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Biological and chemical processes associated with blossom-end rot development in tomato

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Biological and chemical processes associated with blossom-end rot development in tomato

By

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DISSERTATION

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Dissertation abstract

Blossom-end rot is a physiological disorder which causes significant losses in tomatoes, peppers, watermelons, and eggplants. Though blossom-end rot has been associated with calcium deficiency for decades, abiotic and oxidative stress are also significant factors in blossom-end rot development. The cause of cell death during symptom development is not well understood. This dissertation investigates the biological and chemical processes associated with cell death during blossom-end rot development. Due to confounding factors at the whole plant and fruit level, direct treatment and observation of the relevant tissue during blossom-end rot development is difficult. To improve experimentation, chapter 2 demonstrates and validates a new method for studying blossom-end rot using tomato pericarp discs. Chapter 3 uses greenhouse experiments and pericarp disc experiments to investigate blossom-end rot induced by excess calcium application. Chapter 4 uses the pericarp disc system to characterize the importance and timing of calcium and antioxidant related events during blossom-end rot symptom development. Chapter 5 investigates lignification during blossom-end rot as a result of accumulated reactive oxygen species and increased peroxidase activity in whole fruit affected by blossom-end rot. Overall, this research has provided the first causative evidence that calcium, ascorbic acid, and glutathione are protective against BER symptom development. Furthermore, this research shows that calcium and the ascorbic acid-glutathione antioxidant system are directly connected and both reactive oxygen species generation and calcium signaling are critical steps in blossom-end rot induction. Based on these results, the most efficient gains with regards to reduction in commercial blossom-end rot incidence would likely be made through optimizing calcium translocation specifically to the blossom-end pericarp and enhancing the ascorbic acid-glutathione antioxidant system.

Findings

This dissertation investigates the biological and chemical trends associated with blossom-end rot development. Much of the research presented here heavily utilizes a pericarp disc system to study blossom-end rot development in tomatoes. Thus, understanding and validating trends in the pericarp disc system is critical to the validity of further conclusions. My results demonstrated that blossom-end rot symptom development in the disc system resembled blossom-end rot development in fruit in terms of visual appearance, location, and enzymatic trends. Additionally, this system allowed for collection of causative evidence that was previously rare in blossom-end rot research due to experimental constraints. For early demonstration, calcium and ascorbic acid were shown to inhibit blossom-end rot symptom development. Constraints of the disc system include wounding effects and cessation of cell expansion that may confound results. However, the occurrence of BER symptoms in the disc system is particularly interesting considering BER occurs during cell expansion and is associated with larger fruit size. With xylemic and phloemic supplies of water and nutrients halted, cell expansion is halted or greatly reduced in the disc system. Furthermore, discs incubated in air lost weight and developed symptoms during storage, while discs stored in water gained weight but did not develop symptoms during storage. This is strong evidence that a high cell expansion rate itself is not the cause of BER, but rather factors associated with rapid cell expansion. These could include dilution of the calcium supply, increased water and nutrient demand, and increased gibberellin biosynthesis.

The role of calcium in inhibiting blossom-end rot development has been hotly debated over the last two decades. To address this debate, the enigmatic phenomenon of increased blossom-end rot development due to excess calcium application was investigated. Application of excess calcium in the irrigation solution increased BER symptom severity compared to a moderate

irrigation calcium concentration and did not result in increased calcium uptake to the fruit. Conversely, calcium treatment of the pericarp discs resulted in a dose dependent inhibition of blossom-end rot symptoms with no upper limit. These results confirm calcium's protective action against blossom-end rot development and show that overapplication of calcium increases blossom-end rot severity through whole plant effects. Similarly, abscisic acid and gibberellin treatments did not affect blossom-end rot symptom development in the disc system, suggesting the critical effects of these phytohormones may be at the whole plant rather than cellular level.

Further investigation of the mechanism of calcium and ascorbic acid's protective affect was carried out using the disc system. Results demonstrate that directly increasing calcium in the pericarp tissue increases ascorbic acid and glutathione contents. These results, along with the inhibition of BER symptom development by glutathione treatment, indicate that the protective effect of calcium against BER development is at least in part related to enhancement of the ascorbic acid-glutathione antioxidant system. Furthermore, cellular process associated with early BER development can be stopped or slowed with calcium or ascorbic acid treatment. While production of reactive oxygen species is likely directly related to the induction of BER, H_2O_2 accumulation did not cause BER symptom development when adequate calcium was available. Results from whole fruit experiments indicate that lignification occurs during blossom end rot, likely through increased peroxidase activity and H_2O_2 production. Lignification was not increased in the healthy stem-end tissue of BER affected fruit and may function to protect the healthy portion of the fruit from water loss and microbial invasion. Results from calcium channel blockers indicate that calcium signaling is likely also associated with BER induction.

Overall, this research has provided the first causative evidence that calcium, ascorbic acid, and glutathione are protective against BER symptom development. Furthermore, this research shows

that calcium and the ascorbic acid-glutathione antioxidant system are directly connected and both reactive oxygen species generation and calcium signaling are critical steps in blossom-end rot induction. Further research into critical signaling pathways during blossom-end rot induction would improve our knowledge of calcium deficiency in plants as a whole and may lead to more effective treatments in the future. However, the most efficient gains with regards to reduction in commercial blossom-end rot incidence would likely be made through optimizing calcium translocation specifically to the blossom-end pericarp and enhancing the ascorbic acid-glutathione antioxidant system.

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Contents

Chapter 1: Introduction

1.1 Blossom-end rot: overview

Blossom-end rot (BER) is a physiological disorder affecting tomatoes, peppers, eggplants, and watermelons. BER causes significant produce loss and frustration from home gardens to large-scale operations. Tomato processor estimates of blossom-end rot incidence range from 0.5-5% annually, with individual fields exhibiting up to 15% blossom-end rot (Hamilton, Timothy. Interview by Nicholas Reitz, 2017, Hagassou et al., 2019). With an estimated 12 million tons of processing tomatoes produced yearly in California, up to 600,000 tons of processing tomatoes are affected by BER in California alone (United States Department of Agriculture and National Agricultural Statistics Service, 2018). Greenhouse grown tomatoes are also affected, despite the potential to provide optimal environmental conditions and plant nutrition (Nonami et al., 1995; Penn State Extension, 2020). Due to the unsightly appearance of BER fruit and potential interference with processing, affected tomatoes cannot be used for fresh market, whole peeled canned, or diced canned products, and are often discarded. With BER, cell death occurs at the distal end during tomato fruit development. Visual symptoms include water soaking of the tissue that spreads most often from a single point. Low soil calcium, high soil salinity, and water stress are environmental factors often associated with increased BER incidence (de Freitas & Mitcham, 2012).

A large body of background research on tomatoes is available, including investigations of growth and ripening processes, sequencing and annotation of the genome (Hosmani et al., 2019), cataloging of numerous mutants, and development and characterization of extensive germplasm resources. Notably, the Tomato Genetic Resource Center at UC Davis has a wealth of resources

including an annotated database of lines available for research use ("Tomato Genetics Resource Center," n.d.). Well established protocols exist for genetic transformation and regeneration of tomato plants (Rothan et al., 2019). As such, BER in tomatoes is often used as a model for studying calcium deficiency disorders in other horticultural crops, including tipburn in lettuce and bitter bit in apples.

No microorganism is known to be associated with BER development. While BER is commonly assumed to be unrelated to microorganisms in or on the fruit, it is worth noting that the author could not find a specific experiment demonstrating this in 4 years of familiarization with the literature. With an ever-growing body of research supporting the importance of the plant microbiome in general plant physiology, and specifically to BER development (Shao et al., 2018), further investigation may be warranted. Such experimentation is not presented here as it does not align with the purpose of the dissertation research or the expertise of the author.

Calcium deficiency, abiotic stress, oxidative stress, phytohormone regulation, and rapid cell expansion have all been implicated in the development of BER (Figure 1.1). Since increased BER incidence under low calcium was first reported (Lyon et al., 1942), BER has been considered a calcium deficiency disorder. As such, most research during the 20th century on BER was focused on calcium uptake and transport (Adams and Ho, 1993; Ho et al., 1995, 1993; Nonami et al., 1995). Under many conditions, low calcium can reliably induce BER and is often used in research to induce BER. However, fruit calcium concentrations do not always correlate well with BER incidence and increasing soil calcium is not fully effective at reducing BER incidence. This tenuous relationship between calcium and blossom end rot has led some studies to highlight other factors, such as abiotic and oxidative stress, as important for blossom-end rot development (Aktas et al., 2005; Aloni et al., 2008; Casado-Vela et al., 2005; de Freitas et al.,

2017; Mestre et al., 2012). In general, adverse conditions, such as water deficit and salt stress, increase BER incidence. Accumulation of reactive oxygen species and depletion of antioxidant systems are associated with BER affected tissues. The role of abiotic stress and oxidative stress is discussed in detail in sections 1.3 and 1.4, respectively.

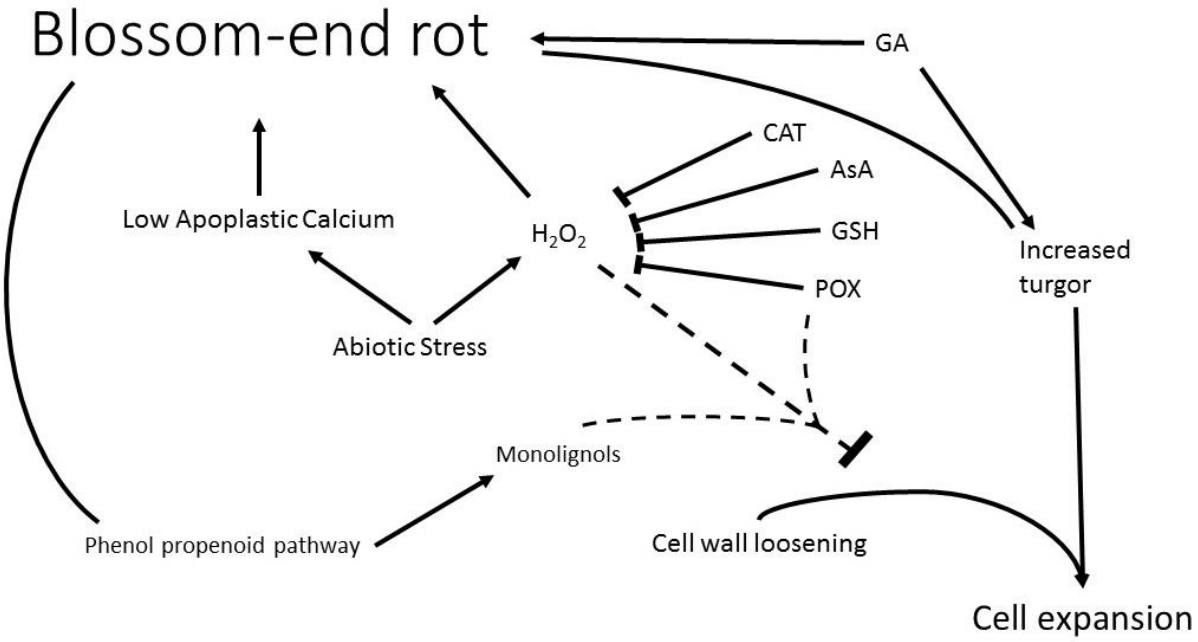

Figure 1.1: Physiological events associated with blossom-end rot development. Arrows indicate an increase, upregulation, or promotional effect. Block end lines indicate a decrease, downregulation, or inhibition. Lines without ending shapes indicate an association. Abbreviations: GA=gibberellic acid, CAT=catalase activity, AsA=ascorbic acid, GSH=glutathione, POX=peroxidase, H_2O_2=hydrogen peroxide.

Fruit and cell expansion rates are also important factors in blossom-end rot development. Tomato fruit development occurs in 3 distinct phases, cell division, cell expansion, and ripening

(Gillaspy et al., 1993). During the cell division phase, the primary mechanism for fruit growth is cell division. During the subsequent phase, cell division largely ceases and cell expansion is the main driver of fruit volume increase until the fruit reaches near its final size. The fruit then reaches maturity and begins the ripening process. Blossom-end rot development occurs exclusively during the cell expansion phase (Ho and White, 2005). Riboldi et al. (2018) found that tomato varieties with elongated fruit had increased BER incidence, likely due to factors reducing calcium transport to the distal portion of the fruit. Specifically, xylem functionality, which is often measured as the number of stainable xylem vessels in the blossom-end of the fruit, has been highlighted as important to reducing BER development. Riboldi et al. (2018) found that xylem functionality was higher in tomato varieties that were less susceptible to blossom-end rot. Additionally, the phytohormones gibberellic acid and abscisic acid have been shown to increase and decrease blossom-end rot development, respectively (de Freitas et al., 2017, 2014). Gibberellic acid promotes rapid cellular expansion, while abscisic acid reduces leaf transpiration and increases the number of functional xylem vessels in the fruit (de Freitas et al., 2011b).

Due to the complex network of factors affecting BER development, causative evidence has been difficult to collect on pathways associated with blossom-end rot. For example, increasing soil calcium can reduce blossom-end rot. However, fruit tissue calcium is often not well correlated with BER incidence (Aslani et al., 2020; Ho and White, 2005), and excess calcium can also result in increased BER incidence (Nonami et al., 1995). Similarly, ROS accumulation and antioxidant depletion are associated with BER development (Aktas et al., 2005; Aloni et al., 2008; Mestre et al., 2012).Whether these are causative factors or downstream effects of cell death has still not been determined. This lack of causative evidence is due in part to the difficulty of treating the affected tissues with compounds of interest without confounding affects at the

fruit and whole plant levels. To improve treatment delivery and observation, a pericarp disc system was developed that allows for direct treatment and observation of the relevant tissue during symptom development (chapter 2). The results presented in chapters 2, 3, and 4 demonstrate that calcium and ascorbic acid have a powerful and interactive protective effect when applied to the fruit system. The nature and timing of these effect is explored, as well as their relation to effects at the whole plant level.

1.2 Role of calcium in blossom-end rot development

Calcium has long been associated with BER development and extensive research has been carried out examining the relationship between BER development and calcium. Recommendations for calcium concentrations in fertigation solutions include 328-360ppm (Kreij, 1996) and 61-105ppm (Bar-Tal et al., 2017). However, because many factors can affect calcium requirements and use, the optimal concentration likely depends on the variety grown, and environmental conditions. Because calcium is phloem immobile and moves through the plant primarily through the xylem, factors affecting transpiration are of particular importance. In a thorough investigation of factors affecting calcium uptake, Adams and Ho (1993) found that root temperature and solar radiation were well correlated with calcium uptake. Salinity reduced fruit calcium uptake in this same study, and calcium concentrations were lower in the distal pericarp and placental tissue compared to the proximal tissue. Ho et al. (1993) found that a BER susceptible variety of tomato had less calcium uptake into the distal part of the fruit at high salinities compared to lower salinities. Additionally, safranin staining showed reduced functional xylem vessels in the distal portion of fruit at high salinities. BER incidence increased with irradiance and temperature. While Nonami et al. (1995) found that a calcium gradient existed between the blossom-end and calyx of the fruit, fruits showing early signs of BER had similar

calcium concentrations and distributions compared to healthy fruits. Furthermore, BER was unrelated to calcium uptake when comparing between varieties. Testing treatments over multiple years, Taylor et al. (2004) found differing results between years. Overall, they found that BER was increased by lower transpiration, increased rainfall, and reduced calcium uptake. Root restriction has been found to decrease BER incidence, possibly due to changes in hormone sythesis (Bar-Tal and Pressman, 1996). Increasing potassium soil concentration increases BER incidence, likely by reducing calcium uptake. Taken together, these results indicate an inconsistent link between soil calcium and blossom-end rot. More important are the environmental factors affecting calcium uptake, such as solar radiation, relative humidity, temperature, and soil salinity.

Sprays of calcium can be partially effective in reducing BER development (Liebisch et al., 2009; Schmitz-Eiberger et al., 2002). The incomplete inhibition is likely due to calcium's phloem immobile nature. Because calcium is transported through the xylem, sprays applied to the leaves would not result in increased fruit calcium concentrations. Calcium absorbed through the fruit exocarp, however, could mitigate BER development. The inclusion of adjuvants improves the effectiveness of foliar sprays, possibly by improving calcium uptake into the fruit tissue (Schmitz-Eiberger et al., 2002).

Morphology and calcium partitioning at the cellular level during blossom-end rot development has also been studied. (Suzuki et al., 2000) found that the cells in the water-soaked area had disrupted plasma membranes, wavy cell walls, breakdown of the endoplasmic reticulum, and swollen plastids. Using antimonate precipitation and transmission electron microscopy, (Suzuki et al., 2003) found that calcium around the plasma membrane was decreased in BER affected cells and increased in healthy cells the further from the BER affected tissue they were located.

Some of the most compelling evidence of calcium's role in BER is from two studies that examined the effect of expressing an Arabidopsis vacuolar H+/cation antiporter (*CAX1*) in tomato plants (de Freitas et al., 2011; Park et al., 2005). *CAX1* expression in tomato fruit increased calcium transport into the vacuole, increasing total fruit calcium. However, this process also drastically increased the incidence of BER. A deeper look at calcium localization found low apoplastic calcium to be a key factor in BER development, rather than total tissue calcium (de Freitas et al., 2011; de Freitas et al., 2012).

Despite decades of research, understanding of calcium's relationship with BER is still incomplete. Of particular interest are reports of increased BER incidence when excess calcium is applied to the soil (Gholipour et al., 2017; Nonami et al., 1995). The lack of a critical calcium threshold for the development of BER has also been cited as evidence of calcium's inconsequential role in BER development (Saure, 2001, 2014). However, tomato fruit are extremely diverse in their size, shape, growth characteristics, and chemical constituents. Fruit can vary greatly even within a single fruit cluster on a single plant of a single variety. This was observed throughout this dissertation research and a good example image can be found in figure 7 of (Schmitz-Eiberger et al., 2002). As such, the expectation that a single critical calcium concentration could be found to predict BER development is unrealistic. Additionally, as discussed below, abiotic stress is well established as another key factor in BER development. Rather than calcium deficiency causing BER induction, it is possible that increased calcium incurs a protective effect against abiotic stresses and thus BER development. The calcium level needed to inhibit BER induction could vary with the level of the stress, further explaining the lack of a single critical concentration.

1.3 Role of abiotic stress during blossom-end rot development

Calcium status of the cell or fruit is not the only factor to consider in the development of BER, with abiotic stress also playing a key role in BER incidence (Aktas et al., 2005; Cantuário et al., 2014; Dekock et al., 1982; Turhan et al., 2006a). Environmental conditions can have a significant effect on BER incidence. (Adams and Ho, 1993) found that high relative humidity decreased calcium uptake to the leaves but increased calcium uptake into the fruit. Conversely, (Kreij, 1996) found that decreased relative humidity favored increased BER incidence. (Turhan et al., 2006a) reduced BER incidence in peppers through ventilation cooling of the greenhouse growing environment. Amount and frequency of irrigation can also affect BER. Silber et al. (2005) found that higher frequency of fertigation increased nutrient uptake despite receiving the same daily quantity of fertigation. Increased fertigation frequency decreased BER development. Manganese was also highlighted as important to reducing BER. Even with low calcium fertigation, de Freitas et al. (2014) found that the induction of BER for research purposes was best accomplished through daily water stress.

Soil salinity can also greatly affect BER incidence. (Adams and Ho, 1992) found a clear trend of reduced calcium uptake due to increased salinity. However, inconsistent trends were observed between 5, 10, and 15 mS cm^{-1} saline treatments in early and late harvests. In early harvests, the 10 mS cm^{-1} treatment exhibited the highest BER incidence, while in the late harvest the 15 mS cm^{-1} had the highest BER incidence. In a follow up study (Adams and Ho, 1993), calcium uptake was found to decrease linearly with salinity. Increased BER with excessive nutrient concentrations, including calcium, was reported. Fruit grown under these conditions did not accumulate additional calcium, despite having additional soil calcium available (Adams and Ho, 1993). In contrast, Rubio et al. (2009) found for pepper plants grown with increased NaCl,

adding more calcium incurred a protective effect against BER. (Willumsen et al., 1996) used cation activity ratios to better understand the effect of ions in the soil on BER incidence, with the comparison between calcium and magnesium levels being particularly important. (Ho et al., 1995) compared calcium-efficient and calcium-inefficient plants and found that BER incidence was not always well correlated with calcium uptake efficiency, particularly when saline stress was applied. These studies combined show that salinity is consistently effective in inducing BER development. Calcium is sometimes, but not always, effective in protecting against salinity induced BER development, and is conspicuously ineffective when calcium itself is one of the salts inducing salt stress.

Application of excessive amounts of calcium can also increase BER incidence, leading some to question the critical nature of calcium concentrations to BER development. When the calcium-zeolite hydroponic solution concentration was increased from an optimal 20g/L to 40g/L, Nonami et al. (1995) found a slight increase in BER incidence in two tomato varieties. The suggested explanation was osmotic effects under high calcium concentrations. The EC of the 40g/L calcium zeolite was ranged from approximately 11mS/cm to 30mS/cm throughout the experiment. In further investigations, Hossain and Nonami (2012) found decreased water potential, osmotic potential, and fruit growth rate when the electric conductivity (EC) of the irrigation solution was raised to 8 mS/cm by adding $CaCl_2$, compared to a more moderate amount of calcium provided in a standard nutrient solution (EC=1 mS/cm). However, it is still not fully understood whether high calcium-induced BER development is due to effects at the whole plant level or high concentrations of calcium ions in the fruit. A better understanding of the limits of calcium's protective effect against BER when applied through irrigation would benefit both the scientific understanding of this disorder and the practical application of calcium

for the prevention of BER. Chapter 3 of this dissertation investigates BER induced by excess calcium at the whole plant and fruit tissue level.

1.4 Role of oxidative stress during blossom-end rot development

Recent studies have investigated the role of reactive oxygen species (ROS) and oxidative stress as it relates to BER and calcium concentrations. Figure 1.2 shows a visual representation of ROS metabolism assembled from a review of the literature.

