^{2+} disorders in fruits.

Transpiration is a factor that directly influences this process since its increase can increase Ca^{2+} transport towards the leaves, reducing its transport into the fruits. In present study a significant reduction in transpiration rates of EBL-treated plants were not observed. EBL2 use was not able to reduce incidence of BER, but such dosage promoted a low decrease in leaf stomatal conductance. Although some studies have shown a decrease in transpiration in response to BRs (Xu et al. 1994) and in tomato higher concentrations of EBL have been shown to led to stomatal

closure (Xia et al. 2014), the data obtained show that there is no clear relationship between transpiration and BER reduction in response to EBL.

Under conditions of high temperature and low air relative humidity, it is possible that a situation of thermal stress in plants occur. Although during the course of the experiment extreme temperatures and low air relative humidity were observed, it is worth mentioning that EBL provides greater tolerance to environmental stresses, as observed by Ahammed et al. (2015). There is evidence that ROS increase BER incidence, since ROS cause direct damages to growing fruits and cell membranes (Schmitz-Eiberger and Noga 2003). BRs are known to increase plant tolerance to stress (Schnabel et al. 2001), improving cells ability to cope with ROS (Liu et al. 2009); reduction in BER incidence may be directly related to this improvement in the antioxidant capacity of fruit cells.

In addition, studies have shown that BRs increase differentiation of vascular tissues, raising the concentration of xylem vessels (Nagata et al. 2001). Since Ca^{2+} moves through xylem, higher xylems differentiation due to BRs may have resulted in the observed increase in fruit Ca^{2+} content. Therefore, the lower percentage of BER observed in BRs treated plants may be explained by both higher fruit stress tolerance and functional xylems.

2.4.3. Effect of bioregulators on the physical-chemical quality of fruits

Although some plant hormones are able to reduce fruit susceptibility to BER, it is desirable that they do not decrease fruit quality. The hormones evaluated had no negative effect on fruit color or number. pH values and soluble solids were also unaffected, except for GA, which reduced the soluble solids of fruits. Treatment with GA is widely used in fruits to increase size and accelerate growth (De Freitas et al. 2012a), which may have diluted soluble solids in the fruit. Total acidity was reduced by hormone treatments, mainly by EBL, with exception to fruits that were treated with ABA.

2.5. Conclusion

Applied bioregulators influenced availability of Ca^{2+}, modifying contribution to fruits to the detriment of leaves. Application of ABA reduced the percentage of BER,

keeping transpiration levels lower when compared with control and GA-treated plants. In addition, EBL treatments reduced the percentage of BER, but did not alter fruit characteristics such as color, pH, and soluble solids, while increasing ratio values, evidencing an improvement in the sensorial quality of fruits.

Despite the mechanisms presented here, more research should be carried out to understand mainly how BRs act in the control of BER in fruits and its relation with ABA. There is a great potential for their use in agriculture; however, the first factors for the consolidation of their use would be to study the ideal doses, periods and place of application, adverse effects they may cause in post-harvest storage, as well as market acceptance of these fruits.

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3. FRUIT SHAPE REGULATES SUSCEPTIBILITY OF TOMATO TO BLOSSOM-END ROT

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Abstract

Calcium (Ca^{2+}) is a nutrient in tomato plants, and its deficiency usually causes several problems such as the physiological disorder known as blossom-end rot (BER) in the fruit. The objective of this study was to evaluate and identify morphological and physiological characteristics related to the susceptibility of tomato varieties to BER. The varieties studied were 'Amalia', 'IPA-6', 'M-82', 'Mara' and 'Nagcarlan', presenting different fruit format. The results show that the 'Mara' and 'Nagcarlan', 'Amalia' and 'IPA-6', or 'M-82' presented low, medium and high susceptibility to BER, respectively. The physiological parameters negatively correlated to BER were plant water potential, leaf area, plant dry mass, relationship between proximal/distal Ca^{2+}, K^{+} content in the proximal and distal portion of the fruits and proximal Ca^{2+} content. The physiological parameters positively correlated to BER were number of trichomes in the abaxial and adaxial portions of the leaves, leaf stomatal conductance, distal Ca^{2+} content bound to the cell wall, leaf transpiration and fruit length. According to the results, total fruit Ca^{2+} concentration, especially in the distal fruit tissue, is not the only factor responsible for the development of BER, but the balance between factors that increase and decrease the susceptibility of each genotype to this disorder.

Keywords: Blossom-end rot; Tomato varieties; Ca^{2+} disorder

3.1. Introduction

Calcium (Ca^{2+}) is a nutrient for plant growth, participating in both metabolic and structural processes. More specifically, Ca^{2+} plays an important role in cell wall composition, membrane structure and integrity (White and Broadley, 2003), as well as secondary messenger in the cytosol (Gilroy et al., 2016; Tuteja and Mahajan, 2007). When bound to pectins, it provides the structural maintenance of the tissues, which in turn provides rigidity, responsible for protection against external agents. When bound to the membrane phospholipids, Ca^{2+} maintains its stability, thus regulating the main intercellular exchange processes (Hepler and Winship, 2010).

In tomato fruits, typical symptoms of Ca^{2+} deficiency are known as blossom-end rot (BER). The characteristic symptoms of this disorder are initially the aqueous aspect, followed by death and darkening of the tissues in the distal region of the fruit. Studies suggest that BER occurs when Ca^{2+} that flows into the distal region of the fruit do not occur in synchrony with cell growth, causing abnormal cellular distribution of Ca^{2+}, leading to cell death and subsequent tissue necrosis of the affected region (De Freitas et al., 2011b; Ho, 1998; Saure, 2005).

The onset of BER may be related to environmental conditions, such as high temperatures and low relative humidity, which promotes high transpiration rates (De Freitas et al., 2011b). The movement of Ca^{2+} in the plant is connected to the movement of xylem sap. Its distribution to the leaves is, therefore, favored in detriment of the fruits, due to the transpiratory rates (De Freitas et al., 2011b; Ho et al., 1993). Ca^{2+} reaching the leaves remains immobilized, not being translocated to the fruits. In situations of high growth rates, deficient Ca^{2+} supply may not be able to meet physiological demands (Dražeta et al., 2004; Ho and White, 2005). As a result, the occurrence of water deficit can affect the translocation of Ca^{2+} within the plant (Guichard et al., 2001).

There is great genetic variability among tomato genotypes for susceptibility to BER. Elongated fruits present greater susceptibility when compared to rounded, flattened fruits and small fruits of the cherry type. Studies suggest that this occurs due to the shape and size of the fruits, which influences the transport of Ca^{2+} to the distal region of the fruits. Other factors such as the venation pattern and degree of xylem maturation and functionality also influence transport (Dražeta et al., 2004) and this may be related to the velocity of growth and blockage of xylem vessels (De Freitas et al., 2011b).

The large number of tomato varieties allows a high diversity that results in greater or lesser susceptibility to BER. However, little is known about which mechanisms act in each variety by inhibiting or stimulating the development of this physiological disorder. Studies on BER should not be restricted only to approaches related to Ca^{2+} deficiencies, but also to other factors that contribute to the greater or lesser susceptibility to this disorder. Knowing the main mechanisms regulating BER in each variety will allow the development of more specific and efficient control approaches.

The objective of this study was to evaluate and identify morphological and physiological characteristics related to the susceptibility of tomato varieties to BER.

3.2. Material and Methods

3.2.1. Plant material, growth conditions and application of treatments

This study was carried out in greenhouses with tomato plants of the cultivars ‘Amalia’, ‘Mara’, ‘Nagcarlan’, ‘IPA-6’, and ‘M-82’, provided by the germplasm bank of the Department of Genetics of the Luiz de Queiroz School of Agriculture. The plants were seeded in trays with a 1:1 ratio of commercial substrate (Plantmax HT, Eucatex Brazil) and expanded vermiculite, supplemented with 1 g L^{-1} of NPK 10:10:10 and 4 g L^{-1} of limestone. Thirty days after planting, the seedlings were transplanted into individual 30L pots containing the same substrate used for planting the seeds. Previous fertilization and cover fertilization were performed according to the recommendations for the crop (Benton Jones, 1998). The plants were fertilized every 20 days, during de growing and fructification time, with 10 g of slow realease fertilizer containing N (16%), P_2O_5 (8%), K_2O (12%), MgO (2%), S (5%), Fe (0.4%), Cu (0.05%), Mn (0.06%), Zn (0.02%), B (0.02%), Mo (0.015%), but without Ca (Basacote Plus; Compo Expert; Soil fertilizer, Agricultural). To stimulate the incidence of BER, no calcium was provided. The average maximum temperature was 32.5°C, and the minimum was 24.4°C. Relative humidity averages were 80% maximal and 55% minimal.

3.2.2. Incidence of BER and growth assessments

The incidence of blossom-end rot was calculated according to the number of fruits with symptoms of rot in relation to the total number of fruits. The number of flowers was counted and after pollination the number of fruits, which were used to determine the percentage of fruit fixation. The fresh and dry mass of the plants were determined at the time of maximum plant growth, approximately 40 days after transplanting the seedlings. Biweekly measurements of height were carried out, starting after transplanting until the maximum growth in height, totaling 4 measurements throughout the experiment.

3.2.3. Determination of stomata and trichrome density

Samples of the first pair of fully expanded leaves were collected for the analysis of epidermal impressions with the use of instant adhesive (Gülcan and Misirli, 1990). Sampling was performed in the median portion of the leaves. The numbers of stomata and trichomes were determined under an optical microscope and the counting was performed from the scanned images of each field considered. The mean replicates (1 leaf per plant for each treatment) were calculated and stomata and trichome densities were expressed as numbers of stomata and trichomes per mm^2.

3.2.4. Determination of leaf stomatal conductance, leaf transpiration and SPAD index

For the determination of leaf stomatal conductance and transpiration, a model LI-COR-1600 (LI-COR Biosciences, Lincoln, NE, USA) was used. The analyses were performed between 9 and 11, in the morning, at 10, 25, 40 and 55 days after pollination of the flowers of the first inflorescence, corresponding to the periods of beginning of flowering, maximum flowering, beginning of fruiting and maximum fruiting, respectively. Subsequently, the mean values of the whole period evaluated were used for the analysis of the data.

Leaf stomatal conductance and transpiration were determined on mature leaves close to the first inflorescence. Leaf chlorophyll content was measured using the chlorophyll meter (SPAD 502, Minolta Co. Ltd., Osaka, Japan). Leaves were selected from the middle third of the plants and measured 3 times to calculate the average SPAD index for each plant.

3.2.5. Determination of Ca^{2+}, Mg^{2+} and K^{+} in leaves and fruits

Fruit collection for determination of Ca^{2+} content was performed 30 days after pollination. The proximal and distal portions of whole fruits and leaves, oven dried at 65ºC until constant weight, were used. Samples of mature leaves were removed from the region close to the fruits of the first inflorescence. 500 mg of the dry material were taken, 6 mL of nitroperchloric acid (2:1) added, gypsum block digested at 240°C and

completed to 15 g with distilled water. The determination was performed by atomic absorption, according to Malavolta et al. (1997). The results were expressed in g of Ca^{2+}, Mg^{2+} and K^{+} per Kg of tissue dry matter.

For the determination of Ca^{2+} bound to the wall, the pericarp tissue of the distal portion of the fruits was used and extraction of the cell wall was performed according to a method developed by Campbell et al. (1990). The determination of Ca^{2+} was performed using the same methodology already described for Ca^{2+} determinations in leaves and fruits.

3.2.6. Experimental design

The experimental design was in randomized blocks with four blocks and two plants per block. The results were submitted to analysis of variance and, when significant, were submitted to the Tukey test at the 5% probability level. For the analysis of the percentage of blossom-end rot, the non-parametric Kruskal-wallis test was performed at 5% probability. The Pearson correlation test was performed for the variables at a 5% probability level.

In order to identify patterns of variation among treatments, principal component analysis (PCA) was performed. For the selection of the factors to be analyzed, the Kaiser criterion (Kaiser, 1960) was used for factors with their own value greater than 1. The analysis was performed through the FactoMineR bookstore (Husson, 2014) in the R Project Team, 2014).

3.3. Results

3.3.1. Percentage of blossom-end rot

According to the results, the incidence of BER was lower in the ‘Mara’ and ‘Nagcarlan’, medium in the ‘Amalia’ and ‘IPA-6’, and highest in the ‘M-82’ (Figure 1). The percentage of fruit fixation was higher in the ‘Nagcarlan’, while the ‘Amalia’ had the lowest amount of fruit at the end of the experiment (data not shown). The other varieties presented intermediate values of percentage of fruit fixation. Fruits of the ‘Amalia’ are larger and flater and the ‘Nagcarlan’ are smaller and rounder, compared with the other varieties.

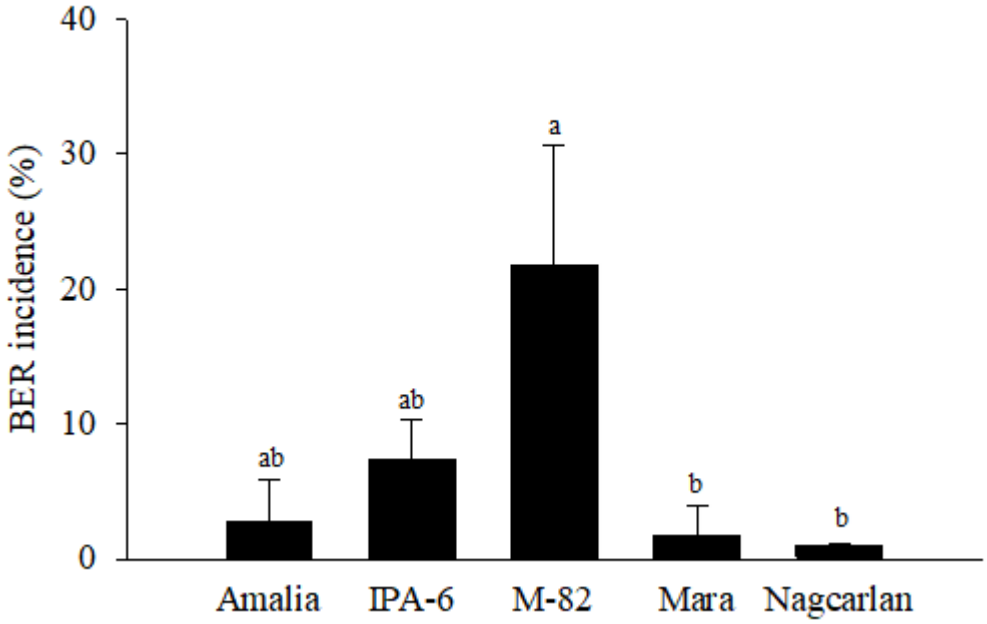

Figure 1. Blossom-end rot (BER) incidence in tomato varities with low (Mara and Nagcarlan), medium (Amalia and IPA-6), or high (M-82) susceptibility to BER. Fruit were harvested at 15 days after pollination. Averages followed by the same letter are not statistically different (Kruskal-wallis 5%). Data shown, mean ± standard deviation.

3.3.2. Correlation between physiological parameters and blossom-end rot

The correlation analyzes shows negative correlation between BER and water potential, leaf area, plant dry mass, proximal/distal Ca^{2+}, total Ca^{2+} in the proximal fruit tissue, and K^{+} in the distal and proximal fruit tissues (Table 1). The physiological parameters of abaxial and adaxial trichomes, leaf stomatal conductance, Ca^{2+} bound to the cell wall in distal fruit tissue, leaf transpiration and fruit length showed a positive correlation with BER incidence (Table 1).

Table 1. Correlation analysis between physiological parameters and BER incidence to determine parameters potentially inhibiting ($-R^2$) or triggering ($+R^2$) BER in tomato varieties with low (Mara and Nagcarlan), medium (Amalia and IPA-6), or high (M-82) susceptibility to BER.

