岡山大学
OKAYAMA UNIVERSITY

Studies on effect of defoliation on blossom-end rot development and calcium transport into tomato fruit

トマト果実への Ca 転流と尻腐れ果発生に及ぼす摘葉処理の影響

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Environmental and Life Science

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Thesis

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ABSTRACT

The main objective of the research was to determine the effect of defoliation on BER incidence and Ca transport into different size tomato fruit as influenced by environmental factors under moderate water stress, provided by root zone restriction. Two studies were conducted in 2017-2018. The objective of the 1st study was to determine the effect of defoliation on BER incidence and Ca transport into different size tomato fruit cultivars. Four experiments were conducted between January 2017 and June 2018. The start and end dates for each experiment were; 14 March–2 May, 22 July–23 August, 30 August–7 October 2017 and 20 May–25 June 2018, for experiment 1, 2, 3 and 4, respectively. Five tomato cultivars including a large ('Momotaro fight (MF)', 3 medium ('Lui 60 (L60)', 'Tio cook (TC)', and 'Cindy sweet (CS)', and a small ('Pepe (PP)', size fruit cultivars, respectively, were grown under moderate water stress controlled by a combination of root zone restriction and solar mediated fertigation. Leaf area of plants was reduced by 20-30% by removing alternate leaflets on all leaves. Defoliation significantly reduced BER in all experiments. Defoliation increased both FGR and CTR and there were significant linear relationships between them. However, degree of increase was apparently larger in CTR than that in FGR especially in the BER sensitive large fruit cultivar MF, and defoliation increased total Ca concentration in fruit accordingly.

In the 2nd study, the objective was to determine the optimum number of whole leaves to retain on a tomato plant for effective BER management in MF and CS and explore the relationship between shoot Ca and fruit Ca in non-defoliated plants. Treatments involved maintaining 18, 15 and 12-leaves on the plant. All lateral shoots were removed regularly throughout the growing period except the shoot closest to the flowering truss in the 18 leaves treatment. At the length of 10cm, this shoot was removed for real time Ca determination using a hand held Ca^{2+} meter. In the 18-leaves, BER was higher in MF at 10% compared to 2% CS. FGR was significantly different in MF, however, no significant difference was observed among treatments in CS. Defoliating to 12-leaves increased CTR by 59% and 37% in MF and CS, respectively. Defoliating to 12-leaves and 15-leaves increased the water soluble Ca concentration in the distal part of fruit. In the plants defoliated to 18-leaves, a significant steady decrease was observed in the concentration of water soluble Ca in the distal part of the fruits with increase in truss order. There was a significant linear relationship between water soluble Ca concentration in the distal part of fruit and shoot Ca concentration in the plant defoliated to 18-leaves. We conclude that under moderate water stress by root zone restriction and also certain other BER inductive conditions, defoliation to 12-15 leaves on a tomato plant should be a promising approach for decreasing BER incidence in susceptible large fruit cultivars.

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It is rightly said that “the journey of one thousand miles begins with one step”. I am humbled that the journey I started 3 years ago for my PhD course has come to an end. A challenging but interesting journey it was, I must confess. Nothing comes easy they say, but with support and determination, anything is achievable. The support I received on this journey, and the experiences I had, cannot be acknowledged on a page or two. It’s a whole book. However, that said, there are people who stood out so prominently in this journey and I will attempt to mention them.

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TO GOD BE ALL THE GLORY!

DEDICATION

First, to God, who enables me to do all things that my hands find to do according to Ecclesiastes 9:10! Then, To my husband; Mr. Simon Alusiola Litembekho, and children; Maxwell Waya, Jewel Baraka and Joy Pendo

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LIST OF ABBREVIATIONS

BER	Blossom-end rot
FGR	Fruit growth rate
CTR	Calcium transport rate
FW	Fruit weight
MF	Momotaro fight
TC	Tio cook
L60	Lui 60
CS	Cindy sweet
PP	Pepe
LAI	Leaf area Index
TDM	Total dry matter

CHAPTER 1. GENERAL INTRODUCTION

Tomato is an important horticultural crop that earns farmers' income as it is consumed in large quantities worldwide and makes a substantial overall health and nutritional contributions to the human diet (Burton–Freeman and Reimers, 2011; FAO, 2015). Greenhouse tomato production is shifting to meet emerging consumer needs. Increasing environmental concerns have pressured growers to supply high-quality vegetables using sustainable production methods (Moya et al., 2017) such as nutrient and water management. In tomato, salinity and water stress can improve fruit quality by influencing the content and composition of soluble sugars, organic acids, and some amino acids (Adams, 1991; Cuartero and Fernandez-Munoz, 1999; Saito et al., 2008; Sakurai and Oyamada, 1995; Zushi and Matsuzoe, 1998). The use of root-volume restriction, a technique that predisposes the plant to moderate water stress, can improve the quality of tomato fruit produced using drip fertigation (Sakurai and Oyamada, 1995; Yamasaki, 1999; Saito et al., 2008). However, on the other hand, water stress is associated with the increased risk of BER occurrence (Adams and Ho, 1992; Dekock et al., 1979; Taylor and Locascio, 2004). Many studies have associated BER with systemic or localized calcium (Ca) deficiency leading to substantial losses in yield and quality (Shear, 1975; Huang et al., 2005; Saure, 2005; Yang and Jie, 2005; De Freitas and Mitcham, 2012).

Ca is an essential plant macronutrient with key structural and signaling roles. Calcium ions (Ca^{2+}) act as: an osmoticum within vacuoles; a stabilizing element of membranes; a strengthening agent in cell walls; and a secondary messenger for a

multitude of signals controlling plant development and responses to biotic and abiotic stresses (Marschner, 1995; White and Broadley, 2003; McAinsh and Pittman, 2009; Dodd et al., 2010; Gonzales-Fontes et al., 2017). Studies have shown that Ca deficiency can be triggered by all factors that can limit plant Ca uptake and translocation to the fruit (Taylor and Locascio, 2004). Factors, such as low Ca content and availability in the soil, inadequate root Ca^{2+} uptake, Ca^{2+} competition with other nutrients in the root, as well as leaf and fruit competition for Ca^{2+} available in the xylem sap, may aggravate BER occurrence (Besford, 1978; Saure, 2001, 2005). Understanding the mechanisms involved in BER development is the key to effectively controlling this disorder in tomatoes (Abdal and Suleiman, 2005).

Despite studies suggesting that Ca deficiency is neither a primary nor an independent factor in the development of BER (Nonami *et al*., 1995; Saure, 2001; Rached, 2018), studies show evidence that low Ca concentration in the fruit is closely associated with BER incidence in tomatoes (Ho and White, 2005; Sun et al., 2013; Ooyama et al., 2017; Vinh et al., 2018). In the literature, the cause of BER development in tomato has been attributed to a low Ca level in the whole plant due to decreased soil Ca supply or root Ca uptake, low transport of Ca to and in the fruit, or an increased demand for Ca due to a high growth rate of the fruit (Ho *et al.,* 1995; Saure, 2001; Ho and White, 2005). Tissue Ca supply is often found to be tightly linked to transpiration and there is wide support that transpiration pull is responsible for the continuous ascent of water and Ca from roots to topmost parts of plants and leaves play an important role (Adams and Ho, 1993; Giliham et al., 2011).

Despite the major role of leaves in plant transpiration and importance of leaf area index which is commonly recognized for photosynthesis, not many studies have been reported on the effect of defoliation on Ca transport and development of BER in tomato. Sato et al. (2004) showed that defoliation could reduce BER incidence in hydroponically grown tomato, however, they did not determine Ca in fruit tissue and no further work has been done to ascertain the Ca transport and concentration in fruit of defoliated plants. Secondly, since previous studies have shown that large fruit cultivars in tomato have a low total Ca concentration and are more susceptible to BER (Yoshida et al., 2014; Vinh et al., 2018), there is need to understand the Ca transporting potential of different size tomato fruit cultivars in relation to environmental changes under moderate water stress.

This research had two main objectives.

1. To investigate the effect of defoliation on BER development and calcium transport into fruits of five tomato cultivars grown under moderate water stress as influenced by changes in environmental conditions
2. To determine the optimum number of whole leaves to retain on a tomato plant for effective BER management and explore the relationship between fruit Ca and shoot Ca as a diagnostic tool for predicting fruit Ca status

Specific research questions were:

1. What is the effect of defoliation on :
    a. BER incidence in different size tomato fruit?
    b. Fruit growth rate in the different size tomato cultivars?

c. Daily Ca transport into tomato fruit?

d. Ca concentration in the distal portions of tomato fruits?

All the above as related to changes in environmental factors.

2. What is the relationship between fruit growth rate and daily Ca transport into fruit in the different size tomato cultivars as influenced by defoliation?

3. What is the optimum number of leaves to retain on a tomato plant grown under root zone restricted system for effective BER management?

4. Can shoot Ca concentration in non-defoliated plants be used as a diagnostic tool for assessing the fruit Ca status in tomato plants grown under root zone restricted system?

CHAPTER 2. LITERATURE REVIEW

2.1 Calcium (Ca): Function, uptake, transport and distribution in plants

Ca is an essential plant macronutrient with key structural and signaling roles. Ca^{2+} act as: an osmoticum within vacuoles; a stabilizing element of membranes; a strengthening agent in cell walls; and a secondary messenger for a multitude of signals (White and Broadley, 2003; McAinsh and Pittman, 2009; Dodd et al., 2010). Within leaves, the current paradigm predicts that Ca^{2+} moves via extracellular pathways and is separated from water when water enters cells (Canny, 1993; Storey and Leigh, 2004). In numerous plant signal transduction pathways, Ca^{2+} is a versatile second messenger which controls the activation of many downstream actions in response to various stimuli. There is strong evidence to indicate that information encoded within these stimulus-induced Ca^{2+} oscillations can provide signalling specificity. Such Ca^{2+} signals, or 'Ca^{2+} signatures', are generated in the cytosol, and in noncytosolic locations including the nucleus and chloroplast, through the coordinated action of Ca^{2+} influx and efflux pathways (McAinsh and Pittman, 2009).

Solute and water transport pathways within plants can be broadly categorized as symplastic (intracellular) or apoplastic (extracellular); a transcellular pathway involving both apoplastic and symplastic compartments has also been defined where solutes and water cross multiple cellular membranes whilst traversing plant tissue (Johansson et al., 2000). Several authors have studied the predominant pathway of water (and Ca^{2+}) flow through plant tissues and found that it differs between species, organs, developmental stage, and with environmental parameters (Johansson et al., 2000; White, 2001; White and Broadley, 2003; Cholewa and Peterson, 2004).

Ca transport is generally believed to be exclusively through the xylem/apoplast pathway because Ca is considered relatively immobile in the phloem/symplast system. However, studies have shown that in some fruits, such as apple and kiwifruit, xylem functionality loss during the late fruit development causes reduction of Ca uptake. In several other studies, though, an indication that the phloem might also be a major pathway for Ca transport in fruits and that both symplast and apoplast pathways participated in Ca movement into fruit has been shown (Song et al., 2018 add more authors here). Hence, the pathway(s) of Ca transport to fruit is still a matter of dispute and might be different among plant species (Song et al., 2018).

The apoplastic or symplastic pathways have distinct characteristics. The Ca^{2+} apoplastic flux is significantly dependent on the transpiration rate, but the pathway is relatively non-selective for divalent cations (White, 1998; White, 2001; White and Broadley, 2003). The symplastic pathway is more selective and controls Ca^{2+} transport into the xylem depending on the demand for Ca^{2+} in the shoot (Clarkson, 1993; White, 1998, 2001; White and Broadley, 2003). It is likely that the proportion of Ca^{2+} transported via the symplast increases as the flux of Ca^{2+} to the shoot decreases. This may occur following increased suberization of the endodermis, low Ca supply, or low transpiration (Clarkson, 1984; Baxter et al., 2009). Ca deficiencies are often manifested in tissues that have low relative rates of transpiration compared with other parts of the plant, which clearly highlights the role of transpiration in supply of Ca^{2+} and the low rate of symplastic transport of Ca^{2+} within most tissues. Experiments show that root pressure and recycled phloem water (Munch water) are capable of delivering sufficient quantities of most

nutrients to the shoot, but not Ca which requires high rates of transpiration (Tanner and Beevers, 2001).

However, even when the leaf transpiration rate is high, Ca deficiency can also occur in adjacent lowly transpiring organs on the same plant, for example fruits (Dayod et al., 2010). Presumably, this is a consequence of insufficient Ca delivery to these tissues because when the transpiration rate of these regions is increased then the severity of Ca deficiency symptoms such as blossom end rot or tipburn is reduced (Chang and Miller, 2004; Frantz et al., 2004). Thus, under some circumstances (e.g. high growth rates and/or impeded transpiration in folded leaves), it appears that long-distance symplastic transport is unable to deliver sufficient Ca^{2+} to tissues that have low transpiration, especially when competing against tissues with higher transpiration rates. Lower transpiration results in lower Ca content of plant tissues, therefore climate change which is predicted to reduce transpiration through the effects of elevated atmospheric CO_2 and increased frequency of drought and salinity is also likely to decrease plant Ca content (Martinez Ballesta et al., 2010).

Water is usually initially taken up from the soil through the plant root system and transported to the shoot via the xylem. However, during leaf development water may also be transported by the phloem (Schmalstig and Geiger, 1985). Water moves down water potential gradients where component osmotic potential gradients require a semipermeable membrane for flow to occur. Expansion growth requires water uptake, with most of the volume increase accounted for by vacuolar expansion (Schmalstig and Geiger, 1985). Growing tissues therefore must develop water potential gradients and water must be conducted to growing tissues. The preferred pathway of water flow within

leaf tissue may involve a combination of pathways at some point depending on leaf developmental stage (Evert et al., 1985; Voicu and Zwiazek, 2010). Cells adjacent to xylem vessels in leaves may have an important role in facilitating water flow between the apoplastic and symplastic compartments (Frangne et al., 2001; Heinen et al., 2009). It has been shown for leaves of some species that apoplastic water movement from the xylem is essentially blocked (Fitzgerald and Allaway, 1991), and water flow can be entirely cell to cell to the epidermis (Ye et al., 2008; Nardini et al., 2010a), while in others the apoplastic pathway seems to predominate (Voicu et al., 2009). This is similar to the variation observed in roots. (Bramley et al., 2009). The bundle sheath in leaves may have suberin lamellae and/or apoplastic barriers on radial walls, thereby decreasing the apoplastic flow of water (Lersten, 1997), and in other cases bundle sheath extensions can allow high connectivity to the epidermis and thence various degrees of connectivity to the mesophyll (Voicu and Zwiazek, 2010). It would be useful to examine species displaying these differences for differences in Ca compartmentation in leaves as this may show interesting correlations, but at present this has not been done.

There are numerous examples of interactions between water flow and Ca^{2+} in the literature. Foremost is the effect of apoplastic Ca^{2+} on stomatal aperture (Ruiz et al., 1993; DeSilva et al., 1996; Webb et al., 2001; Yang et al., 2006). As water flow through the plant is dominated by stomatal conductance, stomatal aperture is the most important factor in drawing water through the plant. It is known that $[Ca^{2+}]_{apo}$ adjacent to the guard cell will regulate stomatal aperture, for example in response to low temperature stress (Wilkinson et al., 2001), and it has been proposed that this may involve an interaction with AQPs in the guard cells (Yang et al., 2006). There has also been a report of a 4-fold

difference in sensitivity to Ca^{2+} between the adaxial and abaxial guard cells of Vicia faba (Wang et al., 1998), which may have implications for delivery of Ca^{2+} to different sides of the leaf. However, it is clear that hydraulic conductance through the leaf can be a substantial fraction of whole-plant hydraulic conductance (Tsuda and Tyree, 2000), and as such the way in which Ca^{2+} interacts with this internal conductance will influence the delivery of Ca^{2+} to different cell types including the guard cells (Gilliham et al., 2011).

In tomato plants developing fruits are such a powerful sink for Ca that developing leaves may become Ca-deficient unless high rates of transpiration are maintained. In plants with large heads of enclosed leaves, e.g. Brassica spp. and lettuce, excessive transpiration by outer leaves diverts Ca from meristems and a variety of necrotic symptoms follow (Bangerth, 1979). Redistribution of many mineral nutrients from older or senescing tissues occurs in the phloem thus reducing the dependence of the plant on external nutrient supply and allowing weakly transpiring meristems and fruits to receive adequate mineral nutrition. The immobility of Ca in phloem and symplast prevents internal redistribution of this kind so that developing tissues require continuous inputs of Ca from the surroundings. These arrive by migration along the xylem walls, as described above, or possibly by root pressure delivery of xylem sap (i.e. free Ca^{2+}) (Clarkson, 1984).