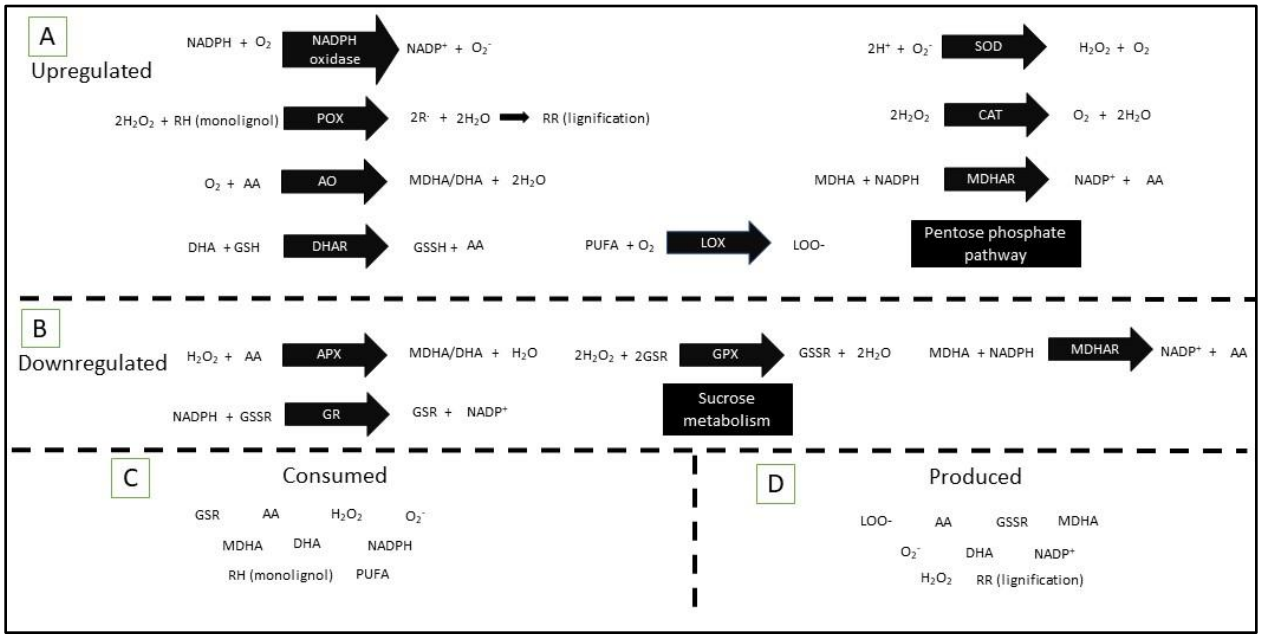

Figure 1.2: Biochemical steps and pathways in affected fruit tissue associated with blossom-end rot development. Arrows indicate individual enzymes and boxes indicate pathways. (A) Biochemical steps and pathways upregulated or increased in blossom-end rot affected tissue compared to similarly located tissue in healthy fruit. (B) Biochemical steps and pathways downregulated or decreased in blossom-end rot affected tissue compared to similarly located tissue in healthy fruit. (C) Substrates that are consumed by upregulated or increased processes. (D) Substrates that are produced by upregulated or increased processes during blossom-end rot

development. Nicotinamide-adenine di-nucleotide phosphate=NADPH (reduced) or $NADP^+$ (oxidized), oxygen=O_2, proton=H^+, superoxide dismutase=SOD, hydrogen peroxide=H_2O_2, hydrogen donors (specifically monolignols)=RH (reduced) or $R^{\cdot}$ (oxidized), POX=peroxidase, lignification=RR, ascorbic acid=AA, ascorbate oxidase=AO, mono/di dehydroascorbate=MDHA/DHA, water=H_2O, mono dehydroascorbate reductase=MDHAR, glutathione=GSH (reduced) or GSSG (oxidized), dehydroascorbate reductase=DHAR, polyunsaturated fatty acids=PUFA, lipoxygenase=LOX, peroxidized lipid=$LOO^{\cdot}$, APX=ascorbate peroxidase, GPX=glutathione peroxidase, GR=glutathione reductase

ROS production can occur through multiple pathways in the plant cell, including from solar energy capture, mitochondrial function, and concerted enzymatic production (Corpas et al., 2015). The latter has been the most studied during BER development, with NAD(P)H oxidases playing a key role. NAD(P)H oxidases, also referred to as respiratory burst oxidase homologs (RBOH), produce reactive oxygen species in response to abiotic and biotic stress. (de Freitas et al., 2017) found upregulation of the RBOH gene RBOHD in fruit susceptible to BER. RBOHD is associated with hydrogen peroxide (H_2O_2) accumulation in the presence of pathogens (Morales et al., 2016) and is part of the calcium and ABA regulated pathway in stomatal guard cells (Kwak et al., 2003). However, overexpression of RBOHD does not necessarily result in ROS accumulation, suggesting posttranscriptional regulation of activity. Specifically, calcium binding and calcium dependent protein kinase activity has been implicated in RBOH activation (Kobayashi et al., 2007). NAD(P)H oxidases shuttle a proton and an electron taken from NAD(P)H over the plasma membrane to protonate a diatomic oxygen molecule, forming superoxide and $NAD(P)^+$. NAD(P)H oxidases were characterized in relation to salinity-

associated BER development by (Aktas et al., 2005). They found that BER fruit and fruit grown under increased salinity exhibited increased NAD(P)H oxidase activity. The addition of zinc, manganese, and high amounts of calcium in the reaction solution reduced NAD(P)H activity. Interestingly, a moderate concentration of calcium increased NAD(P)H oxidase activity compared to low and high calcium concentrations.

ROS-related enzymes are also differentially active during BER development. While (Schmitz-Eiberger et al., 2002) reported reduced leaf superoxide dismutase activity in low calcium treatments, (Mestre et al., 2012) and (Casado-Vela et al., 2005) reported increased activity and protein levels, respectively, in low calcium treatments. Because SOD carries out the conversion of superoxide to hydrogen peroxide, an increase in activity would result in higher H_2O_2 accumulation while a decrease would favor superoxide accumulation. While either may lead to cellular damage, previous reports of accumulated H_2O_2 suggest that H_2O_2 is being produced, likely through SOD activity (Mestre et al., 2012).

While regulated production of reactive oxygen species is involved in stress signaling and mitigation (Baxter et al., 2014), excessive accumulation of ROS in biological systems can be damaging to cellular membranes. Lipid peroxidation is increased in calcium deficient tomatoes and leaves compared to tomatoes and leaves with adequate calcium (Mestre et al., 2012; Schmitz-Eiberger et al., 2002). (Schmitz-Eiberger et al., 2002) found that calcium sprays fully eliminated this increase in lipid peroxidation. Interestingly, these same calcium sprays were not fully effective at reducing BER incidence, indicating the connection between lipid oxidation and BER may not be perfect.

There have been varying reports on the relationship of abscisic acid to BER development. (Schmitz-Eiberger et al., 2002) found reduced amounts of ascorbic acid in the leaves of calcium-

deficient tomato plants (Aloni et al., 2008) found similar results in BER affected peppers. (Rached et al., 2018) found that BER resistant varieties had increased ascorbic acid contents when BER inducing salt stress was applied. However, (Mestre et al., 2012) found similar levels of ascorbic acid between calcium deficient tomatoes exhibiting BER and healthy fruit grown with higher calcium concentrations in the hydroponic solution. The dynamic activity of ascorbic acid-related enzymes discussed below may present a fuller picture of ascorbate-glutathione metabolism as it relates to BER development. (Schmitz-Eiberger et al., 2002) found that tomatoes from treatments with increased BER incidence also had reduced leaf tocopherol levels, demonstrating possible effects of antioxidants outside the ascorbate-glutathione system.

An increase in ascorbate oxidase gene expression (de Freitas et al., 2017) and activity (Aloni et al., 2008) has been reported in BER affected fruit. Interestingly, mono- and di-dehydroascorbate reductase activities were increased in calcium deficient fruit (Mestre et al., 2012). This favors the regeneration of ascorbate. In conjunction with increased ascorbate oxidase, this could form a cyclical reaction of ascorbate oxidation and reduction, with the glutathione being consumed as a result.

Glutathione concentrations and glutathione reductase activity were also decreased in calcium deficient fruit. Furthermore, BER is associated with impairment of sugar metabolism in fruit (Turhan et al., 2006a), which would limit the production of NAD(P)H as a substrate for glutathione reductase. It is worth noting that proteomic analysis uncovered an increase in pentose phosphate related proteins in BER affected fruit (Casado-Vela et al., 2005). Because sugar metabolism is upstream of the pentose phosphate pathway, the production of NAD(P)H as a result of increased pentose phosphate pathway related proteins could be limited. Further research into NAD(P)H levels and metabolism may yield insights into the redox and energy balance

within cells developing BER. Depletion of ascorbic acid and glutathione could lead to a reduction in the cell's ability to mitigate the accumulated H_2O_2. Glutathione is a key component in cell detoxification, and increased glutathione-S-transferase expression may help BER resistant tissues mitigate toxic effects (de Freitas et al., 2011a).

Reductions in ascorbate/glutathione contents and catalase activity (Aloni et al., 2008; Mestre et al., 2012), paired with an increase in peroxidase activity, suggests a peroxidase dependent pathway for consumption of hydrogen peroxide. However, peroxidase requires electron donors to facilitate H_2O_2 consumption. One possible electron donor group is lignin precursors, called monolignols. The radicalization of monolignols by peroxidase causes lignin cross linking and cell wall stiffening (Passardi et al., 2004). Peroxidase activity is greatly increased in the leaves of tomato plants grown under low calcium growth conditions (Schmitz-Eiberger et al., 2002). Additionally, the monolignol biosynthesis pathway and peroxidase genes have been shown to be upregulated in BER susceptible *CAX1* tomatoes (Freitas et al., 2011). (Gholipour et al., 2017) also reported increased levels of a lignin precursor. Lignification during BER development could explain reports of disrupted cell expansion and increased cell wall rigidity during blossom end rot (Gholipour et al., 2017). Figure 1.3 shows the localization and mechanism of ROS production and consumption in relation to possible lignification during BER development. The research presented in chapter 5 investigates the timing and tissue localization of lignification during blossom end rot.

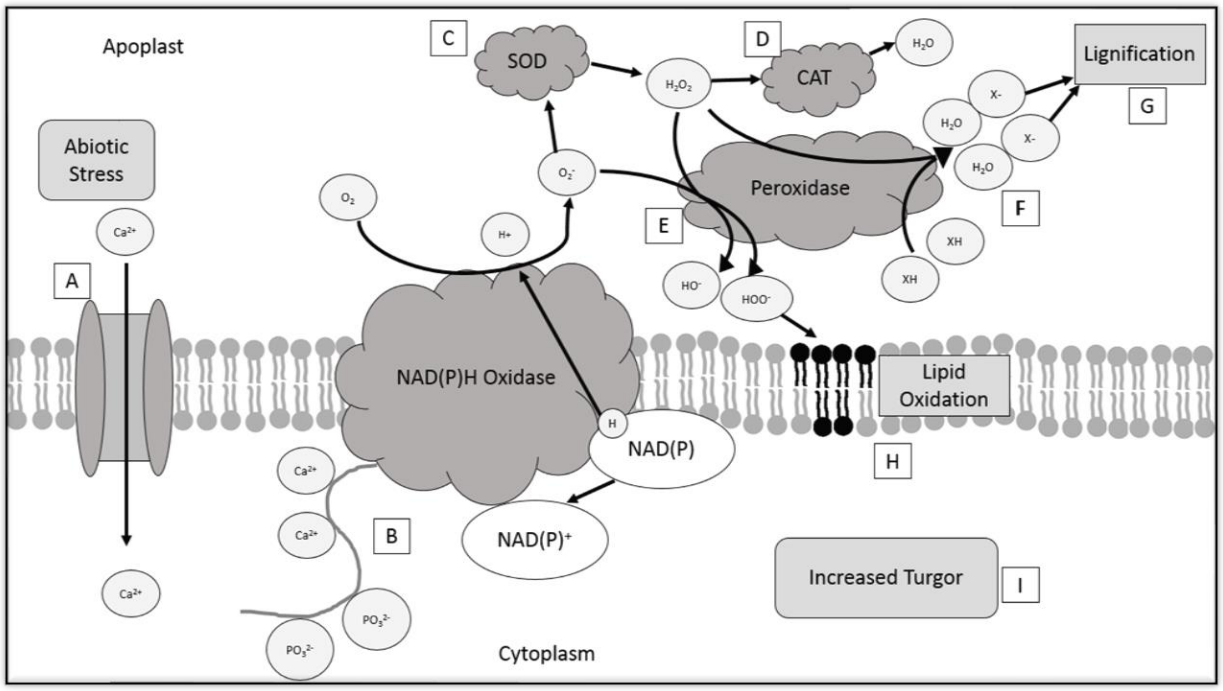

Figure 1.3: ROS metabolism and hypothesized method of lignification in BER. Information compiled from recent studies on BER and calcium deficiency in tomatoes and peppers. A) Abiotic stress and reactive oxygen species induce an influx of calcium into the cytosol. B) NAD(P)H oxidases generate superoxide (O^-) through the oxidation of NADH or NADPH. NAD(P)H oxidase activity is increased in BER tomato pericarp compared to healthy tissue, possibly due to direct calcium binding and calcium dependent phosphorylation (PO_3^{2-}) (Aktas et al., 2005; Mestre et al., 2012). C) Superoxide is reduced to hydrogen peroxide (H_2O_2) through superoxide dismutase. Superoxide dismutase activity is increased in BER tissue compared to healthy tissue and BER tissue exhibits increased apoplastic hydrogen peroxide accumulation (Mestre et al., 2012). D) Catalase reduces hydrogen peroxide to water and oxygen. Catalase activity is decreased in tomatoes grown under low calcium conditions (Mestre et al., 2012). E) Peroxidase facilitates the reduction of superoxide to highly reactive hydroxyl (HO^-) and hydroperoxyl radicals (HOO^-). F) Peroxidase catalyzes the consumption of hydrogen peroxide to

form water, radicalizing two hydrogen donors (hydrogen donors=XH, radicalized hydrogen donors=$X^{\cdot}$). Donors include antioxidants, such as ascorbic acid and glutathione, and monolignols, the biosynthetic pathway of which is upregulated in BER affected tomatoes (de Freitas et al. 2011). G) Peroxidase catalyzed monolignol radicalization leads to monolignol cross-linking, lignification of the cell wall, and increased cell wall rigidity. H) Phospholipid oxidation by reactive oxygen species decreases membrane integrity and increases membrane leakage, a characteristic of BER incidence. I) Cells undergoing cell death processes during BER development are shown to have increased turgor, associated with reduced osmotic potential and increased cell wall rigidity (Gholipour et al., 2017).

1.5 What's in a name?

For decades BER has been considered a calcium deficiency disorder. As such, much of the research associated with BER has been conducted in relation to calcium. While our understanding of blossom-end rot has become more nuanced, most of the popular mitigation recommendations are still related to calcium application. However, salinity and irrigation management are also integral to control of BER. Perhaps most importantly, conversations regarding BER among gardeners, producers, processors, and researchers is dominated by calcium. This is at least partially a function of the ubiquitous association between BER and calcium deficiency, and has led to a misconception that the cause of BER is known and well understood. In conversations with producers, the author has been asked what there was left to study in blossom end rot since it was known to be a calcium deficiency disorder. However, BER still persists in these same producer's fields, and tons of tomatoes are still lost each year. It is well past time to break the word association of blossom-end rot and calcium among horticultural professionals. The phrases "abiotic stress disorder" or "stress related disorder" adequately

summarize the non-microbial nature of the disorder, while leaving space for BER's diverse contributing factors.

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Chapter 2: Validation and demonstration of a pericarp disc system for studying blossom-end rot of tomato

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Abstract

Background: Blossom-end rot in tomatoes is often used as a model system to study fruit calcium deficiency. The study of blossom-end rot development in tomatoes has been greatly impeded by the difficulty of directly studying and applying treatments to the affected cells. This manuscript presents a novel method for studying blossom-end rot development after harvest in immature whole fruit and in pericarp discs.

Results: Pericarp discs removed from the bottom pericarp of immature healthy fruit developed blossom-end rot like symptoms, corresponding to a decrease in L* value and an increase in a* value. Symptoms also developed in columella tissue, but not in stem-end pericarp tissue, similar to patterns observed during blossom-end rot development on the plant. Ascorbate oxidase and peroxidase activity, which are elevated in blossom-end rot affected fruit compared to healthy fruit, were both correlated with colorimetric measures of tissue darkening in discs. Respiration rate was higher in discs that later developed blossom-end rot symptoms, with increased respiration in asymptomatic discs on day 1 of storage being associated with symptom development on day 2. Calcium chloride and ascorbic acid treatments inhibited symptom development, demonstrating the potential of this method to provide causal evidence.

Conclusions: Results indicate that symptom development in this system is consistent with blossom-end rot development with regards to location, color change, and the activity of key enzymes. This system has the potential to be used to elucidate the cause of fruit calcium deficiency and improve knowledge of the biological basis for calcium's diverse effects on fruit.

2.1 Introduction

Blossom-end rot (BER) in tomatoes and peppers causes significant losses in the vegetable industry each year. BER is characterized by water-soaking of the tissue and cell death in the blossom-end pericarp. This is followed by blackening and sometimes drying of the affected tissue. BER occurs during fruit development prior to physiological maturity (de Freitas and Mitcham, 2012). While calcium deficiency in fruit tissue and abiotic stress can increase BER, the biological mechanism of cell death remains unknown and current treatments are not fully effective.

Many previous studies on the biological drivers of BER development in tomatoes and peppers have relied on comparing measurements from BER affected fruit to healthy fruit. This approach has identified several possible causes of cell death. Comparing BER affected tomatoes grown in a low calcium hydroponic solution to healthy fruit grown with a higher calcium hydroponic solution, Mestre et al. (Mestre et al., 2012) found a reduction in glutathione content, glutathione reductase activity, and catalase activity. Increased hydrogen peroxide and lipid peroxidation were also associated with BER affected fruit. Aloni et al. (Aloni et al., 2008) found depleted apoplastic and symplastic ascorbic acid contents and increased apoplastic ascorbate oxidase activities in BER affected peppers compared to healthy peppers. An investigation of intracellular wash fluids by Turhan et al. (Turhan et al., 2006b) found increased hydrogen peroxide concentration and peroxidase activity in BER affected fruit compared to healthy fruit. While

these results strongly support the trend of increased oxidative stress during blossom-end rot development, the conclusions thus far are mainly correlative in nature. Through overexpression of a vacuolar calcium importer, de Freitas et al. (de Freitas et al., 2011a) found that increasing vacuolar calcium and decreasing apoplastic calcium increased BER development in tomatoes. These results suggest that decreased apoplastic calcium can lead to BER development, though the hypothesis that adequate apoplastic calcium incurs a protective effect has not yet been tested. Additionally, confounding factors at the whole plant, fruit, and cellular level make it difficult to determine the sequence of events leading to cell death during BER development. To eliminate these confounding factors and establish causative evidence regarding the biological cause of BER, direct manipulation of the apoplastic calcium concentration and antioxidant capacity is needed.

Pericarp discs have been used previously to study ripening in tomatoes (Campbell et al., 1990; Campbell and Labavitch, 1991). The fruit used in these experiments were at the mature green stage and the discs studied during these experiments followed ripening trends regarding physiology, visual appearance, and original tissue location. This paper describes a new method for studying BER development in immature tomatoes using the pericarp disc system.

2.2 Results

Symptom development in immature whole fruit after harvest

Immature green tomatoes (Variety Harris Moran 4885 (HM)) showing no symptoms of BER were observed to develop BER symptoms during storage after harvest, if harvested just prior to the usual timing of BER development (approximately 8-12 days after pollination) on the plant (Figure 2.1). BER development off the plant occurred within the first 3 days after harvest and the

symptoms resembled BER regarding location and visual development. These results were replicated with 21-day old fruit of the HM variety and in the Rutgers variety.

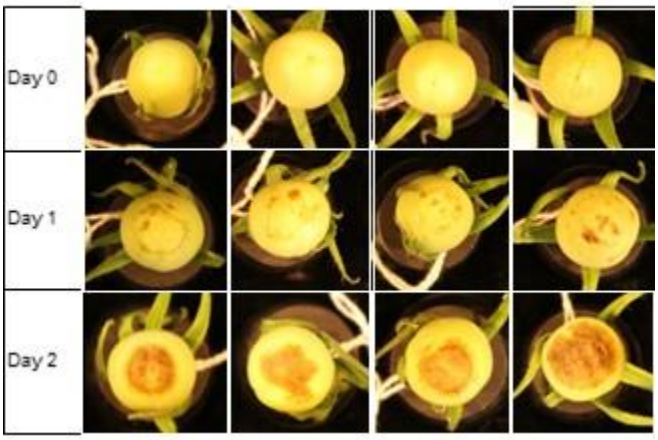

Figure 2.1: Whole HM 4885 tomatoes (n=4 fruit) harvested immature and stored in high humidity developed blossom-end rot over 2 days of storage.

Visual development of symptoms

Pericarp discs were made from the stem-end, blossom-end, and columella tissue of immature HM tomatoes 21 days after pollination (Supplemental Figure 2.1a). During the first 12 hours of storage, disc color lightened slightly and developed a “frosted” appearance as described previously (Campbell et al., 1990). In discs made from stem-end pericarp, this appearance remained the same for 4 days. In blossom-end pericarp discs, symptoms developed resembling the progression of BER development on the plant. Water soaking of the tissue was first observed on day 2 of storage, usually followed by tissue darkening. While water soaking often started on day 2, initiation of water soaking also occurred on day 3 and 4 in some bottom discs. Water soaking and tissue blackening was initially localized to one area of the disc, and subsequently

spread to the rest of the disc, as observed in Supplemental Video 1. In discs made from fruit already showing BER symptoms, the blackening spread from the BER affected area to the non-affected area. An example of these symptoms is shown in (Figure 2.2a). These results were replicated in Rutgers and Ailsa Craig tomatoes (data not shown). Darkening in top discs was observed only when the discs were excessively handled for color or weight loss analysis, and was visually similar to symptom development in blossom-end discs. In columella discs, symptoms developed similarly to that of pericarp discs. Discs taken from closer to the stem-end often maintained a green-white appearance, while discs taken from near the blossom-end darkened over the 4-day storage period (Figure 2.2b). HM and Ailsa Craig tomatoes produced the most consistent results (data not shown). Rutgers tomatoes were more susceptible to symptom development at sites of damage from handling in stem-end discs (data not shown).

Colorimeter measurements

Color was measured in HM discs (Figure 2.2c) and whole fruit harvested with BER symptoms (Figure 2.2d). L* and a* values were used to quantify darkening and loss of green color, respectively. Visual evaluations of symptom development in discs were also completed for comparison (Supplemental Figure 2.1b). Discs exhibiting symptoms and BER tissue in whole fruit harvested with BER symptoms had lower L* values and higher a* values than stem-end discs or the stem-end of whole fruit harvested with BER symptoms. Blossom-end discs that did not develop symptoms had similar values to those of the stem-end discs.

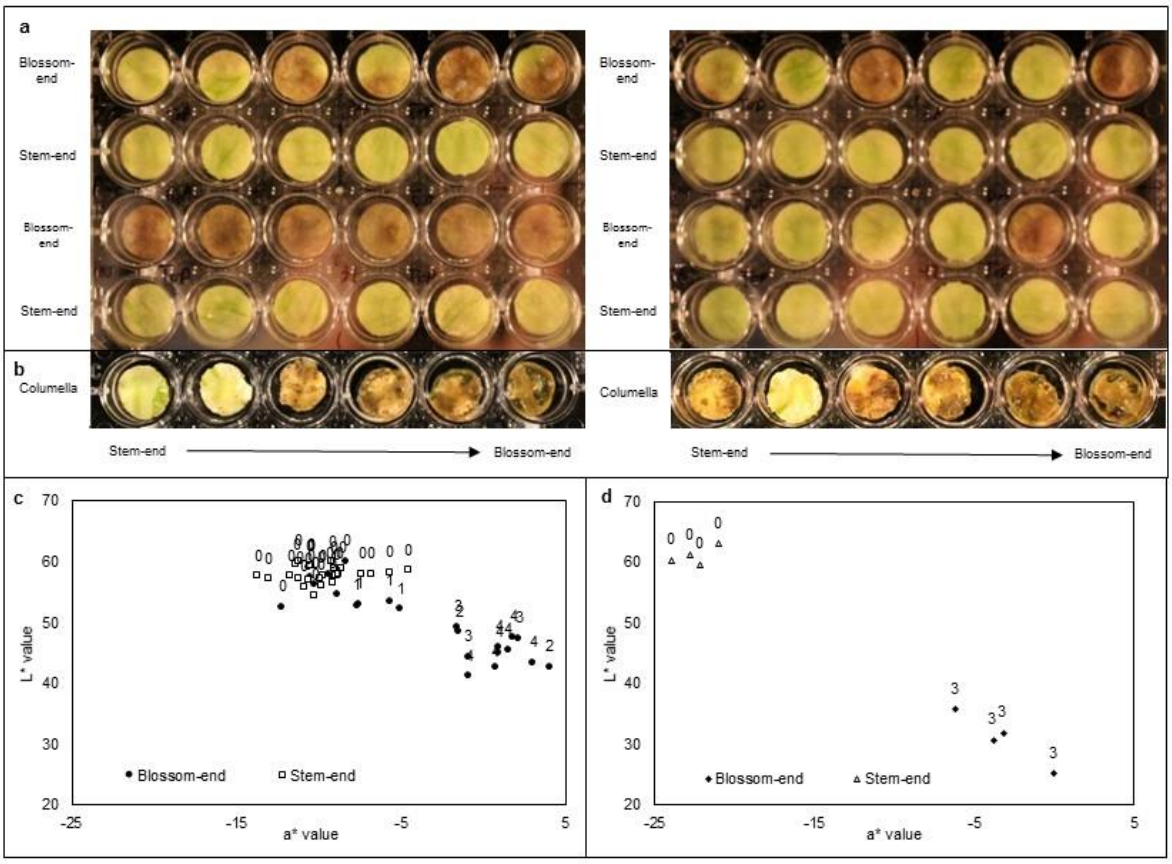

Figure 2.2: Symptom development and color analysis of pericarp and columella discs after 4 days of storage. The top images (a) show pericarp discs from the blossom-end (rows 1 and 3) and stem-end (rows 2 and 4) of a total of 4 fruit. Within each image, rows 1 and 2 containing discs from the same fruit, and rows 3 and 4 contain discs from the same fruit. No fruit were showing blossom-end rot symptoms prior to disc preparation. Columella discs from two fruit (b) were arranged with the disc taken from closest to the stem-end on the left and each subsequent disc being taken from closer to the blossom-end. Colorimetric measurements were made on the pericarp discs shown above (a) on day 4 of storage. Each point represents an individual disc, and data labels indicate the visual BER rating at the time of color measurement. Pericarp color of

healthy stem-end tissue and BER affected blossom-end tissue was measured on 4 whole fruit that developed BER on the plant (d).