Inhibiting BER	R^2	Triggering BER	R^2
Water potential	-0.93	Adaxial Trichome	+0.92
Leaf Area	-0.79	Abaxial Trichome	+0.90
Plant Dry Mass	-0.76	Stomatal Conductance	+0.87
Fruit Proximal/Distal Ca^{2+}	-0.70	Fruit Distal Cell Wall Ca^{2+}	+0.80
Fruit Proximal K^+	-0.63	Transpiration	+0.77
Fruit Distal K^+	-0.58	Fruit Length	+0.61
Fruit Proximal Ca^{2+}	-0.50	Leaf Mg^{2+}	+0.49
Abaxial Stomata	-0.49	Fruit Cell Wall Ca^{2+}/Distal Ca^{2+}	+0.54
Leaf K^+	-0.24	Fruit Set	+0.06
SPAD Index	-0.21	Leaf Ca^{2+}	+0.04
Fruit Distal Mg^{2+}	-0.13	Fruit Distal Ca^{2+}	+0.02
Adaxial Stomata	-0.09	Fruit Proximal Mg^{2+}	+0.01

SPAD index= chlorophyll content index

3.3.3. Parameters of plant growth

At the beginning of the fruiting, sampling was performed to determine the leaf area (Figure 2a). The largest leaf area, greater than 2500 cm², was observed in the ‘Nagcarlan’. The other varieties presented values lower than 2000 cm², and ‘M-82 presented the smallest leaf area with a value below 1500 cm² (Figure 2a). Similarly, the ‘Nagcarlan’ showed the highest dry mass per plant, with mean values of 33 g, while ‘M-82’ had the lowest plant dry weight of 16 g (Figure 2b).

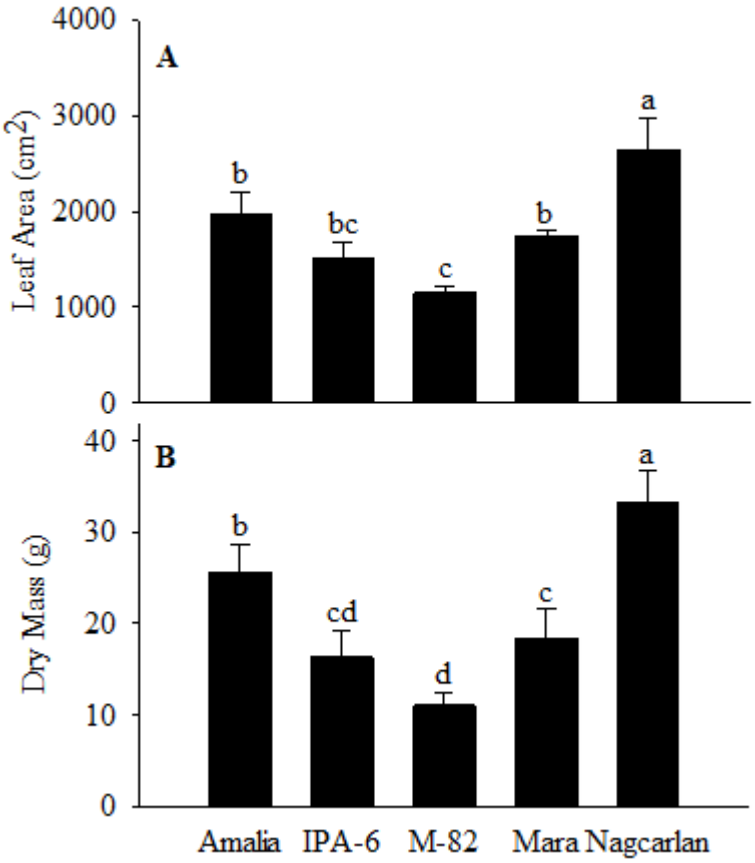

Figure 2. Leaf area per plant (A) and plant dry mass (B) at maximum growth (60 days after planting) of tomato varieties with low (Mara and Nagcarlan), medium (Amalia and IPA-6), or high (M-82) susceptibility to BER. The averages followed by same letter were not statistically different (Tukey 5%). Data shown, mean ± standard deviation.

3.3.4. Stomata and trichome density

'Nagcarlan' showed the highest stomatal density in the abaxial part of the leaves (Figure 3a). 'Amalia', 'M-82' and 'Nagcarlan' presented the lowest stomatal density in the adaxial portion (Figure 3a). 'M-82' showed the highest number of trichomes in the abaxial part, and 'Amalia', 'IPA-6' and 'Mara' had the lowest values (Figure 3b). In the adaxial portion, 'M-82' also showed the highest number of trichomes and the 'IPA-6', 'Mara' and 'Nagcarlan' the smallest (Figure 3b).

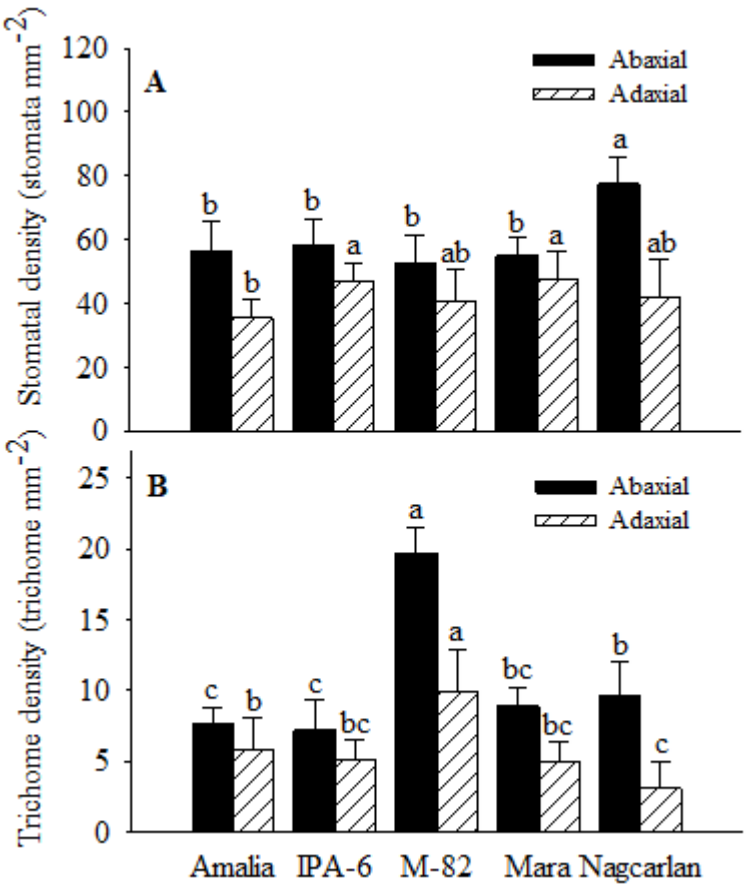

Figure 3. Leaf stomatal density (A) and tricome density (B) of tomato varieties with low (Mara and Nagcarlan), medium (Amalia and IPA-6), or high (M-82) susceptibility to BER. The averages followed by same letter were not statistically different (Tukey 5%). Data shown, mean ± standard deviation.

3.3.5. Concentration of Ca^{2+}, Mg^{2+} and K^{+} in plant and fruit

Leaf nutrient analysis revealed that the highest Ca^{2+} concentration was observed in the 'Nagcarlan', compared to 'Mara' (Figure 4a). For leaf Mg^{2+}, 'IPA-6' showed higher concentration than 'Mara'. In relation to leaf K^{+}, 'Amalia' presented higher concentration than 'M-82' and 'Nagcarlan' (Figure 4a). In fruits, the highest concentration of Ca^{2+} in the proximal portion was observed in 'Nagcarlan' and 'IPA-6', compared to 'Mara' and 'M-82' (Figure 4b). For the distal portion, the highest concentration of Ca^{2+} was observed in 'Amalia' and 'IPA-6' in relation to 'Mara' (Figure 4b). For Mg^{2+} in the proximal portion, the highest concentration was observed in 'Mara' in relation to 'Amalia' (Figure 4b).

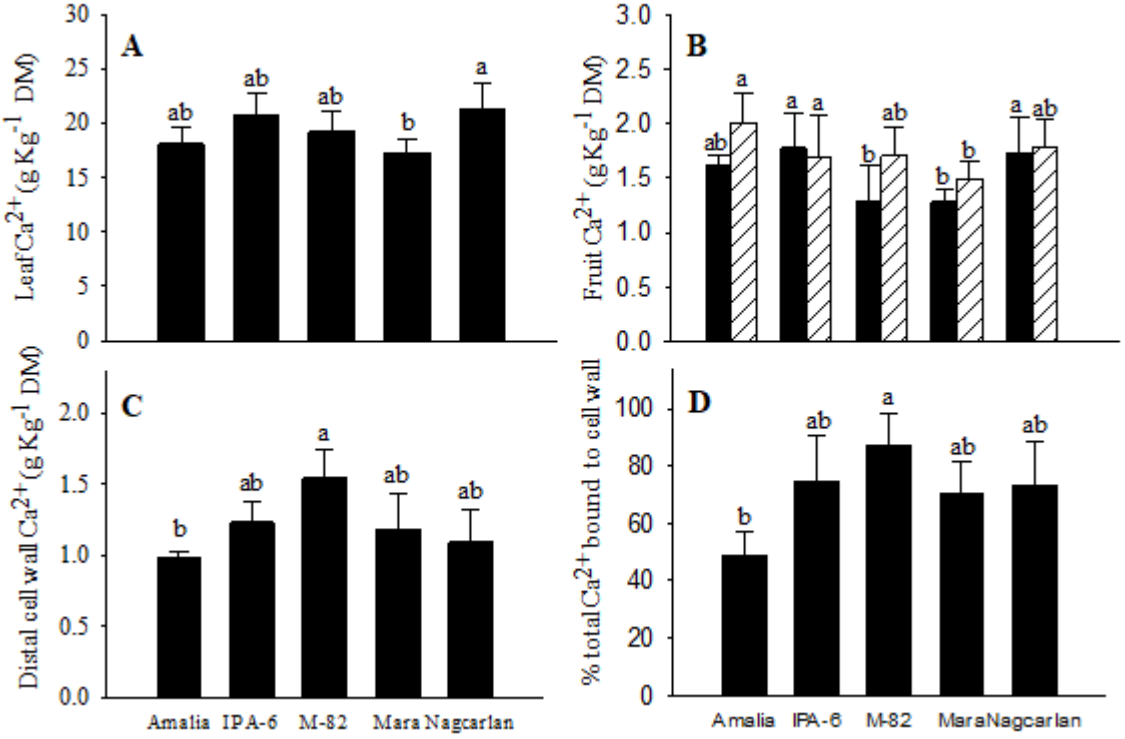

Figure 4. Leaf Ca^{2+} (A), fruit Ca^{2+} (black is proximal and striped bars is distal tissue) (B), cell wall-bound Ca^{2+} in fruit distal tissue (C), and percentage of total fruit Ca^{2+} bound to the cell wall (D) in tomato varieties with low (Mara and Nagcarlan), medium (Amalia and IPA-6), or high (M-82) susceptibility to BER. The averages followed by same letter were not statistically different (Tukey 5%). Data shown, mean ± standard deviation.

In the distal portion, however, 'IPA-6', 'Mara' and 'Nagcarlan' had higher Mg^{2+} concentrations than 'Amalia'. 'Mara' had higher K^{+} concentrations than 'IPA-6' and 'M-82' in the proximal portion and in relation to 'M-82' in the distal portion (Figure 4b). The concentration of Ca^{2+} bound to the cell wall was higher in 'M-82' and lower in 'Amalia' (Figure 4c). There was no significant difference between the other varieties (Figure 4c). The percentage of total Ca^{2+} bound to the cell wall was higher in 'M-82' and lower in 'Amalia' (Figure 4d).

Table 2. Leaf and fruit Mg^{2+} and K^{+} concentrations in tomato varieties with low (Mara and Nagcarlan), medium (Amalia and IPA-6), or high (M-82) susceptibility to BER. Leaf and fruit were harvested at 15 days after pollination. Fruit were analyzed at the proximal and distal tissues.

Varieties	Mg^{2+} (g kg^{-1} DM)			K^{+}(g kg^{-1} DM)		
	Leaf	Fruit tissue		Leaf	Fruit tissue	
		Proximal	Distal		Proximal	Distal
Amalia	4.7 ab*	1.5 b	1.5 b	38 a	39.3 abc	41.2 ab
IPA-6	5.9 a	1.8 ab	1.9 a	34.2 ab	34.3 c	38.4 ab
M-82	5.3 ab	1.7 ab	1.6 ab	28.1 bc	36.1 bc	38 b
Mara	4.1 b	2.1 a	1.7 a	33.3 abc	44.4 a	40 a
Nagcarlan	4.9 ab	1.5 b	1.7 a	24.6 c	41.4 ab	36.7 ab
CV%	13.62	14.55	8.06	12.38	6.94	6.03

*The averages followed by same letter were not statistically different (Tukey 5%).

3.3.6. Physiological parameters

Leaf stomatal conductance was higher in ‘M-82’ and ‘IPA-6’ (Figure 5a). ‘Amalia’ and ‘Mara’ presented the lowest values and ‘Nagcarlan’, presented intermediate values of leaf stomatal conductance. Leaf transpiration rates followed the same leaf tomatal conductance behavior (Figure 5b). ‘M-82’ and ‘IPA-6’ had the highest leaf transpiration rates of the period and only ‘Mara’ presented significantly lower mean values in the period (Figure 5b). Leaf water potential was higher (more positive) in ‘Amalia’, ‘Nagcarlan’ and ‘Mara’. ‘IPA-6’ had the intermediate value and ‘M-82’ had the lowest value. (Figure 5c). The chlorophyll index (SPAD) did not present significant differences among the studied varieties, and they had an average index of 45 (data not shown).

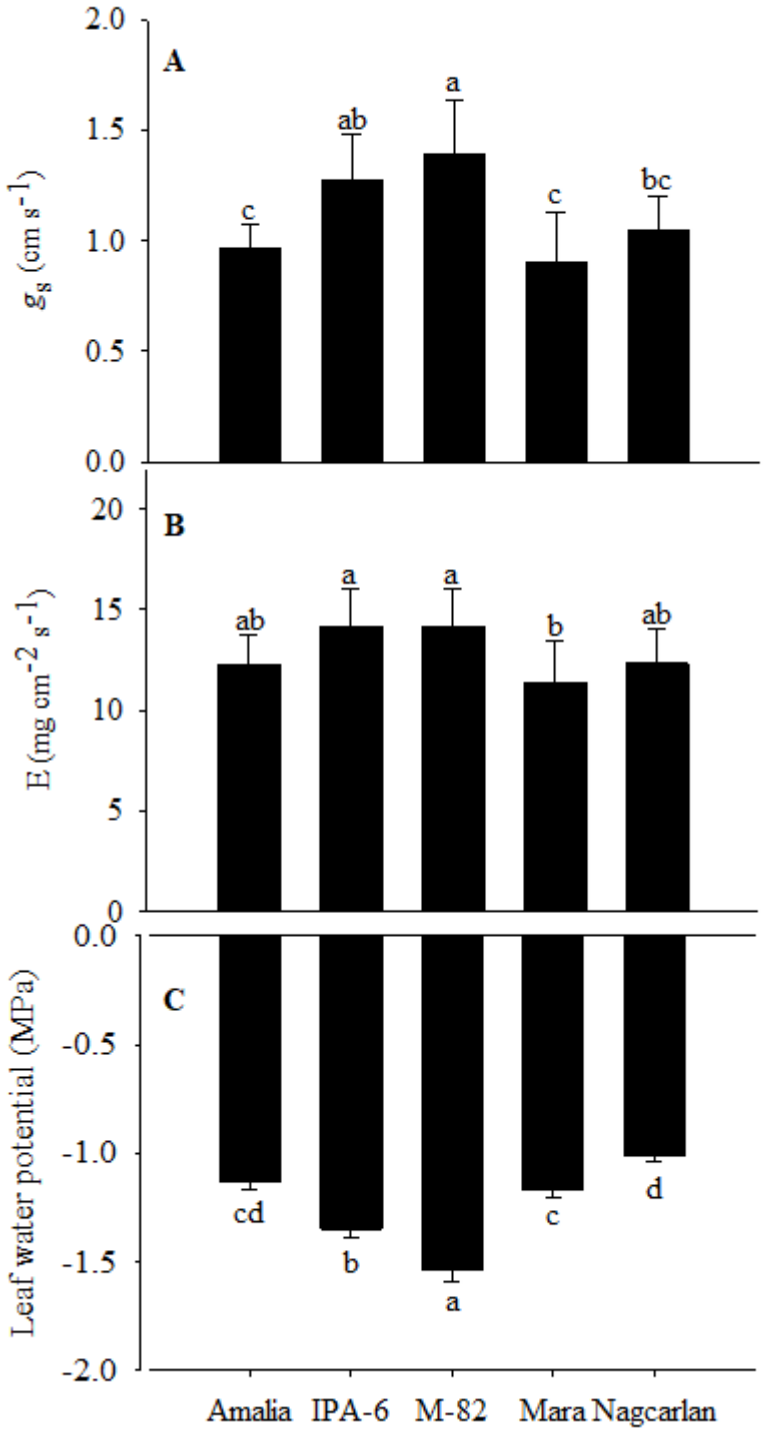

Figure 5. Leaf stomatal conductance (A), leaf transpiration rate (B), and leaf water potential (C) in tomato varieties with low (Mara and Nagcarlan), medium (Amalia and IPA-6), or high (M-82) susceptibility to BER. The averages followed by same letter were not statistically different (Tukey 5%). Data shown, mean ± standard deviation.