2.2 What is BER?

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

brown discoloration of the tissue occurs finally in most Ca-deficiency disorders and this could be brought about by increased leakage of phenolic precursors from the vacuole into the cytoplasm with subsequent oxidation by polyphenoloxidases (Faust and Shear, 1968). Polyphenols, however, also can damage enzymes, mitochondria, etc. Therefore, they not only are a result of these disorders but also may be involved in the development of them. Ca is widely accepted to be the main factor causing BER (Taylor and Locascio, 2004). In fact, BER appears when Ca is lacking in the distal part of tomato fruit (Bradfield and Guttridge, 1984). At the cellular level, BER appears with low levels of Ca^{2+} in plasma membranes (Suzuki et al., 2003). These facts can be explained by the need for Ca^{2+} for cell membrane stability and semi-permeability (Marschner, 1995). Several authors have tried to determine the critical Ca concentration under which BER is triggered in the fruit distal pericarp, but no consensus was established (Saure, 2001). In some cases, total Ca^{2+} concentration was even higher in BER fruits than healthy ones (Nonami et al., 1995). Based on these facts, Saure (2001) concluded that stress factors may be involved in BER appearance rather than Ca^{2+} *per se* and the debate continues.

2.3 Some hypotheses on BER development

The sequence of events preceding BER development is increasing membrane leakage, cell plasmolysis, and membrane breakdown that lead to the water-soaked symptoms on the blossom-end fruit surface (Saure, 2001; Suzuki et al.,2003; Ho and White, 2005). Increased membrane leakage has been reported to result from lower levels of free apoplastic Ca^{2+}, which stabilizes cell membranes by bridging phosphate and carboxylate groups of phospholipids and proteins at the membrane surface (Clarkson and Hanson, 1980; Legge et al., 1982; Kirkby and Pilbeam, 1984; Hirschi, 2004). Previous

studies have shown that apoplastic levels of Ca^{2+} must be maintained at certain thresholds to avoid excessive membrane leakiness and damage (Hanson, 1960; Kirkby and Pilbeam, 1984; Picchioni et al., 1998). Based on these ideas, BER could be triggered by an abnormal regulation of cellular Ca^{2+} partitioning and distribution that depletes the apoplastic pool of Ca^{2+} that otherwise might bind to and stabilize the plasma membrane (De Freitas and Mitcham, 2012). Axelos and Thibault (1991) illustrated as shown in Fig. 1, how Ca combines with pectin to form Ca pectate that keeps cell membranes sturdy and rigid.

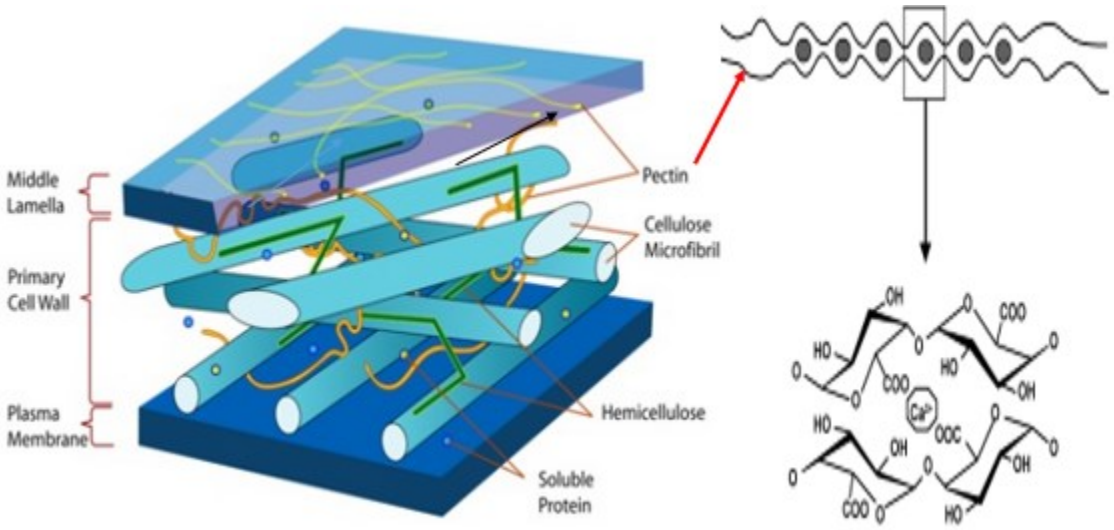

Fig. 1. Schematic representation of calcium binding to pectin sequences (Adapted from Axelos and Thibault, 1991).

Another hypothesis looks at the action of hormones in response to abiotic stress especially those known to stimulate auxin biosynthesis and transport in the plant. Abiotic stress is known to stimulate auxin biosynthesis and transport in the plant that leads to cell expansion and most of these responses take place through cytosolic Ca^{2+} oscillations (White and Broadley,

2003; Hornitschek et al., 2012). Studies suggest that during the process of auxin-induced cell growth (Perrot-Rechenmann, 2010), low levels of cytosolic Ca^{2+} in the tissue could result in abnormal auxin-induced signaling responses, and insufficient apoplastic Ca^{2+} concentrations could lead to excessive cell enlargement, both cases leading to cell death and Ca^{2+} deficiency symptom development in the fruit (Ho and White, 2005).

Suzuki et al., (2003) attempted to clarify the localization of Ca in the pericarp cells and the ultrastructural changes during the development of BER. Ca precipitates were observed as electron-dense deposits by an antimonate precipitation method. They observed that some Ca precipitates were localized in the cytosol, nucleus, plastids, and vacuoles at an early developmental stage of normal fruits. Ca precipitates were increased markedly on the plasma membrane during the rapid-fruit-growth stage compared with their level at the early stage. Cell collapse occurred in the water-soaked region at the rapid-fruit-growth stage in BER fruits. There were no visible Ca precipitates on the traces of plasma membrane near the cell wall of the collapsed cells. The amount of Ca precipitates on plasma membranes near collapsed cells was smaller than that in the cells of normal fruits and normal parts of BER fruits, and the amount on cells near collapsed cells was small. The amount of Ca precipitates on the plasma membranes increased as the distance from collapsed cells increased. On the other hand, Ca precipitates were visible normally in the cytosol, organelles, and vacuoles and even traces of them in collapsed cells. The distribution pattern of the Ca precipitates on the plasma membrane was thus considerably different between normal and BER fruits. On the basis of these observations they concluded that Ca deficiency in plasma membranes is caused by cell collapses in BER tomato fruits. This result shows the importance of Ca in BER studies.

Reactive oxygen species (ROS) were proposed to be involved in the causal mechanism of BER development (Aktas et al., 2003). ROS are a major player in stress related mechanisms. ROS are well known to be involved in triggering cell damage and death by membrane lipid peroxidation, leading to increased membrane leakage and cell lysis (Van Breusegem and Dat, 2006), a very similar mechanism to what happens during BER development. Interestingly, the appearance of BER was found to correspond to the stages of fruit development at which the production of ROS was maximal while scavenging was limited in pepper fruits (Aktas et al., 2003). ROS levels can be controlled by several enzymatic and non-enzymatic mechanisms. For enzymatic control, enzymes like superoxide dismutase (SOD), peroxidase, and catalase can be deployed to control ROS levels, while for non-enzymatic control, two main metabolites, ascorbate and glutathione, are produced and recycled to protect the plant from oxidative damage (Mittler, 2002). Rached et al., (2018) reported similar results supporting the involvement of ROS as a major player in BER appearance because in their study, BER-resistant cultivars showed a larger increase in their ROS scavenging capacity, represented by ascorbate, in response to BER-inductive growth conditions.

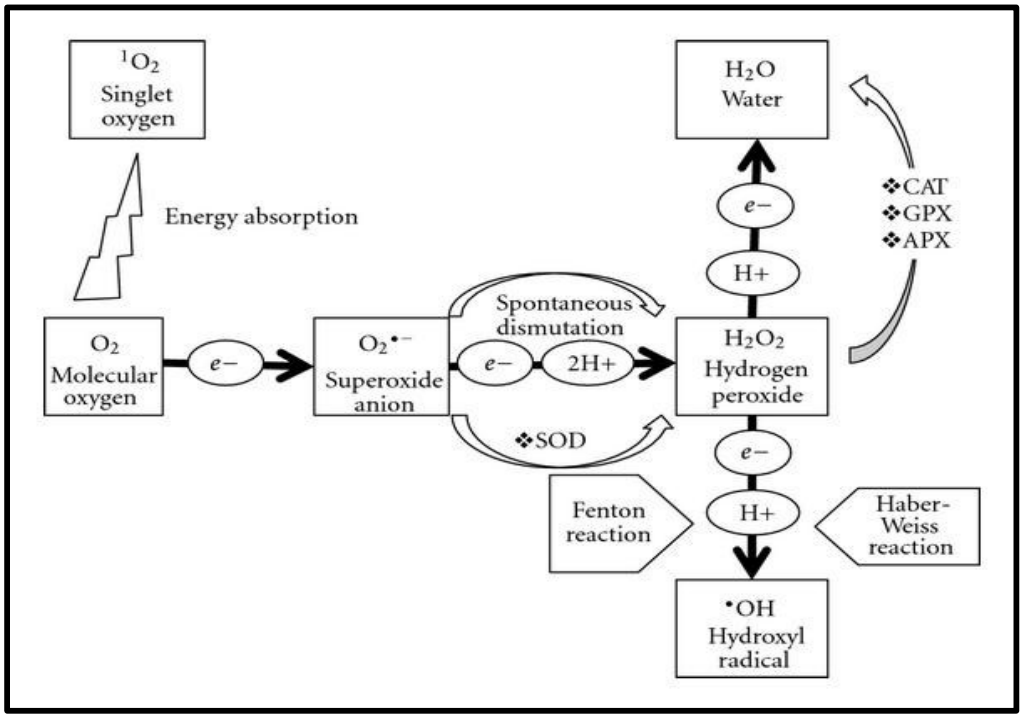

Fig. 2. Schematic representation of generation of reactive oxygen species (ROS) in plants. Activation of O_2 occurs by two different mechanisms. Stepwise monovalent reduction of O_2 leads to formation of $O_2 \bullet -$, H_2O_2, and •OH, whereas energy transfer to O_2 leads to formation of $1O_2$. $O_2 \bullet -$ is easily dismutated to H_2O_2 either non enzymatically or by superoxide dismutase (SOD) catalyzed reaction to H_2O_2. H_2O_2 is converted to H_2O by catalase (CAT), guaiacol peroxidase (GPX), and ascorbate peroxidase (APX) (Sharma et al. 2012).

According to Wiersum hypothesis (1966), a fast growing, low transpiring tissue gets more water via the phloem and less via the xylem and hence less Ca compared to a slow growing organ. There are few detailed experiments investigating this possibility, but they do show that with an accelerated growth rate a decrease in the Ca concentration can indeed be observed. The importance of growth rates on the resulting Ca content is further strengthened by the characteristic Ca uptake curves found e.g. for fruits (Wilkinson, 1968). A rapid increase in Ca was generally noted during the earliest stage

of fruit development, when growth rate is slow. At later stages, however, a remarkable reduction in the rate of Ca accumulation may be observed, when the fruit grows at a much faster rate. Some exceptions to this general rule are, for example, a continuous linear increase (Tromp and Oele, 1972), a rapid increase when the fruit approaches maturity (Oberly, 1973), or even a decrease in Ca at that time (Wilkinson, 1968). Such results show that Ca uptake is not determined by growth rate alone. This could in some way be expected, because conditions that affect the growth rate of storage organs might also affect vegetative plant parts. This probably creates competition between vegetative and storage organs which could well have influenced Ca distribution (Bangerth, 1979).
It was demonstrated for apples (Lewis et al., 1977), however, that increased wind speed reduced bitter pit and increased Ca content of fruit, probably by reducing vegetative growth and the competition for Ca.

Recent studies have suggested the importance of environmental factors in aggravating BER occurrence (Yoshida et al, 2014; Ooyama et al., 2017; Vinh et al., 2018). Tissue Ca concentration is often found to be linked to transpiration, a major driving force of Ca transport in plants. Previous studies have shown that large sized fruit cultivars in tomato have a low total Ca concentration and are more susceptible to BER. However, there is limited understanding on Ca transport into fruits in relation to environmental changes when tomato leaf area is reduced. Bangerth, (1979) posited that since the number of possible interactions that can affect Ca uptake and distribution is so great, it is unlikely to see the development of cultural practices that will completely eliminate Ca deficiency, without a direct application of Ca to the susceptible organ.

Indeed, very few studies have been conducted to find solutions of managing BER using cultural practices.

2.4 Restricted root growth in tomatoes

In tomato, salinity and water stress in the root zone are known to improve the fruit quality by influencing the content and composition of soluble sugars, organic acids, and some amino acids (Adams, 1991; Cuartero and Fernandez-Munoz, 1999; Saito et al., 2008; Sakurai and Oyamada, 1995; Zushi and Matsuzoe, 1998). Sakurai and Oyamada (1995) reported that root zone restriction with a polyester sheet improve TSS and acid content with a decrease of fruit yield. Yamasaki (1999) also reported that restricting the root volume of tomatoes, using non-woven fabric, simplified the management of water in the root zone, thus improving the fruit quality. The use of root-volume restriction can improve the quality of tomato fruit produced using drip fertigation (Saito et al., 2008). However, there is the challenge of physiological disorders under this system as a result of the moderate water stress. Water stress is associated with the increased risk of BER occurrence (Adams and Ho, 1992; Dekock et al., 1979; Taylor and Locascio, 2004).

2.5 Effect of environmental conditions on growth and nutrient translocation in plants

The occurrence of BER is influenced by various factors including cultivar and environmental factors (Adams and Ho, 1992; Ho and White, 2005; Ho et al.,1993; Yoshida et al., 2014). It has been reported that BER is enhanced by water stress (Adams and Ho, 1993; Ho and White, 2005; Kataoka et al., 2017; Pill et al.,1978; Robbins, 1937), high temperature, and high light intensity (Adams and Ho, 1993; De Freitas and Mitcham, 2012; Ho, 1989; Ho and White, 2005; Hoet al., 1993; Yoshida et al., 2014) and it has been widely accepted that BER of tomato is likely to be induced by not only Ca

deficiency but also a variety of factors which disturb the distribution of Ca in the fruit tissues (Dekock et al., 1979; Saure, 2001).

2.5.1 Water Stress

It is well known that water stress is associated with the increased risk of BER occurrence (Adams and Ho, 1992; Dekock et al., 1979; Taylor and Locascio, 2004) by restricting water uptake which is the solvent for Ca^{2+} flux, therefore depressing Ca translocation along vascular vessels and then causing a lack of Ca in fruit required for cell structure maintenance. It further increases the risk of BER development by restricting Ca^{2+} uptake and/or reducing transpiration rate which is known as driving force of transport of Ca^{2+} together with water flow to fruit (Adams and Ho, 1993; De Freitas et al., 2011; Taylor and Locascio, 2004).

2.5.2 Salinity

The effect of high salinity is probably to cause an osmotic effect on roots depressing Ca^{2+} uptake by restricting water uptake, therefore reducing total Ca content of the fruit (Adams and Ho, 1993; Taylor and Locascio, 2004). The high saline condition may increase levels of reactive oxygen species in the apoplast at the time of BER development (De Freitas and Mitcham, 2012), therefore increasing the probability of BER development. In a study to understand role of ROS in BER development, A BER inducing nutrient solution that has elevated salinity was effective (Rached et al., 2018).

2.5.3 Light

Increasing light intensity and temperature can lead to a reduction of relative humidity which can increase the fruit susceptibility to BER occurrence. Ooyama et al., (2017) examined the incidence of blossom-end rot in relation to the Ca concentration in

tomato fruits as affected by a long daily photoperiod using supplemental lighting (60~168μmol·m^{-2}·s^{-1} of PPFD at plant canopies) in autumn cropping. Results showed that supplemental lighting significantly decreased both total and water-soluble Ca concentrations in distal fruit tissue and aggravated the incidence of BER in the tomato. No significant difference was observed in the fruit growth rate; however, a positive relationship was found between leaf stomatal conductance and the intensity of supplemental lighting. They concluded that probably active leaf transpiration after sunset caused by supplemental lighting may have resulted in decreased Ca translocation into the fruit through xylem vessels. In addition to the vigorous growth and increased Ca demand of fruit resulting from a high temperature and strong solar radiation, reduced Ca translocation into the fruit due to a short dark period may aggravate tomato fruit susceptibility to BER in the late spring to mid-summer season.