Weight loss in discs during storage

HM disc weight loss over a 4-day storage period was not significantly different between stem-end discs and asymptomatic blossom-end discs (Figure 2.3a). Discs with a visual symptom rating of 2 had significantly higher weight loss than asymptomatic blossom-end discs and stem-end discs. Weight loss in Rutgers discs was higher than HM discs. Weight loss for stem-end, side, and blossom-end Rutgers discs was slightly higher from day 2 to day 3.5 than day 0 to day 2 (Figure 2.3b). Weight loss in columella discs was similar from day 2 to day 3.5 and day 0 to day 2.

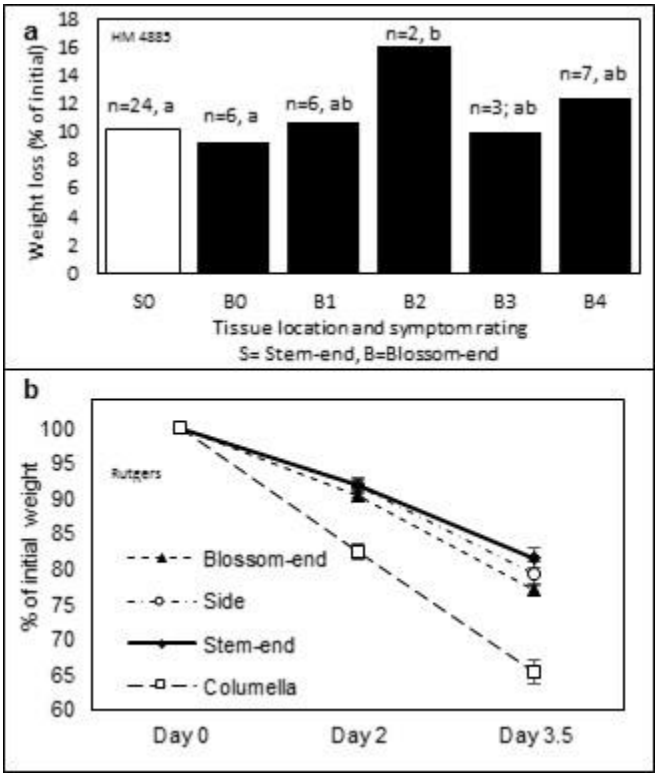

Figure 2.3: a) Percent weight loss for stem-end discs (S0) and blossom-end discs from HM 4885 tomatoes at each visual symptom rating (B0 to B4). Data inside each bar represents the sample size and means separation. Bars with the same letter were not significantly different ($p>0.05$). b) Weight loss over 3.5 days of storage in stem-end, side, blossom-end, and columella discs (n=12, 12, 12, and 11, respectively) from Rutgers tomatoes. Values are presented as the percent of the initial disc weight and error bars represent the standard error of the mean.

Enzyme analysis and color correlation

Enzyme analysis and color measurements were taken after 4 days of storage on 24 top and 24 bottom discs. A Pearson correlation analysis between enzyme activities and color measurements from HM discs showed that both pyrogallol peroxidase and ascorbate oxidase were significantly negatively correlated with L* values and positively correlated with a* values (Table 2.1). The mean ascorbate oxidase activity was 193.8 nmol min^{-1} g^{-1}. The mean pyrogallol peroxidase activity was 11.7 μmol min^{-1} g^{-1}. Both ascorbate oxidase and pyrogallol peroxidase activity means were significantly higher in blossom-end discs compared to stem-end discs.

Table 2.1. Pearson correlation analysis (top value) and p value (bottom value) between enzyme activity and color measurements from HM4885 discs (n=48).

	L*[a]	a*[b]	Ascorbate Oxidase	Pyrogallol POX
L*	1	-0.873 <0.0001	-0.362 0.0115	-0.423 0.0027
a*	-0.873 <0.0001	1	0.438 0.0019	0.516 0.0002
Ascorbate Oxidase	-0.362 0.0115	0.438 0.0019	1	0.5075 0.0002
Pyrogallol POX	-0.423 0.0027	0.516 0.0002	0.508 0.0002	1

[a] L* is a measure of lightness with black at 0 and white at 100,

[b] a* is a measure of red to green coloration, with green in the negative direction and red in the positive direction

Respiration measurements

Blossom-end discs had a higher mean respiration rate than stem-end discs during the first two days of storage (Figure 2.4a). Respiration rates on day 1 were correlated with measurements of tissue darkening and loss of green color on day 2. The Pearson's correlation coefficient was -0.752 and 0.821 when comparing day 1 respiration to day 2 L* values and a* values, respectively (Figure 2.4b).

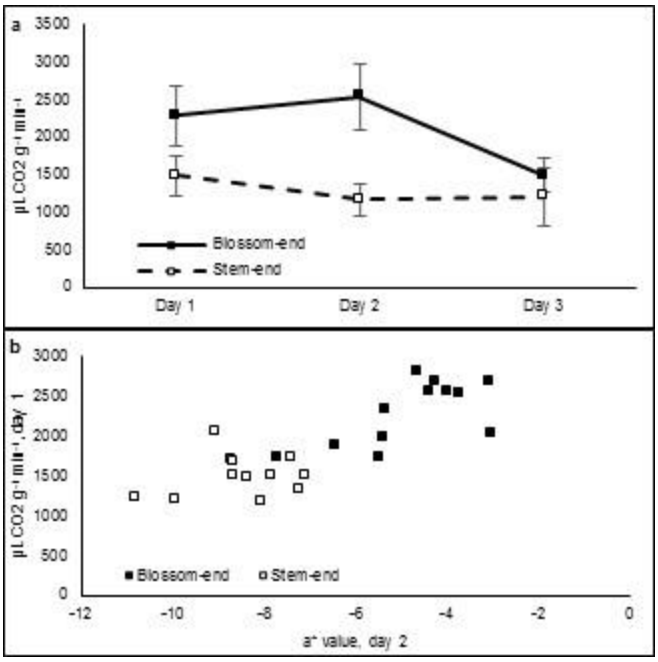

Figure 2.4: Respiration rate of HM 4885 tomato pericarp discs. Respiration rates over three days of storage (a) is presented as the mean of 12 discs with error bars representing the standard deviation. Respiration on day 1 compared to color analysis (a* value) on day 2 (b) is presented with each point representing an individual disc.

Calcium and ascorbic acid treatments

Treating discs with a 15 min soak in either 10g/L calcium chloride or 500mM ascorbic acid was effective in inhibiting the development of visual symptoms. Both visual evaluation (Figure 2.5a and 2.5b) and color data (Figure 2.5c and 2.5d) indicates that symptoms only developed in deionized (DI) water pH 5.58 and DI water pH 2.00 treated discs. Ascorbic acid treatment caused a mild loss of green color as seen in the slight increase in a* value compared to calcium treatments. Aside from this color change, ascorbic acid and calcium treated discs appeared healthy with no water soaking or darkening. Discs treated with DI water pH 2.00 showed water soaking and tissue degradation, and exhibited a gray color. Gray color development became

apparent 3 days after harvest and disc formation, and became more noticeable on day 3.5, similar to the timing of tissue darkening observed in DI water-treated discs. In addition to 15 minute soaking treatments, vacuum infiltration was tested as a method for inducing the uptake of treatment solutions.. However, vacuum infiltration was found to induce a translucent and water-soaked appearance in all treatments, making water-soaked symptoms associated with blossom-end rot hard to identify (results not shown). The vacuum infiltration method was not pursued further.

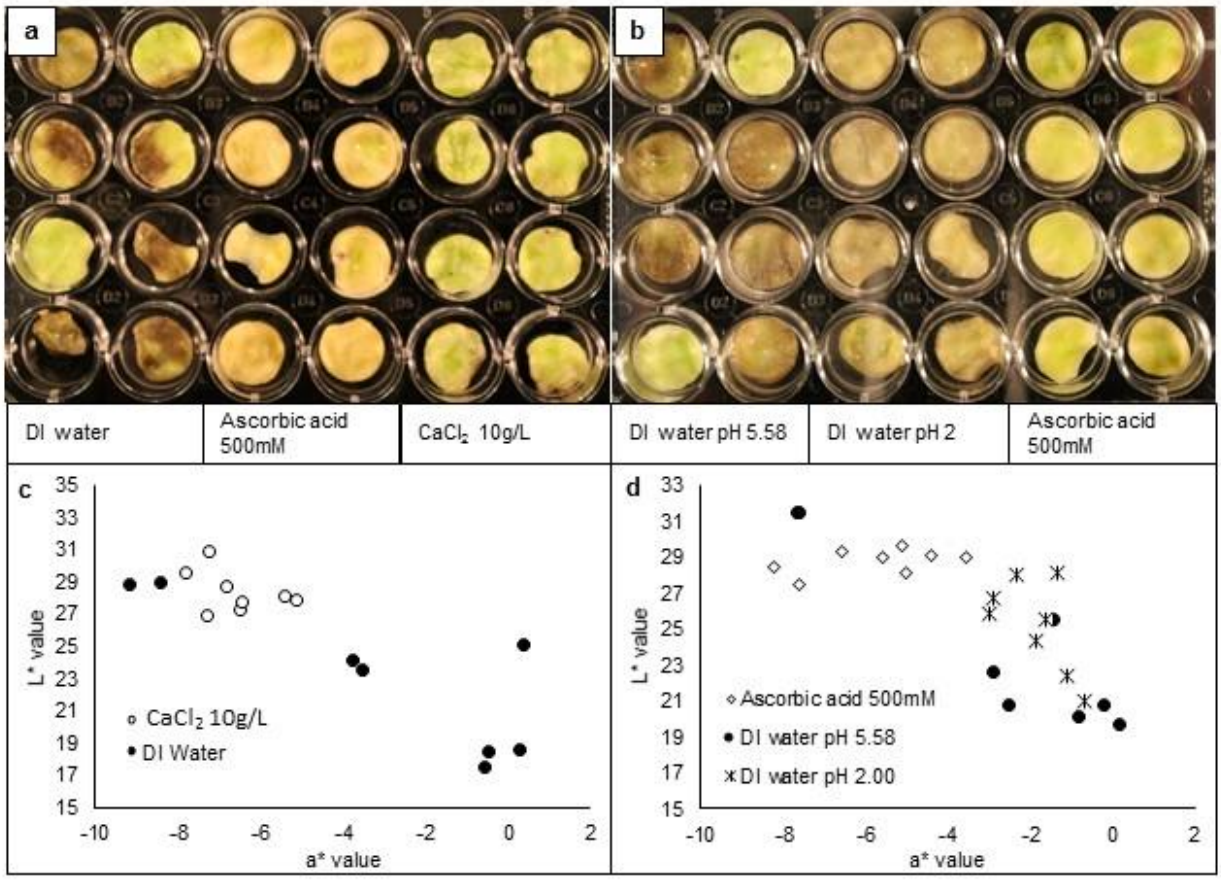

Figure 2.5: Symptom development in pericarp tissue discs 3.5 days after a 15 minute soak treatment in (a) water, 500mM ascorbic acid, or 10g/L (90.1mM) calcium chloride, and (b) DI water (pH 5.58), DI water (pH 2 .00), or 500mM ascorbic acid. Figure 2.5a and 2.5b each show a

total of 24 blossom-end discs from 4 fruit. Each treatment was applied to 2 discs from each of the 4 fruit. Colorimetric data from discs in (a) and (b) is presented in (c) and (d), respectively.

2.3 Discussion

Comparison of symptom development in discs and whole fruit

Symptom development in harvested whole fruit and disc systems closely resembled symptom development in tomato fruit on the plant. Harvested whole fruit developed symptoms only at the blossom-end of the fruit. Similarly, only discs from the blossom-end of fruit developed symptoms in the pericarp disc system. Similar to trends in whole fruit (Suzuki et al., 2000), columella tissue taken from near the blossom-end of the fruit developed BER like symptoms while top columella tissue often did not develop symptoms.

Visual BER symptom development in whole fruit on the plant begins with water soaking of the tissue at the blossom-end, usually in one or a small number of spots. Water-soaked tissue begins to darken until it has a blackened appearance (de Freitas and Mitcham, 2012). In discs, symptoms developed in a similar manner, starting with the development of a water-soaked and somewhat translucent appearance. Water-soaked areas then darkened. Drying of the affected tissue in discs was limited, likely due to the high relative humidity environment, though weight loss evidence suggests that increased water loss can occur during symptom development. Drying of the BER affected area is also common in fruit that develop BER on the plant.

Comparison of enzyme activity

Ascorbate oxidase activity was used as a measure of the overall trend in antioxidant depletion in BER affected fruit. Maintaining ascorbic acid concentrations in fruit has been suggested as key to BER resistance and increasing ascorbic acid concentrations in vulnerable fruit tissues is a

possible treatment to inhibit BER incidence (Aloni et al., 2008; Turhan et al., 2006b). However, conflicting ascorbate concentrations have been reported in BER fruit compared to healthy fruit (Aloni et al., 2008; Mestre et al., 2012). Thus, direct measurement of ascorbic acid is an unreliable indicator for the state of the antioxidant system and was deemed inappropriate for the purpose of comparing BER processes in whole fruit to symptom development in discs. Alternatively, ascorbate oxidase activity has been shown to be increased in BER affected peppers compared to healthy peppers (Aloni et al., 2008). In tomato, ascorbate oxidase was found to be transcriptionally upregulated in fruit treated with the BER-inducing phytohormone gibberellin (de Freitas et al., 2017). Supplemental Figure 2.2 shows ascorbate oxidase activity in healthy top, healthy bottom, and BER affected pericarp tissue from BER affected fruit, and healthy top and healthy bottom pericarp tissue from BER unaffected fruit. This data indicates that the ascorbate oxidase activity from stem-end pericarp tissue of a BER-affected fruit is similar to the stem-end and blossom-end of healthy fruit. In our pericarp disc system, ascorbate oxidase activity was significantly correlated with objective measures of tissue darkening (negatively correlated with L* values and positively correlated with a* values), suggesting that similar antioxidant depletion processes occur during symptom development in discs as in whole fruit.

Peroxidase activity was chosen as a second indicator of enzymatic similarities between symptom development in discs and BER development on the plant. Peroxidase activity measured using an aromatic electron donor was shown to be greatly increased in BER fruit compared to healthy fruit (Reitz and Mitcham, 2021a; Turhan et al., 2006b). This trend has been linked to increased lignification, and peroxidase trends presented by Reitz and Mitcham (Reitz and Mitcham, 2021a) are similar to those found for ascorbate oxidase activity in Supplemental Figure 2.2. In our disc system, peroxidase activity was significantly correlated to measures of tissue darkening

(negatively correlated with L* values and positively correlated to a* values), suggesting a similar trend to that of increased peroxidase activity in BER affected whole fruit on the plant as compared to healthy fruit.

Respiration during symptom development

Regulation of respiration is vital to plant health, with increased respiration indicating an increase in energy consumption and possibly stress. Results presented here indicate symptom development is associated with an increase in respiration rate. Furthermore, the correlation between respiration on day 1 and color measurement on day 2 indicates this increase in respiration rate occurs prior to visual symptoms. Blossom-end disc respiration decreased to near the level of the stem-end discs on day 3, possibly due to cell death or exhaustion of the available chemical energy pool. This also indicates that processes associated with blossom-end rot are likely active prior to visible symptom development.

Calcium and ascorbic acid treatments

Calcium deficiency in soil and fruit tissue has long been associated with BER incidence (de Freitas and Mitcham, 2012; Hagassou et al., 2019). However, abiotic stress can also increase BER incidence, bringing into question the direct relationship between BER occurrence and calcium deficiency. We applied calcium chloride and ascorbic acid to disc tissues to test the direct effect of increased calcium and antioxidant potential on BER development. Both treatments eliminated symptom development as evaluated visually and by colorimeter.

Calcium localization within the cell is a key factor in BER incidence. De Freitas et al. (de Freitas et al., 2011a) directly studied the effect of calcium localization at the cellular level by increasing vacuolar calcium through increased expression of the sCAX1 gene. Increased vacuolar calcium

was associated with decreased apoplastic calcium and increased BER incidence. Calcium applied to the pericarp disc systems would likely diffuse into the apoplast, testing the opposite localization state compared to that of De Freitas et al. (de Freitas et al., 2011a). As expected, the opposite effect (inhibition of BER symptoms) was observed when calcium was applied.

Correlation of BER symptom development with increased ROS accumulation and depleted antioxidant activity has been well established in the literature. Similar to calcium localization, decreased antioxidant potential in the apoplast has been shown to be correlated with BER symptom development (Mestre et al., 2012; Schmitz-Eiberger et al., 2002; Turhan et al., 2006b). Ascorbic acid treatment of discs inhibited BER symptom development, representing the first causative evidence that increasing antioxidant potential in the apoplast incurs a protective effect against BER development.

Potential uses, advances, and limitations

The pericarp disc system used with immature green tomatoes has multiple potential uses for future research. As demonstrated by the respiration data presented here, using this method in combination with non-destructive measurements can allow for active monitoring of the biological changes in the discs as symptoms develop. Similarly, performing destructive analyses on half of each disc while allowing the remaining half of the disc to continue the storage progression may allow for a better understanding of why some discs and tissues develop symptoms while others do not. However, care should be taken to reduce the risk of handling induced symptoms if this technique is pursued. Finally, and most beneficial, treating pericarp tissues directly can provide causative evidence for testing hypotheses that are currently supported by correlative evidence. Ascorbic acid and increased antioxidant capacity generally, have been proposed as incurring a protective effect against BER development (Aloni et al., 2008; Mestre et

al., 2012; Turhan et al., 2006b). Results presented here provide causative evidence to support ascorbic acid's protective effect. Similarly, much of the evidence for calcium's role at the tissue level has been correlative in nature. Results presented here provide the first causative evidence for calcium's protective effect against BER development at the cellular level. This method can be used to explore the mechanisms of BER development. It would be particularly interesting to test the effect of calcium transport and receptor inhibitors. Inhibitors of enzymes involved in ROS metabolism could also be tested. Varied timing of such treatments during symptom development could also improve knowledge of the timing of events involved in BER development.

This method could also be used to screen new BER treatments before larger scale testing in the field or greenhouse. Additionally, fruit phytotoxicity of non-BER specific field treatments could also be assessed using the pericarp disc system. It is worth noting, however, that treatments with effects at the whole plant level may not have the same effects in the pericarp system. Similarly, without access to xylem or phloem nutrient sources, cell expansion in the pericarp disc system is likely halted or abnormal. Thus, any treatment that acts through regulating cellular expansion may not be effective. However, this system is very effective in assessing treatments that disrupt pathways leading to cell death during blossom-end rot development.

2.4 Conclusions

Harvested immature whole tomato fruit and pericarp discs develop BER like symptoms during sterile, high humidity storage. Symptoms that developed in discs resembled BER symptoms in visual appearance, tissue location, and activity of key enzymes. Respiration measurements demonstrate the effectiveness of using nondestructive methods in conjunction with this pericarp disc system. Respiration measurements also indicate that cellular processes associated with

symptom development are occurring prior to the emergence of visual symptoms. Additionally, results provide causative evidence of the protective effects of calcium and ascorbic acid against BER symptom development at the cellular level. This method can be used for further research into the biology and treatment of calcium deficiency disorders.

2.5 Materials and methods

Solanum lycopersicum L. (var. HM 4885) seeds were obtained from Agseeds Unlimited (Woodland, CA, USA) and *Solanum lycopersicum* L. (var. Rutgers and Ailsa Craig) seeds were obtained from the Tomato Genetics Resource Center (University of California, Davis, CA, USA). This variety is recognized by the tomato industry as highly susceptible to BER. Seeds were sprouted in peat pellets using double deionized water (ddH_2O). Approximately 2 weeks after germination, seedlings were moved to a greenhouse and transplanted into 7.6 liter pots with a mixture of 1/3 peat, 1/3 sand, and 1/3 rosewood compost, augmented with 1.56 kg m^{-1} dolomite lime. Plants were irrigated daily until pots were dripping with a solution containing 150 ppm nitrogen, 50 ppm phosphorus, 200 ppm potassium, 175 ppm calcium, 55 ppm magnesium, 120 ppm sulfur, 2.5 ppm iron, 0.02 ppm copper, 0.5 ppm boron, 0.50 ppm manganese, 0.01 ppm molybdenum, 0.05 ppm zinc, and 0.02 ppm nickel. Flowers were manually pollinated and tagged. HM fruit used for whole fruit experiments were harvested 9 days after pollination. HM and Rutgers fruit used for pericarp disc experiments were harvested 21 days after pollination.

BER development in harvested whole fruit

Whole fruit were stored blossom-end up in a closed container under a constant flow of humidified air to reduce the accumulation of ethylene and carbon dioxide. Fruit were photographed daily.

Disc Preparation

Discs were prepared from the stem-end pericarp, blossom-end pericarp, and columella tissue on the day of harvest of 21-day-old tomato fruit. Fruit were surface sterilized in approximately 150 mL of 1% sodium hypochlorite for 1 minute, then thoroughly rinsed in double deionized water. A cork borer (approximately 13.5 mm) was used to excise cylindrical tissue samples. For discs made from pericarp tissue, locular and endocarp tissue was removed using a razor blade. Discs were rinsed in ddH_2O and blotted dry on sterilized filter paper. Discs were weighed and placed skin side down in 24 well tissue culture plates (Cellstar, Greiner Bio-one, Kremsmünster, Austria), noting the original fruit and tissue location.

For discs made from columella tissue, a 13.5 mm diameter cylinder was cut from the stem-scar to the center of the blossom-end of the fruit. The blossom-end pericarp and stem-scar were removed, producing a cylinder containing columella tissue with small amounts of locular and seed tissue. This cylinder was cut into discs approximately 2 mm in height, and these were rinsed in ddH2O and blotted dry using sterile filter paper. The discs were placed in a 24 well tissue culture plate with the disc locations from the stem-end to the blossom-end preserved. Tissue culture plates were stored without lids in a closed container under a constant flow of humidified air to reduce the accumulation of ethylene and carbon dioxide.