3.3.7. Principal Component Analysis (PCA)

Principal component analysis was used to reduce the dimensionality of the database and the relation of the components to BER, with a total of four dimensions explaining 100% of the database variability (Figure 6).

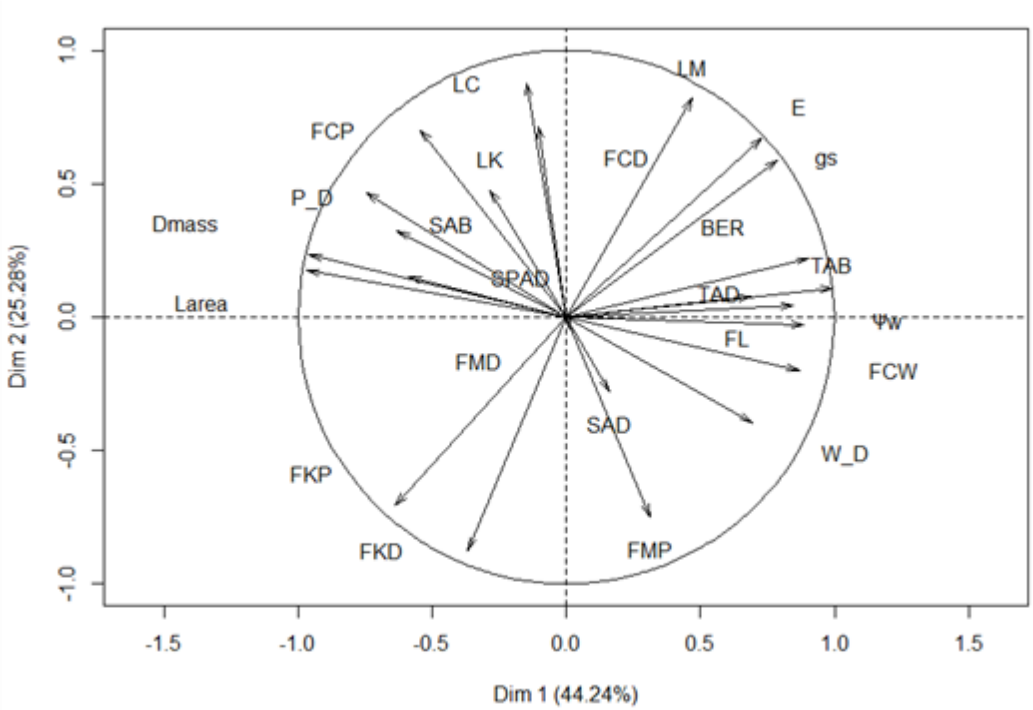

Figure 6. Relation among variables (PCA) shown by scatter plot of the first two principal components based on traits. The variables are BER - blossom-end rot, Dmass - dry mass, E - transpiration, FCD - fruit distal Ca^{2+}, FCP - fruit proximal Ca^{2+}, FCW - fruit distal cell wall Ca^{2+}, FL - fruit length, FMD - fruit distal Mg^{2+}, FMP- fruit proximal Mg^{2+}, FKD - fruit distal K^{+}, FKP - fruit proximal K^{+}, g_s - stomatal conductance, Larea - leaf area, LC - leaf Ca^{2+}, LK - leaf K^{+}, LM- leaf Mg^{2+}, Ψw - leaf water potential, P_D - fruit proximal Ca^{2+} and distal Ca^{2+} relation, SAB - density of abaxial stomata, SAD - density of adaxial stomata, SPAD - SPAD index, TAB - density of abaxial trichome, TAD - density of adaxial trichome, W_D - fruit distal cell wall-bound Ca^{2+} and distal Ca^{2+} relation.

The first component explained 44.24% of the variability of the database and the main variables that contributed to this component were leaf water potential, SPAD chlorophyll index, abaxial stomata, adaxial trichomes, leaf area, dry mass, leaf

stomatal conductance, leaf transpiration, Ca^{2+} concentration in the cell wall in the distal portion of the fruit, K^{+} in the proximal portion of the fruits, BER, fruit length, percentage of total Ca^{2+} bound to the cell wall in the distal portion of the fruit and the relation between proximal and distal Ca^{2+} in fruits These components are those that have the greatest relation to BER.

The second component explained 25.28% of the variability of the database and the main variables were Ca^{2+} in the proximal and distal portion of the fruits, Mg^{2+} and K^{+} in the proximal portion of the fruits, K^{+} in the distal portion of fruits, Ca^{2+} and Mg^{2+} in the leaves. The third component explained 17.74% of the variability of the database and the variables were stomata in the adaxial portion, Mg^{2+} in the distal portion of the fruits and. The fourth component explained 12.73% of the variability of the database and its main variables were abaxial trichomes and leaf K^{+}.

3.4. Discussion

3.4.1. Susceptibility of varieties to blossom-end rot

The BER manifested differently among the evaluated varieties. Considering the reduced percentage of BER, in addition to the high growth rates of ‘Nagcarlan’ in the favorable conditions to the appearance of the disorder, it is possible to classify this variety as being tolerant to BER. ‘M-82’, on the other hand, presented high levels of BER incidence, in addition to reduced growth rates, being able to be classified as highly susceptible to the disorder. The other varieties seem to have an intermediate degree of tolerance to BER. Based on these results, ‘Mara’ and ‘Nagcarlan’, ‘Amalia and IPA-6’, and ‘M-82’ were classified as low, medium and high susceptibility to BER, respectively.

3.4.2. Physiological parameters that inhibit blossom-end rot

The physiological parameters negatively correlated with the incidence of BER were considered inhibitors of the disorder in the studied varieties.

High water potential inhibited BER. A drastic reduction of the leaf water potential leads to a reduction in the transport of xylem sap to the upper plant parts, reducing the Ca^{2+} transport to fruit and consequently increasing the incidence of

BER. In less severe situations in which the transport of xylem sap to the upper plant parts does not present great reductions, the reduction of the leaf stomatal conductance can favor the transport of Ca^{2+} to the fruits.

The occurrence of high diurnal temperatures increases the leaf transpiration rates that can induce water deficiency in the plants, which affects several physiological processes under low soil water content (Tsukaguchi et al., 2003) and in tomato, affects leaf water relations and root hydraulic conductivity (Morales et al., 2003). Such susceptibility is associated to a greater competition between leaves and fruits for Ca^{2+} present in the xylem sap translocated to the upper parts of the plant (Figures 4 and 5). In the occurrence of high transpiration rates due to the high temperatures, an increase of Ca^{2+} transport to the leaves occurs, increasing the incidence of BER. This fact is still favored due to the low transpiration rates presented by the fruits (De Freitas et al., 2011b; Ho and White, 2005).

‘Nagcarlan’ and ‘Mara’, which present low susceptibility to BER and resistance to high temperatures, presented more positive values of leaf water potential. ‘M-82’, on the other hand, presented the most negative value of leaf water potential (Figure 5), which coincided with its high susceptibility to the disorder and sensitivity to high temperatures (Paupière et al. 2017) The degree of tolerance to high temperatures seems to act to improve the water relations of the plant, which directly influences the transport of xylem sap to the upper parts of the plant and consequently the allocation of Ca^{2+} to the fruits.

Dry mass also inhibited BER. The dry mass analysis shows that ‘Nagcarlan’ presented higher and ‘M-82’ lower dry mass values. In general, the other varieties presented intermediate behavior regarding the growth and accumulation of biomass. Similar behavior was observed in relation to the leaf area. This is an important factor regulating BER, as larger leaf areas provide a larger transpiratory surface, thus favoring Ca^{2+} accumulation into the leaves (Ho et al., 1995). However, this is not always the case, since plants such as ‘Nagcarlan’ are more adaptable at elevated temperatures (Paupière et al. 2017), showing lower transpiration rates and higher water potential under the conditions of the experiments, resulting in lower BER incidence.

The ratio between proximal and distal Ca^{2+} is also related to inhibition of BER. Observing the concentrations of Ca^{2+} in both the distal and proximal portions alone, the values were very similar among the varieties, being inferior only in ‘Mara’

tomato fruit. Thus, Ca^{2+} concentrations in these varieties do not fully explain the appearance of BER. However, when the ratio between proximal and distal Ca^{2+} is observed, the values closest to 1 are those observed for the 'Nagcarlan' and 'IPA-6' tomatoes, indicating an efficient transport of the nutrient along the fruit. 'M-82' tomato has the lowest Ca^{2+} partition ratio between the two fruit portions, showing a less efficient transport along the fruit, which coincides with the highest BER incidence.

The Mg^{2+} and K^{+} contents are involved in the competition for sites of uptake and translocation with Ca^{2+} (Taylor and Locascio, 2004). In 'Mara' tomato, the lowest Ca^{2+} contents were observed in the two fruit sections, however, high values of Mg^{2+} and K^{+} were also observed in this variety. However, the incidence of BER in this variety was low, suggesting that Ca^{2+} content in the proximal and distal tissues can not fully explain BER incidence.

The distribution of stomata on the abaxial and adaxial leaf surfaces was also negatively correlated with BER. 'Nagcarlan' tomatoes showed a greater number of stomata on the leaf abaxial surface, which possibly contributed to lower transpiration rates, improving the water relations of the plant under high temperatures and favoring a greater fruit Ca^{2+} uptake.

The effect of the improvement in water relations in reducing the incidence of BER may be related to the reduction of the generation of reactive oxygen species (ROS), which as been suggested to increase fruit susceptibility to BER (Schmitz-Eiberger and Noga, 2003; Saure, 2014). In this way, the varieties that are more tolerant to stress conditions should present lower susceptibility to BER.

3.4.3. Physiological parameters that stimulate blossom-end rot

The physiological parameters positively correlated with BER were considered to stimulate the disorder incidence in the fruit.

Leaf stomatal conductance was being positively correlated with BER. 'M-82' and 'IPA-6' presented higher values of leaf stomatal conductance and higher BER incidence. The same was observed in relation to transpiration, which correlated positively with BER. It is known that stomata regulate leaf transpiration and consequently whole-plant water loss (Farber et al., 2016). Accordingly, higher leaf transpiration rates have been shown to increase leaf Ca^{2+} content and decrease Ca^{2+}

flow into the fruit, which has been linked to higher BER incidence (De Freitas et al. 2014).

Although ‘Nagcarlan’ presented the lowest BER incidence, it was one of the varieties that showed lower leaf stomatal conductance, contrary to what was expected, and high levels of leaf Ca^{2+}. Possibly, each variety presents a different pattern of response between factors triggering and inhibiting BER incidence. These results suggest that Ca^{2+} content in leaves and fruits are not the only factors regulating fruit susceptibility to BER.

The concentration of Ca^{2+} bound to the cell wall in the distal portion of the fruits also correlated positively with BER. Although significant differences were not observed for the ‘Nagcarlan’ and ‘IPA-6’ tomatoes, it was possible to observe that the variety M-82, which has a high percentage of BER, presented high values in relation to the varieties Mara and Amalia. The binding of Ca^{2+} to the cell wall limits its availability to other cellular activities (Conn et al., 2011; De Freitas et al., 2011a, b). It is, therefore, expected that varieties with higher Ca^{2+} bound to the cell wall may have higher susceptibility to BER.

The density of trichomes, both in the adaxial and abaxial leaf portions, was also positively correlated with BER incidence. It is interesting to note that ‘M-82’ tomato plants, which had the highest leaf trichome density, also had the highest percentage of BER incidence. It is possible that trichome density in the ‘M-82’ variety was a response to elevated temperatures as a form of protection against excess water loss via stomata, as observed by Gianfagna et al. (1992).

Fruit length was also positively related to BER. The ‘Nagcarlan’ and ‘Mara’ variety had smaller fruits (3.5 and 5.0 cm, respectively) and lower percentage of BER incidence. Varieties with more elongated fruits, ‘M-82’ (7.0 cm) and ‘IPA-6’ (7.5 cm), presented a higher BER incidence, with higher incidence and severity of this disorder observed in ‘M-82’. The relationship between BER and fruit shape is not fuly understood, but it has been suggested that BER manifests more in elongated fruit (Ho and White, 2005). A possible explanation for this behavior is the fact that the xylem connection in these fruits proves inefficient in bringing Ca^{2+} from the proximal to the distal fruit regions, restricting Ca^{2+} accumulation in the distal tissues where BER symptoms develop (De Freitas et al., 2016).

3.4.4. Summary of the parameters regulating blossom-end rot development in different tomato varieties

Many studies emphasize the importance of total Ca^{2+} concentration in the distal portion of fruits as the main factor involved in the incidence of BER in tomato. However, it is speculated that this physiological disorder is controlled by several factors, not only the nutrient content in the fruits, but also the factors that affect its distribution between leaves and fruits, such as leaf stomatal conductance and leaf transpiration (Figure 7). In fact, the results of the present study described the role of several factors acting both in inhibition and triggering BER in contrasting varieties as well as tolerance to high temperatures, as observed by other authors (Saure, 2014).

The Ca^{2+} transport in the xylem to the aerial part is a central factor for the partition of the nutrient between leaves and fruits. The main factors affecting the Ca^{2+} partition are leaf transpiration and leaf stomatal conductance (Figure 7). High values for such factors are accompanied by an increase in the transport of Ca^{2+} to the leaves and consequently to a reduction of the availability of the nutrient to the fruits and should therefore act as triggers of BER (Abdal and Suleiman, 2005; Guichard et al., 2005; Paiva et al., 1998).

The water relations of the plant are modulated by the leaf stomatal conductance and transpiration and, therefore, the transport of water to the aerial part, which also affects the incidence of BER (Figure 7). The influence of the water potential on the incidence of BER is also dependent on the intensity of the water deficit, and in situations of a great fall of this parameter, there is a drastic reduction of the transport of sap to the aerial part and consequently reduction of Ca^{2+} transport to the fruits. It is important to note that the morphological characteristics of the leaves that affect the transpiration rates, such as leaf area, stomatal density and trichomes, also affect Ca^{2+} transport to the leaves, indirectly affecting the incidence of BER in fruits, as mentioned previously.

The partition of Ca^{2+} in fruits also affects the incidence of BER. The balance between proximal and distal Ca^{2+} plus the Ca^{2+} content bound to the cell wall may differentially affect the incidence of the disorder (Figure 7). In general, high values of distal Ca^{2+}, accompanied by low concentrations of Ca^{2+} bound to the wall and in the distal portion, may inhibit the development of BER.

Finally, based on the morphological characteristics of the fruits of the analyzed varieties, it is possible to infer that genetic components that affect the physiological and morphological characteristics of fruits can also influence the Ca^{2+} partition in them and consequently the incidence of BER (Figure 7). It is possible that larger and more elongated fruits, such as M-82 and IPA-6, or those with a deficient conducting vessel system may present an inefficient distribution of Ca^{2+} between the different parts of the fruit, presenting a higher incidence of BER.