2.5.4 Effects of mineral imbalance on BER incidence

The effects of mineral imbalance on BER incidence have been noted in several papers. Accordingly, nutrient concentration ratio such as N/Ca^{2+}, K/Ca^{2+}, Mg^{2+}/Ca^{2+} has been suggested as more precise parameters to predict the risk of BER development than total Ca^{2+} concentration alone (De Freitas and Mitcham, 2012). Ho and White, (2005) noted that the complex interactive effects between Ca and mineral ions may influence Ca uptake and partially define fruit Ca concentration intake, therefore, affecting the probability of BER development due to promotion or reduction of Ca^{2+} uptake. Depressive effect of high level of K, Mg, and K to Ca uptake have been shown though it depends on their concentration in the soil solution (Bangerth, 1979). For example, the increase of K level from 5 to 10 mmol·L^{-1} in hydroponic solution was reported to

promote the incidence of BER and reduce Ca uptake whereas increasing Ca concentration had no effects on K uptake (Bar-Tal and Pressman, 1996). Freitas and Micham (2012) posit that high levels of K^+ and Mg^{2+} could potentially disturb membrane structure and functions due to replacing Ca^{2+} on binding sites at plasma membrane but not role of Ca^{2+}, leading to a leaky plasma membrane and further increasing risk to BER development.

High level of nitrogen in soil or hydroponic solution may stimulate root growth or favor vegetative growth which can enhance the competition for Ca^{2+} intake due to higher transpiration rate compared to fruit. Besides, high nitrogen may promote fruit growth and its enlargement may dilute fruit Ca content and therefore increasing the risk to BER development (Ho et al. 1999, Sauce 2001; Ho and White 2005). Further, application of NH_4^+-N fertilizers or higher NH_4^+/NO_3^- ratios in soil or hydroponic solution were reported to increase the rate and severity of BER occurrence probably due to interfering with root Ca^{2+} uptake. This implies that the presence of antagonistic ions may not only restrict root Ca intake but also likely result in stimulation of fruit growth which had been associated with development of BER. For a proper mineral balance in optimizing the mineral composition of the solution, avoiding high salinity (i.e. <5 $dS.m^{-1}$) or excessive NH_4^+ (i.e. <10% total N, K^+ and Mg^{2+}) concentrations, whilst maintaining adequate Ca^{2+} concentration have been suggested for adequate root Ca^{2+} uptake (Ho and White, 2005).

2.5.5 Temperature and solar radiation

Ca uptake was found to correlate highly with solar radiation and root temperature (Adams and Ho, 1993; Taylor and Locascio, 2004). Many studies suggest that factors

favoring photosynthesis rates could accelerate fruit enlargement which may dilute Ca content within the fruit and therefore increase probability of BER development (Adams and Ho, 1993; De Freitas and Mitcham, 2012; Saure, 2001, 2005; Taylor and Locascio, 2004). Adams and Ho (1993) pointed that root Ca uptake increased at a temperature from 14 – 26^{o}C, but decreased at lower or higher temperatures. Ca together with water uptake was stimulated as transpiration increased with solar radiation but the rate of absorbed Ca to water may differ. Low temperatures were reported to have a negative effect on root pressure which is associated with Ca transport (Taylor and Locascio, 2004). Temperature and solar radiation are widely known as potential BER-inductive factors. The promotive effects of irradiance and ambient temperature on fruit growth and/or perturbation in Ca uptake and distribution within the whole plant may trigger BER development (Adams and Ho, 1993; De Freitas et al., 2012b; Ho and White, 2005). Other studies have shown that low solar radiation (Yoshida et al., 2014) and also short photoperiod (Ooyama et al., 2017) may reduce water and also Ca competition against leaves caused by transpiration.

2.5.6 Humidity and VPD

Stomatal responses to vapor pressure deficit (VPD) are a principal means by which vascular land plants regulate daytime transpiration. Plants continuously regulate transpiration by controlling the aperture of the stomatal pores on the surface of the leaf. The principal atmospheric determinant of stomatal aperture is the humidity of the air, which can be expressed as the vapor pressure difference between the leaf and the atmosphere. Many authors have studied stomatal responses to atmospheric VPD across the diversity of vascular plant species (Darwin, 1898; Lange et al., 1971; Turner et al., 1984; Franks and Farquhar, 1999; Oren et al., 1999; Brodribb and McAdam, 2011; Mott

and Peak, 2013), with stomata typically closing at high VPD and opening at low VPD. This comprehensive characterization has allowed for the development of highly effective empirical and mechanistic models of leaf gas exchange that provide robust predictions of the responses of transpiration to changes in VPD (Buckley et al., 2003; Katul et al., 2009; Damour et al., 2010; Medlyn et al., 2011). High relative humidity has been reported to both promote or reduce BER incidence (Taylor and Locascio, 2004). Adams and Ho (1993) found that increasing relative humidity may elevate fruit Ca content and decrease that in leaves by reducing transpiration rate and pointed out the high rate of transpiration should be avoided to meet the Ca^{2+} requirement for rapid fruit growth. The experiment by Tadesse et. al (2001) on sweet pepper showed that low relative humidity reduced the incidence of BER and increased Ca concentration in fruits (Taylor and Locascio 2004). Low humidity at night time reduced Ca content and increased the risk of BER development (Adams and Ho, 1992; Bradfield and Guttridge, 1984). In a study that examined the effects of different Ca concentrations in the nutrient solution and of air relative humidity (RH) on the Ca levels and on the incidence of blossom-end rot in tomato fruit cv. Jumbo, results showed that there was a greater Ca accumulation in fruits submitted to low RH with this accumulation occurring at all Ca levels in the solution (Paiva et al., 1998).

In another study, Guichard et al., (2005) examined the influence of air vapor pressure deficit (VPD) and plant fruit load on the expansion and water relations of young tomato fruits grown in a glasshouse under summer Mediterranean conditions. The contributions of phloem, xylem and transpiration fluxes to the fruit volume increase were estimated at an hourly scale from the growth curves of intact, heat-girdled and detached

fruits, measured using displacement transducers. High VPD conditions reduced the xylem influx and increased the fruit transpiration, but hardly affected the phloem influx. Net water accumulation and growth rate were reduced, and a xylem efflux even occurred during the warmest and driest hours of the day. From a farmers' perspective, a larger volume of fruit for large size fruit cultivars would be preferred as this translates to higher yield and subsequently more profit, and therefore strategies to maintain fruit volume would be undesirable.

In conclusion, it seems clear that manipulation of growth conditions such as increasing air humidity that decrease leaf transpiration rate may promote fruit Ca uptake and reduce the risk of BER development. The effects of light and temperature on the BER occurrence are important and fruit enlargement may dilute Ca content within the fruit and therefore increase probability of BER development. Root temperature may affect fruit Ca uptake through the intervention to root uptake and transpiration rates of leaves and fruit. The rate of fruit Ca uptake is almost exclusively dependent on the abundance of functional xylem vessels connecting to the fruits, so tomato genotypes possessing a stronger xylem network are likely to be less susceptible to BER.

2.6 Seasonal changes in Ca concentration in tomato fruit

In a study to understand the factors affecting the incidence of blossom-end rot (BER), the effect of the Ca/K ratio (4/12–12/4, in me·L–1) in nutrient solutions and Ca concentration in fractions in the distal part of young tomato fruits immediately before BER symptoms appear for three seasons, Yoshida et al., (2014) found that seasonally, total Ca concentration in the distal part of the tomato fruit was highest in winter and lowest in summer. Mean values of total Ca in fruits supplied with standard solution (8/8)

were 0.72, 0.53, and 0.35 μmol·g–1 FW in winter, spring, and summer, respectively. Except in winter, the concentration significantly decreased with a decrease in the Ca concentration in the supplied nutrient solution and an increase in the order of inflorescence. Vinh et al., (2018) observed that symptoms of BER appeared and developed most quickly in summer, followed by spring and lastly by autumn.

In an experiment to assess the effects of shoot pruning and inflorescence thinning on plant growth, yield and fruit quality of greenhouse tomatoes in a tropical climate (Max et al., 2016) observed marked seasonal influences on the incidences of the physiological disorders BER and fruit cracking. BER occurrence was higher in the dry season than in the rainy season, the opposite was true for fruit cracking, which was almost negligible during the dry season whereas it contributed significantly to the share of non-marketable fruits in the rainy season. During the rainy season an increase in relative humidity towards the harvesting period resulted in increased fractions of cracked fruits, as this was shown to be caused by large differences in water potentials between leaves and fruits (Lara et al., 2014). The incidence of BER in the rainy season was only 50 % of that in the dry season, which could be attributed to the higher solar irradiation during the dry season, since high light decreases xylem contribution to fruit growth in tomato, according to Hanssens et al. (2015).

2.7 Fruit growth rate and cultivar differences in the susceptibility to BER

Rapid fruit expansion is believed to be a dominant factor to dilute fruit Ca concentration and increase fruit susceptibility to BER during the fruit enlarging period and its effect has been found to be related to the genotype (De Freitas and Mitcham, 2012; Dekock et al., 1982; Ikeda et al., 2017; Ooyama et al., 2016; Wui and Takano, 1995).

Fruit growth is favored under certain conditions of high temperature and solar radiation, perhaps due to accelerated metabolism and increased photoassimilate supply to the fruit (Ho and White,2005; Ho et al., 1993). Rapid fruit expansion may result in a lag in Ca transport to the distal fruit tissue along with an increase in Ca demand (De Freitas and Mitcham, 2012; Ho and White, 2005; Saure, 2005). In a study to clarify the effect of fruit growth rate on the susceptibility to BER. using two different size fruit cultivars, characterized with different susceptibility to BER disorder, Vinh et al., (2018) concluded that cultivar difference in the susceptibility to BER is likely explained by the difference in the growth rate of young fruit, which may closely relate to potential fruit size and majorly defines water-soluble Ca in the distal part of tomato fruit.

'Momotaro Fight' a large fruit cultivar had a high rate of BER incidence while 'Cindy Sweet' a medium fruit cultivar was hardly affected by BER even at low Ca concentration in the supplied solution (Vinh et al., 2018). A vigorous rate of fruit growth in large-sized cultivar, such as 'Momotaro Fight', could work as a dominant factor to decrease water-soluble Ca. Under conditions favoring high fruit growth, such as high temperature and strong irradiation, water-soluble Ca can easily decrease below the critical level and a breakdown of Ca homeostasis likely triggers BER development in the young fruit. When the water-soluble Ca, including apoplastic and cytoplasmic Ca^{2+}, in the distal part of the young fruit is higher than 0.30 μmol·g−1 FW, BER symptoms rarely developed in both cultivars. They further opined that water-soluble Ca can be a useful risk diagnosing parameter of BER incidence, and the level of 0.20 μmol·g−1 FW maybe critical for the frequent development of BER in different sized tomato cultivars grown under various environmental conditions including the rhizosphere.

Kitano et al., (1998) in evaluating the dynamics of fruit growth and photoassimilate translocation in tomato plants as affected by irradiation and day/night air temperature in relation to respiration, photosynthesis and transpiration of the fruit and the leaf, found that fruit growth was explained by about 80% of sap flux imported into the fruit and was scarcely affected by transpirational water loss from the fruit. Irradiation clearly enhanced fruit growth and photoassimilate translocation, and about 70% of fruit growth and about 80% of photoassimilate translocation were brought during the light period with highly activated leaf photosynthesis and fruit respiration under day/night air temperature of 25/15°C. In particular, when air temperature around fruits rose to 25°C in the light period, remarkable increases in fruit growth and photoassimilate translocation were found with the activated fruit respiration. On the other hand, decreases in fruit growth and photoassimilate translocation were found during the dark period without effects of air temperature. From these results, it was suggested that energy-dependent transport process of sugar in fruits is one of the determinant processes regulating fruit growth and photoassimilate translocation in tomato plants under light.

Manipulation of growth conditions such as increasing air humidity that decrease leaf transpiration rate may promote fruit Ca uptake and reduce the risk of BER development (Li *et al*., 2001). Additionally, as the rate of fruit Ca uptake is almost exclusively dependent on the abundance of functional xylem vessels connecting to the fruits, tomato genotypes possessing a stronger xylem network have been found to be less susceptible to BER (Ho *et al.*, 1993). Tomato varieties with elongated fruit usually have a greater susceptibility to BER than other varieties. In a study that evaluated and identified the possible physiological and morphological characteristics related to the

onset of BER development using four varieties of long-shape tomato fruit with different susceptibility to BER: 'San Marzano,' 'Banana Legs,' 'Roma,' and 'Mini-Roma' (Riboldi et al, 2018) results showed that 'San Marzano' and 'Banana Legs' (elongated fruit) had a higher incidence of BER and lower Ca^{2+} concentration in the distal fruit tissue. 'San Marzano' (the most elongated fruit) presented higher electrolyte leakage in the distal fruit tissue. By comparison, 'Roma' and 'Mini-Roma' (less elongated fruit) were less susceptible to BER and had a higher ratio for proximal/distal fruit Ca^{2+} and a lower distal cell-wall bound content of Ca^{2+}. Additionally, xylem functionality (vessels transporting water and solutes) in the distal fruit tissue was also higher in these more-tolerant varieties.

Considerable difference in susceptibility to BER disorder of five cultivars was reported (Vinh, 2018). Five cultivars ('Momotaro fight', 'Tomimaru muchoo', 'Louis 60', 'Cindy sweet' and 'Pepe' with different fruit sizes were evaluated and indeed, difference in susceptibility to BER disorder was highly associated with the difference in fruit growth rate and water-soluble Ca in the distal portion. Large fruit cultivars, 'Momotaro fight' and 'Tomimaru muchoo' presented low water-soluble Ca and high incidence of BER, compared to the medium-sized and the small-sized 'Pepe' which showed moderate and high water-soluble Ca within the distal portion, respectively.

2.8 Effect of defoliation on BER development and plant growth

Leaf is the major source of supplying assimilates to developing organs, young pods and seeds in crops and defoliation may influence TDM production and yield through photosynthate production and distribution into different parts depending on the magnitude of defoliation (Abdi et al., 2007; Mondal, 2007; Barimavandi et al., 2010).

Since vegetative growth, as a powerful sink, consumes produced assimilates, limitation of vegetative growth enhances assimilate transport to fruits. Thus, proper balance between vegetative and reproductive growth could improve fruit quantity and quality (Chauhan and Halima, 2003; Hossain et al. 2006; Gustafson et al., 2006).

Several studies have been done in tomatoes to evaluate effect of defoliation. Sato et al., (2004) investigated defoliation effects on the incidence of blossom-end rot (BER). They removed 50 % of the leaflets from tomato plants (*Lycopersicon esculentum* Mill.) of two cultivars with differing susceptibility to BER, which reduced whole plant transpiration and xylem sap. Both cultivars showed a reduced BER incidence in plants receiving defoliation treatment. Further, defoliation treatment did not decrease the number of marketable fruit or the fresh weight per fruit and they concluded that defoliation treatment of tomato plants could reduce BER incidence without compromising marketable yield. However, this study was done under hydroponics and Ca concentration in the fruit was not analyzed.

In Earl's melon plant, Nashimura et al., (2001) in a study undertaken to clarify the influence of leaf number on the fruit quality and mineral composition, found that Ca content decreased forty days after fruiting in the first leaf on bearing branch in treatment, where number of main stem leaves was over ten. In an attempt to establish the effect of defoliation on dry matter accumulation and distribution to greenhouse tomato fruits (Andriolo et al., 2001), results showed that total dry matter was higher on plants with three leaves per sympod, but fruit dry matter did not differ significantly among treatments. It was concluded the extra dry matter accumulated in non-defoliated plants was not allocated to fruits, remaining mainly in leaves. For commercial purposes, higher

densities of leaf-pruned plants was suggested as a practice to simultaneously maximize light interception and fruit yield per unit soil surface.

In Okra, up to 25% basal defoliation did not reduce yield significantly and interestingly, yield was slightly increased at this thresh hold level, a phenomenon that was attributed to higher total dry matter, greater number of opened flowers and increased pod and seed size (Bhatt and Rao, 2003). However, beyond 25% defoliation, yield significantly reduced. Other authors (Verma et al., 1992; Board and Harville, 1998) have reported similar results in soybean by observing that partial defoliation during flowering and seed filling had no adverse effects on seed yield because ≤ 20-33% defoliation at flower initiation phase attains capacity to compensate leaf loss and reached LAI ≥ 4 immediately after imposed treatment through regrowth of leaves.