Symptom rating, color, and weight loss

After approximately 3.5 days, discs were scored based on their symptom development on a 0 to 5 scale, with 0 being no symptom development and 5 being complete deterioration and blackening of the tissue. Discs were turned upside down and disc color was measured by inverting a Minolta colorimeter (Konica Minolta Inc. Tokyo, Japan) and measuring the color of the pericarp through

the bottom of the plate. Disc color was measured through the bottom of the plate rather than from the top of the well to decrease effects of ambient light. In respiration and treatment testing experiments, disc color was measured through the top to reduce the mechanical damage and potential contamination associated with flipping discs pericarp side down for color measurement from the bottom. Measuring from the top is recommended for future studies. Discs were then weighed, frozen in liquid nitrogen, and stored at -80°C for subsequent enzyme analysis. To determine weight loss trends over time, 6 Rutgers discs from the stem-end pericarp, side pericarp, and blossom-end pericarp were weighed on Day 0, Day 2, and Day 3.5 in a separate experiment.

To compare color changes in discs to the color change of fruit on the plant, 4 fruit exhibiting BER symptoms were harvested. A thin layer of skin was removed from one spot within the BER affected tissue and two spots on the stem-end to expose the pericarp tissue for comparable readings with the discs. Color was measured at these spots as stated above, and the two measurements from the stem-end were averaged for each fruit to create 4 stem-end measurements and 4 blossom-end measurements.

Enzyme extraction and activity measurement

After 4 days of storage, color was measured on 24 stem-end and 24 blossom-end discs from HM fruit. These discs were frozen and stored at -80°C until enzyme extraction. Discs were ground with a pestle as they thawed in a room temperature mortar with 100 mM phosphate buffer, pH 6. The resulting homogeneous mixture was then centrifuged for 20 min at 20,000 g and 4°C, and the supernatant was used as a crude enzyme extract. Enzyme extracts for ascorbate oxidase activity on whole fruit were prepared using the same method.

Peroxidase activity was assayed spectrophotometrically using a BioTek H1 multimode plate reader (Biotek, Winooski VT, USA). The total reaction volume of 100 µL included 70 µL of ultrapure water, 3.33 µL of 100 mM phosphate buffer, 10.66 µL of pyrogallol, 10.66 µL of enzyme extract, and 5.33 µL 0.5% H_2O_2. H_2O_2 was added last to start the reaction. The increase in absorbance at 420 nm, associated with purpurogallin formation, was monitored over 3 minutes. The slope of the regression line for the plot of absorbance over time was used to calculate enzyme activity on a min^{-1} g^{-1} fresh weight basis. The extinction coefficient used for the calculations was 12.0 $(mg/mL)^{-1}$ cm^{-1}.

Ascorbate oxidase was assayed using a total reaction volume of 100µL including 75.33 µL of ultrapure water, 3.33 µL of 100 mM phosphate buffer, 10.66 µL of 250 µM ascorbic acid, and 10.66 µL of enzyme extract. The change in absorbance at 290 nm over 2 minutes was measured, and activity on a min^{-1} g^{-1} fresh weight basis was calculated using 2.8 mM^{-1} cm^{-1} as the extinction coefficient.

Respiration measurement

Respiration rate was measured in HM discs by sealing each of the wells in the 24 well plate with an adhesive PCR plate cover (MicroAmp, Applied Biosystems, Foster City, California, USA) and attaching an adhesive septum (Bridge Analyzers Inc. Alameda, California, USA) to the top. Samples (1mL) were collected by inserting a syringe into the airspace above the discs. Respiration rate was measured 1, 2, and 3 days after harvest. Wells were sealed for 5-11 min. CO_2 production (µl CO_2 min^{-1} g^{-1} fresh weight) was measured using an infrared CO_2 analyzer (Horiba, Irvine, CA).

Calcium and ascorbic acid treatments

Calcium chloride and ascorbic acid treatments were applied to discs as 15 min dips. Discs were prepared from the blossom-end pericarp tissue of 4 HM fruit as stated above. After rinsing and blotting, 2 discs from each fruit (8 total) were placed in 15 mL of either 10 g/L (90.1 mM) $CaCl_2$ or 500 mM ascorbic acid and shaken gently on a rotating platform for 15 min. Controls included 15 min treatments in ddH2O (pH 5.58) or ddH2O adjusted to pH 2.00 on 8 discs each from the same fruit. The discs were blotted dry on sterile filter paper and placed in a 24 well tissue culture plate. The plates were stored in a humidified container as stated above. After 3.5 days the discs were photographed and their color measured. For color measurement of the treated discs the discs were left with the pericarp tissue facing up and the colorimeter was placed directly above each well.

Statistical analysis

Statistical analysis was carried out using SAS On Demand for Academics (SAS Institute Inc., Cary, NC, USA). Pearson's correlation for enzyme analysis and color measurements was completed on a total of 48 discs, 24 from stem-end pericarp and 24 from blossom-end pericarp. A general linear model procedure was used to determine significant differences in weight loss and a Tukey's honest means separation was completed. Pearson's correlation comparing respiration on day 1 with color measurement on day 2 was completed on 24 discs, comprised of 12 stem-end discs and 12 blossom-end discs.

Data availability

The datasets generated during and/or analysed during the current study are available in the Dryad database, using the following information:

Reitz, Nicholas (2020), Validation and demonstration of a pericarp disc system for studying blossom-end rot of tomatoes, Dryad, Dataset, https://doi.org/10.25338/B8DP7R

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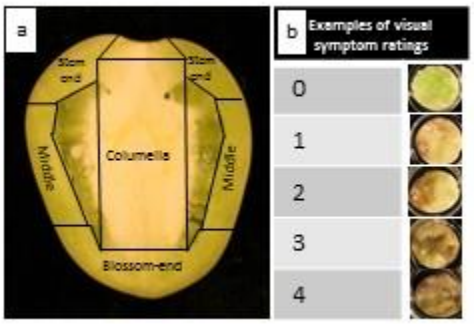
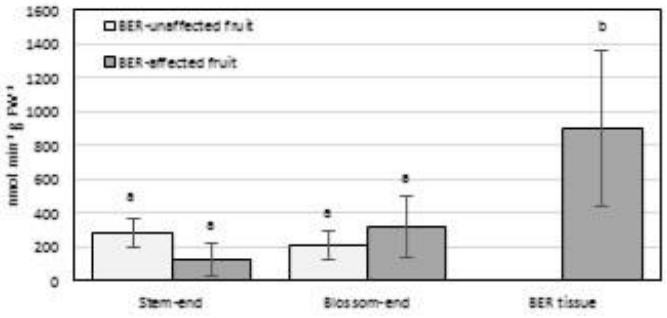
2.7 Supplemental Materials

Supplemental Video 2.1: Video depicting blossom-end rot symptoms spreading during storage of pericarp discs from immature green tomatoes. The first and third rows of discs were obtained from the stem-end of fruit and the second and forth rows of discs were made from the blossom-end of fruit. This can be found accompanying the publication: Reitz, N. F., & Mitcham, E. J. (2021). Validation and demonstration of a pericarp disc system for studying blossom-end rot of tomatoes. Plant Methods, 17(1), 1-10.



Supplemental Figure 2.1: Sampling locations and visual symptom scale. Fruit tissues (a) used for discs included the stem-end, middle, and blossom-end pericarp, as well as columella tissue. Representative (b) discs are shown for each rating on the 0-4 visual symptom rating scale.



Supplemental Figure 2.2: Ascorbate oxidase activity in BER unaffected and BER affected whole fruit harvested 21 days after pollination. Each bar is the mean of 4 replicates, and bars with the same letter were not statistically different ($p>0.05$).

Chapter 3: Differential effects of excess calcium applied to whole plants vs. excised fruit tissue on blossom-end rot in tomato

Abstract

Blossom-end rot can cause serious losses to processing tomato producers, fresh market growers, and home gardeners alike. Blossom-end rot is often associated with calcium deficiency, though salt stress and even high concentrations of calcium salts can also cause blossom-end rot. The goal of this study was to characterize the negative effects of excess calcium compared to inadequate calcium at the whole plant and cellular level. Four calcium levels (0ppm, 10ppm, 100ppm, and 500ppm) were applied daily to highly blossom-end rot susceptible processing tomato plants, starting at first flowering. Fruit were harvested 21-22 days after pollination. Blossom-end rot incidence and severity were lowest in the 10ppm treatment. Blossom-end rot incidence was highest in the 0ppm treatment and blossom-end rot severity was significantly increased in 500ppm and 0ppm treatments compared to 0ppm $CaCl_2$. No significant difference was found in the calcium content of tomato fruit harvested from plants treated with various levels of calcium. The soil drainage solution from the 500ppm treatment had the highest conductivity, which was nearly 10 times higher than that of the 0ppm and 10ppm treatments. Daily plant water use was reduced in the 500ppm treatment. Application of moderate calcium to tomato plants effectively reduced blossom-end rot incidence and severity, but excess levels of calcium did not reduce incidence and further increased blossom-end rot severity. Calcium applied to immature tomato fruit pericarp discs also inhibited blossom-end rot symptom development; however, excess calcium did not induce blossom-end rot symptoms. These results indicate that excess calcium induces BER development due to effects at the whole plant level, but not due to excess calcium in the fruit tissue.

3.1 Introduction

Few horticultural experiences are as disheartening as growing a field or garden of apparently healthy tomato plants, just to lose a large portion of the fruit to blossom-end rot (BER). BER is a physiological disorder causing significant losses to tomato and pepper producers. BER is often associated with a whole plant or fruit-specific deficiency of calcium. Calcium is an important plant nutrient with diverse functions within the fruit cell (Hocking et al., 2016). Regulation of calcium ion concentrations in the cytoplasm, apoplast, and organelles is used by the plant as a signaling mechanism. Furthermore, calcium stabilizes the plasma membrane, and pectin constituents in the cell wall. In addition to incurring a protective effect against blossom-end rot in tomatoes, calcium can help decrease development of other fruit physiological disorders, such as chill injury in loquats and internal browning of pineapple (Li et al., 2020; Youryon et al., 2018). Calcium moves into fruit primarily through the xylem. Reduced leaf transpiration by ABA treatment has been shown to improve the xylemic flow of water and nutrients to the fruit, thus reducing BER incidence (de Freitas et al., 2014). Because calcium is phloem immobile and cannot move from leaves to fruit, foliar sprays are often not recommended. However, reductions in BER in tomato as a result of calcium foliar sprays have been reported (Schmitz-Eiberger et al., 2002), likely due to direct absorption of calcium into the fruit.

Abiotic stresses, such as drought stress and salt stress, are also associated with BER incidence (Hagassou et al., 2019; Turhan et al., 2006a). Several studies have tested varying calcium concentration in soil and hydroponic production systems, with results indicating that increased calcium reduces BER development (Mestre et al., 2012). However, there have been reports of increased BER incidence with increased calcium concentrations in hydroponic solutions (Gholipour et al., 2017; Hossain and Nonami, 2012; Nonami et al., 1995), suggesting that

mitigation of BER may be more complicated than simply applying more calcium. Nonami et al. (1995) found that adding 40g/L calcium-zeolite to hydroponic solutions slightly increased BER incidence in two tomato varieties compared to an optimal concentration of 20g/L. Osmotic effects were suggested as a cause of BER under high calcium concentrations. Hossain and Nonami (2012) further investigated the salt stress effects of increased calcium concentration and its association with BER development. They found that adding $CaCl_2$ to raise the hydroponic solution to an electric conductivity (EC) of 8 mS/cm resulted in fruit with decreased water potential, osmotic potential, and fruit growth rate compared to a moderate calcium concentration (EC=1mS/cm). However, it is still not known whether high calcium-induced BER development is due to effects at the whole plant level or high concentrations of calcium ions in the fruit. Other recommendations for calcium concentrations in fertigation solutions include 328-360ppm (Kreij, 1996) and 61-105ppm (Bar-Tal et al., 2017). However, these varying recommendations and the concentration of solutes due to water use and drying has the potential to induce osmotic stress through excess calcium application, thus increasing BER incidence. A better understanding of the limits of calcium's protective effect against BER when applied through the irrigation solution would benefit both the scientific understanding of this disorder and the practical application of calcium for the prevention of BER.

Though BER has long been associated with calcium deficiency, BER induced by high calcium concentrations has been promoted as evidence to question the protective effect of increased fruit calcium against BER development (Gholipour et al., 2017; Hossain and Nonami, 2012; Nonami et al., 1995). Insights into the cause of BER development associated with high soil calcium concentrations could improve our knowledge of the role of calcium in BER incidence; specifically, knowing whether increased BER incidence associated with high calcium

concentrations is due to effects at the whole plant or tissue level. Our study investigated varying levels of calcium in the irrigation solution to explore the limits of calcium's protective potential against BER. A pericarp tomato disc system was then used to clarify the fruit-specific effects of calcium on BER development.

3.2 Materials and methods

Plant Growth

Solanum lycopersicum (var. HMX 4885, Agseed, Woodland, CA) seeds were germinated in peat pellets and deionized water at 27°C with a 16h/8h light/dark schedule. After approximately 2 weeks, seedlings were transplanted into 7.6 L black plastic pots with 4 drainage holes approximately 3 cm in diameter. The pots were filled with a soil mixture of 1/3 peat, 1/3 sand, and 1/3 rosewood compost, augmented with 1.56 kg m^{-3} dolomite lime. Pots were irrigated with a fertilizer solution containing 150 ppm nitrogen, 50 ppm phosphorus, 200 ppm potassium, 175 ppm calcium, 55 ppm magnesium, 120 ppm sulfur, 2.5 ppm iron, 0.02 ppm copper, 0.5 ppm boron, 0.50 ppm manganese, 0.01 ppm molybdenum, 0.05 ppm zinc, and 0.02 ppm nickel until first pollination. One day prior to pollination, initial water use was determined was by subtracting the weight of 3 individual pots after fertigation from the weight of individual pots prior to fertigation application the next day. At first pollination, 20 g of a calcium-free, slow release fertilizer (Osmocote Plus, The Scotts Company, Marysville, OH, USA) was applied to each plant. The slow-release fertilizer contained 8.4% ammoniacal nitrogen, 6.6% nitrate nitrogen, 9% diphosphate pentoxide, 12% dipotassium oxide, 1.3% magnesium, 6% sulfer, 0.02% boron, 0.05% copper, 0.05% iron, 0.06% manganese, 0.02% molybdenum, and 0.05% zinc. After the first day of pollination, deionized water supplemented with 0ppm, 10ppm, 100ppm, or 500ppm $CaCl_2$ was added daily at a rate of 200% of the average initial water use.

Watering at 200% of the average initial water use resulted in excess water eluting through the drainage holes in the bottom of the pot. Five plants each were treated with 10ppm, 100ppm, and 500ppm $CaCl_2$. Ten plants were treated with 0ppm $CaCl_2$ to produce sufficient fruit for additional experiments not presented here.

Flowers were manually pollinated and tagged 2-3 times per week for 4 weeks for a total of 11 pollination days; fruit were harvested 21-22 days after pollination. On each pollination day, all flowers at or just after anthesis were pollinated. On some pollination days, some plants had no flowers to be pollinated. The first harvest day was deemed day 0 and subsequent harvests occurred on days 2, 7, 9, 11, 14, 16, 18, 21, 23, and 28. All fruit were evaluated visually at harvest for BER on a 0-5 scale, with fruit rated 0 showing no external blossom-end rot symptoms and fruit rated 5 having extensive blackening over a large portion of the blossom-end. The total mass of fruit and number of fruit harvested for each treatment on each day was measured.

Stem water potential

Stem water potential was measured in 3 plants from each treatment. Between 14:00h and 16:00h one day after the last harvest (day 29), one leaf in the mid-section of the main stem of each tomato plant was bagged in a reflective envelope for approximately 20 minutes. Bagged leaves were then cut from the plant and placed in a pressure chamber with the petiole protruding from the chamber (McCutchan and Shackel, 1992). Pressure in the chamber was increased and the pressure at which xylem fluid began eluting from the stem was noted as the stem water potential.

2.3 Elution solution conductivity

On the last day of the experiment, excess solution draining out of the pots after irrigation was collected for conductivity analysis. The same 3 plants that were used for water use and water

potential measurements were used for elution solution conductivity. The solution electrical conductivity was measured using a conductivity meter (SevenCompact Duo, Mettler Toledo, Ohio, USA). The conductivity of the irrigation solution used for each treatment was measured using the same instrument.

Water use and biomass measurements

Water use measurements were repeated with 3 pots from each treatment on days 29 and 30. The average water use over these two days was calculated. Upon completion of the experiment, plant stems were cut approximately 1 cm above the soil surface and the mass of the aboveground plant material was weighed. Water use values were divided by biomass measurements to obtain a biomass adjusted water use value.

Total calcium analysis

Samples of visibly healthy pericarp tissue from halfway between the bottom and top of the fruit was taken from fruit exhibiting BER. Fruit used for total calcium analysis were harvested on day 2 for the 10 ppm, 100ppm, and 500ppm treatments. Fruit from day 0 and day 16 were used from the 0 ppm treatment due to sample limitations. Outer skin was removed, the tissue was lyophilized, and the samples were ground. Tissue from 2 fruit was combined to make 1 replicate, and 3 replicates were analyzed for each treatment. Replicates were analyzed for total calcium content using inductively coupled plasma atomic emission spectroscopy (Meyer and Keliher, 1992) at the UC Davis Analytical Lab (Davis, CA, USA).

Pericarp disc preparation

HM tomato plants were grown and discs were prepared from 21 day old fruit as described in (Reitz and Mitcham, 2021b). Briefly, plants were grown in a complete nutrient solution.

Harvested fruit were surface-sterilized by immersion in a 1% sodium hypochlorite solution for 1 min and rinsed briefly in autoclaved double deionized water. Under sterile conditions, discs were prepared from the blossom-end pericarp and skin tissue with a cork borer. Endocarp and locular tissue was removed using a razor. The pericarp discs were weighed and placed skin-side down in a 24-well plate. Six discs were made from each of 5 fruit (30 discs total). Each treatment group contained one disc from each of the five fruit and an additional disc randomly selected from the remaining discs. $CaCl_2$ was applied at a rate of 100 ng per g disc fresh weight, 10µg per g disc fresh weight, 100 µg per g disc fresh weight, and 1mg per g disc fresh weight using 7.2µM, 720µM, 7.2mM, and 72mM solutions of $CaCl_2$. Because each disc had a different weight, the volume of the solution applied was varied (38.8-70.8 µL/per disc) to accurately produce treatments of the same application rate in g $CaCl_2$ per g disc fresh weight. Discs were stored at 25°C and >90% relative humidity for 4 days. On day 4, discs were photographed and their color was measured using a Minolta colorimeter (Konica Minolta Inc., Tokyo, Japan) to assess BER development. This experiment was repeated on a second set of 5 fruit, for a total of 12 discs per treatment group.

Statistical analysis

Statistical analysis was carried out using Statistical Analysis Software (SAS) On Demand for Academics. An average was taken from ratings of all the fruit collected from each treatment on each day for BER percent and severity (i.e. one average per treatment per day). To determine the significance of treatment effects, a general linear model was used with day in the model to adjust for overall changes in response over time. Tukey's means separation was used within each variable to separate treatment means with an alpha of 0.05. Pearson's correlation was carried out comparing harvest day, % BER, BER severity, and fruit size.

3.3 Results

Blossom-end rot incidence, severity, and calcium analysis

Fruit were harvested 21-22 days after pollination and evaluated for BER incidence and severity. The overall incidence of BER ranged from 71% to 85% (Figure 3.1). Similar trends were observed between BER incidence and BER severity, with a reduction in means when increasing $CaCl_2$ application 0ppm to 10ppm, followed by an increase when calcium chloride was increased to 100ppm and 500ppm. BER incidence was highest in the 0ppm treatment and significantly reduced in the 10ppm treatment. BER incidence in 100ppm and 500ppm treatments were not significantly different from either the 0ppm or 10ppm treatments. BER severity was highest in 0 ppm and 500 ppm $CaCl_2$-irrigated plants, and lowest in 10 ppm irrigated plants. Plants irrigated with 100 ppm $CaCl_2$ were not significantly different from other treatments. No significant difference was found in total fruit calcium concentrations between calcium treatments.

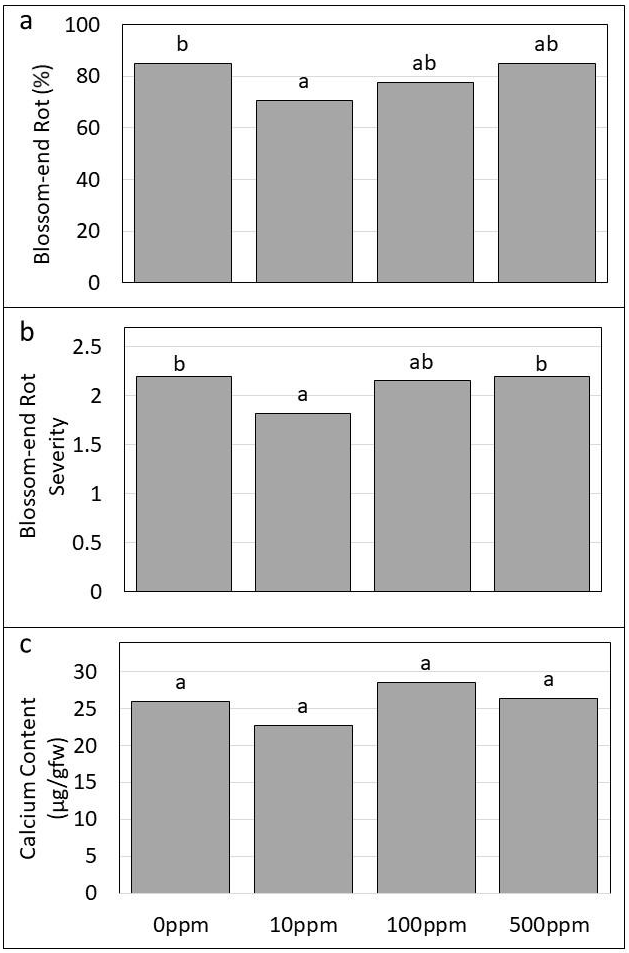


Figure 3.1: Blossom-end rot percent (a) and severity (b) in HM 4885 fruit harvested 21-22 days after pollination from plants grown under 4 different irrigation calcium concentrations. Severity was rated on a 0-5 scale with fruits rated 0 showing no external blossom-end rot symptoms and fruit rated 5 having extensive blackening over a large portion of the blossom-end. Each bar represents the mean of the percentage or average severity score on 11 harvest days. Total fruit tissue calcium (c) was measured using atomic absorption spectroscopy. Each bar represents 3 replicates with 2 fruit combined per replicate. Bars with the same letters are not significantly different (α=0.05).

Growth parameters

Drained solution conductivity was not significantly different between pots irrigated with 0 ppm, 10 ppm, or 100 ppm $CaCl_2$ (Figure 3.2a). Conductivity was significantly higher in pots irrigated with 500 ppm $CaCl_2$, with an average conductivity almost 10 times higher than that of the 0 ppm and 10 ppm irrigated pots. The mean conductivity of the drained solutions followed the trend of the conductivity of the irrigation solutions (Figure 3.2a), and the conductivity of the irrigation solution was lower than that of the drained solution in all treatments. The difference between the mean drained solution conductivity and the irrigation solution conductivity was approximately 3 times greater in the 500ppm treatment compared to the 0 and 10ppm treatment. While the mean stem water potentials followed the trend of BER incidence and severity, no significant differences were found between the measured water potential at different irrigation concentrations (Figure 3.2b). No significant difference was found in above-ground biomass, but water use per biomass was lowest in 500 ppm irrigated plants and highest in 10 ppm irrigated plants (Figure 3.2c,d).