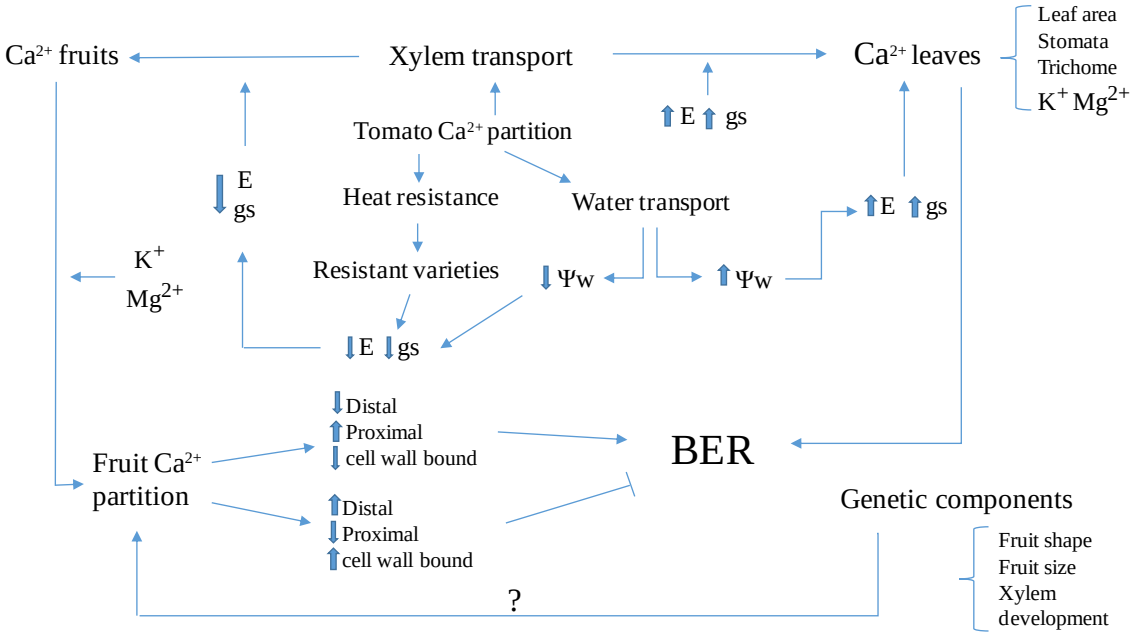

Figure 7. Summary of the parameters involved in BER incidence in different tomato varieties. BER - blossom-end rot, E – leaf transpiration, g_s – leaf stomatal conductance, Ψ_W – leaf water potential.

3.5. Conclusion

The varieties evaluated in this experiment showed differences in relation to the factors possibly regulating BER development in the fruit. Varieties with smaller and/or flattened fruits such as ‘Nagcarlan’, ‘Amalia’ and ‘Mara’ presented lower incidence of BER. In other way, elongated variety ‘M-82’, presented higher incidence of BER.

The physiological conditions of the plants, such as low transpiration and low leaf water potential could help susceptible varieties to maintain higher levels of Ca^{2+} in the fruits, like M-82. Future studies should focus in finding new elongated varieties more resistant to BER in this u favorable conditions and to understand the physiological mechanisms by which they present greater tolerance to BER development.

Acknowledgements

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4. FRUIT MORPHOLOGY CONTROLLING BLOSSOM-END ROT INCIDENCE IN ELONGATED TOMATO FRUITS

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Abstract

Blossom-end rot (BER) is a complex physiological disorder that might reach 100% of the fruit depending on the variety and environmental conditions during cultivation. Tomato varieties with elongated fruit, in general, have a greater susceptibility to BER than other varieties. Although possible mechanisms regulating BER have been addressed in many tomato varieties, those regulating BER in elongated fruits are still poorly understood. In this study, four varieties of long-shape fruits – 'San Marzano', 'Banana Legs', 'Roma', and 'Mini-Roma' – were used to evaluate and identify the possible physiological and morphological characteristics related to the onset of BER development in the fruit. According to the results, varieties that have more elongated fruits, such as 'San Marzano' and 'Banana Legs', had higher BER incidence. The varieties with higher susceptibility to BER had lower Ca^{2+} concentration in the distal fruit tissue. Elongated varieties more tolerant to BER, such as 'Roma' and 'Mini-Roma', had higher fruit proximal/distal Ca^{2+} content ratio, as well as lower cell wall bound/total tissue Ca^{2+} content ratio in the distal fruit end. Xylem functionality in distal fruit tissue was also higher in more tolerant varieties. 'San Marzano', which has the most elongated fruit, presented higher electrolyte leakage in distal fruit tissue. These results support the idea that total fruit Ca^{2+} content is not the only factor determining fruit susceptibility to BER, but rather a balance between physiological and morphological factors that influence Ca^{2+} transport and allocation in the fruit.

Keywords: Elongated fruits; Ca^{2+} disorder; Blossom-end rot

4.1. Introduction

Although blossom-end rot (BER) is a physiological disorder believed to be triggered by low fruit Ca^{2+} content, many other factors can also regulate fruit susceptibility to this disorder (De Freitas et al., 2014). BER may be associated with an unfavorable environmental condition (Guichard et al., 2005) along with a manifestation of genetic components (Ho, White, 2005). However, the mechanisms that regulate the appearance of BER are much more complex and vary among varieties.

In vegetables, imbalances related to Ca^{2+} are associated with burning of leaf edges and BER in fruit distal end of fruit, which occur in the most active growing tissues (White, Broadley, 2003), where the lack of this structural nutrient may cause tissue damage and cell death that leads to tissue browning (Suzuki et al., 2003)

According to Adams (1994), BER is associated not only with nutrient uptake, but also with factors that affect Ca^{2+} distribution in the plant, such as low mobility of this nutrient and high fruit growth rate (Nonami et al., 1995). Calcium translocation in plant is believed to take place exclusively through the xylem vessels in response to water potential gradient triggered by transpiration and growth rates (Ho et al., 1993; De Freitas et al., 2014). Thus, factors that alter xylem flow should also affect Ca^{2+} transport to the upper part of the plant (Ho, White, 2005). Some morphological factors alter transpiration rates, such as leaf area, presence of epidermal adaptations, including trichomes and cuticular waxes, venation pattern, and degree of maturation of the xylem vessels, besides the amount and arrangement of stomata in the leaves (Gutschick, 1999; Kim et al., 2014; Roth-Nebelsick et al., 2001).

Some physiological alterations in biotic and abiotic stresses also affect Ca^{2+} transport by reducing transpiration rates. Such alterations include reduction in water potential and increase in vapor pressure deficit, both contributing directly to stomatal closure, reduction of leaf stomatal conductance, and hormonal responses (De Freitas et al., 2011b). Therefore, this set of morphological and physiological factors should influence susceptibility to BER.

After reaching the fruit through xylem vessels, Ca^{2+} may remain in the proximal tissue or be translocated to the distal tissue. At the cellular level, Ca^{2+} may bind to the cell wall and membranes, representing an insoluble fraction, or remain free in the apoplast, cytosol, and storage organelles, representing a soluble fraction in the tissue (De Freitas et al., 2011a). Studies have shown that Ca^{2+} distribution at the cellular level is more important than total tissue Ca^{2+} content in determining fruit susceptibility to BER (De Freitas et al., 2011b).

Although studies on the mechanisms involved in the development of BER have focused on factors such as Ca^{2+} transport to aerial parts according to transpiration rates and total Ca^{2+} concentration in fruits (Taylor, Locascio, 2007), little is known about the factors that influence its distribution within the fruit. Increased concentration of cell wall-bound Ca^{2+} is known to reduce the pool of soluble apoplastic Ca^{2+} and increase susceptibility to BER (De Freitas et al., 2014). It is

possible that the size and shape of fruits as well as the pattern of development of xylem vessels influence the distribution of the nutrient among different parts of the fruit (Ho, White, 2005; Hocking et al., 2016). It is important, therefore, to know the different pools of Ca^{2+} in fruits and how they relate to morphology.

According to some studies, the San Marzano variety is one of the most affected by BER, presenting incidence rates greater than 50% (Elmer, Ferrandino, 1991). However, elongated tomato varieties are highly cultivated. Although tomato varieties with elongated fruit are more susceptible to BER (Ho, White, 2005), mechanisms regulating BER in fruit are still poorly understood. It is possible that elongated fruit have a lower number of functional xylems due to lower vessel development and/or higher loss of xylem functionality during growth (Drazeta et al., 2004; Saure, 2005; De Freitas et al., 2011). Taking into account all the factors involved in the development of BER, it is clear that the study of this disorder should not be restricted to approaches resulting from Ca^{2+} deficiency, but other variables and environmental conditions should also be considered (Fontes, 2003; Saure, 2001).

No information is available about physiological and morphological characteristics related to the susceptibility of elongated tomato fruit to BER. Knowledge on these characteristics is essential to develop differentiated management practices to inhibit BER development in fruit. Alternatively, breeding programs may use this information to select new varieties less susceptible to BER.

The objective of this study was to characterize varieties of tomato with elongated fruits to identify possible physiological and morphological characteristics related to fruit susceptibility to BER.

4.2. Material and Methods

4.2.1. Plant material, growth conditions and application of treatments

This study was carried out with ‘San Marzano’, ‘Banana Legs’, ‘Roma’, and ‘Mini-Roma’ tomatoes. Seeds were obtained from the tomato germplasm bank of the Department of Genetics at “Luiz de Queiroz” College of Agriculture.

The experiment was performed in a greenhouse with average solar radiation of 1000 μmol m^{-2} s^{-1}, temperature of 17.5ºC, and relative humidity of 74%. Seeds of

different varieties were seeded separately in trays with a 1:1 (v/v) substrate mixture of commercial organic substrate (Plantmax HT, Eucatex Brazil) and expanded vermiculite, supplemented with 1g L^{-1} NPK (10:10:10) fertilizer and 4g L^{-1} dolomitic limestone.

Thirty days after planting, seedlings were transplanted into individual 30-L pots containing commercial organic substrate. Previous fertilization and cover fertilization were carried out according to recommendations for the crop (Benton Jones, 1998). The plants were fertilized every 20 days, during de growing and fructification time, with 10 g of slow realease fertilizer containing N (16%), P_2O_5 (8%), K_2O (12%), MgO (2%), S (5%), Fe (0.4%), Cu (0.05%), Mn (0.06%), Zn (0.02%), B (0.02%), Mo (0.015%), but without Ca (Basacote Plus; Compo Expert; Soil fertilizer, Agricultural). Fruit samples were harvested in the first cluster at 15 days after pollination (DAP).

4.2.2. BER incidence and growth parameters

BER incidence was calculated by multiplying the number of fruit with BER symptoms by 100 and dividing it by the total number of fruit in the first cluster. The plant dry weight was determined by drying the samples (65°C) to constant weight. Leaf area was determined through 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, as well as pericarp thickness were determined using a caliper.

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 ºC under ≤20% relative humidity. After 24 h, fruit were cut into three equal sections at a 90º 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.

4.2.3. Density of leaf stomata and trichomes

Samples from the first pair of fully expanded leaves were collected – on the same day that stomata conductance and transpiration were analyzed – for the analysis of epidermal impression with the use of instant adhesive (Gülcan, Misirli, 1990). Sampling was performed in the median portion of the leaves. The numbers of stomata and trichomes were determined under an optical microscope, and the counting was performed from scanned images of each sampled leaf area. The replicates (1 leaf per replicate [plant] for each treatment) were averaged, and the stomata and trichome densities were expressed as numbers of stomata and trichome per mm^2.

4.2.4. Determination of leaf stomatal conductance, leaf transpiration rate and leaf water potential

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

4.2.5. Total tissue Ca^{2+}, Mg^{2+}, and K^+ contents in leaf and fruit and cell wall-bound Ca^{2+}

Nutrient analysis was accomplished in proximal and distal fruit tissues, as well as in fully expanded leaf close to the first fruit cluster. Samples were oven dried at 65ºC until constant weight. About 500 mg of dry material were 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.

4.2.6. Apoplastic and cytoplasmic electrolytic leakage and soluble Ca^{2+} content in fruit tissue

Fruit electrolyte leakage was performed according to the protocol described by De Freitas et al. (2011a). Three fruit pericarp discs (1-cm 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). Conductivity in the mannitol solution was recorded for 6 hours at 1-hour intervals. Subsequently, the samples were frozen and thawed three times to determine the total conductivity (Saltveit, 2002).

Apoplastic electrolyte leakage was determined using the first three hours of conductivity, in which only the apoplastic ions was lost to the solution. Cytoplasmic electrolyte leakage was determined in the last three hours, representing the ions lost through the membrane. 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 hours, 1-mL sample solution 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 fresh weight.

4.2.7. Experimental design

The experiment followed a randomized blocks design with six blocks and two plants per block. The results were submitted to analysis of variance. Averages were compared by Tukey test at 5%. The variables without normal distribution were analyzed by Friedman's non-parametric test at 5%. Pearson correlation test was performed at 5%. Data were also subjected to principal component analysis (PCA). To select the factors to be analyzed, the Kaiser criterion (Kaiser, 1960) was applied

for factors with values greater than 1. The analysis was performed through the FactoMineR bookstore (Husson, 2014) in the R Project Team, 2014.

4.3. Results

BER incidence was markedly different among the varieties. BER levels in ‘San Marzano’ and ‘Banana Legs’ were higher than in ‘Roma’ and ‘Mini-Roma’ tomatoes (Figure 1).

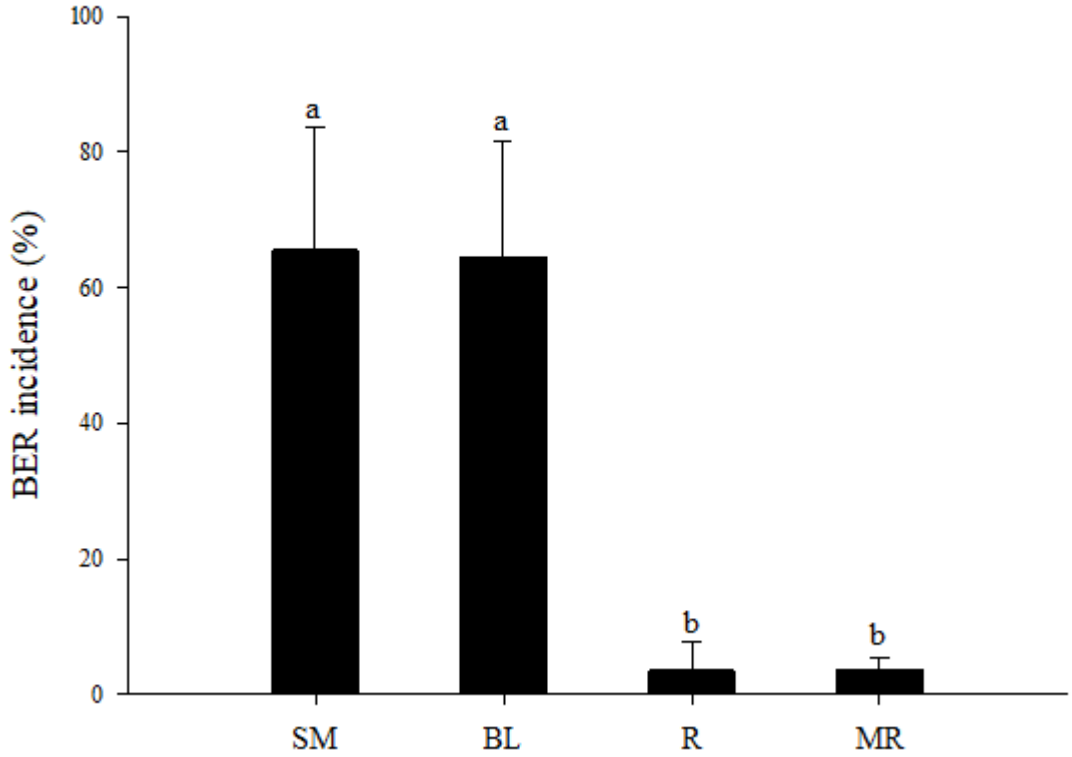

Figure 1. Blossom-end rot (BER) incidence in tomato varieties with low (‘Roma’ and ‘Mini-Roma’) or high (‘San Marzano’ and ‘Banana Legs’) susceptibility to BER. Fruit were harvested at 15 days after pollination. Averages followed by the same letter are not statistically different according to Friedman’s test (5%). Data shown mean ± standard deviation.

A correlation analysis between variables evaluated in the study revealed that some of the physiological and morphological variables correlated positively with BER

(Table 1). The variables that had the highest positive correlations with BER were K^+ in the proximal portion of the fruit, fruit length, Mg^{2+} content in proximal fruit tissue, K^+ and Mg^{2+} content in distal fruit tissue, and fruit diameter. The variables that had the highest negative correlations with BER were Ca^{2+} content in distal fruit tissue, soluble Ca^{2+} in distal fruit tissue, water potential, adaxial leaf trichome density, foliar K^+ and Mg^{2+} contents, and abaxial leaf stomata density.

Table 1. Correlation analysis between physiological/morphological parameters and BER incidence to determine parameters potentially inhibiting ($-R^2$) or triggering ($+R^2$) BER in tomato varieties with low ('Roma' and 'Mini-Roma'), or high ('San Marzano' and 'Banana Legs') susceptibility to BER.