A study conducted on the effect of pruning on qualitative and quantitative characteristics of tomato show that pruning limits vegetative growth and allows more light penetration and so improves qualitative and quantitative characteristics of tomato fruits (Preece & Read, 2005). There is some evidence that pruning not only improves fruit quality but also increases plant health against pests and diseases (Kanyomeka & Shivute, 2005). However, in another study, that sought to assess the effect of different levels of debranching on morpho-physiological, reproductive and yield contributing characters in determinate tomato cultivar cv. Binatomato-5, no significant different in chlorophyll content, photosynthesis rate, total sugar content in leaves, Vitamin C and total soluble solid in fruits due to different levels of debranching was observed (Mondal et al, 2016).

In commercial greenhouse tomato production, indeterminate tomato cultivars are predominantly cultivated with one main stem only and axillary shoots are customarily removed on a regular basis (Navarrete & Jeannequin, 2000; Maboko et al., 2011). In a study that investigated the extent to which and what portion of the defoliation during the beginning of reproductive phase affects fruit yield under field conditions and to identify the yield components responsible for yield reduction in tomato, results showed that weight per fruit decreased only when plants were severely defoliated in tomato and differences in compensation capacity of fruit yield due to leaf loss have been reported (Salful et al., 2016). Hayashida et al., (2006) in an attempt to produce turnip rape (*Brassica napus* L.) with high concentrations of total and water-soluble Ca,conducted a study to investigate the effect of defoliation on the form and levels of Ca (water-, 1N-NaCl-, 2% CH_3COOH- and 5% HCl-soluble Ca) in the lateral shoots of turnip rape. Results showed that defoliation after pinching in November, inhibited elongation of lateral shoots and the yield of lateral shoots was reduced to 84~91% of the control without defoliation. Defoliation significantly increased total and water-soluble Ca concentrations in lateral shoots of turnip rape from November to December, but not those after January. The production of vegetables with high nutritional values is increasingly important. Maintaining a high water-soluble Ca content in vegetables needs to be explored because this form of Ca is highly digestible. From the literature, it's evident that most defoliation studies have focused on dry matter accumulation and there is limited studies on mineral transport.

CHAPTER 3: EFFECT OF DEFOLIATION ON BLOSSOM-END ROT INCIDENCE AND CALCIUM TRANSPORT INTO FRUIT OF TOMATO CULTIVARS UNDER MODERATE WATER STRESS

3.1 Introduction

Ca plays an essential role in processes that preserve the structural and functional integrity of plant membranes, stabilize cell wall structures, regulate ion transport and selectivity, and control ion-exchange behavior as well as cell wall enzyme activities (Marschner, 1995; Rengel, 1992). However, these functions can be seriously impaired due to reduced Ca availability, as Ca can be readily displaced from its membrane binding sites by other cations leading to development of physiological disorders in some fruit. Blossom-end rot (BER) is a physiological disorder in tomato (*Solanum lycopersicum* L.) that occurs under conditions of low apoplastic Ca^{2+}. The plasma membrane can become leaky, leading to cell plasmolysis and eventually death (Suzuki et al., 2003; De Freitas et al., 2011). BER symptoms thus appear as a black sunken decay on the blossom-end of the tomato fruit.

Ca transport in the xylem occurs by mass flow of Ca^{2+} and some organically complexed Ca, and by chromatographic movement along Ca-exchange sites in the xylem walls. Competition between sinks is intensified when Ca^{2+} in xylem is low and transpiration is great (Collier, 1983). As tissues grow, they provide sinks in the xylem exchange column to which Ca migrates. Clarkson (1984) previously demonstrated that under certain environmental conditions, buds, developing leaves and fruit provide major sinks for Ca delivery by the xylem. Plants continuously regulate transpiration by controlling the aperture of the stomatal pores on the surface of the leaf. The principal

atmospheric determinant of stomatal aperture is the humidity of the air, which can be expressed as the vapor pressure deficit (VPD) (McAdam, 2015).

BER has been shown to occur in plants with an adequate Ca supply when grown in environmental conditions that reduce Ca transport to rapidly growing distal fruit tissues (Saure, 2001; Ho and White, 2005). Other studies have shown that fruit growth rate can play a role in inducing BER. Large fruit cultivars have a low total Ca concentration than small fruit cultivars resulting in severe and frequent BER incidence (Ho and White, 2005; Vinh et al., 2018). The authors have found significant relationship between the concentration of water soluble Ca in the distal portion of the tomato and BER incidence under inductive conditions, such as root zone restriction (Ooyama et al., 2016; Yoshida et al., 2014) or elongated photosynthetic light period (Ooyama et al., 2017). Therefore, promotive effects of irradiance and ambient temperature on fruit growth and/or perturbation in Ca uptake and distribution within the fruit may trigger BER development (Adams and Ho, 1993; De Freitas et al., 2012; Ho and White, 2005). Clarkson (1984) noted that Ca deficiency seldom arises because of a failure of Ca supply to plant roots and is more frequently explained by problems arising from its internal distribution and its allocation in mature and growing regions of the plant.

Despite the many studies on relationship between Ca deficiency and BER development, the translocation of Ca within the plant and the causes of Ca deficiency in fruit are still a matter of conjecture (Saure, 2005). Several studies have given suggestions of strategies that can be used to control BER such as reducing excessive gibberellin levels (Saure, 2005), whole plant and fruit specific abscisic acid spray treatments (Barickman et al., 2014; De Freitas et al., 2014) and control of potential BER inductive factors

especially environment (Yoshida et al., 2014) among others. However, few studies have investigated cultural practices that can reduce BER incidence in tomatoes, such as defoliation. Despite the major role of leaves in plant transpiration and importance of leaf area index which is commonly recognized for photosynthesis, not many studies have been reported on the effect of defoliation on Ca transport and development of BER in tomato. Sato et al. (2004) showed that defoliation could reduce BER incidence in hydroponically grown tomato, however, they did not determine Ca in fruit tissue and no further work has been done to ascertain the Ca transport and concentration in fruit of defoliated plants. Secondly, since previous studies have shown that large fruit cultivars in tomato have a low total Ca concentration and are more susceptible to BER (Yoshida et al., 2014; Vinh et al., 2018), there is need to understand the Ca transporting potential of different size tomato fruit cultivars in relation to environmental changes. We were able to repeatedly control BER incidence by growing tomato plants with a combination of restricted root zone volume and solar mediated fertigation control. Here we report the effect of defoliation on Ca transport into different size tomato fruit as influenced by environmental conditions under moderate water stress provided by root zone restriction.

3.2 Materials and methods

As shown in Table 1, four experiments were carried out in a plastic house (6 m wide × 19 m long × 4 m high) in the Faculty of Agriculture, Okayama University from January 2017 to June 2018. Temperature in the plastic house was maintained above 12°C by a warm-air heater and adequate ventilation was applied with a fan and windows when temperature exceeded 28°C. Daily environmental data was recorded for each

experimental period and means were calculated from anthesis to sampling for each analyzed fruit.

Table 1. Mean growing conditions of five tomato cultivars from the beginning of anthesis to the end of sampling for four experimental periods (2017-2018).

	Experiment 1 2017	Experiment 2 2017	Experiment 3 2017	Experiment 4 2018
Date of sowing	18 JAN	12 JUN	20 JUL	2 APR
Beginning of anthesis	14 MAR	22 JUL	30 AUG	20 MAY
End of sampling	2 MAY	23 AUG	7 OCT	25 JUN
Greenhouse temperature (°C)	18.3	31.4	24.7	23.2
Vapor pressure deficit (KPa)	0.56	0.83	0.37	0.70
Solar radiation ($MJ \cdot m^{-2} \cdot day^{-1}$)	6.11	7.49	5.40	7.60
Day length (hh:mm)[z]	12:45	13:39	12:17	14:16

[z] Sunrise to sunset in Okayama (National Astronomical Observatory of Japan)

Five tomato cultivars including a large ('Momotaro fight (MF)', ≥200g), 3 medium ('Lui 60 (L60)', 40–80g; 'Tio cook (TC)', 40–80g; and 'Cindy sweet (CS)', 30-80g) and a small ('Pepe (PP)', ≤ 20g) fruit cultivars, respectively, were grown under moderate water stress controlled by a combination of root zone restriction and solar mediated fertigation. The seeds were sourced from Takii Co., Ltd., Kyoto, Japan (MF, TC, L60 and PP) and Sakata Seed Corp., Yokohama, Japan (CS), respectively. The seeds were then sown on vermiculite moistened with water in trays and placed in a growth

chamber set at 25°C and 12 h day length. At the 3rd true leaf stage, the seedlings were transplanted into plastic pots (12 cm in diameter) filled with tomato growing medium. The seedlings were then moved to the plastic house and fertigated daily with half strength Enshi solution having an EC of 120–130 $mS \cdot m^{-1}$. At the growth stage where flower buds on the plants were visible, the plants were transferred to a solar mediated fertigation system within the plastic house as previously described (Yoshida et al., 2007, 2014). Supplying amount of nutrient solution and fertigation frequency were adjusted to 33-240 mL per 1-4 $MJ \cdot m^{-2}$ to ensure that 10-20% of supplied solution was discharged in the control plant of MF.

Six plants were maintained for each cultivar in each experiment. All lateral shoots were removed regularly throughout the growing period. Half the plants were used as untreated control and the other half were used for the treatment. The treatment involved removal of alternate leaflets on all the three leaves above a flowering truss (Fig. 3) henceforth referred to as 'defoliation' in this study. This treatment was conducted on all leaves between the 1st and the 5th truss to ensure the 1st fruit on the 3rd truss had reached sampling stage. The terminal leaflet on each leaf was not removed. Defoliation treatment commenced at anthesis of the 1st flower on the truss for three leaves just above each truss. Six leaves below the 1st truss were retained on all the plants. A day after end of sampling the 1st fruit on the 3rd truss, leaf area of all leaves (including the midrib) above the 1st and below the 4th truss, respectively, was measured using an area meter (Model L1-3100, Li-COR, Inc. Lincoln, Nebraska USA).

Date of anthesis of the 1st fruit on each truss was recorded. Sampling was conducted for Ca analysis at 14 (large fruit cultivar), 18 (medium size fruit cultivars),

and 20 (small fruit cultivar) days after anthesis, respectively, to obtain sufficient amount of fruit tissue for Ca extraction. Flowers on each truss were thinned to retain only 4, 8 and 20 fruits for the large, medium and small fruit cultivars, respectively. A minimum of 4 and maximum of 9 well-developed and not BER affected 1st fruits of 1st –3rd trusses were sampled for Ca and growth analysis.

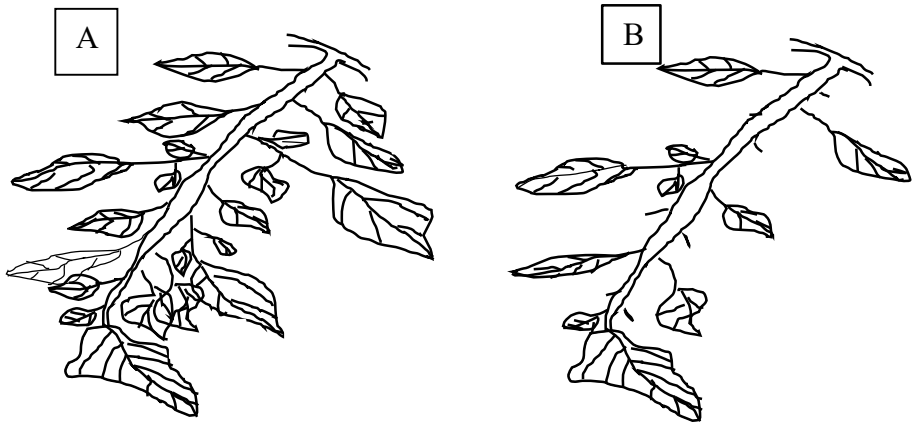

Fig. 3. Sketch showing **A**, a tomato leaf without leaflet removal (Control) and **B**, with alternate leaflets removed, referred to as 'defoliation' treatment in this study.

At sampling, fruit fresh weight without calyx was measured to determine fruit growth rate. The fruit was then divided equatorially into two portions- proximal and distal, and prepared for Ca extraction. Sequential extraction to water and hydrochloric acid (HCl)-soluble Ca fractions that served as representatives for (1) apoplastic and cytoplasmic Ca^{2+}, loosely wall-bound Ca; (2) residual insoluble Ca, respectively, was done, as described in Yoshida et al. (2014). Ca concentration was determined using atomic absorption spectrometry (SPCA-6210, Shimadzu, Kyoto, Japan) and described as $\mu mol \cdot g^{-1}$ FW. BER incidence was recorded when observed.

Fruit growth rate was calculated as fresh weight divided by the number of days after anthesis. Daily Ca transport rate was determined by dividing the Ca amount in fruit by the number of days after anthesis. The incidence of BER in 1st–3rd trusses was recorded until leaf area measurement and calculated as a percentage of BER-affected fruits in the total number of fruits on each truss excluding sampled fruit and young small fruit on the truss at the end of sampling, which had not reached sampling stage (below 1cm and approx. less than 3g) especially common for the small fruit cultivar, PP (Table 2). Microsoft Excel spreadsheets were used for data analysis and multiple comparison of means was done using Tukey's test ($P<0.05$). A summary of the statistical analysis results is shown in Table 3.

Table 2. Number of fruits used in the calculation of BER incidence for five different size tomato fruit cultivars in four experimental periods.

	Actual number of fruits set (A)	Number of fruits sampled for Ca analysis(B)	Number of small fruits at end of sampling(C)	Total number included in BER calculation (A-(B+C))
Cultivar				
Momotaro fight	258	44	10	204
Tio cook	461	52	12	397
Lui 60	546	46	6	494
Cindy sweet	567	54	5	508
Pepe	1299	53	112	1134
Experiment				
1	736	58	30	648
2	724	60	66	598
3	812	71	36	705
4	859	60	13	786
Treatment				
Control	1555	116	86	1353
Defoliation	1576	133	59	1384

Table 3. Leaf area, BER incidence (values were arcsine converted), fruit growth rate, daily Ca transport rate and Ca concentration in the distal part of the fruit as influenced by defoliation in five tomato cultivars grown under four different experimental periods.

	Leaf area (cm^2/plant)	BER incidence (%)	Fruit growth rate ($g \cdot day^{-1}$)	Daily Ca transport rate ($\mu mol \cdot day^{-1}$)	Ca conc. in distal part of the fruit ($\mu mol \cdot g^{-1}FW$)		
Cultivar					HCl-soluble	Water-soluble	Total
Momotaro fight (MF)	3555 a[z]	19.14 a	1.23 a	1.54 a	0.58 d	0.19 c	0.77 d
Tio cook (TC)	2989 b	7.88 b	0.97 b	1.67 a	0.91 c	0.21 c	1.12 c
Lui 60 (L60)	3064 b	4.85 bc	0.74 c	1.53 a	1.22 b	0.26 b	1.48 b
Cindy sweet (CS)	2549 c	2.77 c	0.59 d	1.21 b	1.19 c	0.21 c	1.39 b
Pepe (PP)	2433 c	2.19 c	0.32 e	0.74 c	1.39 a	0.34 a	1.73 a
Experiment (Season)							
1	2828 c	9.25 a	0.52 c	0.79 c	0.82 c	0.24 b	1.06 b
2	988 d	6.82 ab	0.79 b	1.60 a	1.20 a	0.20 c	1.39 a
3	3451 b	4.10 b	0.74 b	1.29 b	1.19 a	0.22 c	1.42 a
4	4405 a	9.50 a	0.95 a	1.61 a	1.04 b	0.29 a	1.33 a
Treatment							
Control	3333 a	10.2 a	0.69 b	1.15 b	1.04 b	0.23 a	1.27 b
Defoliation	2503 b	4.7 b	0.81 a	1.48 a	1.19 a	0.24 a	1.43 a
Significance[y]							
Cultivar (C)	**	**	**	**	*	*	**
Treatment (T)	**	*	**	**	*	NS	*
Experiment period (E)	**	**	**	**	*	*	*
C x T	NS	**	*	*	*	NS	*
C x E	**	NS	**	**	**	**	**
T x E	**	**	*	**	*	NS	NS
C x T x E	NS	NS	NS	NS	NS	NS	NS

[z] Different letters within a category of a column indicate significant mean differences by Tukey's test at $P<0.05$ (n=44, 52, 46, 54 and 53 for MF, TC, L60, CS and PP, respectively and n=58, 71, 60, 60 for experiment 1, 2, 3 and 4, respectively except for leaf area and BER; leaf area n=24 for each cultivar and n=20 for each experiment; BER n=204, 397, 494, 508, 1134 for MF, TC, L60, CS and PP, respectively and n=648, 598, 705, 786 for experiment 1, 2, 3 and 4, respectively). [y] *$P<0.05$; **$P<0.01$; NS, not significant (three-way ANOVA)