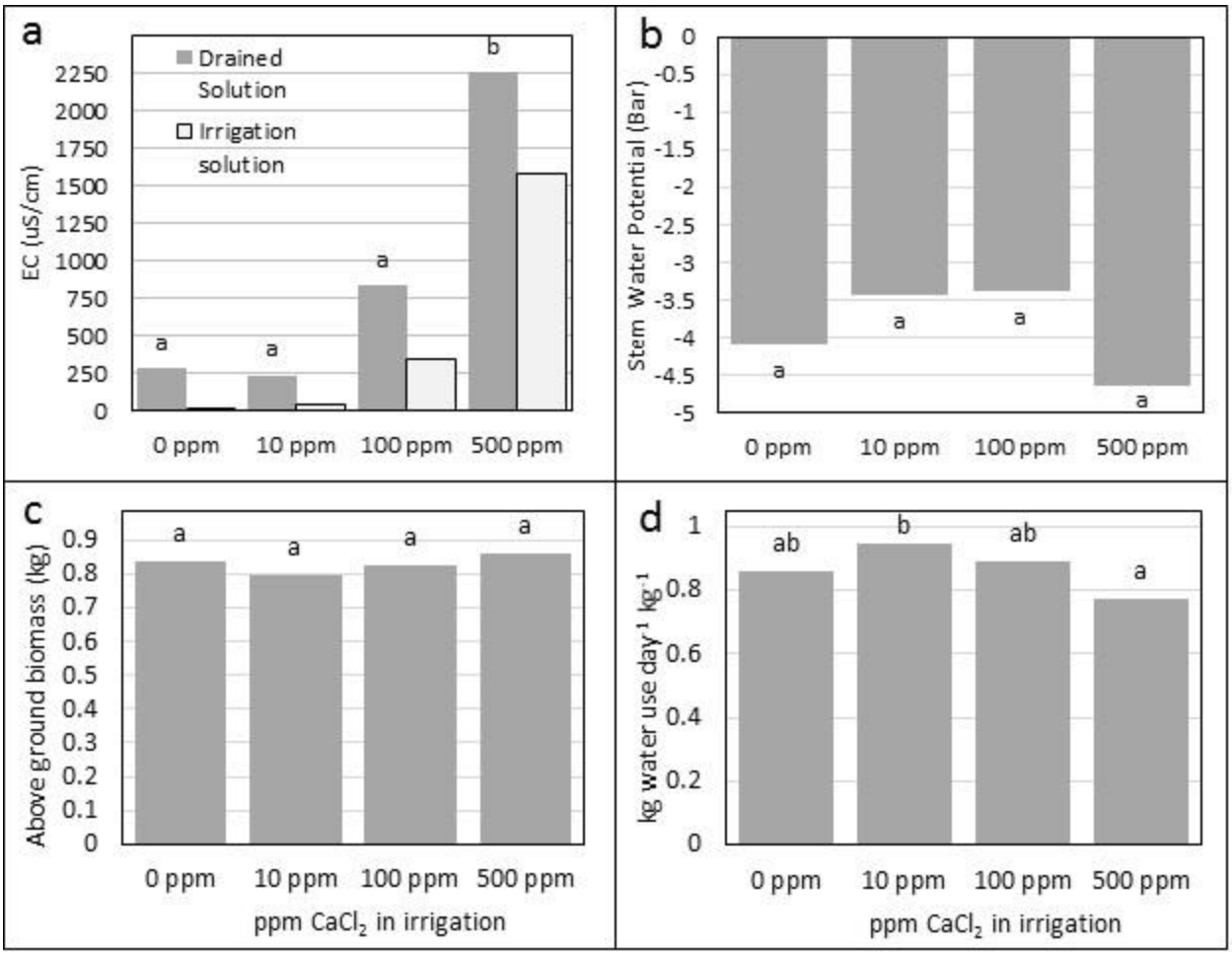


Figure 3.2: Drained solution and irrigation solution conductivity (a), stem water potential (b), above ground biomass (c), and water use per day per kg biomass (d) in tomato plants irrigated with different concentrations of $CaCl_2$. Each bar represents the mean of 3 plants, with the exception of irrigation solution, which was measured once and prepared the same each day. Bars with the same letters are not significantly different (α=0.05).

BER incidence and severity over time

100% BER incidence was observed on the day 0 harvest from plants of all treatments, but the incidence declined in fruit of the same age (21-22 days after pollination) harvested on subsequent harvest days. This resulted in a significant negative correlation between harvest day and BER

incidence and severity (Table 3.1). The average fruit size (grams) at 21-22 days after pollination was significantly negatively correlated with harvest day and significantly, positively correlated BER % and BER severity.

Table 3.1. Pearson correlations between BER incidence and severity, fruit size, and harvest day.

	Harvest Day*	**BER %****	**BER severity****	**Fruit size (g)****
Harvest Day*	1	-0.619 <0.0001	-0.797 <0.0001	-0.888 <0.0001
BER %**		1	0.903 <0.0001	0.693 <0.0001
BER severity**			1	0.827 <0.0001
Fruit size**				1

Upper value: Pearson correlation
Lower value: p value; n = 44
* day value calculated as days after first harvest (day 0)
** correlations performed on the mean values within each treatment and harvest day

Effect of $CaCl_2$ on pericarp discs

Tomato discs treated with $CaCl_2$ showed increasing inhibition of BER symptom development with increased calcium application (Figure 3.3). A reduced number of discs with BER symptoms were observed as calcium application rate increased, ranging from 9 out of 12 symptomatic discs in the 0g/g $CaCl_2$ application to 1 out of 12 in the 1mg/g application (Figure 3.3A). Average color measurements showed increased darkness (lower L* value) with decreased calcium concentrations (Figure 3.3b). A 15 min soak in 1M $CaCl_2$ induced visible shrinking of the disc tissue, but did not induce BER symptoms (Figure 3.3c). A 15 min soak in 10mM $CaCl_2$ inhibited symptom development without tissue shrinking.

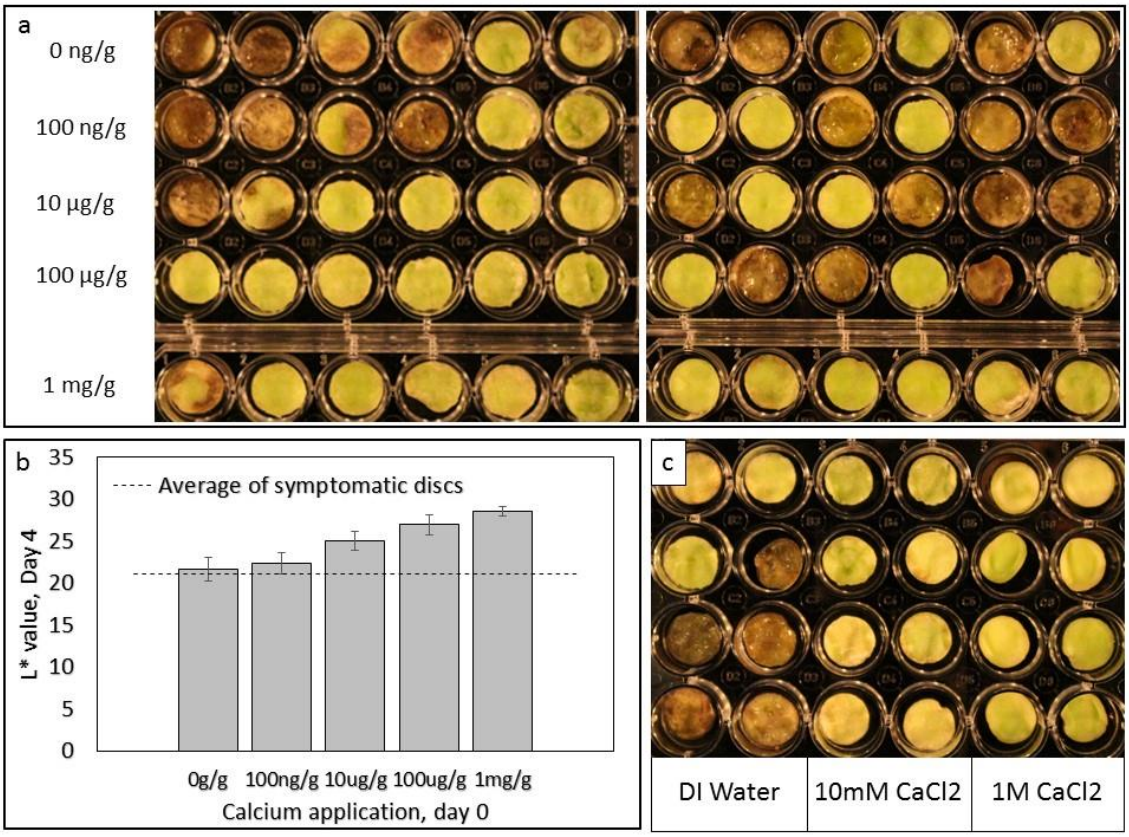


Figure 3.3: Effect of calcium treatments on immature tomato pericarp discs. (a) Appearance of tomato pericarp discs collected from the blossom-end of fruit harvested 21 d after pollination, 4 days after disc preparation and treatment. Each horizontal row contains discs treated with the same weight of $CaCl_2$ per gram of disc by dripping $CaCl_2$ solution onto the disc. The first 5 vertical columns starting on the left in each photo contain discs from the same fruit and each disc in the sixth column is from a different fruit (5 fruit per photo, 10 total). (b) L* value color measurements of the disc shown in (a) above. Each bar represents the mean of 12 discs with error bars representing the standard error of the mean. The dashed line indicates the mean L* value of all symptomatic discs. (c) Appearance of blossom-end pericarp discs prepared from 4 additional fruit 4 days after disc preparation and treatment. Discs were soaked 15 min in DI water (column 1 and 2), 10mM $CaCl_2$ (column 3 and 4), or 1M $CaCl_2$ (columns 5 and 6).

3.4 Discussion

Our results indicate that applying low and excessively high concentrations of calcium (0ppm and 500ppm $CaCl_2$, respectively) to tomato plants similarly increased BER severity compared to applying a moderate calcium treatment of 10ppm. BER induced by excess calcium application to the soil has been reported previously (Gholipour et al., 2017; Hossain and Nonami, 2012; Nonami et al., 1995). Nonami et al. (1995) found an increase from about 25% BER to approximately 45% BER when applying 20 g/L and 40 g/L Ca-zeolite, respectively. Hossain and Nonami (2012) found that increasing the calcium concentration of Otsuka-house nutrient solution by 32.3 mM resulted in induction of BER, while the unaltered Otsuka-house solution resulted in no BER symptoms. Gholipour et al. (2017) used this same 32.3 mM increase to induce BER for their study of cellular water relations and metabolomics during BER development.

A lack of significant difference between treatment groups in fruit tissue calcium concentration in our study suggests that increased application of calcium in irrigation solutions did not lead to increased calcium uptake into the fruit. While water use in plants treated with 500ppm was reduced compared to plants treated with 10ppm $CaCl_2$, this reduction would likely not outweigh the drastic difference in calcium concentration in the soil. To estimate the daily plant calcium uptake (supplemental figure 3.2), the daily calcium lost through drainage was subtracted from the total calcium added through daily treatments. Calcium lost through drainage was calculated based on the EC measurements of the drained solution shown in figure 3.2 and the response in EC to increased $CaCl_2$ concentration shown in supplemental figure 3.1. The average of the 0ppm treatments was used as a blank to account for the EC increase due to the slow release fertilizer applied to each pot. Based on these estimations, plants in the 500ppm treatment likely had much

higher calcium uptake than the 10ppm treatment. The similar calcium contents measured in the fruit and differing estimated calcium uptake highlights the importance of calcium transport to the fruit, rather than total plant calcium uptake. De Freitas et al. (de Freitas et al., 2011b) demonstrated that application of abscisic acid can reduce BER incidence though a similar mechanism. In abscisic acid treated plants, the total plant calcium uptake was decreased, but the fruit calcium uptake was 10-fold higher than that of water treated plants. Complementing the results of De Freitas et al. (de Freitas et al., 2011b), the results presented here show that increasing plant calcium uptake through increasing soil calcium does not necessarily result in increased fruit calcium uptake or decreased BER. Conversely, our results showed increased BER severity in 500ppm treated plants compared to 10ppm treated plants. This increase in severity may be associated with the effects of increased $CaCl_2$ on plant water relations and stress. Water use was reduced in 500ppm treated plants compared to 10ppm treated plants. While no significant differences were found between treatments in water potential, the trends of the means of these measures mirror those of plant water use and are opposite those of BER development.

Rapid fruit growth and cellular expansion have been proposed previously as factors leading to BER development (Ho and White, 2005; Marcelis and Ho, 1999). Aslani et al. (2020) found a significant correlation between BER incidence and tomato fruit growth rate, but fruit calcium content was not significantly correlated with BER incidence. Because tomatoes in our study were harvested 21-22 days after pollination, fruit size presented here was a measure of the early fruit growth. We found that increased fruit size was positively correlated with BER development, supporting rapid fruit growth's association with BER development, even in the absence of effects on total fruit calcium.

The role of calcium in BER development has been discussed in the literature for decades, and the nature of calcium's protective effect against BER is still debated. Nonami et al. (1995) found that increasing calcium concentrations in irrigation solutions did not reduce BER incidence in a linear manner, with BER incidence higher in their highest calcium concentration (40 g/L Ca-zeolite) compared to the moderate treatment (20g/L Ca-zeolite). From these results, they suggested that calcium deficiency was not the sole cause of BER. This assertion has been repeated in subsequent publications, with abiotic stress, oxidative stress, and phytohormonal signaling emerging as additional key factors in BER development (Aktas et al., 2005; de Freitas et al., 2011b; Sergio Tonetto de Freitas et al., 2012; Ho and White, 2005; Saure, 2001). More recently, the importance of these additional factors have been supported through transcriptional and metabolic analysis (de Freitas et al., 2017; Mestre et al., 2012; Reitz and Mitcham, 2021a). De Freitas et al. (2017) found that expression of two reactive oxygen species related genes, respiratory burst oxidase homolog (Rboh) and ascorbate oxidase (AO), were associated with BER development. These results support the work of Aloni et al. (2008) and Aktas et al. (2005), who found increased activity of AO and Rboh, respectively, in BER fruit. In a thorough investigation of the relationship between calcium deficiency and antioxidant systems, Mestre et al. (2012) found that calcium deficiency induced blossom-end rot was associated with a reduction in glutathione. Reitz and Mitcham (2021) found that accumulation of H_2O_2 and subsequent lignification was limited to only the blossom-end of affected fruit. This highlights the importance of tissue and location in blossom-end rot development, even within affected fruit. De Freitas et al (2017) also found involvement of a number of genes related to abiotic stress in BER. Reduction of BER incidence was associated with upregulation of NAC/NOR and CIPK, which are associated with tolerance to salt stress. Upregulation of abiotic stress response genes

including DREB and ethylene response factors (ERF109 and ERF010) was associated with treatment groups exhibiting lower blossom-end rot.

Through the use of pericarp discs, (Reitz and Mitcham, 2021b) showed that both calcium and ascorbic acid provide a protective effect against BER symptom development. Results presented here using the same pericarp disc system indicate that calcium inhibits BER symptom development in a concentration-dependent manner, with increasing concentrations being more effective in reducing symptoms. Excessively high treatment concentrations did not induce symptom development when applied to discs, but did increase symptom severity when applied to whole plants in irrigation solutions. While the effects of adding various levels of calcium to irrigation solutions on BER incidence in the present study closely mirrors the results of Nonami et al. (1995), the pericarp disc results reaffirm calcium's protective effect against BER, even in the presence of excessive calcium concentrations. These results indicate that the BER-inducing effect of excessive calcium in irrigation solutions is due to effects at the whole plant level.

In this study, BER was negatively correlated with the harvest day, meaning BER was more prevalent in the first fruits to develop on each plant. Results suggest that over-compensating for observation of early season BER with excessive calcium application may result in similar or increased BER severity. Moderate application of calcium at a rate of 10 ppm to container grown tomato plants in irrigation solution was sufficient in reducing BER incidence and severity, despite being lower than previous recommendations (Bar-Tal et al., 2017; Kreij, 1996). Foliar application of calcium has also been shown to be effective in reducing BER incidence (Schmitz-Eiberger et al., 2002; Wada et al., 1996), and may also be an effective mid-season option. This could facilitate direct absorption of calcium into the fruit, while reducing the likelihood of overconcentration of calcium in the soil compared to calcium application to the soil.

3.5 Conclusions

Our results provide strong evidence that BER incidence in tomato fruit is strongly linked to calcium concentrations in the fruit. Although excess calcium applied through irrigation water increased the severity of BER, we were able to demonstrate a clear concentration-dependent relationship between the calcium concentration applied to pericarp disc tissue and inhibition of BER, even at very high levels of calcium. Excess calcium induction of BER on the plant appears to be due to water use and salinity effects at the whole plant level.

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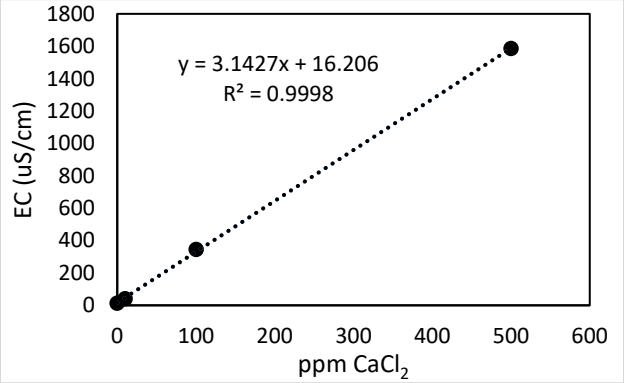
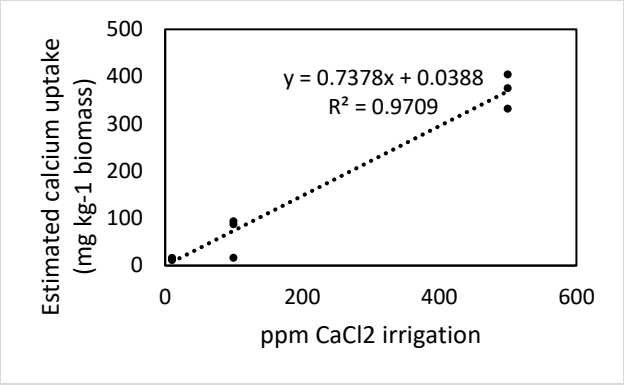
3.7 Supplemental figures



Supplemental figure 3.1: Effect of $CaCl_2$ concentration on EC. Each point indicates the EC of the irrigation solution for 0ppm, 10ppm, 100ppm, and 500ppm $CaCl_2$ treatments. Irrigation solutions contained deionized water and $CaCl_2$.



Supplemental figure 3.2: Estimated calcium uptake in 10ppm, 100ppm, and 500ppm $CaCl_2$ treated plants. Each point represents the estimated calcium uptake per kg biomass of one plant, with 3 plants at each irrigation concentration.

Calcium uptake was calculated by assuming calcium either drained from the pot during daily irrigation or was taken up by the plant. The calcium drained from each pot was calculated using the regression equation from supplemental figure 1 and the drained solution EC measurements presented in figure 2. This value was subtracted from the calcium added to each pot through irrigation to get the daily calcium uptake for each plant. The EC of the drainage solution could also be affected by the slow release fertilizer applied to each pot. To account for this, the average drainage EC of the 0ppm treatment, which should contain minimal calcium, was subtracted from the drainage EC of plants from other treatments. The resulting calcium uptake for 3 plants each of the 10ppm, 100ppm, and 500ppm treatments was plotted against calcium concentration for each treatment.

Chapter 4: Investigating cellular events surrounding blossom-end rot development in tomato using a pericarp disc system

Abstract

Blossom-end rot in tomatoes and peppers has been associated with oxidative stress, abiotic stress, calcium deficiency, and phytohormone regulation. A recent study using pericarp discs demonstrated that direct calcium treatment of the tomato pericarp tissue inhibits BER symptom development. However, the biological mechanism of this inhibition remains unknown. The present study uses the same pericarp disc system to investigate the central biological factors associated with blossom-end rot, specifically in the affected pericarp tissue. Calcium treated discs did not develop blossom-end rot symptoms and were higher in ascorbic acid and glutathione than symptomatic water treated discs. Treatment of discs with ascorbic acid and glutathione also inhibited symptom development. Results from hydrogen peroxide measurement in water and calcium treated discs suggest that hydrogen peroxide accumulation does not induce blossom-end rot symptoms when adequate calcium is present. However, treatment with a reactive oxygen species production inhibitor, diphenyleneiodonium chloride, prevented symptom development, suggesting that reactive oxygen species likely play a direct role in initiation of blossom-end rot. Delayed treatment with either calcium or ascorbic acid reduced respiration rate one day after treatment, suggesting that some cellular processes associated with blossom-end rot development may be reversible. Disrupting calcium signaling with a calcium channel blocker had a similar inhibitory effect, although it also resulted in whitening of the tissue. While whole plant treatments with gibberellins and abscisic acid have been shown to influence BER development, application of gibberellins, a gibberellin biosynthesis inhibitor, or abscisic acid to pericarp discs did not affect symptom development. These results suggest that the critical effects of phytohormone treatments

may be at the whole plant level, rather than in the blossom-end pericarp tissue. Overall, these results indicate that maintaining tissue calcium concentrations and antioxidant potential inhibits blossom-end rot symptom development, and that treatment with either calcium or ascorbic acid can reverse or slow cellular processes associated with blossom-end rot symptom development. The inhibitory effect of calcium treatment may be due in part to enhancement of the ascorbic acid-glutathione antioxidant system.

4.1 Introduction

Blossom-end rot (BER) is a physiological disorder of tomato, pepper, eggplant, and watermelon fruit that causes significant losses to the produce industry. Tomatoes are often used for the study of BER due to well establish growth and transformation methods (de Freitas et al., 2011a), ease of disorder identification and sampling, and extensive previous research (Hagassou et al., 2019). Symptoms of BER in tomato include tissue water soaking, cell death, and tissue darkening during the cell expansion phase of fruit grown. Previous research on BER development in tomatoes and peppers has identified calcium deficiency, oxidative stress, abiotic stress, and phytohormone metabolism as key factors associated with BER development (Hagassou et al., 2019).

A complex network of interacting factors affects blossom end rot development, with both environmental and cellular conditions playing critical roles. Additionally, direct treatment of the affected tissue is difficult without potentially confounding effects at the whole plant and whole fruit level. As a result, the existing research on BER has mainly developed strong associative links rather than causative evidence. Recently, we developed a pericarp disc system that enables direct treatment of the affected pericarp tissue and in-depth study of the subsequent effects (Reitz and Mitcham, 2021a). Using this pericarp disc system, Reitz and Mitcham (2021a) demonstrated

that direct calcium treatment of the tomato pericarp tissue inhibits BER symptom development. However, the biological mechanism of this inhibition remains unknown.

Calcium's effect on BER development has been the focus of much research in the past 30 years. Simply supplying calcium in the soil in high quantities is not fully effective in eliminating BER development, as has been shown by Nonami et al. (1995) and in chapter 3 of this dissertation. An important factor for reduction of BER development is the transport of calcium to the blossom-end of the fruit (de Freitas et al., 2011b) and maintaining apoplastic calcium within the susceptible blossom-end pericarp tissue (de Freitas et al., 2011a). Reitz and Mitcham (2021a, chapter 1 of this dissertation) demonstrated that direct treatment of pericarp tissue with calcium chloride inhibited BER symptoms. Calcium applied to pericarp discs inhibited symptoms in a concentration dependent manner (Chapter 2 of this dissertation). In contrast with applications of calcium to the soil, applying calcium to pericarp discs inhibited BER symptoms with no upper limit on calcium concentration.