Inhibiting BER	R^2	Triggering BER	R^2
Fruit Distal Ca^{2+}	-0.94	Fruit Proximal K^+	+0.99
Soluble Ca^{2+}	-0.92	Fruit Length	+0.95
Adaxial Trichome	-0.91	Fruit Proximal Mg^{2+}	+0.85
Abaxial Stomata	-0.81	Fruit Distal K^+	+0.82
Water potential	-0.75	Fruit Distal Mg^{2+}	+0.82
Leaf K^+	-0.75	Fruit diameter	+0.81
Leaf Mg^{2+}	-0.73	Leaf Area	+0.78
Cytoplasm proximal leakage	-0.71	Adaxial Stomata	+0.77
Distal Xylem functionality	-0.70	Pericarp thickness	+0.76
Fruit Proximal Ca^{2+}	-0.69	Cytoplasm distal leakage	+0.67
Leaf Ca^{2+}	-0.62	Apoplast distal leakage	+0.60
Abaxial Trichome	-0.56	Soluble K^+	+0.50
Soluble Mg^{2+}	-0.53	Transpiration	+0.32
Fruit Distal Cell Wall Ca^{2+}	-0.49	Apoplast proximal leakage	+0.25
Plant Dry weight	-0.36	Stomatal Conductance	+0.01
Proximal Xylem functionality	-0.21		

'San Marzano' had the largest leaf area, compared with the other varieties (Table 2). Dry weight was higher in 'San Marzano' and 'Roma', compared with 'Banana Legs' (Table 2).

Table 2. Leaf area per plant, plant dry weight at maximum growth (60 days after planting), fruit length, fruit diameter and pericarp thickness of tomato varieties with low ('Roma' and 'Mini-Roma') or high ('San Marzano' and 'Banana Legs') susceptibility to BER.

Varieties	Leaf area (m^2)	Dry weight (g)	Fruit Length (cm)	Fruit Diameter (cm)	Pericarp thickness (cm)
San Marzano	1.20±0.09 a*	165.1±3.6 a	7.52±0.12 a	5.13±0.12 a	0.99±0.10 a
Banana Legs	0.83±0.12 b	113.9±58.7 b	6.97±0.14 b	4.22±0.19 b	0.65±0.05 ab
Roma	0.74±0.09 bc	163.0± 24.1a	4.83±0.23 c	3.86±0.30 b	0.57±0.08 b
Mini-Roma	0.62±0.10 c	146.4±19.9 ab	3.57±0.08 d	3.01±0.30 c	0.52±0.04 b
CV %	9.14	14.15	2.58	5.99	10.49

* Averages followed by same letter are not statistically different according to Tukey's test (5%). Data shown mean ± standard deviation.

Fruit length was greater in 'San Marzano', followed by 'Banana Legs'. The lowest fruit length values were found in 'Mini-Roma', followed by 'Roma'. Fruit diameter was higher in 'San Marzano', compared with the other varieties (Table 2). The lowest fruit diameter was observed in 'Mini-Roma'. Pericarp thickness was higher in 'San Marzano' and 'Banana Legs', compared with the other varieties (Table 2).

Xylem functionality in the proximal fruit tissue was higher in 'Mini-Roma' compared with 'Roma' (Figure 2). In distal fruit tissue, 'Mini-Roma' also showed higher xylem functionality than other varieties, whereas 'San Marzano' and 'Banana Legs' showed lower numbers of functional xylem vessels in the tissue (Figure 2).

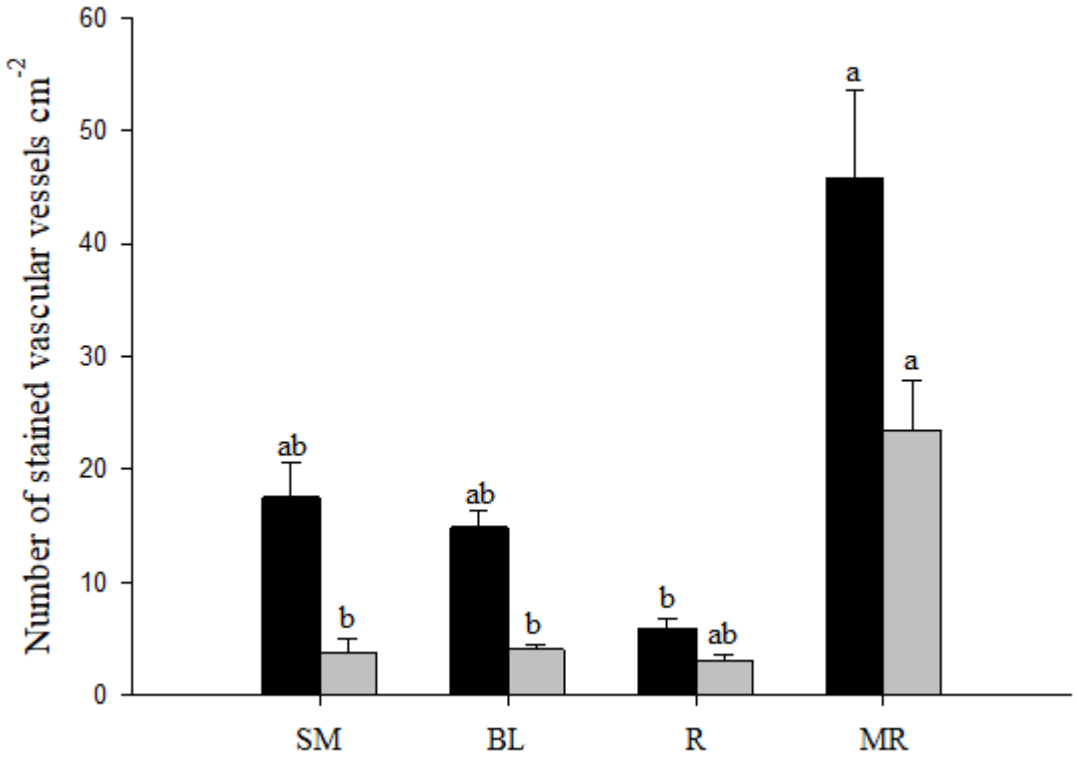

Figure 2. Xylem functionality in tomato varieties with low ('Roma' and 'Mini-Roma') or high ('San Marzano' and 'Banana Legs') susceptibility to BER. Fruit were harvested at 15 days after pollination and xylem functionality was determined in the proximal (black) and distal (grey) fruit regions. Averages followed by the same letter are not statistically different according to Friedman's test (5%). Data shown mean ± standard deviation.

Leaf water potential was less negative in 'Mini-Roma', 'Banana Legs' and 'Roma' (Table 3). The highest transpiration rates were observed in 'San Marzano', compared with 'Roma' (Table 3).

Table 3. Leaf stomatal conductance (g_s), leaf water potential (Ψw) and leaf transpiration (E) of tomato varieties with low ('Roma' and 'Mini-Roma') or high ('San Marzano' and 'Banana Legs') susceptibility to BER.

Varieties	g_s (mol H_20 m^{-2} s^{-1})	Ψw (MPa)	E (mmol H2O m^{-2} s^{-1})
San Marzano	0.13±0.01 a*	-0.93±0.06 b	1.65±0.07 a
Banana Legs	0.14±0.02 a	-0.81±0.10 a	1.45±0.26 ab
Roma	0.13±0.02 a	-0.74±0.88 a	1.44±0.12 b
Mini-Roma	0.14±0.02 a	-0.80±0.07 a	1.56±0.23 ab
CV %	11.35	9.26	8.19

* Averages followed by same letter are not statistically different according to Tukey's test (5%). Data shown mean ± standard deviation.

Leaf stomatal density in the abaxial side was higher in 'San Marzano' and 'Banana Legs' than in 'Mini-Roma' (Table 4). In the adaxial leaf side, stomatal density was higher in 'Banana Legs', 'Roma', and 'Mini-Roma'. There was no significant difference in leaf trichome density in either sides among varieties (Table 4).

Table 4. Leaf stomatal density and trichomes density of tomato varieties with low ('Roma' and 'Mini-Roma') or high ('San Marzano' and 'Banana Legs') susceptibility to BER.

Varieties	[a]SAB	[a]SAD	[b]TAB	[b]TAD
San Marzano	104.0±15.9 a*	10.1±3.7 b	7.95±2.44 a	2.59±1.0 a
Banana Legs	103.9±17.1 a	22.6±5.7 a	0.00±0.00 a	1.80±1.1 a
Roma	90.8±25.7 ab	29.0±3.4 a	8.41±4.61 a	3.66±0.98 a
Mini-Roma	66.9±13.2 b	25.3±4.5 a	7.51±4.26 a	3.40±1.33 a
CV %	14.60	21.32	31.44	36.40

* Averages followed by same letter are not statistically different according to Tukey's test (5%). Data shown mean ± standard deviation. [a]SAB and SAD – Stomatal density in the abaxial and adaxial face, respectively; [b]TAB and TAD – Trichomes density in the abaxial and adaxial face, respectively.

Ca^{2+} partitioning between leaves and fruits varied among varieties (Table 5). In leaves, Ca^{2+} concentration was higher in 'Mini-Roma', compared with 'Banana Legs'. In fruit, the highest concentrations of soluble Ca^{2+} were observed in 'Roma', followed by 'Mini-Roma', and the lowest concentrations were observed in 'San Marzano' and 'Banana Legs'. In proximal fruit tissue, the highest Ca^{2+} concentration

was observed in ‘Mini-Roma’, compared with the other varieties. In distal fruit tissue, the highest Ca^{2+} concentration was observed in ‘Roma’ and ‘Mini-Roma’. These varieties showed the highest ratios between Ca^{2+} concentrations in proximal and distal fruit tissues (P/D), which was almost double the values presented by the other varieties. Cell wall-bound Ca^{2+} in distal fruit tissue was higher in ‘Roma’, compared with the other varieties. The ratio between cell wall-bound Ca^{2+} and distal Ca^{2+}, both in distal fruit tissue (CW/D), was higher in ‘San Marzano’ and ‘Banana Legs’, followed by ‘Roma’ and finally ‘Mini-Roma’.

Table 5. Ca^{2+} concentration in leaves (g Kg^{-1} DW), soluble, proximal and distal fruit tissue (g Kg^{-1} DW), cell-wall bound Ca^{2+} (g Kg^{-1} DW) of tomato varieties with low (‘Roma’ and ‘Mini-Roma’) or high (‘San Marzano’ and ‘Banana Legs’) susceptibility to BER.

Varieties	Leaf	[a]Soluble	Proximal	Distal Ca^{2+}	[b]P/D	[c]Cell Wall	[d]CW/D
SanMarzano	17.0±2.0 ab*	0.09±0.01 c	0.66±0.06 b	0.31±0.06 b	2.09±0.13 ab	0.22±0.16 b	0.71±0.08 a
BananaLegs	13.0±2.9 b	0.14±0.03 c	0.7±0.12 b	0.32±0.06 b	2.19±0.21 b	0.21±0.12 b	0.66±0.13 a
Roma	16.7±3.1 ab	0.26±0.04 a	0.72±0.04 b	0.65±0.06 a	1.11±0.14 bc	0.32±0.04 a	0.49±0.10 b
Mini-Roma	18.5±3.3 a	0.20±0.02 b	0.90±0.11 a	0.84±0.06 a	1.08±0.09 c	0.24±0.11 b	0.24±0.03 c
CV %	16.85	17.93	11.92	35.95	9.38	20.30	15.33

* Averages followed by same letter are not statistically different according to Tukey’s test (5%). Data shown mean ± standard deviation. [a]soluble: soluble concentration in distal end; [b]D/P: distal/proximal (Friedman’s test 5%); [c]Cell wall: Ca^{2+} bound to the cell wall of the distal end; [d]CW/D: cell wall/distal

Partitioning of Mg^{2+} between leaves and fruits also varied among varieties (Table 6). Foliar Mg^{2+} concentration was higher in ‘Mini-Roma’ and lowest in ‘Banana Legs’. Concentrations of soluble Mg^{2+} in fruits were higher in ‘San Marzano’, ‘Roma’, and ‘Mini-Roma’. Mg^{2+} concentration in proximal fruit tissue was higher in ‘San Marzano’, compared with ‘Roma’ and ‘Mini-Roma’. In distal fruit tissue Mg^{2+} concentration was higher in ‘San Marzano’, ‘Banana Legs’, and ‘Roma’.

Concentrations of foliar and fruit soluble K^{+} did not show significant differences among the varieties (Table 6). Similar to that observed for Mg^{2+} concentrations, K^{+} concentration in proximal fruits was higher in ‘San Marzano’, compared with ‘Roma’ and ‘Mini-Roma’, and in the distal portion it was higher in ‘San Marzano’, ‘Banana Legs’, and ‘Roma’.

Table 6. Mg^{2+} and K^{+} concentration (g Kg^{-1} DW) in leaves, soluble, proximal and distal fruit tissue with low (‘Roma’ and ‘Mini-Roma’) or high (‘San Marzano’ and ‘Banana Legs’) susceptibility to BER.

Varieties	Leaf	[a]Soluble	Proximal	Distal	P/D
			Mg^{2+}		
San Marzano	5.8±0.40 b*	0.99±0.19 a	1.05±0.17 bc	1.45±0.13 a	0.72±0.11 b
Banana Legs	4.3±0.75 c	0.37±0.16 b	1.39±0.16 a	1.34±0.09 a	1.04±0.14 a
Roma	6.0±0.81 ab	1.08±0.23 a	1.21±0.09 ab	1.11±0.06 b	1.10±0.10 a
Mini-Roma	7.3±1.35 a	0.88±0.08 a	0.91±0.07 c	0.89±0.08c	1.04±0.18 a
CV %	14.06	23.30	9.98	8.23	12.76
			K^{+}		
San Marzano	27.8±3.0 a	40.5±3.4 a	24.6±4.6 a	28.3±3.94 a	0.87±0.10 a
Banana Legs	20.0±3.1 a	35.7±3.7 a	20.4±1.3 ab	28.5±1.35 a	0.72±0.05 b
Roma	30.1±3.8 a	37.3±8.4 a	17.8±1.2 b	25.3±1.87 a	0.71±0.07 b
Mini-Roma	30.8±3.9 a	34.1±2.8 a	18.3±1.4 b	19.9±1.83 b	0.92±0.04 a
CV %	10.88	14.70	12.85	10.24	6.98

***** Averages followed by same letter are not statistically different according to Tukey’s test (5%). Data shown mean ± standard deviation. [a]soluble: soluble concentration in distal end; [b]D/P: distal/proximal

Cytoplasmic electrolyte leakage in proximal fruit tissue was statistically equal among all varieties (Table 7). Apoplastic electrolyte leakage in proximal fruit tissue was higher in ‘San Marzano’, compared with ‘Banana Legs’ and ‘Roma’. In distal fruit tissue, the apoplastic and cytoplasmic electrolyte leakages were higher in ‘San Marzano’, compared with the other varieties.

Table 7. Cytoplasmic and apoplastic fruit tissue electrolytic leakage (% h^{-1}) in tomato varieties with low (‘Roma’ and ‘Mini-Roma’) or high (‘San Marzano’ and ‘Banana Legs’) susceptibility to BER.

Varieties	[a]CP	[a]AP	[b]CD	[b]AD
San Marzano	0.85±0.10 a*	1.71±0.53 a	1.7±0.75 a	2.41±0.55 a
Banana Legs	0.79±0.36 a	1.33±0.50 b	1.05±0.28 b	1.60±0.72 b
Roma	0.92±0.45 a	1.42±0.39 b	0.99±0.12 b	1.57±0.18 b
Mini-Roma	0.85±0.20 a	1.49±0.28 ab	0.97±0.28 b	1.62±0.33 b
CV %	10.11	11.61	11.73	10.11

* Averages followed by same letter are not statistically different according to Tukey’s test (5%). Data shown mean ± standard deviation. [a]CP and AP: cytoplasmic and apoplastic electrolyte leakage in proximal fruit tissue, respectively; [b]CD and AD: cytoplasmic and apoplastic electrolyte leakage in distal fruit tissue; respectively

PCA (Figure 3) was used to represent the dimensionality of the database and the relation of the components with BER. PCA showed a total of three dimensions that explains 100% of the variability of the database. The first component explained 54.11% of the variability of the database, and the main variables that contributed to this component were BER, leaf area, fruit length, fruit diameter, pericarp thickness, xylem functionality in the distal portion, water potential, soluble Ca^{2+} in distal fruit tissue, Ca^{2+} in proximal and distal fruit tissue, Mg^{2+} content in leaves, Mg^{2+} content in proximal and distal fruit tissue, soluble K^{+}, and cytoplasmic and apoplastic electrolyte leakage in proximal and distal fruit tissue. These components have the greatest relation to BER incidence. The second component explained 28.62% of the variability of the database, and the main variables were plant dry weight, stomatal conductance, transpiration, stomatal density in the abaxial leaf side, Ca^{2+} content in leaves, soluble Mg^{2+} in distal fruit tissue, K^{+} content in leaf, and cytoplasmic and apoplastic electrolyte leakage in proximal fruit tissue. The third component explained 17.25% of the variability of the database, and the variables were xylem functionality in proximal fruit tissue and cell wall-bound Ca^{2+}.