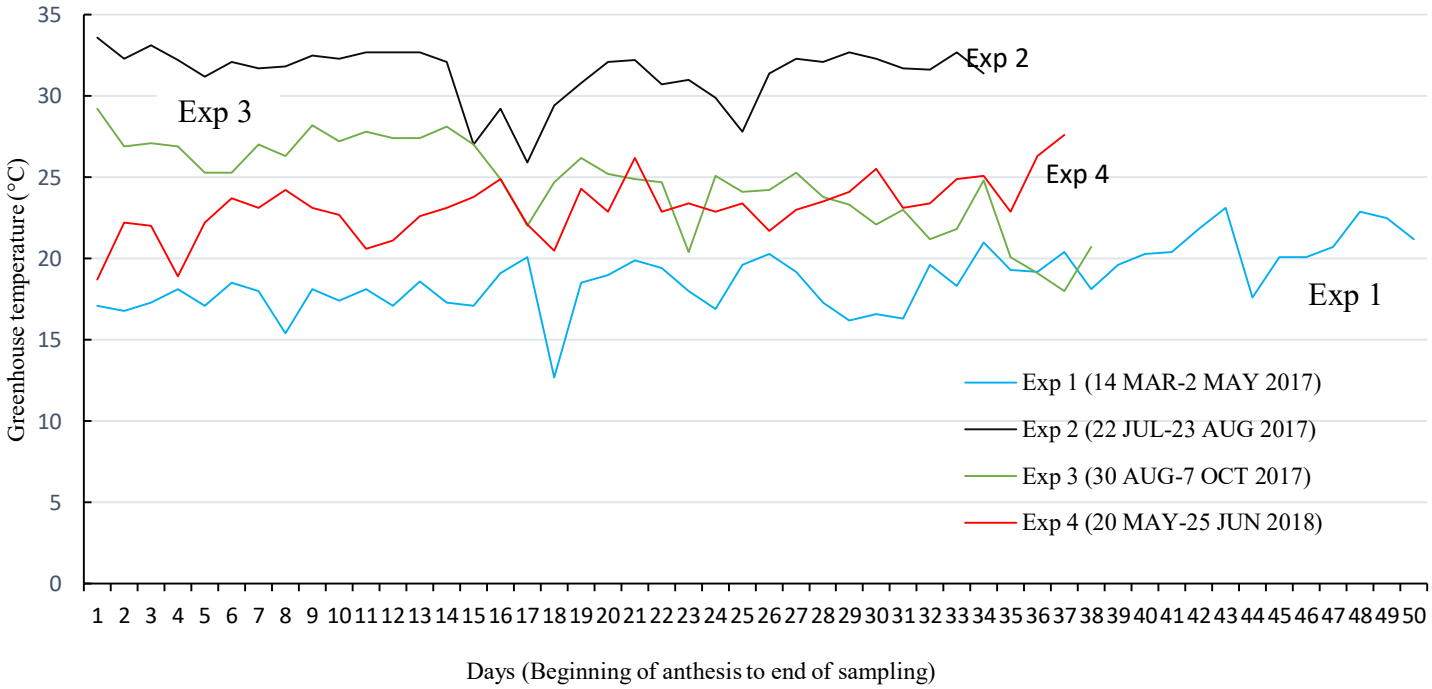

Fig. 4. Daily mean greenhouse temperatures for four experiments from beginning of anthesis to end of sampling.

3.3 Results

3.3.1 Environmental conditions and leaf area

Mean values of the environmental conditions and changes in daily mean temperature for the four experiments from the beginning of anthesis to the end of sampling are shown in Table 1 and Fig. 4, respectively. In experiment 1, the lowest and highest daily mean temperatures during the experimental period were 12.7°C and 23.1°C, respectively. Solar radiation and VPD were medium and day length was shorter than in experiment 2 and 4. In experiment 2, temperatures were high throughout the period with the lowest and highest daily means of 25.9°C and 33.6°C, respectively. Solar radiation and VPD were high in this experiment and day length was long. In experiment 3, daily mean temperature steadily decreased from 29.2°C to 18°C. However, solar radiation was low and day length was short. In experiment 4, a steady increase in temperature from 18.7°C to 27.6°C was recorded. Solar radiation and VPD were the highest, and day length was long.

Defoliation consistently reduced leaf area by 20-30% in all the cultivars in all the experimental periods (Table 3 and Fig. 5). Leaf area of control plant was largest in experiment 4 where temperature was medium and solar radiation and day length were maximum, and it was smallest in experiment 2 where temperature and VPD were highest. Large and medium fruit cultivars had larger leaf area than the small cultivar PP in all the experimental periods. L60 had the highest average leaf reduction of 31%, while MF had the lowest average leaf reduction of 21%.

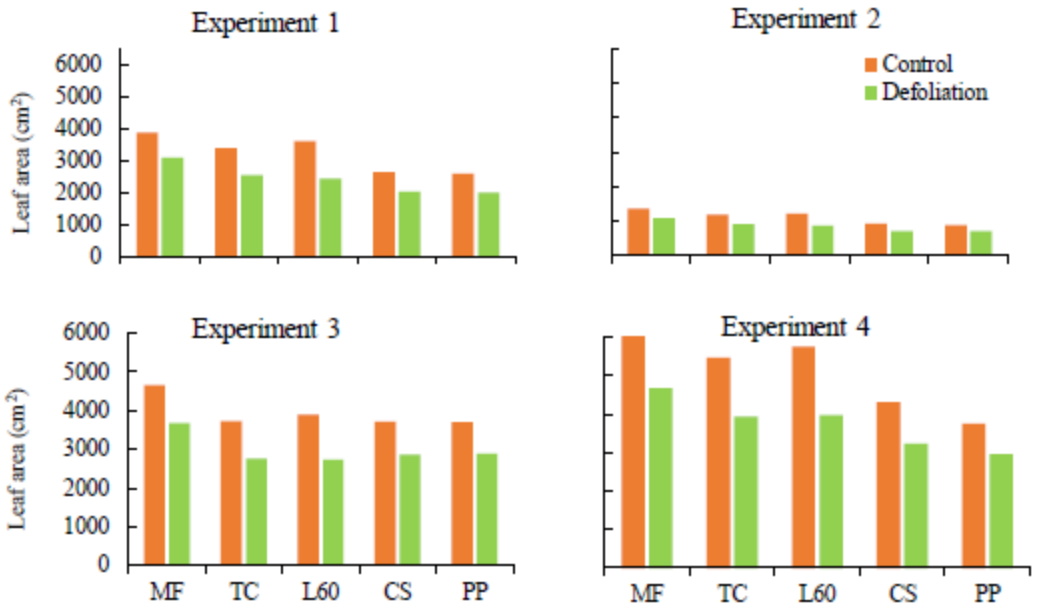

Fig. 5. Effect of defoliation on total leaf area (cm^2) between the 1st and 4th trusses of five different size tomato fruit cultivars at the end of sampling for four experimental periods.

3.3.2 Effect of defoliation on BER incidence

BER incidence was significantly different between cultivars, experimental periods and treatments as shown in Table 3. The large fruit cultivar MF, consistently had the highest BER incidence and the small fruit cultivar PP had the lowest incidence in all the experiments (Fig. 6) The highest BER incidence was observed in MF in experiment 4 at 47%. The lowest BER incidence in our experiments was observed in experiment 3 with all the cultivars showing incidence of below 10% except for MF which had 19%.

Defoliation corresponded to a reduction in incidence of BER in each of the experiments. However, defoliation had the smallest and largest effects on BER in experiments 3 and 4. In the latter experiment, no BER was observed in defoliated plants of L60 and PP and BER

incidence reduced to a quarter of control in the BER susceptible large fruit cultivar MF and medium fruit cultivar TC.

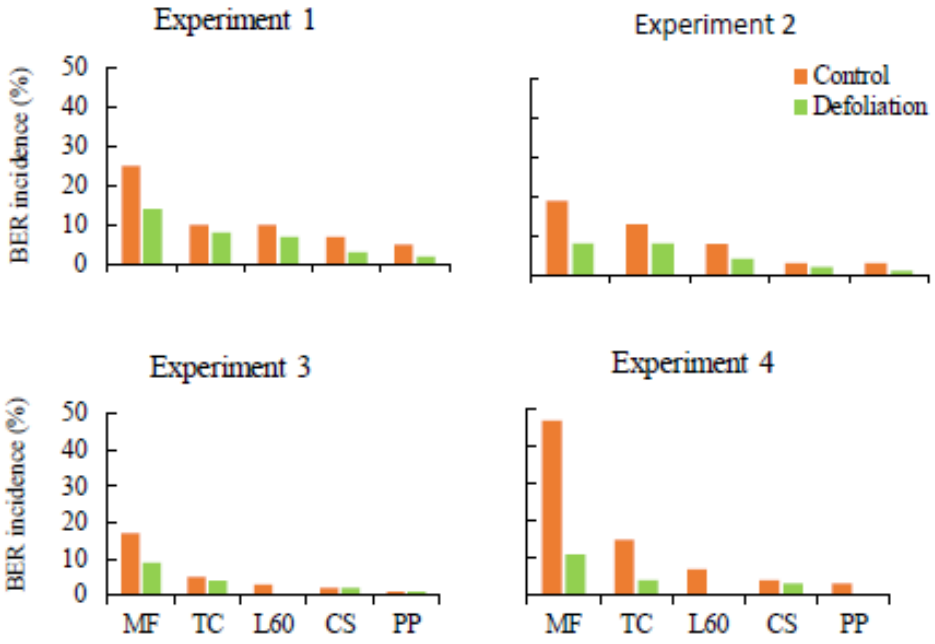

Fig. 6. BER incidence (%) as influenced by defoliation in five different size tomato fruit cultivars grown under four experimental periods.

3.3.3 Effect of defoliation on fruit growth rate

Fruit growth rate was larger in defoliated plants compared to the control plants throughout the experiments (Table 3 and Fig. 7). The smallest fruit growth rate was observed in experiment 1 and differences among the other 3 experiments were little. However, it increased much by defoliation in experiment 1 and 4 by ratios of 1.42 and 1.39, where temperature was lowest in the former and radiation and day length were largest in the latter, respectively. In experiment 3, which had low radiation and short day length, defoliation increased fruit growth rate by the lowest ratio of 1.07. On average, defoliation increased fruit growth rate in MF, TC, L60, CS and PP by a ratio of 1.31, 1.29, 1.22, 1.21,

and 1.19, respectively. The large and medium fruit cultivars consistently had a larger fruit growth rate than the small fruit cultivar PP.

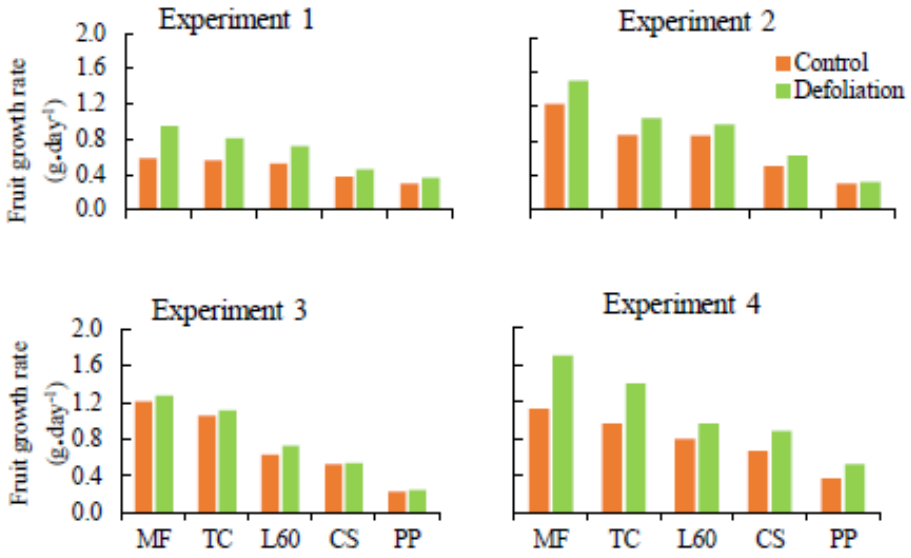

Fig. 7. Fruit growth rate ($g \cdot day^{-1}$) as influenced by defoliation in five different size tomato fruit cultivars grown under four experimental periods.

3.3. 4 Effect of defoliation on daily Ca transport rate

Daily Ca transport into fruits increased in defoliated plants in all the cultivars and in all the experimental periods (Table 3 and Fig. 8). In control, the Ca transport rate was highest in experiment 2 and lowest in experiment 1. The increase in Ca transport rate in defoliated plants was highest in experiment 4 by a ratio of 1.68 followed by 1.37, 1.33 and 1.28 in experiments 1, 3 and 2, respectively. The increase in Ca transport rate in defoliated plants was higher in large and medium fruit cultivars than in the small fruit cultivar. Defoliation increased daily Ca transport rate in MF, L60, TC, CS and PP by an average ratio of 1.64, 1.55, 1.35, 1.30, 1.13, respectively.

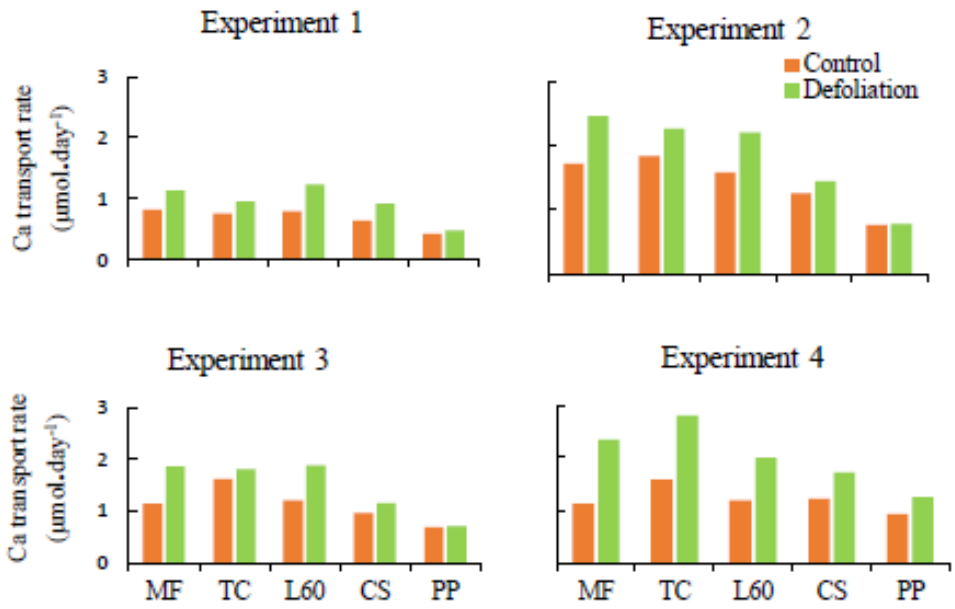

Fig. 8. Rate of Ca transport into fruit (μmol·day^{-1}) as influenced by defoliation in five different size tomato fruit cultivars grown under four experimental periods.

3.3.5 Influence of defoliation on relationship between daily Ca transport rate and fruit growth rate

There were significant linear relationships between fruit growth rate and daily Ca transport rate into fruits and the trends differed among cultivars (Fig. 9). The slope value of TC, one of the medium fruit cultivars, was the highest at 1.87 in the control and 1.79 in the defoliated plants, and the other medium fruit cultivars showed a similar trend. MF had the lowest slope value at 0.74 in the control and 1.19 in the defoliated plants and the effect of defoliation on Ca transport rate was larger than that on fruit growth rate. The slope ratio (defoliated/control) of 1.61 in MF was the highest, followed by PP, L60, TC and CS at 1.31, 1.15, 0.94 and 0.82, respectively.