The ascorbic acid-glutathione antioxidant system has also been implicated in BER development. Reduced ascorbic acid content and increased ascorbate oxidase activity had been previously reported to be associated with BER fruit (Aloni et al., 2008; de Freitas et al., 2017). A reduction in glutathione concentration and glutathione reductase activity has also been associated with BER development (Mestre et al., 2012). Reitz and Mitcham (2021a) found that exogenous supplementation of ascorbic acid in pericarp discs inhibited BER symptoms. It remains unknown, however, if glutathione can also inhibit BER symptom development. Furthermore, the relationship between calcium and the ascorbic acid-glutathione antioxidant system remains unknown.

Phytohormone treatment with abscisic acid (ABA) and gibberellins (GA) decreases and increases BER development, respectively. Abscisic acid has been shown to be a powerful inhibitor of BER development when applied at the whole plant level (de Freitas et al., 2011b, 2014, 2017). Interestingly, fruit dipped in ABA had reduced, but not eliminated, BER incidence. This change was associated with increased xylem function and increased calcium concentrations in the blossom-end of the fruit. GA foliar sprays have been shown to drastically increase BER incidence, with increased electrolyte leakage and decreased apoplastic calcium concentrations in fruit from GA treated plants compared to controls (de Freitas et al., 2012). These decreased apoplastic calcium concentrations were associated with an increase in *CAX* gene expression which is responsible for calcium transport into storage organelles. In this same study, prohexadione-calcium (PH), a GA biosynthesis inhibitor, eliminated BER development. Treatment with prohexadione was associated with increased fruit pericarp calcium concentrations and xylem functionality. However, it is not well known if ABA, GA, and PH have critical effects at the cellular level, or if their important effects are on the plant as a whole. This research investigates the effects of direct phytohormone treatment of the affected tissue without confounding effects at the whole plant level.

Abiotic stress, including water deficit and increased soil or nutrient solution salinity, has been shown to induce BER. Adams and Ho (1993) found that increased salinity reduced fruit calcium uptake. Similarly, Ho et al. (1993) demonstrated that calcium uptake to the blossom end of fruit was lower when grown at high salinity compared to lower salinity conditions. Similarly, Silber et al. (2005) found that increasing the frequency of fertigation improved nutrient uptake and decreased BER development. Even with low calcium fertigation, de Freitas et al. (2014) found that the induction of BER for research purposes was best accomplished through daily water

stress. Water deficit or osmotic stress may also be a factor in symptom development in the pericarp disc system. Because only healthy fruit were used during the experiments of Reitz and Mitcham (2021a), symptom development in the pericarp disc system was likely instigated by the stress of disc preparation and storage. Of particular interest is weight loss during disc storage, which was likely due, at least in part, to water loss, and may simulate water stress on the plant. Understanding the effect of water loss and osmotic effects in the disc system may offer insights into the relationship between BER and water relations overall.

The research presented here uses the pericarp disc system to investigate the effects and critical timing of calcium, reactive oxygen species (ROS), and the ascorbic acid-glutathione antioxidant system in BER symptom development. The effects of phytohormones at the tissue specific level are also investigated. Other calcium deficiency disorders, such as bitter pit in apples and tip burn in lettuce, cause significant losses to the produce industry. In addition to directly addressing questions related to BER development in tomatoes, results from this study may be applicable to other calcium deficiency disorders and could enhance our understanding of the role of calcium in plant cells generally.

4.2 Materials and methods

Plant growth

Solanum lycopersicum L. seeds of the variety HM 4885 (HM) were obtained from Agseeds Unlimited (Woodland, CA, USA) and *S. lycopersicum* L. seeds of the variety Ailsa Craig (AC) were obtained from the Tomato Genetic Resource Center (UC Davis, Davis, CA, USA). The HM variety is highly susceptible to BER and HM fruit were used in the experiment utilizing discs with partial BER affected tissue. HM fruit were also used for delayed treatment experiments

because a similar aspect of BER was being tested. Ailsa Craig fruit were used for the remaining experiments because of the prevalence of BER-unaffected fruit and the ease of disc preparation from the rounded fruit shape. Seeds were planted in peat pellets and germinated with double deionized water. Seedlings were transplanted approximately 2 weeks after germination into 7.57 liter pots with a mixture of 1/3 peat, 1/3 sand, and 1/3 rosewood compost, augmented with 1.56 kg m^{-3} dolomite lime. An irrigation solution of 150 ppm nitrogen, 50 ppm phosphorus, 200 ppm potassium, 175 ppm calcium, 55 ppm magnesium, 120 ppm sulfur, 2.5 ppm iron, 0.02 ppm copper, 0.5 ppm boron, 0.50 ppm manganese, 0.01 ppm molybdenum, 0.05 ppm zinc, and 0.02 ppm nickel was applied until saturation to each pot daily. At anthesis, flowers were pollinated and tagged. Fruit were harvested 21 days after pollination for all experiments.

Disc preparation

Discs were prepared following the methods of Reitz and Mitcham (2021a). Briefly, fruit harvested 21 days after pollination were surface sterilized in 10 g L^{-1} sodium hypochlorite and rinsed thoroughly with sterile water. Cylinders of pericarp tissue were cut from the blossom-end of the fruit with a 13.5mm cork borer and the endocarp tissue removed with a razor. The discs were rinsed in autoclaved, ultrapure water, and placed skin side down in a 24 well plate. Plates of discs were stored at 25°C in closed containers under constantly flowing >90% RH air for up to 4 days.

Calcium chloride treatment time course

A time course of calcium and water treated discs was completed to investigate the effect of calcium treatments on hydrogen peroxide accumulation and the ascorbic acid-glutathione antioxidant system. Discs (24 per treatment) were treated with a 15-minute soak (with gentle

agitation) in 10 mL ultrapure water or 10g L^{-1} calcium chloride. On days 1, 2, and 3, disc color was measured on 8 discs per treatment using a Minolta colorimeter (Konica Minolta Inc. Tokyo, Japan). L* color was used to measure tissue darkening and a* color was used to measure loss of green color. Half of each of the 8 discs was sampled for hydrogen peroxide measurement. The unused half of each disc was frozen, and 4 of these halves for each day and treatment were used for ascorbic acid and glutathione analysis.

Hydrogen peroxide content

Hydrogen peroxide was assayed by measuring the oxidation of potassium iodide at A_{350}, compared to a standard curve (Junglee et al., 2014), using a BioTek H1 multimode plate reader (Biotek, Winooski VT, USA). Tissue was ground in 0.25 g mL^{-1} reaction solution using a mortar and pestle. Each 1mL of reaction solution consisted of 250 μL 100 mM potassium phosphate buffer (pH 6), 250 μL 0.1 % trichloroacetic acid, and 500 μL 1M potassium iodide. Samples were ground with the reaction mixture and centrifuged for 15 min at 12,000 **g**. The supernatant (100 μL) was added to a microplate well (Fisherbrand, Pittsburg, PA, USA) and the absorbance at 350 nm was measured. Controls for each sample were prepared in the same manner, but with ultrapure water added instead of the potassium iodide solution. Samples were compared to a standard curve of 1 μM to 5 mM H_2O_2 in potassium phosphate buffer and presented on a per gram fresh weight basis.

Ascorbic acid and glutathione analysis

Ascorbic acid and glutathione concentrations were measured using reverse phase high performance liquid chromatography. Tomato samples were ground in liquid nitrogen and vortexed with an extraction mixture of 6% (w/v) metaphosphoric acid containing 2 mM EDTA

and 1% (w/v) insoluble polyvinyl polypyridone. The extraction mixture was added at a rate of 0.2 g tissue mL^{-1} extraction mixture. The extracts were centrifuged at 20,000 **g** for 12 min at 4°C and the supernatant was filtered through a 0.45 µm syringe filter. Filtered samples were injected (5 µL) into an Agilent 1260 HPLC. The column was an Agilent eclipse XDB-C18 5 um 4.6x150 mm and the mobile phase was 400 µl L^{-1} o-phosphoric acid, pH of 2.5, and 0.1 mM EDTA. The flow rate was 0.8 mL min^{-1} and a gradient of 0-30-0% acetonitrile was run for 12-15-18 minutes, followed by 7 minutes with 0% acetonitrile for a total run time of 25 minutes. Samples were analyzed at 243 nm and 197 nm for ascorbic acid and glutathione, respectively, with retention times of approximately 2.9 and 4.3 min. A standard of 1 mM ascorbic acid and 1 mM glutathione in 3% (w/v) metaphosphoric acid, stabilized with 200 mM dithiothreitol, was used for quantification.

Delayed calcium and ascorbic acid treatment

The effect of treatment timing on disc color and respiration rate was evaluated by treating discs from HM fruit on day 0, 1, or 2 with water, 10 g L^{-1} calcium chloride, or 500 mM ascorbic acid. For each treatment type, 24 discs were prepared from 4 fruit, and 2 discs from each fruit were treated on each day. Treatments were applied directly to each well at a rate of 1.25 mL per well. After 15 minutes of gentle shaking, the solution was removed and the discs were blotted dry. Color and respiration rate were measured on days 1, 2, and 3 on all discs. Respiration rate was measured by sealing the well of each plate with an adhesive PCR plate cover (MicroAmp, Applied Biosystems, Foster City, California, USA). An adhesive septum (Bridge Analyzers Inc. Alameda, California, USA) was applied to the PCR plate cover above each well. After 4-6 minutes of sealing time, a syringe was used to extract 1mL of the headspace air from each well.

This was injected into an infrared CO_2 analyzer (Horiba, Irvine, CA) and respiration rate was calculated on a μl CO_2 min^{-1} g^{-1} fresh weight basis.

Ascorbic acid, glutathione, verapamil, and diphenyleneiodonium chloride treatments

On the day of harvest, discs were treated with 15-minute dips as described above. Each experiment included 24 discs from 4 fruit, and each treatment was applied to 2 discs from each fruit (8 discs per treatment). Discs treated with 500 mM glutathione or 500 mM ascorbic acid were compared to water treated discs from the same fruit. The calcium channel blocker verapamil hydrochloride (VER) was used to disrupt calcium signaling in pericarp tissue (Iriti et al., 2006; Singh et al., 2011). The NADPH oxidase inhibitor diphenyleneiodonium chloride (DPI) was used to inhibit the production of ROS by NADPH oxidase (Distéfano et al., 2017). Discs treated with 10 mM VER dissolved in water or 1 mM DPI dissolved in water with 0.1% (v/v) dimethyl sulfoxide were compared to control discs treated with 0.1% (v/v) dimethyl sulfoxide. Treatment concentrations were selected to promote rapid diffusion of treatments into the apoplast. Color was measured as previously described 1, 2, 3, and 4 days after treatment for all experiments. Electrolyte leakage was measured in 4 VER, DPI, and water treated discs by placing each disc in 10 mL of an isotonic solution of 0.2 M mannitol. This was gently shaken at room temperature and the electrical conductivity (EC) was measured at 0.5, 1, 1.5, 4, 4.5, and 5 hours. After freezing and thawing three times, the total EC was measured. The electrolyte leakage was calculated as the increase in percent of total EC over time during the linear phase from 4 to 5 hours.

Electrolyte leakage in differing tissues throughout storage and in calcium treated discs

Electrolyte leakage was measured to test the effect of tissue location and calcium treatment on membrane integrity. Discs were prepared from the stem-end and blossom-end tissue of 4 AC fruit, with a total of 12 discs per tissue type. On days 1, 2, and 3 of storage, 1 disc from each tissue type of each fruit was removed and the electrolyte leakage was analyzed as previously stated. The blossom-end tissue from an additional 4 AC fruit were used to prepare 16 discs. From each fruit, 2 discs were treated with a 15-minute soak in either water or 10 g L^{-1} calcium chloride, with a total of 8 discs per treatment. These discs were analyzed for color and electrolyte leakage after 4 days of storage.

Incubation in mannitol solutions and water

To investigate the effect of stress due to water loss on symptom development, discs were incubated in mannitol solutions. Discs were prepared from 4 AC fruit, weighed, and placed in a 24 well plate. Ultrapure water (1 mL) was added to the wells of 2 discs per fruit (8 wells total), and a mannitol solution (0.4 M, 1 mL) was added to the wells of 2 discs per fruit (8 wells total). No solution was applied to the remaining 2 discs from each fruit (8 total). On day 2, discs were removed from the solution, blotted dry, weighed, and returned to the same solution. On day 4, discs were removed from the solution, blotted dry, weighed, and the color was measured. To test the effect of increased water loss due to reduced storage solution osmotic potential, an additional experiment was completed with 4 additional AC fruit, adding 0.3 M and 0.6 M mannitol to 2 discs from each fruit (8 discs total per concentration).

Phytohormone treatment

Phytohormone treatments were applied to test for tissue specific hormone effects related to BER tissue development. Discs were treated with phytohormones on the day of disc preparation. Treatments of 10 mL per 8 discs were applied with a 15-minute soak with gentle shaking (approximately 120 rpm). Phytohormone treatments included 1mM ABA (Protone®, Valent Biosciences, Libertyville, IL, USA), 1 mM gibberellic acid (GA_3, Arcos Organics, Geel, Belgium), and 1 mM PH (Apogee®, BASF, Ludwigshafen, Germany). To test the effect of GA_3 in the presence of adequate calcium, calcium chloride was included with a GA_3 treatment at a rate of 10 g L^{-1}, with 10 g L^{-1} sodium chloride and GA_3 as a control. Each experiment contained 24 discs from 4 fruit, with 2 discs from each fruit (8 total) within each of the three treatments. Each experiment included two phytohormone treatments and a water control. Each experiment was carried out on both AC and HM fruit. Treatments were grouped as shown in table 5.1. Disc color was measured 4 days after treatment. Follow up experiments with the same design were carried out using 2 mM PH, 5 mM PH, 100 µM ABA, 2 mM ABA, 1mm GA_{4+7} (Phytotech Labs, Lenexa, Kansas, USA), 100µM GA_{4+7}, 1mM GA_{4+7} with 10 g L^{-1} calcium chloride, and 100µM GA_{4+7} with 5 g L^{-1} calcium chloride.

Statistical analysis

Statistical analyses were carried out using SAS On Demand for Academics (SAS Institute Inc., Cary, NC, USA). To test for significant differences between means, analysis of variance was carried out with Tukey's means separation.

4.3 Results

Relationship between calcium, ascorbic acid, glutathione, and BER development

Ascorbic acid and glutathione were significantly higher overall in calcium treated discs compared to water treated discs, with no treatment by day interaction (figure 4.1a, 4.1b). Treatments were not significantly different overall in H_2O_2 content (figure 4.1c). Calcium treated discs increased in H_2O_2 content from days 2 to 3 and water treated discs decreased over the same time period. This resulted in a significant treatment by day interaction. Calcium, ascorbic acid, and glutathione treated discs appeared healthy after three days of storage, while water treated discs exhibited water soaking of the tissue and subsequent browning (figure 4.1e). Measurements of a* value were not significanly different overall between calcium, ascorbic acid, glutathione, and water treatments (figure 4.1d, 4.1f). However, a significant treatment by day interaction was observed in both experiments, indicating that water treated discs increased in a* value faster than calcium, ascorbic acid, or glutathione treated discs.

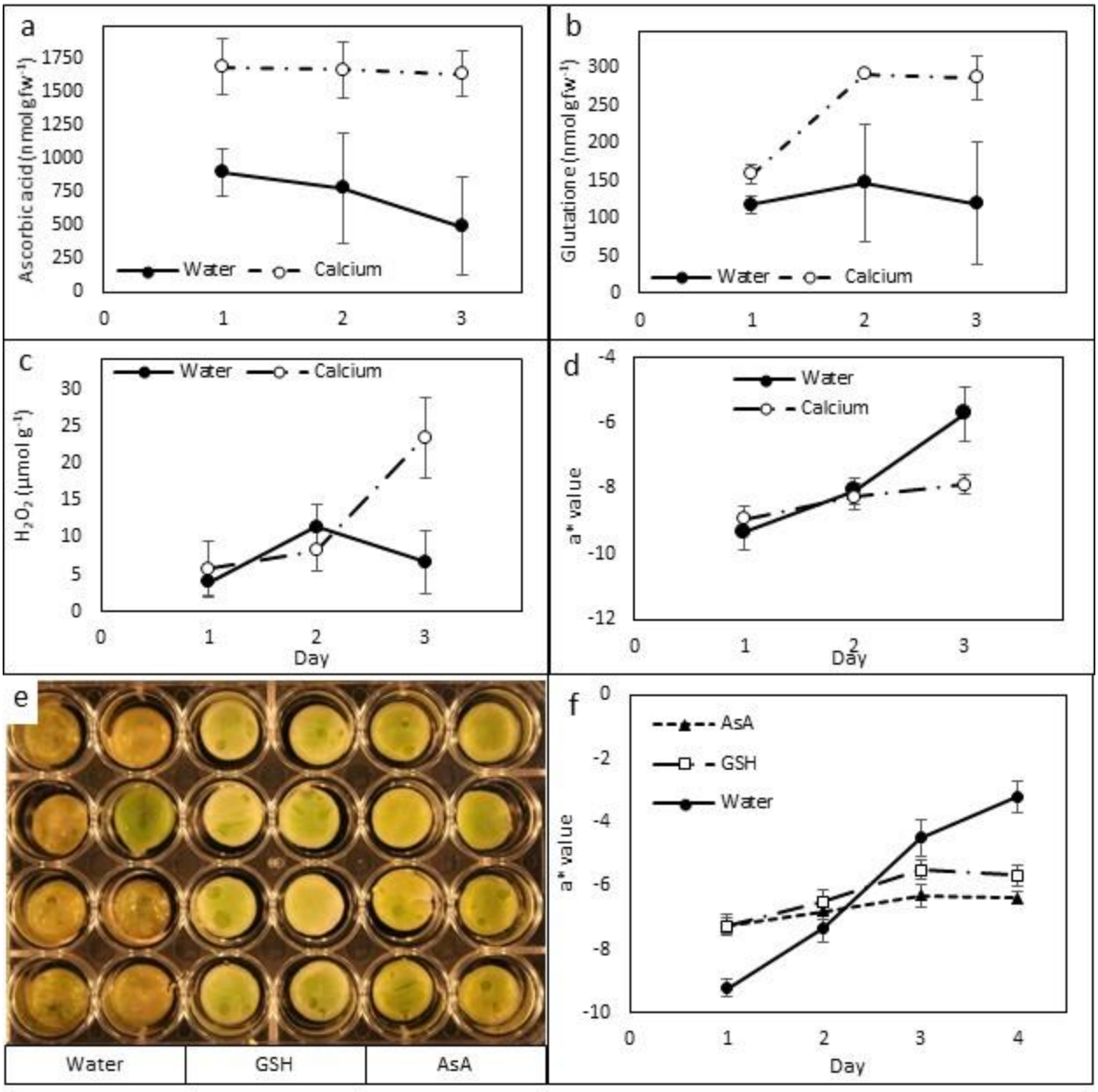


Figure 4.1: Effect of calcium chloride treatment on ascorbic acid (a), glutathione (b), and hydrogen peroxide (c), and change in a* value disc color (d) over 3 days of storage. Color values presented in (d) were measured on the discs sampled for subsequent H_2O_2, ascorbic acid, and glutathione content analysis. A photo (e) was taken of discs 4 days after treatment with water, 500mM glutathione (GSH), or 500mM ascorbic acid (AsA). The change in a* value color (e) of these same discs was measured over 4 days of storage. Each point in (a) and (b) is the mean of 4

discs. Each point in (c), (d), and (f) is the mean of 8 discs from . Error bars represent the standard error of the mean. Discs were prepared from 4 Ailsa Craig fruit for (a), (b), (e), and (f), and 8 Ailsa Craig fruit for (c) and (d).

Effect of delayed calcium and ascorbic acid treatment

Treatment with calcium chloride on day 0 resulted in significantly lighter tissue color (higher L* values) and reduced loss of green coloration (lower a* means) on day 3 compared to day 2 treatement (figure 4.2). Treatment with calcium chloride on day 1 resulted a moderate reduction in tissue darkening and loss of green coloration on day 3, with mean L* and a* values not significantly different from day 0 and day 2 treatments. Treatment with calcium on day 0 or 1 resulted in a reduced respiration rate on day 2 compared to discs treated on day 2. Disc treated with ascorbic acid on day 0 and day 1 had similar L* values throughout storage. However, day 1 and day 2 ascorbic acid treatments has an increased loss of green color on days 2 and 3 compared to day 0 treated fruit. Respiration rate decreased on the day after treatment for day 1 and 2 ascorbic acid treatments. Day 0 ascorbic acid treatment reduced respiration rate on day 1 compared to to discs from the day 1 and day 2 treatment, which had not yet been treated. Water treatments on any day did not induce significant differences in L* or a* color. Day 2 water treatment increased respiration on day 3 compared to day 1 and day 2 treatments.

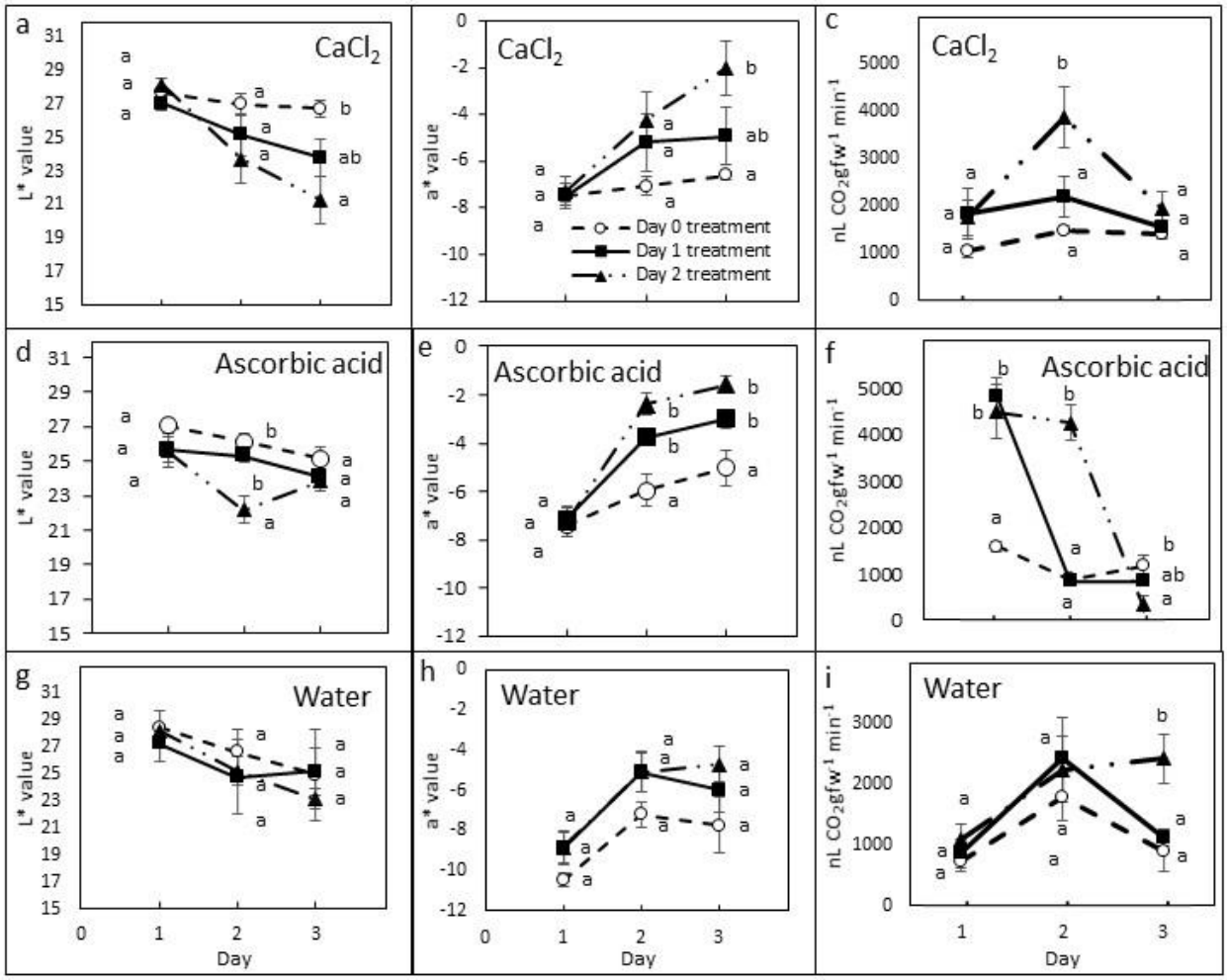


Figure 4.2: Effect of delayed treatment of tomato pericarp discs with 10 g L^{-1} calcium chloride (a, b, c), 500 mM ascorbic acid (d, e, f), and water (g, h, i) on L* values (a, d, g), a* values (b, e, h), and respiration rates (c, f, i) over 3 days of storage. Discs were prepared from Ailsa Craig fruit, and treatments were applied on day 0, 1, or 2 after disc preparation. Evaluations were made on days 1, 2, and 3 after disc preparation. Each point is the mean of 8 discs with standard error of the mean. Points with the same letter on the same day within each treatment were not significantly different (p>0.05).