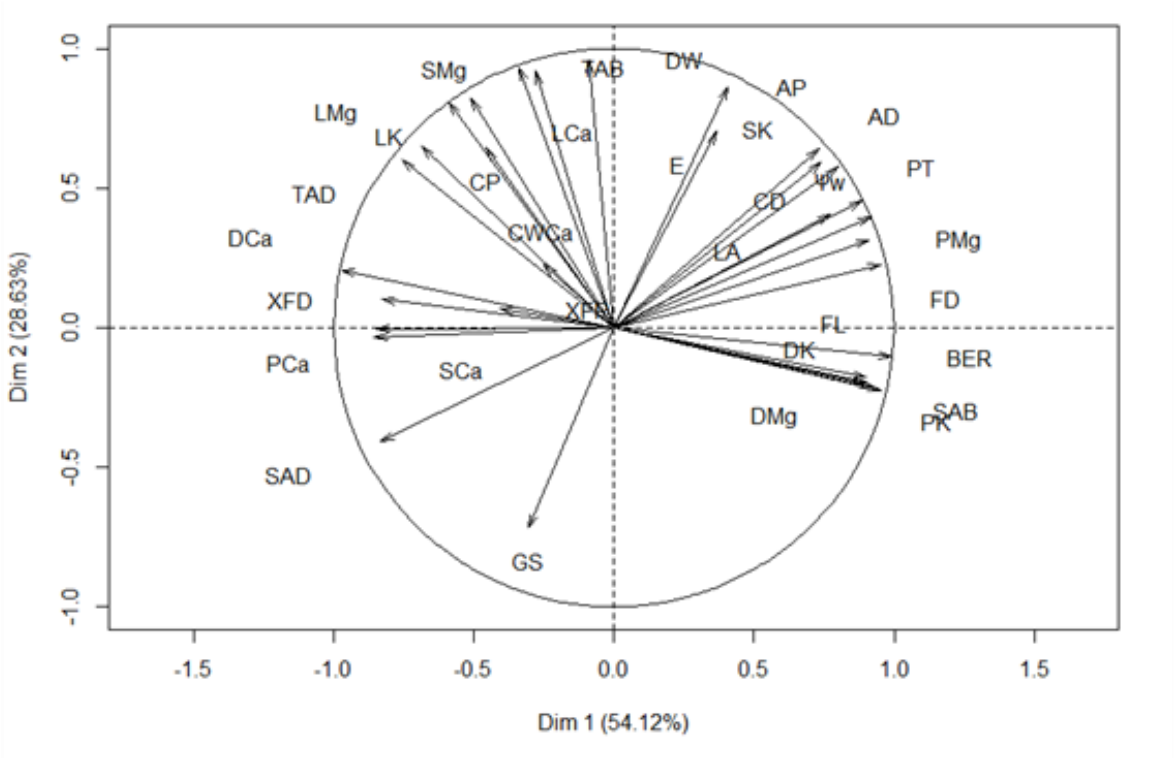

Figure 3. Relation among variables (PCA) shown by scatter plot of the first two principal components based on traits. The variables are BER - blossom-end rot, DW - plant dry weight, E - transpiration, DCa - fruit distal Ca^{2+}, PCa - fruit proximal Ca^{2+}, CWCa - fruit distal cell wall Ca^{2+}, SCa, fruit soluble Ca^{2+}, FL - fruit length,, FD - fruit diameter FMD - fruit distal Mg^{2+}, PMg- fruit proximal Mg^{2+}, DK - fruit distal K^{+}, PK - fruit proximal K^{+}, g_s - stomatal conductance, LA - leaf area, LCa - leaf Ca^{2+}, LK - leaf K^{+}, LMg- leaf Mg^{2+}, Ψw - leaf water potential, SAB - density of abaxial stomata, SAD - density of adaxial stomata, TAB - density of abaxial trichome, TAD - density of adaxial trichome, PT - pericarp thickness, XFP - proximal xylem functionality, XFD - distal xylem functionality.

4.4. Discussion

4.4.1. Variety susceptibility to BER

BER manifested differently among the evaluated varieties. Considering the high percentage of BER, in addition to the high growth rates of ‘San Marzano’ under conditions favorable to the appearance of the disorder, it is possible to classify this variety as being highly susceptible to BER. ‘Banana Legs’ also presented high levels of incidence and could be classified as highly susceptible to BER. Both varieties

present long-shaped fruits, especially when compared with the other two varieties studied. The other varieties, ‘Roma’ and ‘Mini-Roma’, presented low susceptibility to BER. Therefore, we can classify ‘San Marzano’ and ‘Banana Legs’ as highly susceptible to BER, and ‘Roma’ and ‘Mini-Roma’ as being less susceptible to BER, under the conditions of this study.

4.4.2. Physiological parameters inhibiting BER

The variables that correlated negatively with the incidence of BER in fruit were classified as parameters that inhibit BER development. Concentrations of distal and soluble Ca^{2+} are among the physiological factors that presented the highest negative correlations with BER. Lower negative correlations were also observed for Ca^{2+} in proximal, leaf, and cell wall.

Ca^{2+} distribution was different among the varieties studied (Table 5). Although only ‘Banana Legs’ showed lower Ca^{2+} concentration in leaves, Ca^{2+} concentration in the soluble, proximal, and distal fraction of both ‘San Marzano’ and ‘Banana Legs’ fruits were lower than in smaller fruit varieties, i.e., ‘Roma’ and ‘Mini-Roma’. Therefore, although the plants were supplied with Ca^{2+} absorbed from the soil and transported to the aerial parts and fruits, its distribution did not occur in the same way, and the distal portion of ‘San Marzano’ presented lower Ca^{2+} concentration, xylem integrity, and consequently higher BER incidence.

Leaf water potential was a factor that inhibited BER. A decrease in the incidence of BER was related to less negative values of water potentials. BER incidence in ‘San Marzano’ was high, also showing more negative water potential. A drastic reduction of the water potential leads to a reduction in the transport of sap to the aerial part and in the transport of Ca^{2+} to the fruits and consequently increasing the incidence of BER.

The results obtained for Ca^{2+} proximal/distal (P/D) concentration ratio suggest that the Ca^{2+} transport efficiency is almost twice as high in the varieties with low BER incidence. In addition, Ca^{2+} fraction bound to the cell wall was also higher in more elongated fruit varieties, representing 71% Ca^{2+} in the distal portion of ‘San Marzano’ and 66% in the distal portion of ‘Banana Legs’. For the less susceptible varieties, cell wall-bound Ca^{2+} was 49% in ‘Roma’ and 24% in ‘Mini-Roma’, considering total tissue Ca^{2+} content as 100%. These results suggest that Ca^{2+}

binding to the cell may represent an important mechanism determining elongated variety susceptibility to BER.

4.4.3. Physiological parameters triggering BER

The variables that correlated positively with BER incidence were classified as parameters that trigger BER development. K^+ and Mg^{2+} concentrations in fruits, which compete with Ca^{2+} for the binding sites, were the physiological factors that presented the highest positive correlations with BER. Lower positive correlations with BER incidence were also observed for leaf transpiration, leaf stomatal conductance, and electrolyte leakage in fruit tissue.

There was no significant difference in the leaf stomatal conductance, although there were differences in the transpiratory activity between ‘San Marzano’ and ‘Roma’. ‘San Marzano’ leaves showed high transpiratory activity, which may be correlated to the high incidence of BER in this variety. Similar results were found in several studies (De Freitas et al., 2011b; De Freitas et al., 2014).

Mg^{2+} and K^+ concentrations were high in the distal portion of the fruits of ‘San Marzano’, ‘Banana Legs’, and ‘Roma’, compared with ‘Mini-Roma’. These nutrients are well known as triggers of BER, and hence higher concentrations in distal tissue could help to understand the role of BER, especially in ‘San Marzano’ and ‘Banana Legs’, which presented higher BER incidence than ‘Roma’ and ‘Mini-Roma’. It may be observed that, in ‘San Marzano’, Mg^{2+} and K^+ concentrations in the leaf, soluble and proximal portions are also high when compared with ‘Mini-Roma’, which presented lower BER incidence.

Finally, electrolyte leakage in ‘San Marzano’ fruit tissue was higher compared with other varieties, both in the cytoplasmic and apoplastic fractions. Therefore, our results suggest that these fruits presented high membrane permeability in distal portion and can partly explain why this variety displayed higher BER incidence. For ‘Banana Legs’, however, it seems that the incidence of BER may not be directly linked to the increase of membrane leakage, but rather to the combination of the other factors already discussed.

As previously discussed, Ca^{2+} is an ion necessary to stabilize the plasma membrane, and its lack causes an ionic imbalance, which makes it leakier,

deregulating all cellular functioning. Thus, higher concentrations of Mg^{2+} and K^{+} could favor an imbalance. However, concentrations of these cations alone do not determine whether or not the fruit could develop BER, but they are one of several indicators that we might use to predict the appearance of the symptoms.

4.4.4. Morphological parameters inhibiting BER

Morphological variables that correlated negatively with the incidence of BER in fruits were classified as parameters that inhibit BER development. Content of stomata in the abaxial portion and trichomes in the adaxial portion had the most negative correlation coefficients, possibly strongly inhibiting BER development in the fruit. According to the negative correlations observed, other parameters such as xylem functionality and dry weight may also inhibit BER development.

The greater number of stomata in the abaxial portion instead of the adaxial portion provides a lower water loss, as plants that have the highest concentration of stomata in that part of the leaf tend to be more adapted to excess transpiration. Therefore, this characteristic, together with leaf stomatal conductance and transpiration, collaborates to inhibit BER by helping to maintain a balance between water loss and xylem flow towards the fruits, to the detriment of the leaves.

Nevertheless, xylem vessels, which were functional for solute transport and water flow in the distal portion of the conducting vessels, showed significant differences. In ‘San Marzano’, there was a lower functionality of the xylem in the period of BER development, which usually starts 15 days after pollination. Clearly, loss of functionality causes a negative effect to the fruits, bearing in mind that the incidence was greater than 60% in this variety. Several studies try to understand the reason for this loss of functionality (Bondada et al., 2005). Some hormones, such as abscisic acid, play an important role in maintaining xylem functionality throughout fruit growth and development (De Freitas et al., 2014).

However, regarding the varieties studied, there is no information related to the reason why the number of functional vessels is much smaller in the distal portion, or why these vessels lose their functionality during fruit growth and development. In ‘San Marzano’, mainly due to its well elongated shape, this characteristic may be important to understand the reasons why degeneration of the distal portion begins approximately 15 days after pollination.

Plant dry weight was similar among varieties, except for ‘Banana Legs’, which presented lower values. Thus, there was no influence of plant growth on the incidence of BER. Density of trichomes is another factor understudied, but that is closely related to plant adaptation to conditions of cultivation. Some studies have reported that higher density of trichomes could act as a protective shield against excess transpiration (Manetas, 2003). As a consequence, a microclimate is created in the foliar surface that inhibits excess transpiration, modifying the xylematic flow towards the fruits.

4.4.5. Morphological parameters triggering BER

Morphological variables that correlated positively with BER incidence in fruit were classified as parameters that trigger BER development. The parameters that positively correlated with BER were fruit length, fruit diameter, pericarp thickness, and the presence of adaxial stomata.

The varieties studied showed visible differences in fruit anatomy. The ‘San Marzano’ has approximately 10-cm elongated fruits. ‘Banana Legs’ fruits are elongated, but smaller than those of ‘San Marzano’. ‘Roma’ and ‘Mini-Roma’ are very similar, presenting only differences in fruit size. ‘San Marzano’ presented the most elongated fruits and, at the same time, greater incidence of BER, whereas ‘Banana Legs’ displayed smaller fruits but, similarly, high incidence of BER.

Likewise, fruit diameter and ‘San Marzano’ pericarp thickness showed a significant difference in relation to the other varieties. There are few studies that relate characteristics of fruits as the main cause of BER. However, our results clearly show a positive correlation between fruit length and BER incidence. Studies have suggested that Ca^{2+} transport along the fruit might be limited to the xylem vessels (De Freitas et al., 2014), which rapidly become non-functional early during fruit growth and development (Bondada et al., 2005).

Water flow in the xylem in early stages of fruit development is linked to a hydrostatic gradient that is developed as a result of growth and transpiration rates. However, during development, some modifications may occur in this flow, caused by vessel blockage and/or a change in the hydrostatic gradient. The process of blocking vessels is unclear, but some substances such as thyloses or sugars may be

deposited in them, preventing the movement of water and salts (Knipfer et al., 2015, Choat et al., 2009).

Nevertheless, due to negative potentials caused by high transpiration rates and accelerated growth of fruit tissues, vessels may be affected by embolism. If this process is not reversed, the bubbles might completely obstruct the vessels, causing them to become unusable as a water flow path.

As accelerated growth stops, however, this xylem flow begins to change, becoming less intense. The largest quantity of sap is supplied to the fruit via phloem, which initiates cellular supply with photosynthetic products, mainly sugars. Furthermore, studies suggest that, in addition to decreased xylem flow and increased flow of phloem sap, accumulation of solutes occurs in the apoplast of the distal region of fruits, possibly due to a high contribution of the phloem in solute accumulation in the fruit (Bondada et al., 2005).

Ca^{2+} concentration was lower and Mg^{2+} higher, in the distal portion, in the soluble fraction, in ‘San Marzano’, compared with the treatments. These results suggest that loss of xylem functionality in fruit resulted in a lower accumulation of mobile Ca^{2+} in the xylem and a higher accumulation of Mg^{2+} in the xylem and phloem, leading to nutritional imbalance that increased the membrane permeability observed in the distal region of ‘San Marzano’ fruits. Lower concentration of soluble Ca^{2+} and of Mg^{2+} increased the evidence that these two nutrients may affect the susceptibility of tomato fruits to BER.

4.4.6. Suggested mechanisms regulating BER development in tomato varieties with elongated fruits

BER is a disorder studied for many years, but hitherto, all studies have focused on understanding Ca^{2+} regulation, the relation between cations in the soil, nutrient solution, and environmental effects. It is evident how transpiration and water potential may affect Ca^{2+} distribution in leaves and fruits, triggering BER, but there is a clear difference among varieties stomatas spread in the leaf area and that showed us how these varieties could control environmental effects.

However, it is still unclear how the differences in fruit shape and growth may affect BER incidence. In our study, during fruit growth, a great percentage of fruits beginning to develop BER symptoms was noticed and, after a certain growth, the

severity of damaged tissue was clearly high, compared to previous studies. Most of distal part of fruit was completely necrotic, showing a high susceptibility of these varieties to BER.

Previous studies tried to understand how and why the xylem stopped being functional in the distal part of fruit. In this study, in some elongated fruits, as in ‘San Marzano’, the xylem functionality was entirely different in both parts of the fruit. Consequently, with less Ca^{2+} reaching the distal area in these developing fruits and the distance between proximal tissues and the distal end, it was possible to start understanding that Ca^{2+} partitioning between the tissues and cell was completely different and evident when verifying Ca^{2+} concentration.

Furthermore, Ca^{2+} had an effect on the membranes. With less Ca^{2+} reaching the distal end tissues, less Ca^{2+} was binding to the cell wall and plasmatic membranes (Figure 4). As known, lack of Ca^{2+} could destabilize the membranes, changing their normal functioning and the ability to exchange ions, which leads to Ca^{2+} aberrant signals. The end of this process could lead these tissues to cell death, and the first signs of BER could be noticed in the fruits.

From an agronomic point of view, improvement of factors that inhibit BER as well as attenuation of factors that induce BER may be fundamental to optimize management practices of tomato plants containing elongated fruits. Controlling the growing environment in order to minimize transpiration and thus curb high water deficits may help control BER incidence. Recent studies also show that application of abscisic acid might minimize the incidence of BER by reducing leaf transpiration and maintaining a higher number of functional xylem vessels during early stages of fruit growth and development (De Freitas et al., 2014). Therefore, hormone treatments and control of culture medium could be used to inhibit BER incidence in elongated tomato varieties. In addition, the parameters triggering BER could be used as markers in breeding programs to select more resistant cultivars.