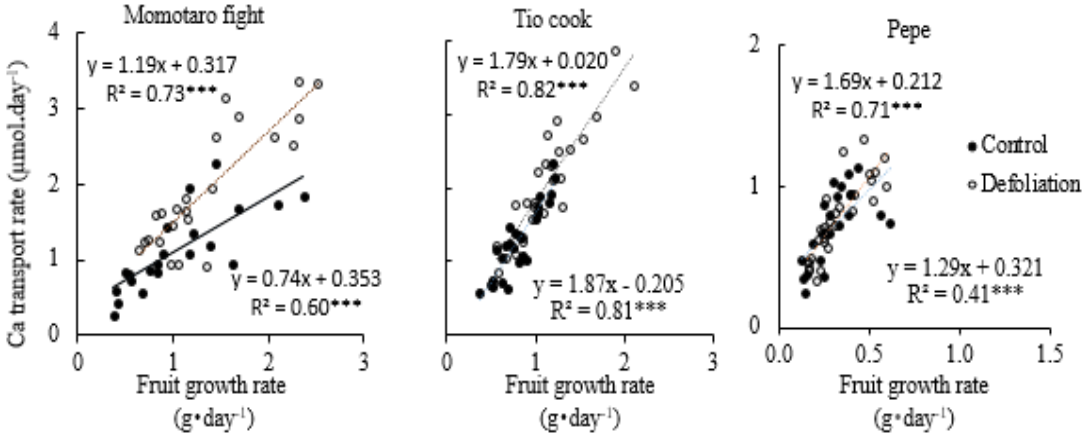

Fig. 9. Comparison of influence of defoliation on the relationship between rate of Ca transport into fruit and fruit growth rate in large ('Momotaro fight'), medium ('Tio cook') and small ('Pepe') size tomato fruit cultivars.

*** indicate significance at $P<0.001$, respectively.

3.3.6 Effect of defoliation on Ca concentration in distal part of tomato fruit

Total Ca concentration in the distal part of the fruit significantly differed among cultivars, treatments and experiments (Table 3) and it was slightly higher in defoliated plants. However, the difference caused by defoliation was not significant statistically (Table 3). As shown in Fig. 10, MF, a large fruit cultivar, had the lowest Ca concentration in the distal part of the fruit with 0.62, 0.88, 0.57 and 0.75 μmol·g^{-1}FW, in experiments 1, 2, 3 and 4 in the control plants. PP, a small fruit cultivar, had the highest Ca concentration in the distal part of the fruit among the cultivars with 1.08, 1.45, 2.06 and 1.78 μmol·g^{-1}FW, in experiments 1, 2, 3 and 4, respectively. Among medium fruit cultivars, L60 had the highest Ca transport rate and exhibited the highest Ca concentration in the distal part of fruit. Water-soluble Ca concentration was also the highest in the small fruit cultivar PP in all the

experiments and the lowest in MF in the control, except for experiment 2. However, the effect of defoliation was highest in MF in experiment 1 by a ratio of 1.43.

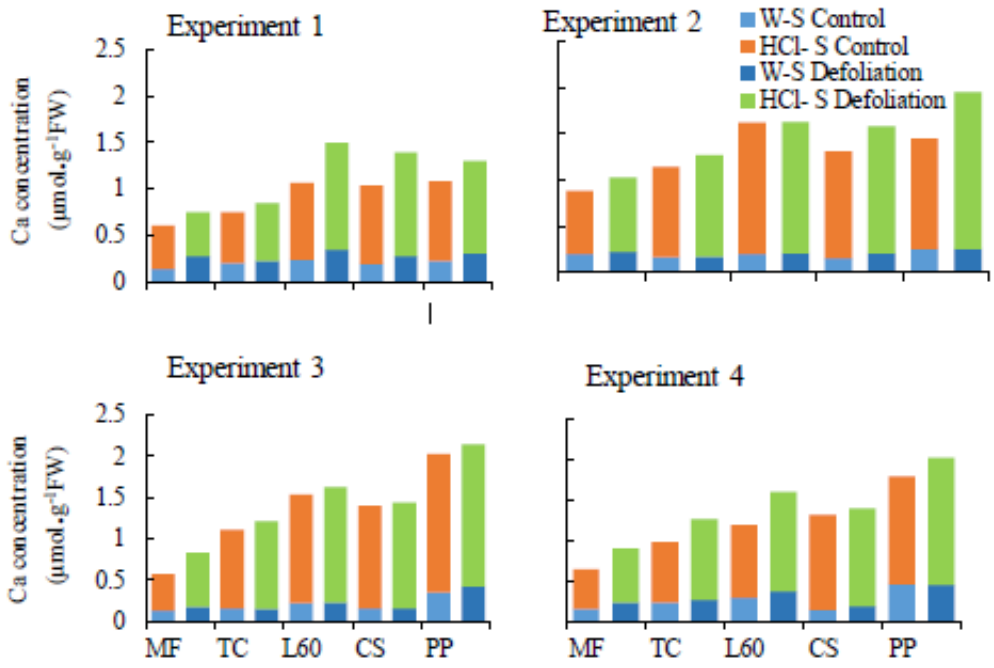

Fig. 10. Water-soluble (W-S) and insoluble (HCl-S) Ca concentration (µmol·g^{-1}FW) in the distal part of fruit as influenced by defoliation in five different size tomato fruit cultivars grown under four experimental periods.

3.4 Discussion

The objective of this study was to determine the effect of defoliation on BER incidence and Ca transport into different size tomato fruit cultivars under moderate water stress. Many studies have established the relationship between Ca deficiency and BER in fruit (Adams and Ho, 1993, De Freitas et al., 2014; Ooyama et al., 2016, 2017; Vinh et al., 2018; Yoshida et al., 2014). Development of BER in tomato fruit has been known to occur mostly under conditions of dryness or an inadequate supply of Ca in the root zone (De Freitas and Mitcham, 2012; Ho and White, 2005; Saure, 2001, 2005; Yoshida et al., 2014).

In this study, we used the combination of restricted root zone volume and solar-mediated fertigation control. Under this system, moderate water stress levels were maintained continuously and BER symptoms were observed in all the seasons and in all the cultivars at varying severity levels (Table 3 and Fig. 6). This result shows that this system is an effective tool in BER studies as previously reported (Yoshida et al., 2007, 2014).

Leaf area analysis revealed that alternate leaflet removal reduced leaf area of tomato plants by 20-30% and cultivars varied in the response to defoliation (Fig. 5). Large and medium fruit cultivars had larger leaves than the small fruit cultivar in this study. Interestingly, alternate leaflet removal did not reduce leaf area above 31% irrespective of the cultivar. The reduction far smaller than 50% was probably due to the midrib, terminal leaflet, and the compensatory expansion of retained leaflets on the leaf. Sato et al. (2004) also applied similar treatment by removing half number of leaflets from a leaf, but they did not measure leaf area. The compensatory expansion of leaflets should vary depending on cultivar and growing season, and finally result in the differences in the reduction of leaf area.

In this study, defoliation significantly reduced BER in all the experiments. This result is similar to that of Sato et al. (2004). Competition for Ca between the leaves and fruits with Ca moving preferentially to the leaf rather than to the fruit under rapid transpiration (Ho, 1989) may cause inadequate distribution of Ca in the fruit at periods of critical demand leading to a Ca deficiency associated with BER disorder (Adams and Ho, 1993). The reduced BER incidence by defoliation may have been as a result of the increased Ca transport rate into fruit, leading to the increased Ca concentration in the fruit.

BER reduced considerably in the large and medium fruit cultivars that had the largest increase in Ca concentration due to defoliation (Fig. 10), which probably became available for structural functions in the fruit at a point before BER was triggered, unlike in the control plants. Cultivars differed in their susceptibility to BER. Large and medium fruit cultivars were more susceptible than the small fruit cultivar. This result agrees with several other studies that have associated difference in susceptibility to BER among cultivars to genetic characteristics regulating the potential size and growth rate of fruit (Adams and Ho, 1992; Ho and White, 2005; Marcelis and Ho,1999).

Fruit growth rate and potential fruit size are important parameters in understanding Ca transport into tomato fruit. Previous studies have suggested that rapid fruit expansion may result in a lag or reduction in Ca transport to rapidly growing distal fruit tissue along with an increase in Ca^{2+} demand by fruit tissue (De Freitas and Mitcham, 2012; Ho and White, 2005; Saure, 2005). In this study, defoliation increased fruit growth rate (Fig. 7) in the large fruit cultivars than in the small fruit cultivars under conditions of low temperature, high solar radiation and long day length. This result differs from that of Sato et al. (2004) whose study found no significant effect on fruit growth. However, they conducted the experiment only in one season and also under hydroponics system. Therefore, our result may imply that probably under root zone restriction, thus moderate water stress, defoliation improved water relations within the fruit. Even though fruit growth rate was increased by defoliation thus expected to be more susceptible to BER, the simultaneous increase in Ca transport rate and Ca concentration in the fruit, may have caused the lower BER observed in defoliated plants.

It is widely known that Ca cannot be readily remobilized once unloaded from the xylem, and in particular, to points downstream of transpiration flow. Since Ca is transported via the transpiration stream, leaves, as highly transpiring organs, usually have high Ca than the low transpiring organs such as fruit. Higher transpiration and growth rates can reduce water potential and increase tissue strength as sinks for xylem Ca^{2+} (White and Broadley, 2003; Conn and Giliham, 2010). Under conditions which favor high transpiration of leaves, within the greenhouse, competition for Ca between leaves and fruits can occur leading to reduced Ca concentration in fruit at critical times resulting in development of BER. In our study, the observed increase in Ca transport rate and Ca concentration in defoliated plants (Fig. 8 and 10) is probably a result of reduced transpiration in the leaves and this led to a lesser competition for water between the leaves and fruits causing more Ca to be distributed into the fruit especially in the large and medium fruit cultivars.

Both physiological and molecular mechanisms are involved in the uptake and transport of Ca that affect the distribution and accumulation of Ca in plant tissues. Ca accumulation in tomato fruit has been shown to be dependent on rates of xylem sap flow, influenced by transpiration and growth rates, however, cultivar differences in these parameters have been reported (Ho et al., 1993; De Freitas et al., 2014; Vinh et al., 2018; Riboldi et al., 2018). Our study showed a significant linear relationship between Ca transport into tomato fruit and fruit growth rate (Fig. 9) in all the different size fruit cultivars with the large and medium fruit cultivars showing a higher Ca transport rate than the smaller fruit cultivar. Defoliation had a larger effect on the Ca transport rate in the large and medium fruit cultivars than in the small fruit cultivar (Fig. 8). This could imply that

the Ca transporting potential into tomato fruit is proportional to fruit expansion and defoliation may have caused a compensatory physiological effect enabling the fruit to draw in more water hence increasing the Ca transport rate into the fruit.

However, in addition to fruit growth rate, multiple regression analysis showed that environmental factors affecting BER development including temperature, radiation, VPD and day length (Adams and Ho, 1993; De Freitas et al., 2012; Ho and White, 2005; Ooyama et al., 2016; Yoshida et al., 2014) play an important role in explaining Ca transport (Table 4 and Fig. 4). As a whole, temperature and day length are positively, and radiation is negatively related to Ca transport. For BER development, these environmental factors also play a significant role except day length (Table 5). Converse to Ca transport, temperature is negatively and radiation is positively related to BER incidence as a whole. Although elongated photoperiod significantly increased BER development in our previous study (Ooyama et al, 2017), day length showed no significant effect on BER incidence in this study. Length of photoperiod and/or dark period may be less important compared to cumulative solar radiation.

Table 4. Regression analysis of Ca transport rate into fruit against fruit growth rate (FGR, FW/No. of days after anthesis) and environmental parameters namely, greenhouse temperature (GHT), solar radiation (SR), vapor pressure deficit (VPD), day length (DL). FGR and environmental parameters were calculated for each analyzed fruit from anthesis to sampling.

Parameter	Control (n=116)	Defoliation (n=133)	Total (n=249)
Adjusted R Square	0.54**	0.84**	0.71**
Intercept	-1.97**	-2.27**	-2.34**
VPD	0.12NS	-0.05*	0.09NS
SR	-0.07NS	-0.04NS	-0.07*
GHT	0.04**	0.03**	0.03**
DL	3.57NS	4.08*	4.44*
FGR	0.77**	1.27**	1.13**

NS, *, ** indicate non-significance or significance at $P< 0.05$ and $P<0.01$, respectively

Table 5. Regression analysis of BER incidence (%, mean values of 3 plants x 3 trusses for 2 treatments x 5 cultivars x 4 experiments) against environmental parameters namely; greenhouse temperature (GHT), solar radiation (SR) and vapor pressure deficit (VPD), and fruit growth rate (FGR) and water-soluble Ca concentration in the distal part of fruit (WS Ca).Environmental parameters were calculated for each experiment from anthesis of 1st flower of 1st truss to sampling of 1st fruit of 3rd truss as shown in Table 1.

Parameters	Control n=20	Defoliation n=20	Total n=40 [z]
Adjusted R^2	0.34*	0.55**	0.36**
Intercept	-3.16NS	77.22*	-32.77NS
VPD	-1.89NS	39.31*	-24.79NS
SR	4.18*	-0.14NS	6.67*
GHT	-0.92NS	-0.36 NS	-0.61*
DL	0.42NS	-8.79NS	5.43NS
FGR	16.60NS	8.27*	2.87*
WS Ca	-41.46*	-2.46NS	-73.73**

NS, *, ** indicate non-significance or significance at $P< 0.05$ and $P<0.01$, respectively.

When control and defoliated plant were analyzed dividedly, only VPD represented adverse effect for defoliated plant compared to control, and the effect was significant only in defoliated plant in both Ca transport and BER. Solar radiation and day length were occasionally significant statistically, however temperature which is closely correlated to fruit growth rate was steadily significant in explaining Ca transport rate into fruit. This result may indicate that rapid fruit growth may be a dominant factor affecting Ca transport rather than environmental factors except temperature. Therefore, environmental factors directly affecting leaf transpiration may be not effective for control plant subjected to moderate water stress, but occasionally effective for less stressed defoliated plant in explaining Ca transport rate in tomato. For BER incidence, environmental factors and also fruit growth rate and water-soluble Ca concentration in the distal part of fruit which have been highly significant parameters in our previous studies (Ooyama et al., 2016; Vinh et al., 2018; Yoshida et al., 2014) were only occasionally, not steadily, effective parameters in this study. Here, we did not take BER affected fruit as a sample, and number of samples fluctuated especially in control plant. Thus fruit rapidly growing and/or containing very low water-soluble Ca may have developed BER and been omitted from fruit sample. Nonetheless, even though the effect of defoliation on the water-soluble Ca concentration was not significant, the *P* value was less than 0.1.

Several studies have reported that Ca allocation among tissues, between symplastic and apoplastic spaces, or among various cell compartments determine the availability of Ca for its structural function (De Freitas and Mitcham, 2012; Yoshida et al., 2014; Vinh et al., 2018). Vinh et al., (2018) suggested that water soluble Ca, including apoplastic and cytoplasmic Ca^{2+} which are closely related to cell physiological processes compared to cell

wall bound or insoluble Ca fractions in the distal part of young tomato fruit can be useful risk diagnosing parameter to BER incidence. In this study, defoliation increased the Ca concentration in the distal part of fruit in all cultivars (Table 3 and Fig. 10). This increase could be a probable reason why there was decreased BER incidence in the defoliated plants compared to the control. Most likely, in defoliated plants, sufficient water-soluble Ca accumulated in time within the distal part of fruit, thus maintaining the Ca homeostasis within the fruit.

In conclusion, alternate leaflet removal applied for moderately water stressed plants significantly increased fruit growth rate, increased Ca transport into tomato fruit and decreased BER in susceptible large fruit cultivars (Table 3). Compared to small fruit, the higher Ca transport rate into fruit presented by the large fruit cultivar was probably, due to a faster fruit growth rate. However, in the large fruit cultivar, Ca amount often cannot meet the demand, resulting in BER incidence. We therefore hypothesize that defoliation probably decreased the competition for water between the leaves and fruits due to reduced transpiration causing sufficient Ca to be transported into the fruits at a critical time before BER is triggered. We would therefore recommend defoliation as a simple cultural practice that can be used to mitigate against BER development in large fruit cultivars not only under root restricted condition but also under other BER inductive conditions. However, the defoliation technique used for this study can be labor intensive. Further research with the aim of establishing a practical number of whole leaves that can be removed to increase Ca within the fruit without consequently stressing the plant and /or compromising on fruit quality is proposed.

CHAPTER 4: THE EFFECT OF DEFOLIATING WHOLE LEAVES ON BLOSSOM-END ROT INCIDENCE AND CALCIUM CONCENTRATION IN FRUIT OF TWO TOMATO CULTIVARS GROWN UNDER MODERATE WATER STRESS

4.1 Introduction

In tomato production, BER may cause severe economic losses because of the deterioration of fruit quality and market acceptability (Yoshida et al., 2014). Many studies have associated BER with systemic or localized calcium (Ca) deficiency De Freitas and Mitcham, 2012; Saure, 2005). In the previous chapter, we reported that alternate leaflet removal for tomato plant grown under restricted root zone volume increased Ca transport into fruit and reduced BER incidence (Indeche et al., in press; Sato et al., 2004). We noted that this technique can be laborious in commercial tomato production. Here, first, we report results of a more practical defoliation technique suitable for commercial production for tomato plants grown under a system combining restricted root zone volume and solar-mediated fertigation control.