Effect of calcium chloride and ascorbic acid treatment on discs with partial BER tissue

Symptoms spread from the BER affected region of water treated discs made from discs that included a small portion of BER affected tissue until the entire disc was affected with BER (figure 4.3). The spread was associated with a loss of green color (higher a* value) and darkening (lower L* values) of the tissue. Calcium chloride inhibited the spread of BER symptoms and maintained a green color in the disc tissues. Ascorbic acid treated discs did not exhibit symptom spreading from the BER affected tissue, but darkened and lost green color over the 4 days of storage. On day 4, ascorbic acid treated discs had a notably less healthy appearance compared to the calcium chloride treated discs. The BER affected area of calcium chloride treated discs shriveled while the unaffected area maintained a healthy appearance. In general, calcium chloride treated discs had lower a* value measurements and higher L* value measurements compared to the other treatments.

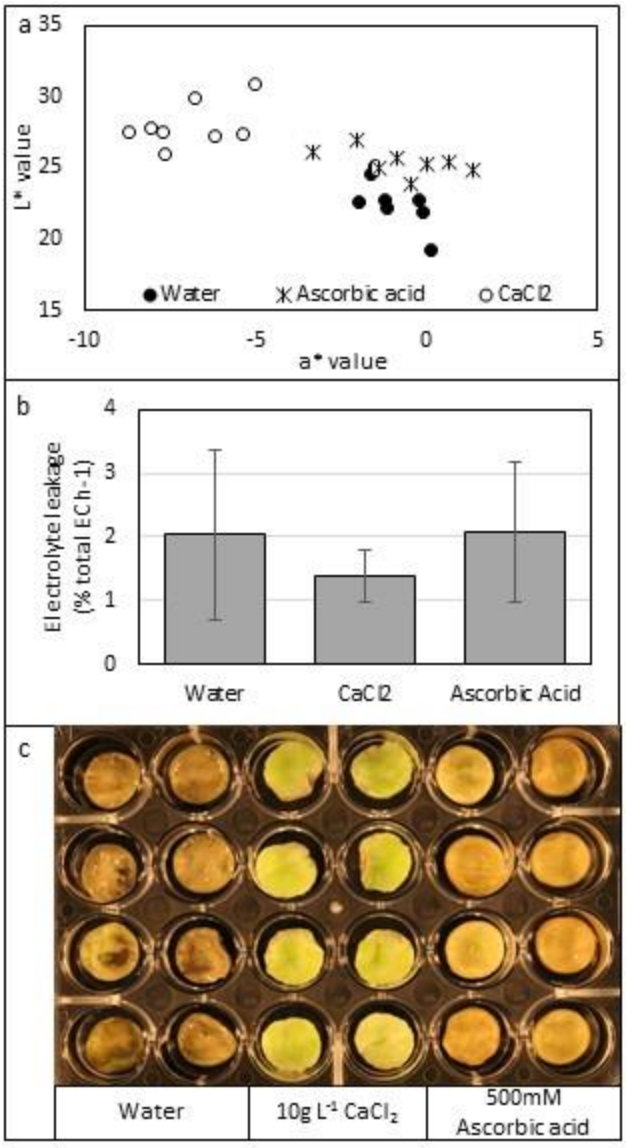


Figure 4.3: Effect of 10 g L^{-1} calcium chloride and 500 mM ascorbic acid treatments on blossom-end rot symptom development in pericarp discs that included a small portion of blossom-end rot tissue. Discs were made from HM 4885 tomato fruit. Color (L* and a* value) (a) and electrolyte leakage (b) were measured on discs 4 days after treatment. Each 15-minute dip treatment was completed on 2 discs from 4 fruit (8 discs total). A photo was taken (c) on day 4 of storage.

Effect of superoxide production and calcium signalling inhibitors on BER development

Water treated discs developed BER symptoms, including water soaking, loss of green color, and tissue darkening (figure 4.4). Discs treated with the NADPH oxidase inhibitor (DPI) or calcium channel blocker (VER) appeared healthy with no water soaking after 4 days of storage (figure 4.4a). Verapapmil treatment induced a bleaching effect causing the discs to turn white or very light green. Upon close observation, it was evident that the VER treated discs remained firm and healthy, with no water soaking. Color analysis (figure 4.4b) indicated increased lightness (higher L* value) and similar loss of green color (a* value) in VER treated discs compared to water treated discs. DPI treated discs retained a healthy appearance with no water soaking throughout storage. Color measurment indicated that DPI treated discs were darker than water treated discs, but retained green coloration better than water treated discs. Both VER and DPI treated discs had lower mean electrolyte leakage than water treated discs, although differences were not significant (figure 4.4c).

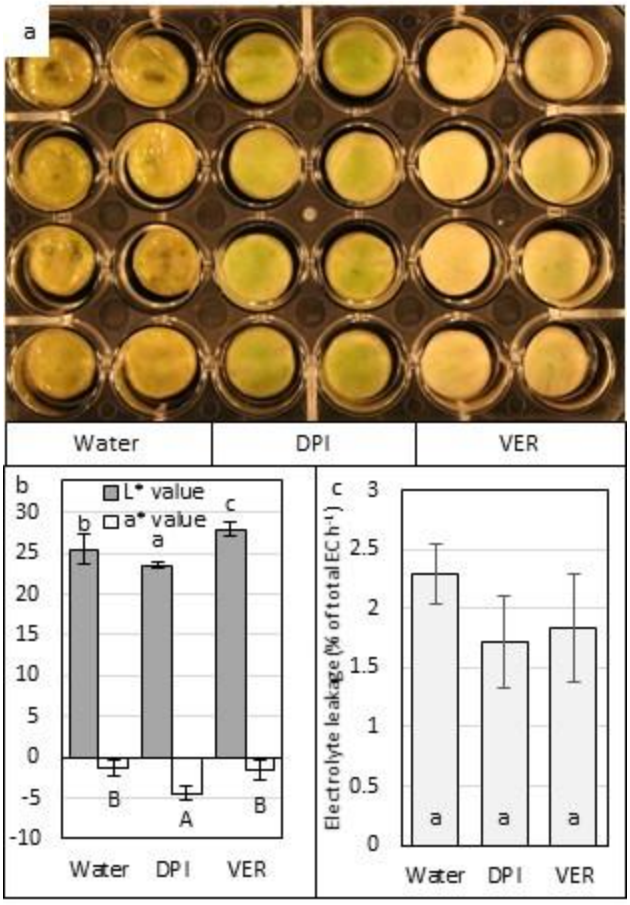


Figure 4.4: Effect of reactive oxygen species (DPI) and calcium (VER) signaling blockers on blossom end rot symptom development in pericarp discs from Ailsa Craig fruit. Discs were photographed (a) 4 days after treatment with water, 1 mM diphenyleneiodoneum chloride (DPI), or 10mM verapamil (VER). Disc color (b) and electrolyte leakage (c) was measured on 8 and 4 discs, respectivly, 4 days after treatment. Error bars represent the standard deviation of the mean. The same letter within each measurement (L*, a*, and electrolyte leakage) indicates that means were not significantly different (p>0.05).

Effect of tissue location and calcium treatment on electrolyte leakage

Electrolyte leakage was higher throughout storage in discs prepared from blossom-end pericarp compared to discs prepared from stem-end pericarp (figure 4.5a). Both stem-end and blossom-end discs increased in electrolye leakage from day 1 to day 2, and decreased from day 2 to day 3. Calcium chloride treated discs prepared from blossom-end pericarp had significanly lower electrolyte leakage compared to water treated discs on day 4 of storage (Figure 4.5b). Electrolyte leakage and disc L* color (indicative of BER symptoms) were significantly correlated with Pearson's correlation coefficients of -0.631 and 0.571 for L* and a* values compared to electrolyte leakage (p=0.009 and 0.021).

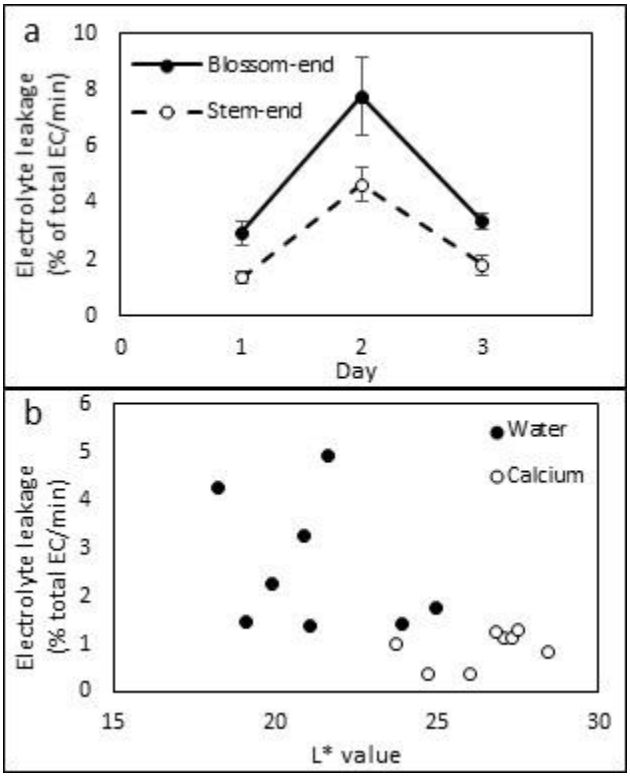


Figure 4.5: Effect of tissue location (a) and calcium chloride treatment (b) on electrolyte leakage in pericarp discs from Ailsa Craig fruit. Electrolyte leakage was measured on pericarp discs prepared from the stem-end and blossom-end of tomato fruit on days 1, 2, and 3 after disc

preparation (a), with each point representing the mean of 4 pericarp discs. Error bars represent the standard error of the mean. Electrolyte leakage measurements 4 days after disc preparation from blossom end pericarp (b); Blossom-end discs were treated with a 15-minute dip of either water or 10 g L^{-1} calcium chloride immediately after preparation.

Effect of incubation in water and mannitol solutions

Discs incubated in water or mannitol solutions did not develop BER symptoms and appeared healthy at the end of 4 days of storage (figure 4.6). Some, but not all, discs incubated in air developed BER symptoms by 4 days of storage. Discs incubated in air has significatnly decreased green color (higher a* mean) compared to discs incubated in water or 0.4 M mannitol. Incubation in mannitol resulted in significantly greener discs than discs stored water. Weight increased overall in discs incubated in water and 0.3 M mannitol. Weight decreased slightly in discs incubated in 0.4 M mannitol. In discs incubated without solution, weight decreased continuously to 89.5% of the initial weight. Discs incubated in 0.6 M mannitol lost a similar amount of weight as discs incubated without solution from day 0 to day 2, but weight was similar from day 2 to day 4 in discs incubated in 0.6 M.

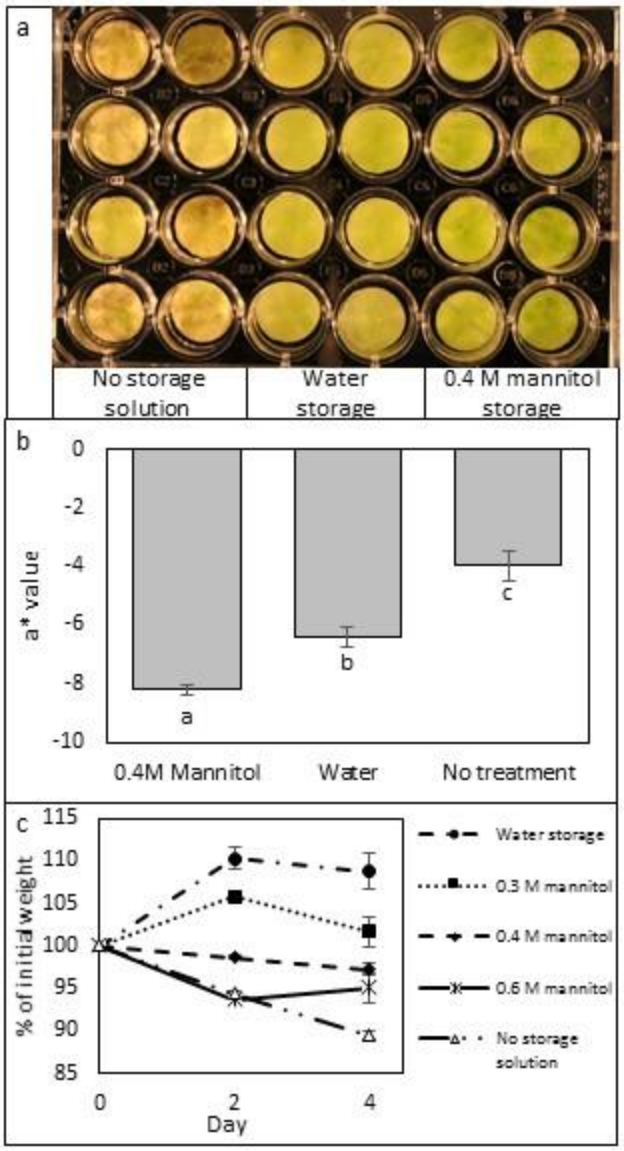


Figure 4.6: Effect of incubation solution on the appearance (a), color (b), and weight (c) of pericarp discs from Ailsa Craig fruit. Each treatment was applied to 2 discs from 4 fruit (8 discs total). The discs were photographed (a) on day 4 after removal of the storage solutions. Color (a* value) was measured (b) on day 4 of storage. Each bar indicates the mean of 8 discs and error bars represent the standard error of the mean. Symptoms were only observed in discs incubated without a solution. Disc weight (c) was measured prior to the application of incubation solutions

(day 0), and on days 2 and 4 after blotting dry. Each point represents the mean of 8 discs with error bars representing the standard error of the mean.

Effect of phytohormone treatment on BER development in discs

Application of the phytohormones ABA and GA_3 did not have significant effects on BER development in AC and HM tomato varieties as measured by changes in the mean L* and a* values compared to water treated discs after 4 days of disc storage (Table 4.1). Application of calcium chloride with GA_3 in HM discs significantly reduced BER symptoms as measured by L* and a* values compared to treatments of NaCl with GA. Application of NaCl with GA_3 significantly increased a* values compared to water or calcium chloride and GA_3 treatments. L* value and a* value means for discs treated with PH were notably increased and decreased, respectively, compared to water treatments in HM fruit, although variation was high and differences were not significant. Additional experiments on discs from Ailsa Craig fruit using 2 mM PH, 5 mM PH, 100 μM ABA, and 2 mM ABA produced similar results, with no inhibitory effects observed (data not shown). Results from experiments using GA_{4+7} (data not shown) were similar to results from GA_3 treatment presented in table 5.1.

Table 4.1: Phytohormone treatment effects on blossom-end rot symptom development in pericarp discs.

Variety	Experiment	Treatment[z]	L*	±SD		a*	±SD	
AC[x]	1	Water	24.30[y]	1.50	A[y]	-3.03[y]	2.12	a
		ABA[x]	23.77	1.94	a	-2.86	2.09	a
		GA_3[x]	23.92	1.25	a	-3.61	2.25	a
	2	Water	25.14	0.59	a	-4.03	0.69	a
		PH[x]	24.08	1.29	a	-4.33	1.04	a
		PH+GA_3	23.89	1.71	a	-4.56	0.72	a
	3	Water	22.26	2.60	ab	-2.61	2.60	ab
		GA_3+$CaCl_2$	23.87	0.72	b	-5.03	0.88	a
		GA_3+NaCl	21.39	1.83	a	-0.79	2.16	b
HM[x]	1	Water	25.56	4.84	a	-5.35	4.67	a
		ABA	27.27	3.13	a	-4.48	3.89	a
		GA_3	26.41	2.99	a	-5.29	4.84	a
	2	Water	24.98	6.57	a	-1.46	3.38	a
		PH	27.50	2.32	a	-3.51	3.09	a
		PH+GA_3	29.20	0.86	a	-4.27	1.31	a
	3	Water	27.85	3.20	a	-5.70	2.27	a
		GA_3+$CaCl_2$	28.84	0.87	a	-5.17	0.95	a
		GA_3+NaCl	26.13	3.36	a	-2.13	3.09	b

[z] means within each experiment with the same letter were not significantly different ($p \geq 0.05$)

[y] n=8 discs per treatment within each experiment

[x]ABA= 1 mM abscisic acid, GA_3= 1 mM gibberellic acid, PH= 1 mM prohexadione-calcium, $CaCl_2$= 10 g L^{-1} calcium chloride, NaCl= 10 g L^{-1} sodium chloride, AC=Ailsa Craig variety, HM=HM 4885 variety

4.4 Discussion

Timing of cellular events leading to symptom development in the disc system

The fruit used in this study were healthy at the time of disc preparation, and the AC variety used for many of these experiments rarely developed BER as whole fruit under this study's growth

conditions. Therefore, stresses during disc preparation and early disc storage likely induced BER symptom development. The first visual symptoms of BER were most often observed on day 2 after disc preparation. Respiration rate, electrolyte leakage, and H_2O_2 content in untreated discs trended up from day 1 to day 2 and down from day 2 to day 3. Disc preparation and storage likely induces a significant stress on the tissue, possibly related to wounding, water loss, or loss of nutrient supply. Results from delayed treatment with calcium and ascorbic acid suggest that events occurring on day 0 and day 1 can be either reversed, as in the case of increased respiration rate, or slowed, as in the case of color change (BER symptom development). These results suggest that the key cellular events leading to BER development in the disc system occur on day 0 and day 1 of storage.

Relationship between calcium, ascorbic acid, and glutathione in BER development

Our results indicate that calcium treatment increases the concentrations of both ascorbic acid and glutathione. Furthermore, both ascorbic acid and glutathione treatments inhibited symptom development. Reported levels of ascorbic acid in BER affected fruit compared to healthy fruit have varied. Aloni et al. (2008) found that ascorbic acid was reduced in BER affected peppers compared to healthy fruit, likely due to increased ascorbate oxidase activity. Schmitz-Eiberger et al. (2002) found that calcium treatment, applied as sprays to calcium deficient tomato plants, increased ascorbic acid concentrations and reduced ascorbic acid oxidation in leaf tissue. Conversely, Mestre et al. (2012) found that BER affected and healthy fruit had similar ascorbic acid content, but that glutathione content was decreased in BER fruit compared to healthy fruit. While results between these studies do not fully agree, a clear association between BER development and reduction in the ascorbic acid-glutathione antioxidant system is evident. Building on this, Reitz and Mitcham (2021a) demonstrated that direct treatment of tomato

pericarp tissue with ascorbic acid or calcium inhibited BER symptom development. It was also shown that a spike in respiration rate preceded visual symptom development (Reitz and Mitcham 2021a). Calcium's inhibitory effects on BER may be linked to the ascorbic acid-glutathione antioxidant system. Calcium treated discs were higher in ascorbic acid 1 day after treatment compared to water treated discs. This increased ascorbic acid concentration was maintained throughout disc storage in calcium treated discs, while ascorbic acid concentration decreased in water treated discs. Glutathione was also higher on day one of storage in calcium treated discs compared to water treated discs of the same fruit, and this difference increased throughout storage. Because calcium treatments increased ascorbic acid and glutathione within the first day after treatment, and treatment of discs with ascorbic acid and glutathione inhibit BER symptom development, calcium's inhibitory effect may be at least partially due to enhancement of the ascorbic acid-glutathione antioxidant system.

Our results indicate that treatment of discs with calcium or ascorbic acid on day 1 after disc preparation reduces respiration rate and slows color change. Results from calcium and ascorbic acid treatments on discs containing BER affected tissue and BER adjacent tissue confirms that calcium application can inhibit symptom development after these processes have begun. However, ascorbic acid was not fully effective in preserving the healthy appearance of the tissue. While the loss of green color may be attributed to the low pH of the ascorbic acid treatment (Reitz and Mitcham, 2021a), the slight tissue darkening and possible water soaking resulted in a noticeably less healthy appearance compared to discs showing no BER symptoms treated with ascorbic acid. Reitz and Mitcham (2021b) found that apparently healthy tissue adjacent to the BER affected area was more similar to BER affected tissue than healthy tissue from the stem-end of BER affected fruit or stem-end and blossom-end of healthy fruit. This trend was apparent in

H_2O_2 accumulation, ferulic acid peroxidase activity, and autofluorescence measurement of lignin. Results presented here indicate that calcium was much more effective than ascorbic acid at maintaining healthy tissue adjacent to the BER affected area of discs with partial BER affected tissue. This suggests that cellular events associated with calcium deficiency may be downstream or more easily halted or reversed than those affected by ascorbic acid. Alternatively, ascorbic acid treatment may be inhibiting the visual manifestation of cellular events (i.e. color change and water soaking), but not affecting the underlying cause.

Calcium and ROS signaling pathways may conduct BER induction signals

Our finding of increased H_2O_2 content in calcium treated, asymptomatic discs generally disagrees with previous research into BER development. Multiple studies have found increases in H_2O_2 in BER affected tissue compared to healthy tissue (Mestre et al., 2012; Reitz and Mitcham, 2021b; Turhan et al., 2006). Turhan et al. (2006) found that H_2O_2 accumulation was more pronounced as BER symptoms became more severe. Reitz and Mitcham (2021b) replicated these results and demonstrated that H_2O_2 is likely a key substrate in the deposition of lignin during BER development. In addition to having a strong link with BER development, H_2O_2 accumulation may also be an artifact of the disc system itself. Preparation of discs induces a wound response that likely includes H_2O_2 production (Orozco-Cardenas and Ryan, 1999), possibly explaining the accumulation of H_2O_2 in asymptomatic, calcium treated discs. While this makes investigating the role of H_2O_2 in BER development using the pericarp disc system difficult, it is evident from our results that when sufficient calcium is present, H_2O_2 accumulation does not induce symptom development.

Increased production of superoxide through NADPH oxidase has also been associated with BER development (Aktas et al., 2005; Mestre et al., 2012). While production of superoxide can lead to

H_2O_2 accumulation through the activity of superoxide dismutase, it is also part of the ROS signaling pathway (Suzuki et al., 2011). The NADPH oxidase inhibitor DPI has been used previously to reduce ROS production in the study of programmed cell death processes (Distéfano et al., 2017). Similarly, in our study, the calcium channel blocker Verapapmil inhibited symptom development in discs. VER has been previously shown to reduce programmed cell death and increase virus resistance by reducing the influx of calcium into the cytosol (Iriti et al., 2006; Singh et al., 2011). Inhibition of symptom development in DPI and VER treated discs suggests that both the production of ROS and calcium signaling are critical in BER development.