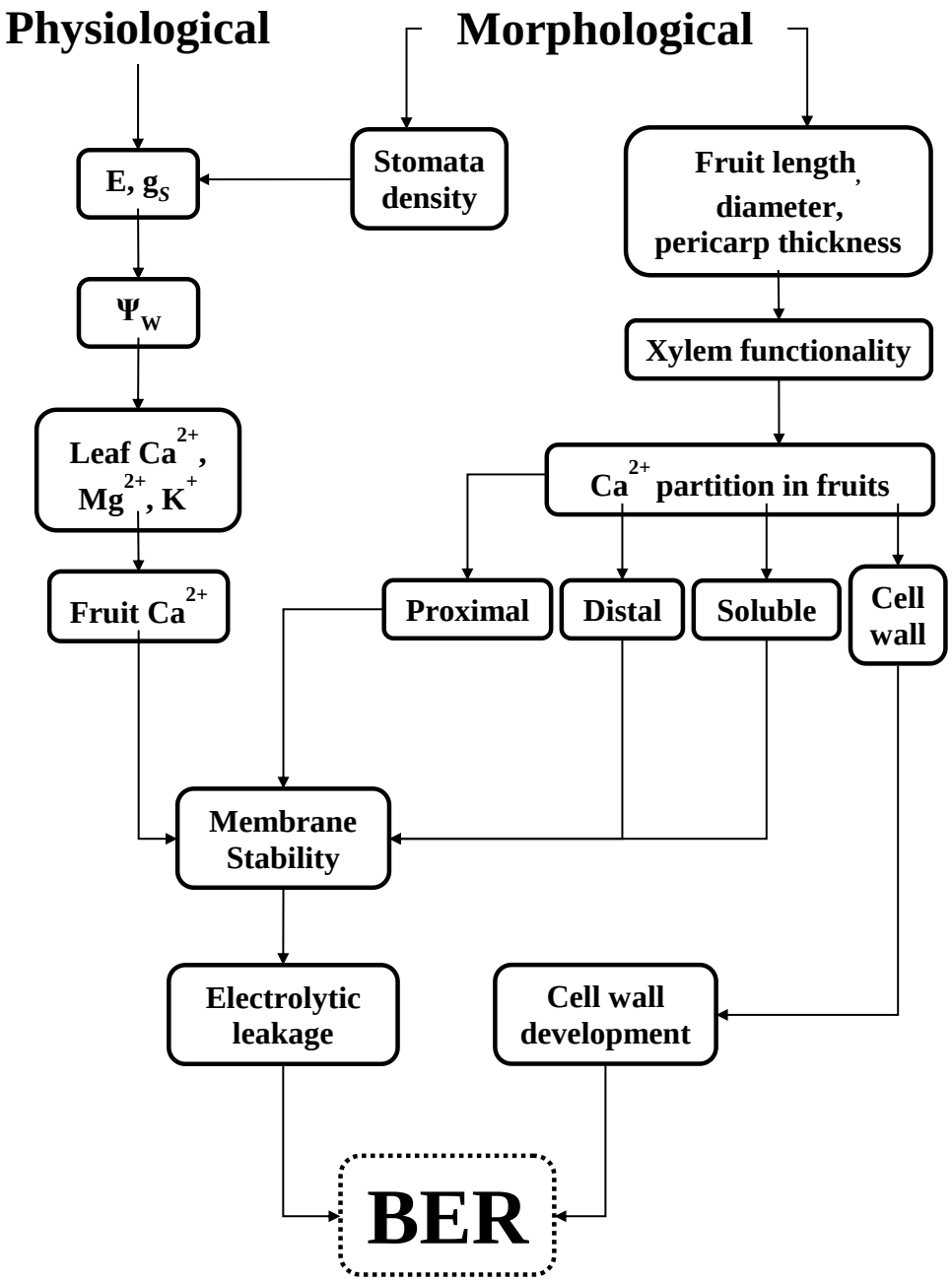

Figure 4. Suggested physiological and morphological mechanisms regulating BER incidence in tomato varieties with elongated fruits. E: leaf transpiration; gs: leaf stomatal conductance; Ψw: leaf water potential.

4.5. Conclusions

Elongated tomato varieties ‘San Marzano’ and ‘Banana Legs’ showed higher susceptibility to BER, whereas ‘Roma’ and ‘Mini-Roma’ had lower susceptibility to the disorder.

The parameters possibly triggering BER were Mg^{2+} content in proximal and distal fruit tissue, K^{+} content in proximal fruit tissue, fruit length and diameter, leaf area, pericarp thickness, and cytoplasmic distal leakage. The parameters possibly inhibiting BER were water potential, distal fruit distal Ca^{2+}, distal soluble Ca^{2+} and Ca^{2+} content in leaves, adaxial trichome, abaxial stomata, proximal cytoplasmic leakage, and distal xylem functionality.

In elongated varieties, like San Marzano, the environmental conditions should be maintained favorable to Ca^{2+} accumulation in the fruits, such as low leaf transpirations and less negative leaf water transpiration.

Future studies should try to understand the motives by which in distal part, xylem, loses its functionality and why more Ca^{2+} remains bounded to cell wall. BER incidence in elongated tomato varieties is not only triggered by exogenous factors or nutrient concentrations, but also by a combination of exogenous and endogenous factors.

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5. 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) reduce 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. Plant and fruit 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 concentration of soluble Ca^{2+} and lower concentration of cell-wall bound Ca^{2+}. EBL-treated fruit also had higher concentration of ascorbic acid and lower concentration 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: Brassinoesteroids; Oxidative stress; Blossom-end rot; Calcium deficiency

5.1. 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 (De Freitas et al., 2017).

In fact, Ca^{2+} deficiency and environment interaction, as well as genetic predisposition of the genotypes, are factors that increase the percentage incidence of BER (Ho and White, 2005). However, few studied factors such as fruit shape and loss of xylem functionality in the distal portion (De Freitas et al., 2011b) have emerged as key factors for the onset of young fruit BER.

Brassinoesteroids (BRs) have been shown to induce stress tolerance in plants (Schnabel et al., 2001), 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 adaptation of plants to stresses. However, studies in tomato are scarce and there is none exploring BR function to reduce BER in fruits.

In these 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 BR has been reported to activate plant antioxidant defenses 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 (Liu et al., 2009). Thus, stress tolerance conferred through the exogenous application of this hormone was different from all the mechanisms already analyzed in studies on Ca^{2+} disorders.

One of the main effects of Ca^{2+} in plants is maintenance of membrane function and stability (Moran et al., 1994). Thus, its deficiency triggers a membrane degradation, which can be evidenced by the peroxidation of its lipids and generation of oxidative radicals (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).

BRs are also known for their function on xylem vessel development. Plants grown in a medium containing brassinazole, which is a BRs biosynthesis inhibitor,

were shown to have slight predominance of phloem differentiation and inhibition in the development of secondary xylem, indicating that BRs play an important role in xylem development (Nagata et al., 2001). Based on this evidence and in the fact that Ca^{2+} is a xylem mobile nutrient, higher levels of BRs, or proper BRs homeostasis with other growth regulators, could favor xylem development and Ca^{2+} translocation in the plant and fruit, possibly increasing fruit Ca^{2+} uptake, and reducing fruit susceptibility to Ca^{2+} deficiency disorders.

Exposure to many abiotic stresses, including cold, drought, or high light can exacerbate ROS production. Such ROS can damage the protein subunits, membranes, and pigments of photosystem I (PSI) and photosystem II (PSII), resulting in protein degradation, inactivation of reaction centers, and inhibition of the subsequent repair mechanisms of the reaction centers (Nishiyama et al., 2006). The increased production of H_2O_2 under stress conditions, such as exposure to salt or water deficit, is the major contributor to the damage experienced by plants (Mittler, 2002). As H_2O_2 passes readily through cell membranes, it can cause damage at locations far from its site of generation (Foyer et al., 1997).

Plant cells have a number of protective mechanisms to eliminate or reduce ROS (Cuypers et al., 2016), one of them is through the activation of the enzymatic antioxidant system, which operates by the sequential and simultaneous actions of enzymes including SOD, CAT, APX, among many others (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 H2O (Gratão et al., 2005)

Plant cells treated with a BR known as 24-epibrassinolide (EBL) were shown to increase the activity of enzymes such as APX, CAT, SOD, as well as to enhance the synthesis of antioxidant substances such as ascorbic acid and reduced glutathione, resulting in lower contents of ROS and lipid peroxidation in the cells (Liu et al., 2009)

Vitamin C or L-ascorbic acid (AsA) found in plants and in fruits is generated through fructose, being the most present plant antioxidant, because of its high concentration in tissues. Ascorbic acid can directly scavenge ROS produced during aerobic metabolic processes such as photosynthesis or respiration although the extent to which the direct reduction of ROS occurs in plants remains to be determined (Gallie, 2013).

Independent of fruit Ca^{2+} content (Saure, 2001; Saure, 2014), it is possible that neither Ca^{2+} nor ROS alone can fully explain Ca^{2+} deficiency disorder development, but rather the interaction between Ca^{2+} and ROS concentrations in the tissue. The objective of this study was to understand the mechanisms through which EBL inhibits BER development in tomato fruit.

5.2. Material and Methods

5.2.1. Plant material, growth conditions, and application of treatments

This study was conducted using the 'BRS Montese' (EMBRAPA) with hybrid long-shape tomatoes and seeds donated by Agrocinco Seeds of Value. The experiment was accomplished in a greenhouse with average solar radiation of 1000 μmol m^{-2} s^{-1}, temperature of 17.5ºC, and relative humidity of 74%. Seeds were seeded separately in trays with a 1:1 (v/v) substrate mixture of commercial organic substrate (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.

Thirty days after planting, the seedlings were transplanted into individual 30 L pots containing commercial organic substrate. Previous fertilization and cover fertilization were carried out according to the recommendations for the crop (Benton Jones, 1998). The plants were fertilized every 20 days, during de growing and fructification time, with 10 g of slow realease fertilizer containing N (16%), P_2O_5 (8%), K_2O (12%), MgO (2%), S (5%), Fe (0.4%), Cu (0.05%), Mn (0.06%), Zn (0.02%), B (0.02%), Mo (0.015%), but without Ca (Basacote Plus; Compo Expert; Soil fertilizer, Agricultural).

When plants started blooming, they were sprayed weekly with a 125-mL solution per plant containing water (control) and 24-epibrassinolide - EBL (Sigma-Aldrich, Saint Louis, MO, USA) (0.01 μM). Fruit samples were harvested on the first cluster at 15 days after pollination (DAP).

5.2.2. BER incidence and growth parameters

The incidence of BER was calculated by multiplying the number of fruit with BER symptoms by 100 and dividing by the total number of fruit in the first cluster. The plant dry weight was determined drying the samples (65°C) until constant weight. Leaf area was determined through 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 ºC under ≤20% relative humidity. After 24 h, fruit were cut into three equal sections at a 90º 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.

5.2.3. Determination of leaf stomatal conductance, leaf transpiration rate, and leaf water potential

An infrared gas analyzer (IRGA) model LCpro+ (ADC BioScientific 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 psicrometric technique using a microvoltmeter model HR-33T (Wescor, Logan, UT, USA) coupled to Wescor C-52 chambers.

5.2.4. Total tissue Ca^{2+}, Mg^{2+}, and K^{+} contents in leaf and fruit and Ca^{2+} bound to the cell wall

Nutrient analysis was accomplished in proximal and distal fruit tissues, as well as in fully expanded leaf close to the first fruit cluster. Samples were oven dried at

65°C until constant weight. About 500 mg of dry material were 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.

5.2.5. Apoplastic and cytoplasmic electrolytic leakage and soluble Ca^{2+} 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 hours at 1 hour 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 three hours 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 three hours 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 hours, 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.

5.2.6. Ascorbic acid, MDA, H_2O_2 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 (DCFI) 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 μmol^{-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 μmol^{-1} min^{-1} mg protein (Nakano; Asada, 1981).

5.2.7. Experimental design

The experiment followed a randomized blocks design with five blocks and two plants per block. The results were submitted to analysis of variance. Averages were compared by T test at 5%. The variables without normal distribution were analyzed by Friedman's non-parametric test at 5%. Pearson correlation test was performed at 5%. Soluble Ca^{2+}+APX, Soluble Ca^{2+}+AsA, Soluble Ca^{2+}+SOD, Soluble Ca^{2+}+CAT, correspond to the mains values related to the appearance of BER added and Soluble Ca^{2+}-H_2O_2, the values subtracted. Everything was centered on the 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.

5.3. Results

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 (Figure 1).

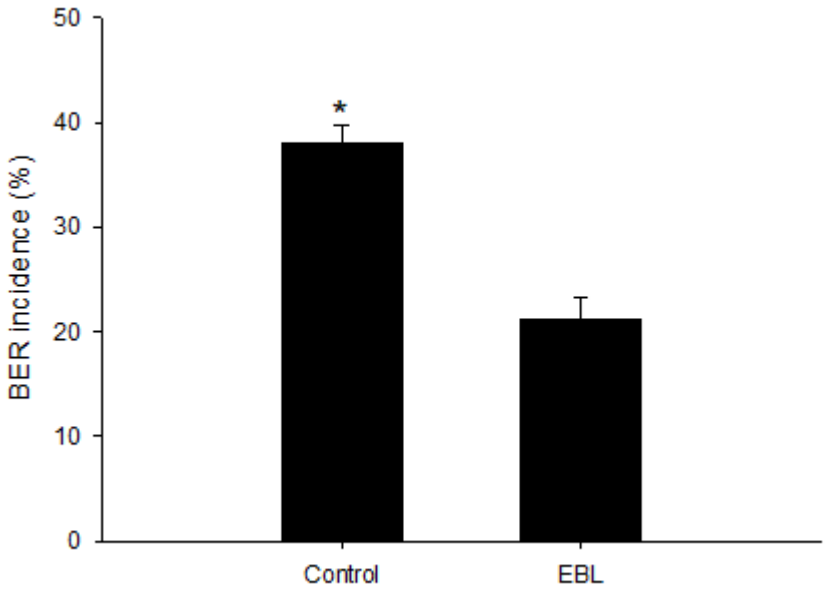

Figure 1. Blossom-end rot (BER) incidence in tomato subjects to EBL treatment. Fruit were harvested at 15 days after pollination. *Averages are statistically different according to the T test (5%). Data shown mean ± standard deviation.

There were 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).

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

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

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

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

Treatments	g_s (mol H_2O m^{-2} s^{-1})	Ψw (MPa)	E (mmol H_2O 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

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

The Ca^{2+} partitioning between leaves and fruits varied in the treatments (Table 3). In leaves, a 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 cell wall in distal fruit tissue was higher in 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).

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	[a]Soluble	Proximal	Distal Ca^{2+}	[b]P/D	[c]Cell Wall	[d]CW/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	-

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

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

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	[a]Soluble	Proximal	Distal	[b]P/D
			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	-

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

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

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

Treatments	[a]CP	[a]AP	[b]CD	[b]AD
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

[a]CP and AP: cytoplasmic and apoplastic leakage from proximal part, respectively; [b]CD and AD: cytoplasmic and apoplastic leakage from distal part; respectively
Averages are statistically different according to the T test (5%). Data shown mean ± standard deviation.

The concentration of AsA was 8% higher in EBL-treated plants (Figure 2).

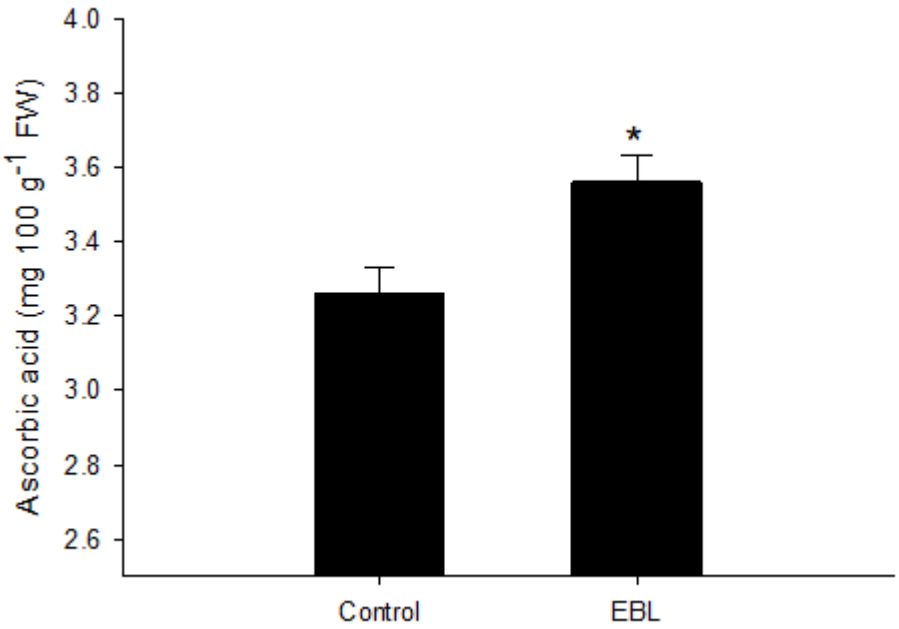

Figure 2. Ascorbic acid concentration in tomato fruits subjected to EBL treatment. Fruit were harvested at 15 days after pollination. *Averages are statistically different according to the T test (5%). Data shown mean ± standard deviation.