It has been reported repeatedly that excessive nitrogen nutrition and subsequent vigorous vegetative growth often causes severe BER incidence. Our result revealed that competition for water and Ca between leaves and reproductive organs may be a major cause of BER development. In a previous study, we observed tip-burn, another Ca deficiency symptom that develops in young leaves on apical and also lateral shoots in Ca starved plants developing severe BER fruits. These facts may represent that young shoot and floral organs are similarly influenced by competition for Ca against mature leaves. It

was supposed that Ca status of young lateral shoot may relate to that of young fruit sensitive to BER and reflect or indicate the risk of BER development. Cultural practices such as lateral shoot removal, defoliation, pruning, pinching and staking are some of the common practices done in tomato production to ensure high yield and quality. The relationship between lateral shoot Ca and Ca in fruit as a diagnostic measure for fruit Ca status has not been explored. Thus, secondly, we tried to explore the relationship between Ca status in lateral shoot and neighboring young fruits.

4.2 Materials and methods

An experiment was carried out in a glasshouse in the Field Science Center, Okayama University from January 2018 to July 2018. Temperature in the glasshouse was maintained above 12°C by a warm-air heater and adequate ventilation was applied with windows when temperature exceeded 28°C. Two tomato cultivars 'Momotaro fight' (Takii Co., Ltd., Kyoto, Japan) and 'Cindy sweet' (Sakata Seed Corp., Yokohama, Japan) were examined. On 5th January, the seeds were sown on vermiculite moistened with water in trays and placed in a growth chamber set at 25°C and 12 h day length. At the third true leaf stage, the seedlings were transplanted into plastic pots (12 cm in diameter) filled with 600 ml of tomato growing medium. The seedlings were then moved to a small plastic house within the glasshouse and fertigated daily with half strength Ohtsuka A solution having an EC of 120–130 $mS \cdot m^{-1}$. At the growth stage where flower buds on the plants were visible, the plants were transferred to a solar mediated fertigation system within the glasshouse as previously described (Yoshida et al., 2007, 2014).

Four plants were maintained for each treatment in each cultivar. Six leaves below the 1st truss were retained on all the plants before treatments commenced. All lateral shoots were removed regularly throughout the growing period except the shoot closest to the flowering truss in the control plants. At the length of 10 cm, these shoots were sampled for real time Ca determination using a hand held Ca^{2+} meter. At anthesis of 5th truss, defoliation treatments were commenced and continued until the 1st fruit on the 10th truss had been sampled for 'Momotaro fight' and 1st fruit on the 15th truss for 'Cindy sweet'. Treatments were:

1. Control where no defoliation was done except for the dead and yellowing leaves to maintain 18–21 leaves at any given time on the plant
2. 15 leaves maintained on the plant
3. 12 leaves maintained on the plant

In this study, the treatments are henceforth referred to as 18-leaves, 15-leaves and 12-leaves, respectively. Sampling was done on the 1st fruit on the 5th–10th truss at 14 and 5th–15th truss at 18 days after anthesis in 'Momotaro fight' and 'Cindy sweet', respectively. Defoliation treatment was done at anthesis of the 1st flower on the youngest truss. Flowers on each truss were thinned to retain 4 and 8 fruits on 'Momotaro fight' and 'Cindy sweet', respectively. Nutrient solution supplied to the plants during the day was monitored within the sampling period and drained solution within 6 hours recorded (Fig.11). At sampling, fruit fresh weight without calyx was measured to determine fruit growth rate. The fruit was then divided equatorially into two portions- proximal and distal, and prepared for Ca extraction. Sequential extraction to water- and hydrochloric acid (HCl)-soluble Ca fractions that served as representatives for (1) apoplastic and cytoplasmic

Ca^{2+}, loosely wall-bound Ca; (2) residual insoluble Ca, respectively, was done, as described in Yoshida et al. (2014). Ca concentration was determined using atomic absorption spectrometry (SPCA-6210, Shimadzu, Kyoto, Japan) and described as µmol· g^{-1} FW.

For shoot Ca determination, sap from 1 cm of the shoot of 18-leaves treatment was squeezed onto a hand held Ca^{2+} meter (LAQUA twin B-751, Horiba Ltd. Kyoto, Japan) and the concentration in ppm was recorded. Fruit growth rate was calculated as fresh weight divided by the number of days after anthesis. Daily Ca transport rate was determined by dividing the Ca amount in fruit by the number of days after anthesis. Concentration of the water-soluble Ca in the distal part of the fruit was determined as the quotient of the Ca concentration in the distal part of the fruit in µmol and the fresh weight of the distal tissue in grams. Number of fruits that showed BER symptoms was recorded. Microsoft Excel spreadsheets were used for data analysis and comparison of means was done using Tukey's test.

4.3 Results

4.3.1 Nutrient absorption and BER incidence

As shown in Figure 11, the plants in all the treatments absorbed over 50% of the nutrient solution supplied. 18-leaves treatment drained less nutrient solution than the 15-leaves and 12-leaves treatments, however, the difference was non-significant. The rate of BER occurrence in this study was generally low, however, the 18-leaves in both cultivars had the highest rates (Fig. 12). No BER incidence was observed in the 12-leaves, and in the 12 and 15-leaves treatments in 'Momotaro fight' and 'Cindy sweet', respectively. In the 18-leaves, BER was higher in 'Momotaro fight' at 10% compared to 2% 'Cindy sweet'.

The earliest BER incidence was observed on the 7th truss of 'Momotaro fight' in the 18-leaves and 15-leaves treatment. The highest rate of BER incidence was observed in 18-leaves, 10th truss. In 'Cindy sweet', the earliest BER incidence was observed in the 10th truss, 18-leaves treatment.

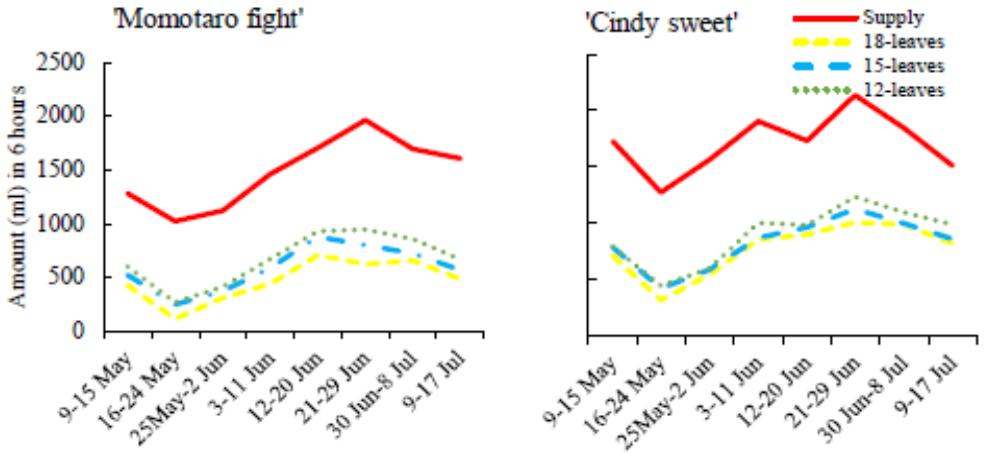

Fig. 11. Changes in the amount of nutrient solution supplied and drained during daytime (6hours) within the sampling period between 9th to 17th July 2018 for two tomato cultivars

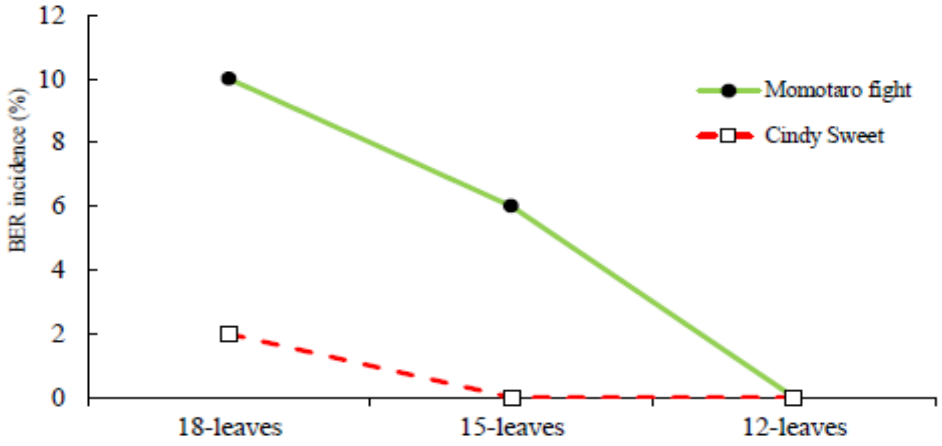

Fig. 12. BER incidence in two tomato cultivars.'Momotaro fight' and 'Cindy sweet' as influenced by number of retained leaves on plants in Spring 2018.

4.3.2 Effect of number of retained leaves on fruit growth rate

Fruit growth rate was higher in 'Momotaro fight' than in 'Cindy sweet' (Fig. 13). In increase in fruit growth in 12-leaves treatment was 21% and 6% in 'Momotaro fight' and 'Cindy sweet', respectively. However, this increase was not significant in 'Cindy sweet' at $P<0.05$.

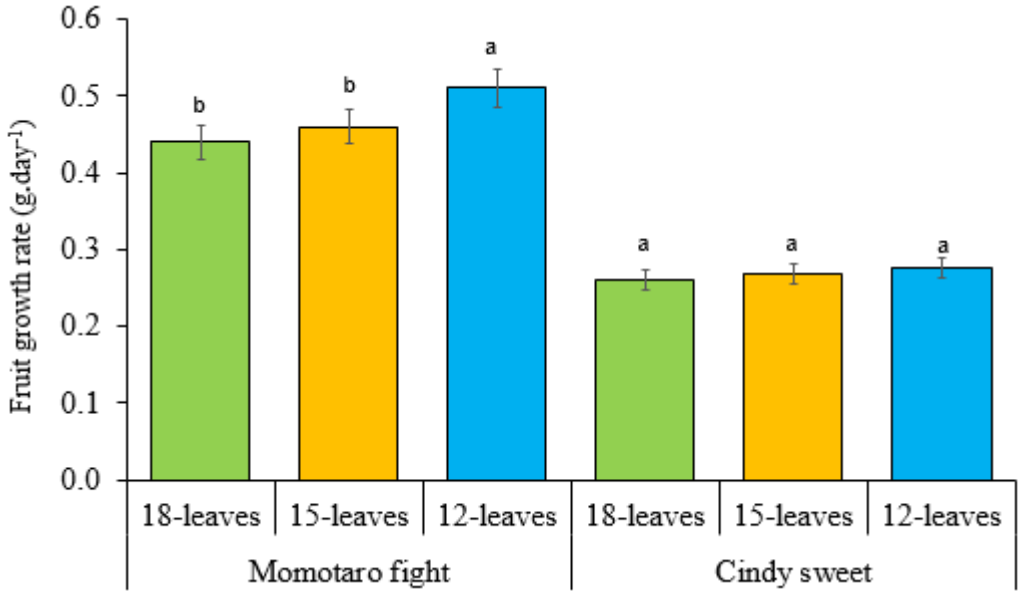

Fig. 13. Fruit growth rate of two tomato cultivars 'Momotaro fight' (n=24, 6 trusses × 4 plants) and 'Cindy sweet' (n=44, 11 trusses × 6 plants) as influenced by number of leaves on a plant. Different letters indicate significant mean differences among treatments within a cultivar by Tukey's test at $P < 0.05$.

4.3.3 Daily Ca translocated into fruit

Daily amount of Ca transported into fruits increased as number of leaves decreased in both cultivars; 'Momotaro fight' and 'Cindy sweet' (Fig. 14). The Ca transport rate was highest in 12-leaves 'Momotaro fight' and lowest in 18-leaves 'Cindy sweet'. There was a significant difference in Ca transport rate between 18-leaves and 12-leaves in 'Momotaro fight'. However, there was no significant difference between 18-leaves and 15-leaves. Defoliating to 12-leaves increased Ca transport rate by 59% and 37% in 'Momotaro fight' and 'Cindy sweet', respectively.

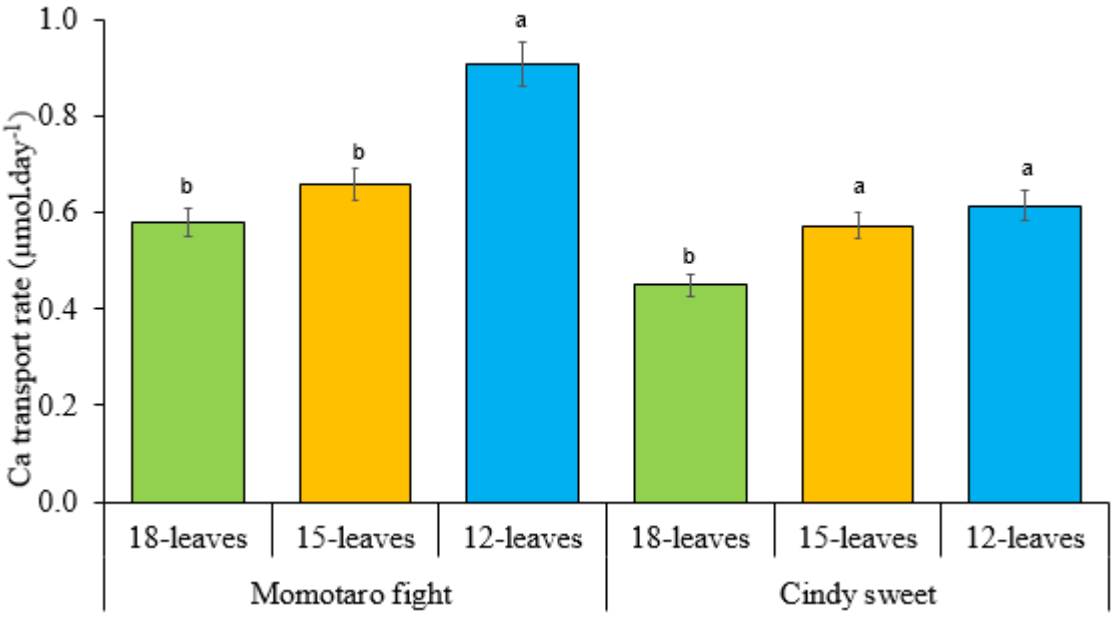

Fig. 14. Daily amount of Ca translocated into fruit in two tomato cultivars 'Momotaro fight' (n=24, 6 trusses × 4 plants) and 'Cindy sweet' (n=44, 11 trusses × 4 plants) as influenced by number of leaves on a plant. Different letters indicate significant mean differences among treatments within a cultivar by Tukey's test at $P < 0.05$.

4.3.4 Concentration of water soluble Ca in the distal part of fruit

Defoliation increased Ca concentration in both cultivars (Fig. 15). The increase was significant among treatments in 'Momotaro fight' but not significant in 'Cindy sweet'. Defoliating to 12-leaves and 15-leaves increased the water soluble Ca concentration in the distal part of fruit by 34% and 23% in 'Momotaro fight', and 14% and 9% in 'Cindy sweet', respectively.

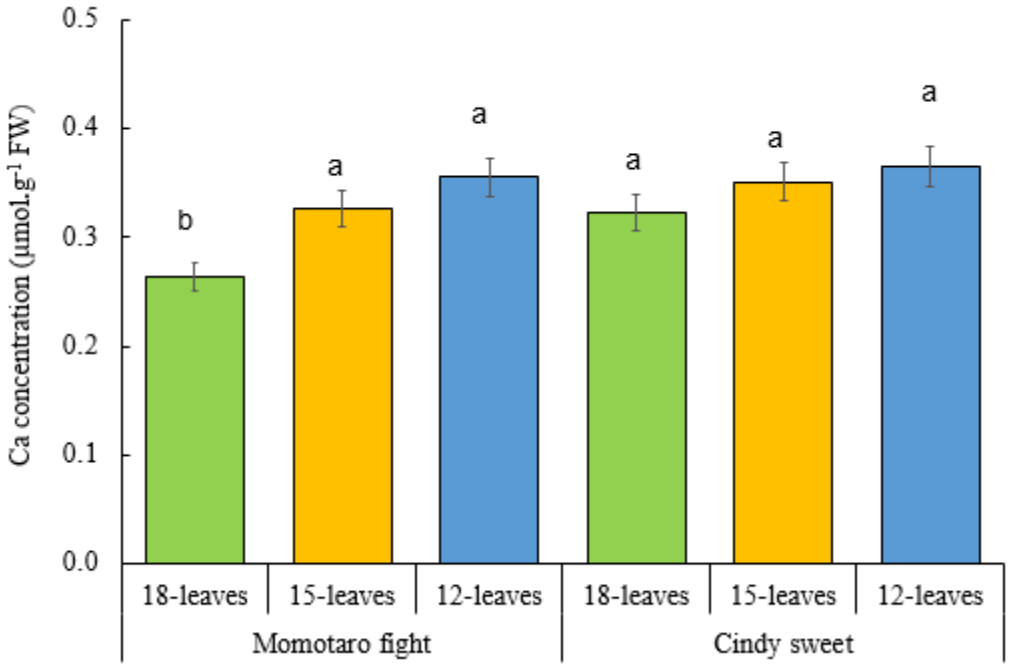

Fig. 15. Water-soluble Ca concentration in the distal part of fruit in two tomato cultivars 'Momotaro fight' (n=24, 6 trusses × 4 plants) and 'Cindy sweet' (n=44, 11 trusses × 4 plants) as influenced by number of leaves on a plant. Different letters indicate

significant mean differences among treatments within a cultivar by Tukey's test at $P < 0.05$.