There was no significant difference in malondialdehyde (MDA) content in both leaves and fruits, using EBL-treatment, compared to control (Figure 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 (Figure 3).

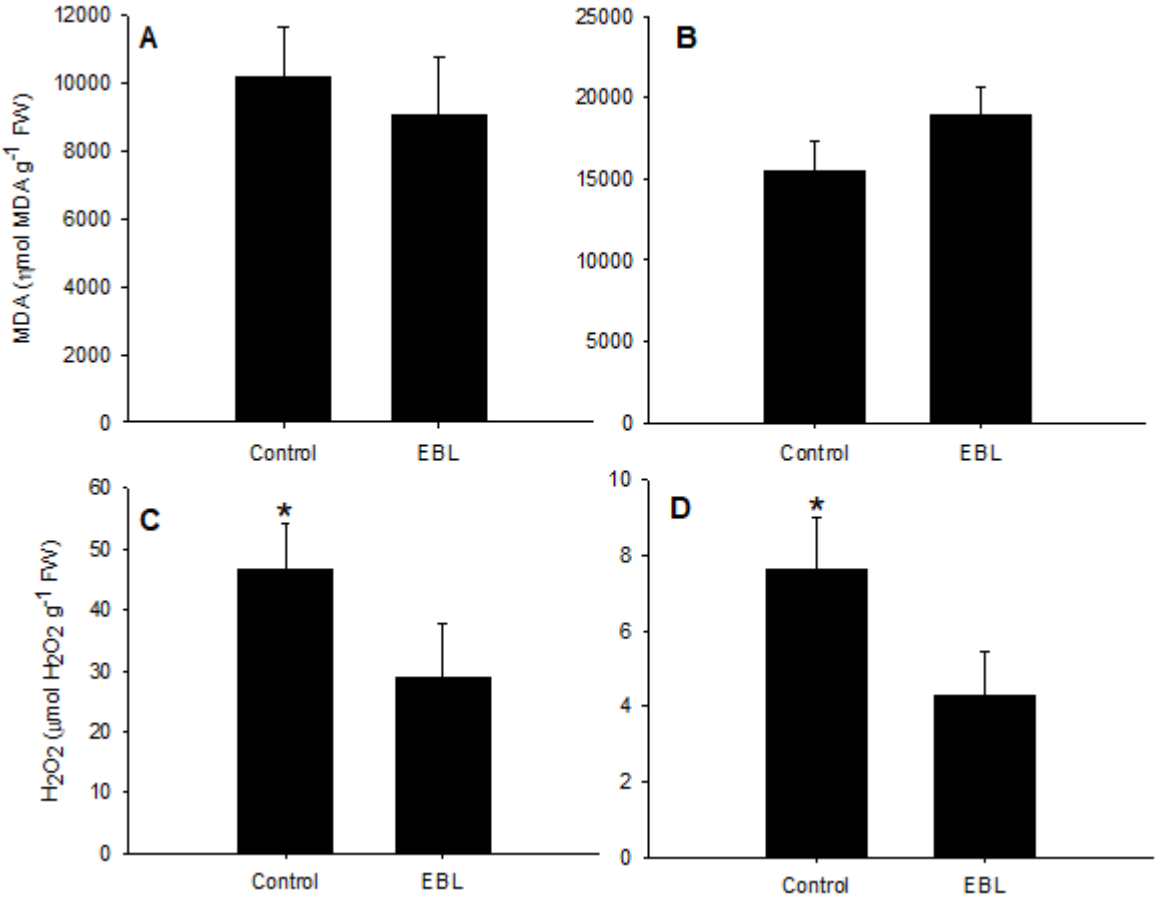

Figure 3. Oxidative damage induced by EBL treatment in tomato tissues expressed as MDA content (nmol g^{-1} fresh weight) and H_2O_2 (µmol g^{-1} fresh weight. A. MDA content in leaves; B. MDA content in fruits; C. H_2O_2 content in leaves; D. H_2O_2 content in fruits. Fruit were harvested at 15 days after pollination. *Averages are statistically different according to the T test (5%). Data shown mean ± standard deviation.

The activity of SOD, CAT, and APX was higher in leaves and fruits of EBL-treated plants (Figure 4). Focusing on the effects of their activity on fruits, SOD, CAT, and APX had 16, 12.5 and 18.4% of increase after EBL treatment, respectively.

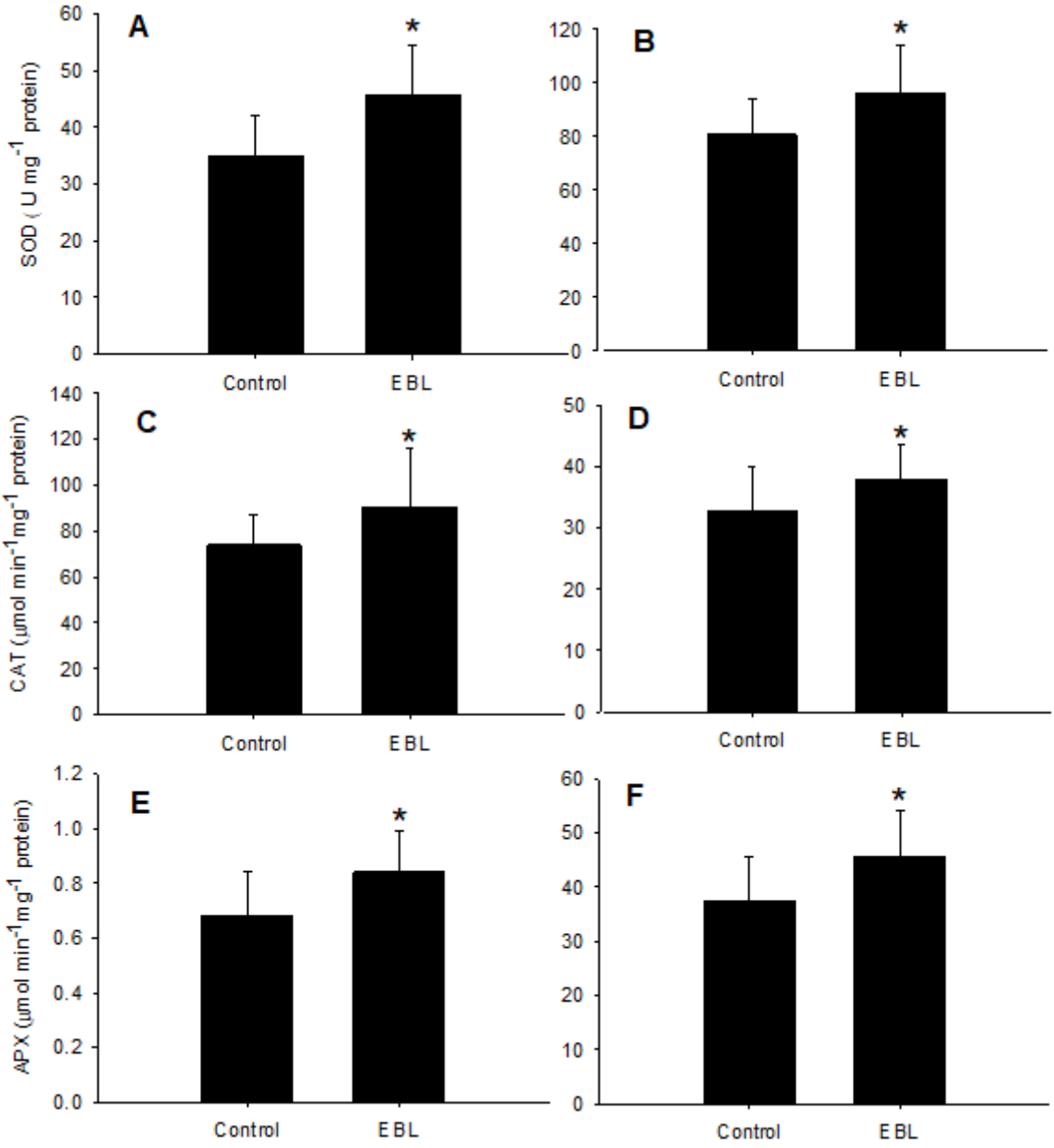

Figure 4. Oxidative stress related enzymes activity in tomato plants and fruits subjected to EBL treatments. A. Superoxide dismutase activity in leaves; B. Superoxide dismutase activity in fruits; C. Catalase activity in leaves; D. Catalase activity in fruits; E. Ascorbate peroxidase activity in leaves; 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 mean ± standard deviation.

The correlation analysis between the variables evaluated in the study revealed that some of the physiological variables correlated positively with BER (Table 1). The variables that had positive correlations with BER were cytoplasmic leakage, H_2O_2, soluble Ca^{2+}-H_2O_2, Cell Wall 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.

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^{2*}**	**Triggering BER**	**R^{2*}**
AsA	-0.32	Cytoplasmic leakage	+0.50
Apoplastic leakage	-0.30	H_2O_2	+0.32
Soluble Ca^{2+}+APX	-0.28	Soluble Ca^{2+}-H_2O_2	+0.32
Soluble Ca^{2+}+AsA	-0.27	Cell Wall Ca^{2+}	+0.29
APX	-0.24	CAT	+0.19
Soluble Ca^{2+}	-0.24	MDA	+0.12
Soluble Ca^{2+}+SOD	-0.06	SOD	+0.11
Soluble Ca^{2+}+CAT	-0.03	Total	+0.54
Total	-0.34		

*Positive correlations mean a proportional correlation between two variables and negative correlations mean inversely proportional correlation between the variables

5.4. 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 (White and Broadley, 2003). Some hormones, such as ABA, are being used to reduce BER damages, but focusing in adapting the plants to a restrictive environment.

Recently, some hormones started to be tested, with new approaches, such as brassinoesteroids. 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 showed to have a great effect on reducing BER incidence in developing tomato fruit by about 44.2%. Due to some well-known evidences 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 increasing stress-related defenses, like antioxidants 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 on determining fruit susceptibility to BER.

5.4.1. 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 and plant dry weight. Ca^{2+} is transported through transpiratory current, reaching the fruit and aerial parts of plants via vascular system, especially through the xylem vessels. As suggested in other studies, functional xylem vessels are required to maintain proper fruit Ca^{2+} uptake in order to support the active growing cells at the fruit distal end (Bondada et al., 2005; De Freitas et al., 2014).

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 also important to consider that xylem and phloem vessels differentiation depend 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 the 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. It is possible to notice that in proximal and distal fruit parts, water-treated fruit showed greater tissue Ca^{2+} content. However, recent studies have shown that concentration of total Ca^{2+} in the tissue cannot fully explain BER incidence, since other pools of Ca^{2+} at the cellular level are also involved (De Freitas et al., 2016).

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 a negative correlation with BER. Furthermore, the Ca^{2+} bound to cell wall was higher in water-treated plants. In that case, higher Ca^{2+} bound to cell wall reduces Ca^{2+} available to other cellular function such as membrane structure and stability, potentially increasing fruit susceptibility to BER. The observed lower Ca^{2+} bound to 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 (De Freitas et al., 2012; 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, it was observed 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 a greater leakage in the control plants and at the same time a higher concentration of Ca^{2+} bound to the wall and lower of 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 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 (Saure, 2001; Suzuki et al., 2003; Ho and White, 2005). So, the loss of integrity of these membranes leads to a 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 $CWCa^{2+}$, 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.

5.4.2. Oxidative stress related factors regulating BER development

BRs can stimulate growth, as well as enhance the plant 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 is producted by cell metabolism, which is aggravated under stress conditions. Generally, in these conditions, there is an increase in the rate of the 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, leading to the liberation of an important rate of the malondialdeyde (MDA) (Yamauchi et al., 2008).

Brassinoesteroids are well known acting increasing antioxidante defense. In this study, EBL treatment increased ascorbic acid (AsA) content in the fruit, which

could help explaining the reduction on 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 to inhibit BER development.

Furthermore, our results show a great increase in the activity of the enzymes SOD, CAT, and APX, both in 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 also 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 in less dangerous compounds, such as water.

Indeed, studies have shown that high expression and activity of these enzymes in response to BRs 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. An ongoing research in our laboratories are considering these possibilities particularly in the case of SOD whose distinct isoenzymes reallocate din distinct cell compartments, and therefore responding 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.

5.4.3. 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 symptoms development (Saure, 2014).

Under stress conditions, increasing tissue ROS production to levels greater than 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, since the main symptoms observed during the development of the disorder are the loss of membrane stability and tissue necrosis (Saure, 2014).

In this study, it was possible to observe that 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 adition, EBL reduced cell wall bound and increased water soluble Ca^{2+} content, as well as increased AsA leves, both showing 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 disrupting membranes, increasing membrane leakage and triggering BER in the fruit.

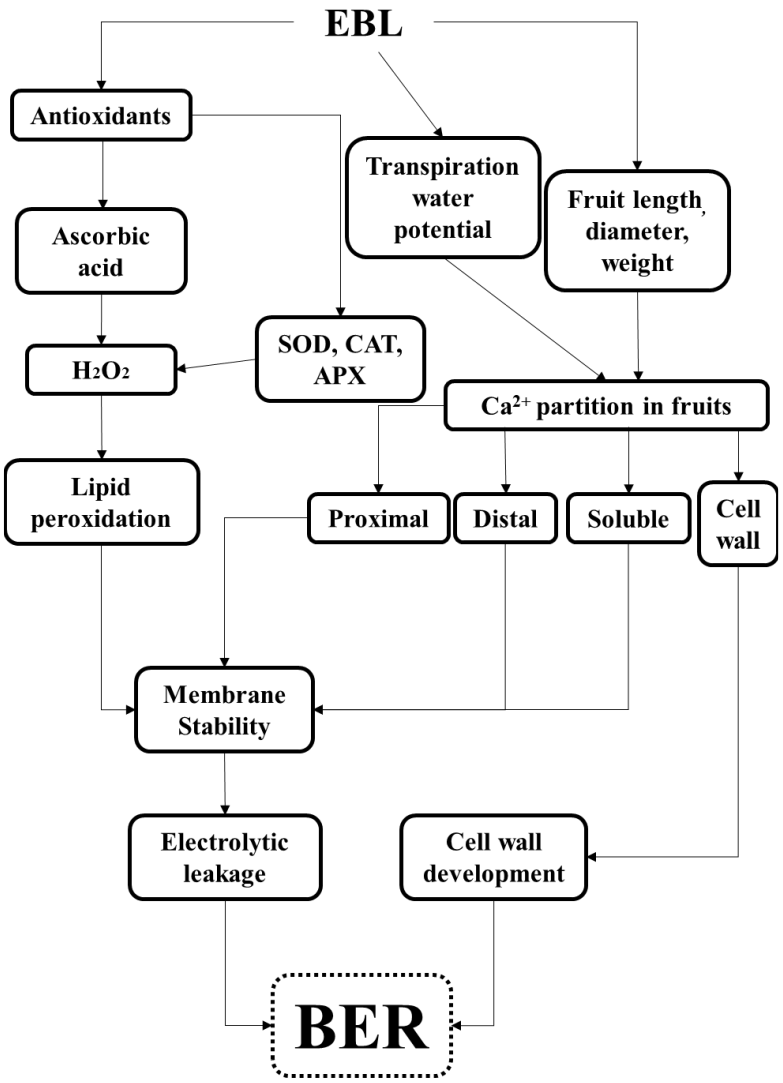

Figure 5. Suggested physiological and morphological mechanisms regulating BER incidence in tomato varieties with elongated fruits

5.5. Conclusions

Brassinoesteroids are still not commercially used in agriculture, but there is a great potential for using it, mainly in stress conditions. Studies should be carried out to optimize doses, timing of applications and possible interactions with other hormones. Furthermore, it is necessary to better understand which mechanisms control the enzymatic activity, increasing soluble Ca^{2+} and decreasing Ca^{2+}.

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.

EBL increase ascorbic acid content and decrease 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.

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