4.3.5 Concentration of water soluble Ca in the distal part of fruit in non-defoliated plants as influenced by order of inflorescence

There was a significant steady decrease in the concentration of water soluble Ca in the distal part of fruit in 18-leaves plants with increase in truss order (Fig.16). The Ca concentration was highest in the 5th truss and lowest in the uppermost last sampled truss in both cultivars. The decrease in water-soluble Ca concentration between the 5th truss and the 10th truss was 55% and 43% in 'Momotaro fight' and 'Cindy sweet', respectively.

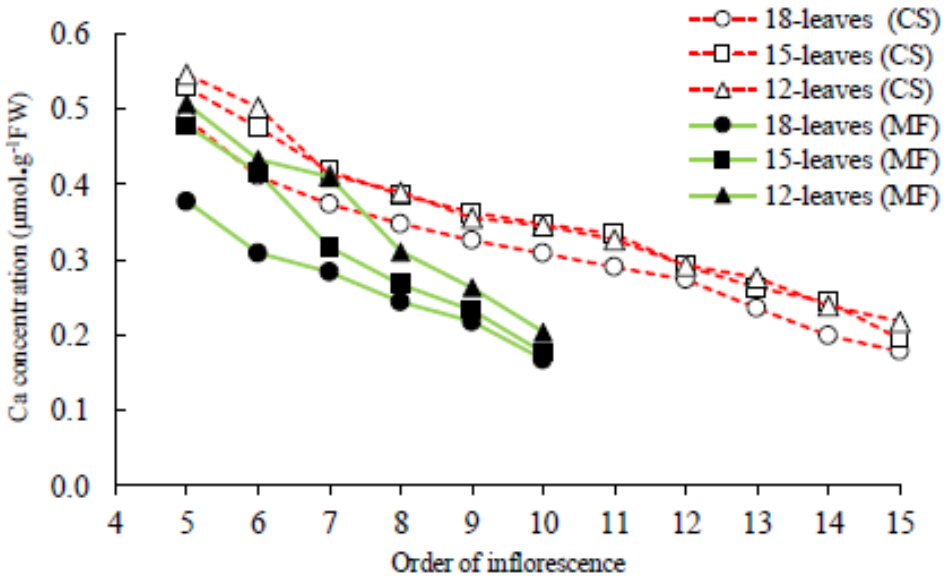

Fig. 16. Water-soluble Ca concentration in the distal part of individual fruit in two tomato cultivars 'Momotaro fight' (MF) and 'Cindy sweet' (CS) as influenced by number of retained leaves on a plant and order of inflorescence.

4.3.6 Real time shoot Ca concentration in 18-leaves tomato plants

Results of the shoot Ca concentration in 18-leaves plants showed that there was more water-soluble, non-structural Ca^{2+} in the shoots of 'Cindy sweet' than that in 'Momotaro fight' (Fig. 17). The Ca^{2+} meter recorded significantly lower concentrations in 'Momotaro fight' with a reading of as low as 1ppm in the 10th truss compared to 4 ppm in the 15th truss of 'Cindy sweet'. However, in both cultivars, shoot Ca concentration and order of inflorescence revealed a significant linear relationship.

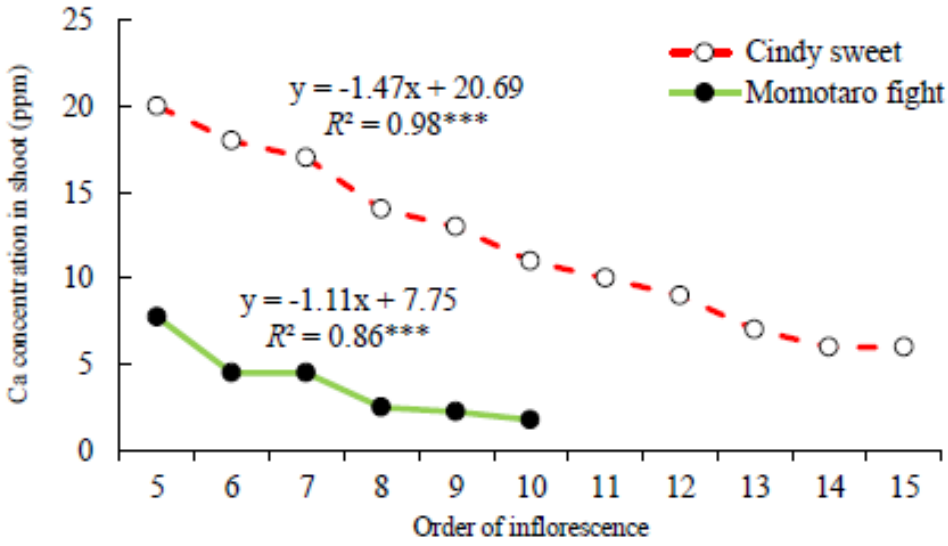

Fig. 17. Shoot Ca concentration of individual plants in two tomato cultivars in 18-leaves plants as influenced by order of inflorescence. *** indicate significance at $P<0.01$

4.3.7 Relationship between shoot Ca and concentration of water-soluble Ca in the distal part of individual fruit in 18-leaves plants

There was a significant linear relationship between shoot Ca concentration and water soluble Ca concentration in the distal part of fruit in non-defoliated plants (Fig. 18). The

coefficient of determination was 64% and 81% in ‘Momotaro fight’ and ‘Cindy sweet’, respectively.

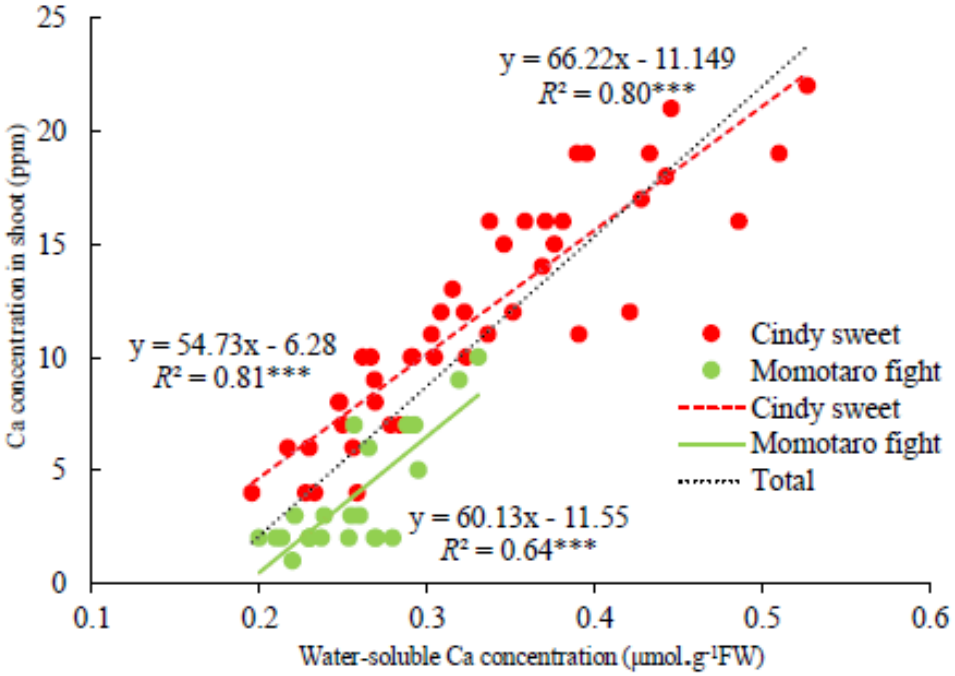

Fig. 18. Relationship between water-soluble Ca concentration in distal part of individual fruit and neighboring shoot Ca concentration in two tomato cultivars in 18-leaves plants. *** indicate significance at $P<0.01$

4.4 Discussion

The objective of this study was first, to determine the effect of number of whole leaves retained on a plant on Ca transport into fruit and BER incidence and second, to assess the relationship between shoot Ca concentration and water-soluble Ca concentration in the distal part of fruit in two tomato cultivars under moderate water stress. In this study, tomato plants were grown under 600 ml/plant of root zone volume in a solar mediated

fertigation system (moderate water stress) and 18-leaves, 15-leaves and 12-leaves were retained on the plant after anthesis of 1st fruit on the 5th truss. It is well known that water stress is associated with the increased risk of BER occurrence (Adams and Ho, 1992; Dekock et al., 1979; Taylor and Locascio, 2004) by restricting water uptake which is the solvent for Ca^{2+} flux, therefore depressing Ca translocation along vascular vessels and then causing a lack of Ca in fruit required for cell structure maintenance. It further increases the risk of BER development by restricting Ca^{2+} uptake and/or reducing transpiration rate which is known as driving force of Ca^{2+}transport together with water flow to fruit (Adams and Ho, 1993; De Freitas et al., 2011; Taylor and Locascio, 2004). However, water stress in the root zone is known to improve the fruit quality by influencing the content and composition of soluble sugars, organic acids, and some amino acids Saito et al., 2008; Sakurai and Oyamada, 1995; Zushi and Matsuzoe, 1998). On the other hand, defoliation has been found to reduce BER incidence in tomato plants grown under moderate water stress (Indeche et al., in press) and also non stressed hydroponic condition (Sato et al., 2004). In this study, BER incidence was below 10%, and the incidence was more prevalent in non-defoliated plants (18-leaves) than in the defoliated plants (15-leaves and 12-leaves) in both cultivars (Fig. 12). Notably, BER incidence was observed in higher trusses as the season progressed which coincided with a decrease in Ca concentration in fruit (Fig. 15). BER development in tomato has been attributed to a low Ca level in the whole plant due to decreased soil Ca supply or root Ca uptake, low transport of Ca to and in the fruit, or an increased demand for Ca due to a high growth rate of the fruit (Ho et al*.,* 1993; Ho and White, 2005). Although the decrease in Ca concentration in the upper trusses was expected to induce BER, BER incidence was low. Studies have shown that for BER to occur, Ca

concentration must fall below a certain threshold (Marcelis and Ho, 1999). The water-soluble Ca concentration in the fruit ranged from 0.53-0.18 μmol·g^{-1} FW in both cultivars, and this may be within the threshold values reported by Vinh et al., (2018).

Cultivar differences in susceptibility to BER were also observed in this study. The large fruit cultivar 'Momotaro fight' which had a faster fruit growth rate was more susceptible than the medium fruit cultivar 'Cindy sweet' (Fig. 13). The importance of growth rate on the resulting Ca concentration of fruit is well demonstrated by several authors in BER development in tomato (Indeche et al., in press; Ooyama et al., 2016; Vinh et al., 2018). In this study, Ca transport rate was higher in the large fruit cultivar than in the medium fruit cultivar and subsequently higher in defoliated plants than in the control (Fig. 14). In comparing the Ca transport rate of different size tomato fruit, the authors found that large fruit cultivars transported potentially more Ca than the small fruit cultivars and suggested that the Ca transporting potential into tomato fruit is proportional to fruit expansion and defoliation may have caused a compensatory physiological effect enabling the fruit to draw in more water hence increasing the Ca transport into the fruit (Indeche et al., in press).

In the 12-leaves plants, fruit had the highest water-soluble Ca concentration in both the large and medium fruit cultivars (Fig. 15). In terms of Ca^{2+} partitioning, conditions that affect the growth rate of storage organs might also affect vegetative plant parts. In other defoliation studies, photosynthate production and distribution into different parts depends on the magnitude of defoliation (Chauhan and Halima, 2003; Hossain et al. 2006). Since vegetative growth, as a powerful sink, consumes produced assimilates, limitation of

vegetative growth enhances assimilate transport to fruits. Bangerth (1979) showed that competition between vegetative and storage organs could well have an influence on Ca distribution. The leaf possesses a higher transpiration rate than the fruit, and often acts as a competing sink with the fruit for directional Ca flow and accumulation (Taylor et al., 2004).

Ca accumulation in tomato fruit has been shown to be dependent on rates of xylem sap flow influenced by transpiration and growth rates (De Freitas and Mitcham, 2012; Ho et al., 1993). In an effort to explore the relationship between shoot Ca and fruit Ca in plants defoliated to 18-leaves, this study revealed that both water-soluble Ca concentration of fruit (Fig. 16) and shoot Ca concentration (Fig. 17) decreased in upper trusses. Since there was a relationship between shoot Ca and fruit Ca (Fig. 18), shoot Ca may be used as a useful tool to predict the fruit Ca status and allow growers to improve the status before BER is triggered. This however is a preliminary study and more experiments need to be done to ascertain the model and further understand the changes in Ca concentration that were observed.

In conclusion, retaining 12–15 leaves on a tomato plant grown under moderate stress reduces BER incidence and increases daily Ca transport rate into fruit and water-soluble Ca concentration of both large and medium fruit tomato cultivars. There is a relationship between shoot Ca and fruit Ca that can be explored in determining the fruit Ca status for management of BER. Even though the number of possible interactions that can affect Ca uptake and distribution is so great, defoliation is one of the simple sustainable cultural practice that can be adopted by growers for management of BER.

CHAPTER 5: CONCLUSIONS AND RECOMMENDATIONS

5.1. Conclusions

1. Defoliation reduced BER incidence, increased fruit growth rate and increased both Ca transport rate and Ca concentration in the fruit in all cultivars and in all seasons. However, the effect of defoliation on these parameters was considerable in large and medium fruit cultivars. This may imply that Ca probably became available for structural functions in the fruit at a point before BER was triggered.
2. Increased Ca concentration in the distal part of fruit due to defoliation was a probable reason why there was decreased BER incidence in the defoliated plants. Most likely, in defoliated plants, sufficient water-soluble Ca accumulated in time within the distal part of fruit, thus maintaining the Ca homeostasis within the fruit.
3. Since defoliation had a larger effect on the Ca transport rate in the large and medium fruit cultivars than in the small fruit cultivar, this could imply that the Ca transporting potential into tomato fruit is proportional to fruit expansion and defoliation may have caused a compensatory physiological effect enabling the fruit to draw in more water hence increasing the Ca transport rate into the fruit.
4. Temperature which is closely correlated to fruit growth rate was steadily significant in explaining Ca transport rate into fruit. This result may indicate that rapid fruit growth may be a dominant factor affecting Ca transport rather than environmental factors except temperature. Other environmental factors directly affecting leaf transpiration may be not effective for non-defoliated plants subjected to moderate water stress.
5. This study supports the theory that under conditions which favor high transpiration of leaves, within the greenhouse, competition for Ca between leaves and fruits can occur

leading to reduced Ca concentration in fruit at critical times resulting in development of BER. The observed increase in Ca transport rate and Ca concentration in defoliated plants is probably a result of reduced transpiration in the leaves and this led to a lesser competition for water between the leaves and fruits causing more Ca to be distributed into the fruit especially in the large and medium fruit cultivars.

5.2 Recommendations

1. To understand effect of defoliation on BER development under moderate water stress, more studies should be done to provide evidence on water and assimilate influxes into fruit.
2. Further studies are required to determine the number of leaves to retain on tomato plant in the other growing seasons.
3. The relationship between water-soluble Ca in the fruit and shoot Ca could be explored in defoliated plants to compare with that in non-defoliated plants, to develop a non-destructive fruit Ca status diagnostic tool.
4. Studies focusing on developing a practical system that can precisely control environmental conditions related to risk of BER occurrence within a greenhouse should be conducted.
5. At cellular level, expression and activity of Ca transporters in fruit of defoliated plants could be determined to further understand the low BER incidence observed.

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