Calcium reduces electrolyte leakage

Asymptomatic calcium treated discs exhibited reduced electrolyte leakage compared to water treated discs which developed BER symptoms (lower L* color). Interestingly, calcium treated discs also had lower electrolyte leakage compared to water treated discs of similar, high L* values. This suggests that calcium treatment reduces electrolyte leakage beyond inhibiting the processes leading to BER symptom development. Throughout storage, asymptomatic stem-end discs exhibited lower electrolyte leakage than blossom-end discs. Interestingly, stem end and blossom-end discs had a similar trend of increasing electrolyte leakage from day 1 to day 2 and decreasing electrolyte leakage from day 2 to day 3. Increased calcium content in the stem-end compared to the blossom-end of tomatoes has been reported previously (Adams and Ho, 1992) and is likely a factor explaining why stem-end tissue is unaffected by BER. Increased calcium in the stem-end discs may explain the decreased electrolyte leakage in stem end discs compared to blossom-end discs. Further research into the timing of calcium's effect on electrolyte leakage could help determine the nature of calcium's effect on membrane integrity.

Water and mannitol storage solutions inhibit symptoms

Our results in this study showed that the presence of an aqueous liquid surrounding the discs during storage inhibited BER symptom development. However, this does not appear to be related to water stress because inhibition of symptom development occurred even when water loss was similar between discs stored in air and mannitol. As mentioned above, both calcium signaling and ROS signaling may be associated with BER development. One hallmark of BER symptom development in both whole fruit on the plant and in the disc system is the progressive spreading of symptoms. Storage in a solution may dilute any cell to cell signals responsible for the spread of symptoms throughout the disc. It is also worth noting that disc weights were generally between 0.3 and 0.4 grams and the storage solutions were applied at 1 mL per disc. If calcium concentrations in the apoplastic space and the surrounding storage solution reached equilibrium, the apoplastic calcium concentration would likely be diluted by at least a factor of two. Because the apoplastic space comprises only a portion of the total weight of the cell, this dilution factor is likely much higher. Low apoplastic calcium concentrations are often associated with increased BER symptom development; dilution of the apoplastic calcium may reduce the calcium gradient between the apoplast and cytoplasm. This could disrupt calcium signaling and inhibit symptom development in a similar manner as the VER treatment.

Phytohormone treatments were less effective on discs than at the whole plant level

In this study, phytohormone treatments did not significantly affect the development of BER symptoms as measured by color. There have been numerous reported effects of phytohormones on BER development. ABA was suggested to affect stomatal closure in leaves and xylem development in the blossom-end of fruit, thus improving calcium flow to the fruit (de Freitas et al., 2011b, 2017). This hypothesis of ABA action is consistent with a lack of effect of ABA

treatment on discs in our study, as de Freitas' effects were at the whole plant and the whole fruit level.

Similarly, GA and PH are suggested to have diverse effects on the affected cells, fruit, and plant. In a study of the effects of foliar sprays of GA_{4+7} on BER, de Freitas et al. (2012) attributed the increase in BER incidence to a reduction in functional xylem elements, and associated reduction in calcium uptake to the blossom-end of the fruit. Any effects of GA applied to discs in this current study would be independent of xylem development and calcium uptake. Thus, the lack of effect of GA_3, GA_{4+7}, and PH treatments on the discs is consistent with the modes of GA action on BER being related to xylem development and calcium uptake.

Another possible explanation put forward for the increase in BER in GA_{4+7} treated fruit was the movement of calcium from the apoplast to cellular storage organelles (de Freitas et al., 2012). This would decrease the critically important apoplastic calcium concentration, thus inducing BER development as has been reported in plants expressing the vacuolar calcium antiporter *CAX1* (de Freitas et al., 2011a). These results were supported in the work of de Freitas et al. (2012), which reported increased expression of *Ca-ATPase* genes in GA_{4+7} treated fruit compared to controls. In subsequent work, de Freitas et al. (2017) identified genes associated with BER development in response to GA_{4+7} treatment through transcriptome analysis of PH, GA_{4+7}, and ABA treated plants. Results indicated a diverse set of genes potentially triggering BER development including transcriptional regulation, hormone metabolism, oxidation-reduction, protein metabolism, and cell wall modification. While these genes can have effects at tissue specific, whole fruit, and whole plant levels, results from the disc experiments presented here indicate that the effects of GA, ABA, and PH are at the whole fruit and/or whole plant level. Phytohormone effects at the tissue specific level (i.e. in the BER affected pericarp tissue) would

likely have resulted in significantly different symptom development in phytohormone treated discs compared to untreated discs. Finally, it is notable that cell expansion is an effect of GA treatment and a factor in BER development. Because the supply of nutrients and water is cut off in the disc system, cell expansion is likely minimal. As such, cell expansion effects related to phytohormone treatment may not be evident in the pericarp disc system.

Our results provide direct evidence that increasing calcium in the pericarp tissue enhances the ascorbic acid-glutathione antioxidant system during the critical early stages of BER symptom development. These results, combined with evidence of inhibition of symptom development by ascorbic acid, glutathione, and the ROS production inhibitor DPI, are strong evidence that calcium's inhibitory effects may be at least in part due to antioxidant system enhancement. In addition, cellular events associated with BER symptom development can be reversed or slowed with calcium or ascorbic acid treatments. This work also provides the first evidence that calcium signaling likely plays a role in initiation of BER development. Finally, the effect of the phytohormones GA and ABA on BER are likely at the whole plant or whole fruit level.

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Chapter 5: Lignification of tomato (*Solanum lycopersicum*) pericarp tissue during blossom-end rot development

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Abstract

Blossom-end rot is a physiological disorder causing significant losses in the tomato industry each year. Accumulation of reactive oxygen species has been established as a key characteristic of blossom-end rot development. An increase in peroxidase activity and lignin precursor content are also associated with blossom-end rot symptoms, leading to the hypothesis that lignification may be occurring during blossom-end rot development. To investigate the potential involvement of lignification, hydrogen peroxide content, catalase activity, and peroxidase activity were measured in the top, bottom, and blossom-end rot affected tissue of blossom-end rot affected fruit, and the top and bottom of healthy fruit. Lignin was assayed using histochemical staining, autofluorescence, and thioglycolic acid degradation methods. Hydrogen peroxide content was increased in blossom-end rot affected and blossom-end rot adjacent tissues compared to healthy fruit and the top of blossom-end rot affected fruit. Catalase activity was significantly reduced and ferulic acid peroxidase activity was increased in the blossom-end rot affected and unaffected tissue at the bottom of blossom-end rot affected fruit compared to the bottom of healthy fruit. Lignin analyses showed increased lignin content in blossom-end rot affected tissue compared to tissue from the bottom of healthy fruit. These results show that lignification occurs during blossom-end rot development, likely through a peroxidase-mediated pathway.

5.1 Introduction

Blossom-end rot (BER) in tomatoes is a physiological disorder characterized by water soaking, blackening, and cell death in the blossom-end tissue during fruit development. Tomatoes are an economically and nutritionally important horticultural crop, with BER causing loss of profit for producers and increases in food loss. BER can also cause losses in peppers and watermelon. While the cause of BER is often attributed to calcium deficiency (Ho and White, 2005), abiotic stresses such as soil salinity and drought stress have also been implicated in BER development (Turhan et al., 2006b). BER symptoms start approximately 10 days after pollination and can continue until the fruit reaches physiological maturity, corresponding with a period of rapid cellular expansion (Ho and White, 2005). However, BER-affected tissue has been reported to have reduced cell size, possibly due to disrupted cellular expansion and an increase in cell wall elastic modulus (Gholipour et al., 2017). The exact cause of disrupted cell expansion and cell death during BER development remains unknown.

Both calcium deficiency and abiotic stress can induce BER development and both are also associated with an accumulation of reactive oxygen species (ROS). It has been suggested that the accumulation of ROS may cause cellular damage and death (Mestre et al., 2012; Saure, 2014; Turhan et al., 2006b). Superoxide, a highly reactive but short-lived ROS, can be produced in the apoplast by membrane bound NAD(P)H oxidase enzymes. Superoxide is then converted to hydrogen peroxide (H_2O_2) by superoxide dismutase. The activity of both of these enzymes is increased in BER-affected fruit, with their combined effect leading to an accumulation of H_2O_2 in BER-affected fruit (Aktas et al., 2005; Mestre et al., 2012). H_2O_2 can be quenched in the cell through antioxidant systems, such as glutathione, ascorbic acid, and catalase. Each of these systems is decreased during BER development, resulting in a reduced ability for the plant to cope

with the damaging effects of ROS (Aloni et al., 2008; Mestre et al., 2012). Though cell death has been attributed to the damaging effects of ROS, more research is needed to establish how ROS accumulation causes damage to cells during BER development, and how this leads to cell death and visual symptoms.

Another potential H_2O_2 quenching enzyme is peroxidase. Unlike catalase, which requires only H_2O_2 as a substrate, peroxidase requires 2 electron donors to convert H_2O_2 to water. When measuring peroxidase in BER fruit, the electron donor used greatly affects the measured peroxidase activity. Ascorbic acid can act as an electron donor in the degradation of H_2O_2 by ascorbate peroxidase, thus reducing oxidative stress in the tissue. However, ascorbate peroxidase activity in BER-affected fruit is reduced compared to healthy fruit (Mestre et al., 2012). Peroxidase activity measured in BER-affected fruit using the aromatic compound tetramethylbenzidine, exhibits drastically increased activity compared to healthy tissue (Turhan et al., 2006b). These differing results suggest that different substrate-specific peroxidases may play diverse roles during BER development.

One possible function of peroxidase during BER development is in the lignification of cell walls. Lignification through the action of peroxidase requires H_2O_2 and aromatic monolignol compounds as substrates. Monolignol biosynthesis, through the phenylpropenoid pathway, is upregulated in BER fruit compared to healthy fruit, and an increase in the monolignol precursor coumarin-glycoside concentration in BER tissue has been reported (Freitas et al., 2011; Gholipour et al., 2017). With an accumulation of H_2O_2, upregulation of the monolignol synthesis pathways, and increased peroxidase activity, it is possible that lignification is occurring during BER development. Lignification would provide a possible explanation for reduced cell size and increased cell wall elastic modulus previously reported (Gholipour et al., 2017). The purpose of

our study was to investigate the presence of lignification during BER development in tomatoes. Additionally, we investigated the locational specificity of ROS accumulation, catalase activity, and lignification-related processes to better understand why BER symptoms occur exclusively in the blossom-end of fruit.

5.2 Materials and methods

Plant growth and tissue sampling

Solanum lycopersicum L. seeds (var. HM 4885) were obtained from Agseeds Unlimited (Woodland, CA, USA) and sprouted in peat pellets and with double deionized water during the summer of 2018. Approximately 2 weeks after germination, seedlings were transplanted into 7.57 liter pots with a mixture of 1/3 peat, 1/3 sand, and 1/3 rosewood compost, augmented with 1.56kg m^{-3} dolomite lime. Plants were irrigated with a solution of 150ppm nitrogen, 50ppm phosphorus, 200ppm potassium, 175ppm calcium, 55ppm magnesium, 120ppm sulfur, 2.5ppm iron, 0.02ppm copper, 0.5ppm boron, 0.50ppm manganese, 0.01ppm molybdenum, 0.05ppm zinc, and 0.02ppm nickel until one day prior to the opening of the first flower. One day prior to the opening of the first flower, daily water use (DWU) was determined by weighing individual pots (including plants) after application of the nutrient solution and prior to nutrient solution application the next day. Upon flowering, nutrient solution application was discontinued, and 20 grams of a calcium free slow release fertilizer (Osmocote Plus, The Scotts Company, Marysville, OH, USA) was mixed with the top 1.5cm of soil. This fertilizer contained 15% nitrogen, 9% phosphate, 12% soluble potash, 1.3% magnesium, 6% sulfur, 0.02% boron, 0.05% copper, 0.46% iron, and 0.06% manganese. After the opening of the first flower, deionized water was added at 200% of the DWU daily. The first flower on each cluster was removed, and each subsequent flower was tagged with the date and manually pollinated.

Fruit were harvested 21 days after pollination during September and October of 2018. Each fruit was rated as healthy, moderately affected, or severely affected based on a 0-5 rating scale, where 0 represented healthy fruit, 2 represented moderately-affected fruit, and 3-4 represented severely affected fruit. All skin was removed from the tissue prior to sampling. Approximately 2 grams of sample was taken from the top and bottom pericarp of healthy fruit. In BER-affected fruit, three samples were taken: unaffected top pericarp tissue, unaffected bottom pericarp tissue (adjacent to BER symptoms), and bottom pericarp tissue exhibiting BER symptoms. These tissues were designated Top, Bottom, and BER tissue. Samples from severely affected fruit were frozen in liquid nitrogen, and then stored at -80°C for subsequent use in enzyme activity analysis.

During the spring of 2019, germination and growth were completed as previously described. Pollination and harvest were completed in June and July of 2019. Tissue from healthy and BER affected fruit was sampled as stated previously and immediately frozen for later use in thioglycolic acid lignin determination. Unfrozen samples were used immediately for H_2O_2 determination and toluidine blue staining microscopy, and whole tomatoes were held overnight before UV autofluorescence microscopy.

Hydrogen peroxide content

Hydrogen peroxide was assayed by measuring the oxidation of potassium iodide at A_{350}, compared to a standard curve (Junglee et al., 2014), using a BioTek H1 multimode plate reader (Biotek, Winooski VT, USA). Tissue (250 mg) was ground in 1mL of reaction solution using a mortar and pestle. Each 1mL of reaction solution consisted of 250µL 100mM potassium phosphate buffer (pH 6), 250µL 0.1% trichloroacetic acid, and 500µL 1M potassium iodide. Grinding fresh samples, rather than frozen, was found to be imperative in obtaining accurate measurements, with frozen samples having little or no H_2O_2 content. Samples were ground with

the reaction mixture and centrifuged for 15 min at 12,000g. The supernatant (100 µL) was added to a microplate (Fisherbrand, Pittsburg, PA, USA) and the absorbance at 350nm was measured. Controls for each sample were prepared in the same manner, but with ultrapure water added instead of the potassium iodide solution. Samples were compared to a standard curve of 1µM-5mM H_2O_2 in potassium phosphate buffer and presented on a per gram fresh weight basis. Hydrogen peroxide content was determined on 4 BER-affected fruit and 4 heathy fruit. Data is presented as a mean of 4 fruit with error bars representing the standard deviation.

Enzyme extraction

Tissue samples were ground into a fine white powder with a mortar and pestle with liquid nitrogen. In a 2 mL centrifuge tube, 0.2 grams of sample was vortexed with 1mL of 100mM potassium phosphate buffer, pH 6, and centrifuged for 20 min at 20,000g. The supernatant was frozen in liquid nitrogen for later use as the water-soluble enzyme extract.

Catalase

Catalase was assayed spectrophotometrically using a BioTek H1 multimode plate reader (Biotek, Winooski VT, USA). Soluble enzyme extract (8.33µL) was added to the reaction mixture (241µL) containing 0.036% H_2O_2 in 100mM potassium phosphate buffer pH 6. This was allowed to react for 20 min. The reaction was stopped by adding 500µL of 1M potassium iodide and 250µL 0.1% trichloroacetic acid (TCA), and incubated for 10 min in the dark. Sample blanks were assayed by substituting ultrapure water for potassium iodide. The initial H_2O_2 concentration for each reaction was determined by adding potassium iodide and TCA immediately after adding sample. The absorbance at 350nm was compared to a 1µM to 1mM H_2O_2 standard curve. Catalase activity was determined on top, bottom, and BER tissue of 4 BER-affected fruit and top

and bottom tissue of 4 heathy fruit. Data is presented as a mean of 4 fruit with error bars representing the standard deviation.

Peroxidase activity

Peroxidase activity was assayed spectrophotometrically using two different electron donor substrates. The total reaction volume of 100µL included 70µL of ultrapure water, 10.66µL of 100mM potassium phosphate buffer pH 6, 10.66µL of either 5% pyrogallol or 1mM ferulic acid, 3.33µL of the enzyme extract, and 5.33 µL 0.5% H_2O_2. H_2O_2 was added last to start the reaction. The increase in absorbance at 420nm, associated with purpurogallin formation, was monitored for pyrogallol peroxidase activity. For ferulic acid peroxidase activity, the decrease in absorbance at 310nm was measured. Measurements were taken at 11 sec intervals for 16 measurements of each well over 2.93 min. The average change in absorbance over 2.93 min was used to calculate enzyme activity on a per minute basis. The extinction coefficients used for the calculations was 12.0 $(mg/mL)^{-1}$ cm^{-1} for pyrogallol ("Enzymatic Assay of Peroxidase (EC 1.11.1.7)," n.d.) and 8400 M^{-1} cm^{-1} for ferulic acid (Garcia et al., 2002). Pyrogallol peroxidase and ferulic acid peroxidase activity was determined on top, bottom, and BER tissue of 4 BER-affected fruit and top and bottom tissue of 4 heathy fruit. Data is presented as a mean of 4 fruit with error bars representing the standard deviation.

Thioglycolic acid lignin

Thioglycolic acid lignin (TGA lignin) determination was completed based on the methods of Cai et al. (Cai et al., 2006) with modifications. Healthy, moderately affected, and severely BER-affected fruit were harvested 21 days after pollination, frozen in liquid nitrogen, and stored at -80°C until analysis. Samples were ground to a fine white powder in liquid nitrogen. Samples

(0.5g) were combined with 1.666 mL 95% ethanol, vortexed thoroughly, and centrifuged at 4°C, 20,000g, for 20 min. The supernatant was discarded and the samples allowed to dry overnight at room temperature. The next day, 0.025g of dried sample was weighed into 15mL centrifuge tubes, and 2.5 mL 2M hydrochloric acid and 0.25mL thioglycolic acid were added and mixed. Tubes were incubated at 95°C for 4 hours, cooled, and centrifuged at 12,000g for 20 min at 4°C. The supernatant was discarded and the pellet was washed with DI water. The pellet was resuspended in 2.5mL 1M sodium hydroxide and held for 18 hours. The mixture was centrifuged at 12,000g for 20 min at room temperature and the supernatant was transferred to a separate tube. Concentrated hydrochloric acid (0.5mL) was added to each tube and the mixture was allowed to precipitate overnight at room temperature before the samples were centrifuged for 20 min at 12,000g and room temperature. The pellet was suspended in 1M sodium hydroxide and the absorbance read at 280nm with 1M sodium hydroxide used as a blank. Thioglycolic acid lignin was determined on top, bottom, and BER tissue of 4 BER-affected fruit, and on top and bottom tissue of 4 heathy fruit. Data is presented as a mean of 4 fruit with error bars representing the standard deviation.

Toluidine blue O staining

Freehand slices of approximately 500μm thickness were made from the top and bottom of a BER-affected fruit using a razor. Bottom slices spanned healthy and BER-affected tissue. Slices were incubated in 0.1% toluidine blue O for 1 min, then rinsed and mounted on slides with water under a cover slip at 4X magnification. Stained samples were imaged using an Evos FL Auto microscope in the color transmitted light setting.

Lignin autofluorescence

Lignin has been shown to autofluorescence when excited in the UV spectrum, with emission in the blue visible light region. Lignin autofluorescence has been suggested to be a sensitive measurement tool in tissues with weak lignification (Donaldson and Williams, 2018). Autofluorescence of fresh tomato tissue was measured using an EVOS FL Auto fluorescence microscope (Thermo Fisher, Waltham, MA, USA). Slices of tomato tissue approximately 5mm by 5mm by 500µm were cut from the top and bottom of fruit with and without BER on the day after harvest. To make each 500µm slice, a subsection of tomato (approximately 1cm x 1cm x 4cm) was placed in a graduated handheld microtome, and the protruding section was removed to create a flat surface. Vertical incisions were made to create a 5mm x 5mm square section that included the skin, perpendicular to the movement of the microtome. Slices of 500µm thickness were made by hand using a razor. Bottom slices from BER-affected fruit were cut at the water-soaking boundary, including both healthy tissue and tissue exhibiting moderate BER symptoms. Slices were mounted on glass slides with a solution containing 50µL 0.2 M mannitol and 1µM Fluo-4 pentasodium salt (Thermo Fisher, Waltham, MA, USA), and a cover slip. Slices were imaged using a EVOS^{TM} DAPI filter cube (excitation: 357nm with a bandpass of ±22nm, emission: 447nm with a bandpass of ±30nm, Thermo Fisher) at 4X magnification. Fluo-4 impermeant was included for calcium analysis (data not presented here) and did not affect autofluorescence in the fluorescence range analyzed when compared to samples not treated with Fluo-4 impermeant (data not shown). Images were analyzed for mean fluorescence of the pericarp tissue using ImageJ software (Schneider et al., 2012). The analysis excluded the thin layer of smaller cells near the skin surface and the highly autofluorescent cuticle. Two slices

were analyzed from each location on four healthy fruit and four BER-affected fruit, with the mean fluorescence of the two slices being averaged into one final value for each fruit.

Statistical analysis

Statistical analyses were completed using SAS On Demand for Academics (SAS Institute Inc., Cary, NC, USA). A general linear model and Tukey's honest means separation were used to test for significant differences between means at $p<0.05$.

Data availability

The data used to generate figures for this manuscript is available from at the following location:

Reitz, Nicholas (2020), Lignification of tomato (Solanum lycopersicum) pericarp tissue during blossom-end rot development, UC Davis, Dataset, Dryad, https://doi.org/10.25338/B88S4D.
Access during review at:
https://datadryad.org/stash/share/z2UIWzILq21DhRi10vF5MwWakmHua3PX5XwLsaMz7XE

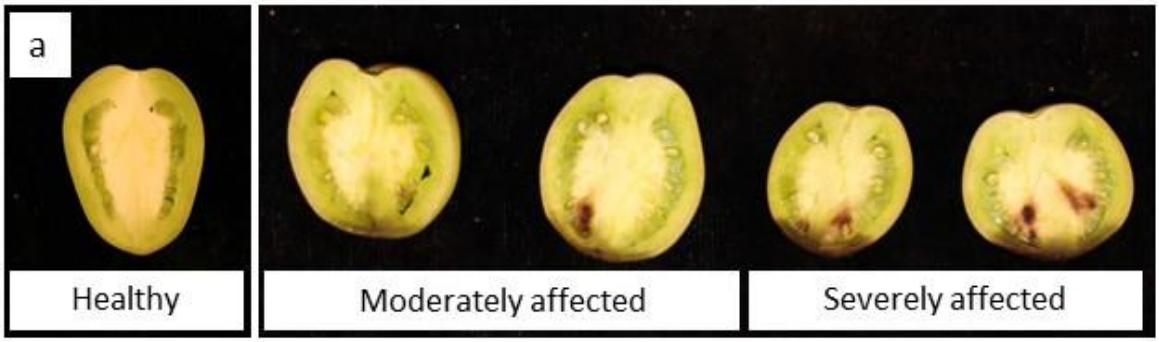
5.3 Results

Plant growth, BER metrics, and sample selection

Solanum lycopersicum variety HM 4885 was recommended by a California tomato processor due to its high prevalence of BER in the field. During 2019 experiments, BER incidence on the first harvest day was 100% and declined over the harvest period, with an overall average of approximately 85%. The earliest observed BER symptom emergence was 8 days after pollination. This variety is useful for BER research due to its easily manageable plants, which reliably produce fruit affected by BER. Additionally, the fruit pericarp of this variety is thick and

easily sampled, and the determinate nature of this variety was more manageable under greenhouse conditions than indeterminate varieties.

Fruit harvested 21 days after pollination had an average weight of 18 grams. Each fruit was rated as healthy, moderately affected, or severely affected with BER (Figure 5.1a). Healthy fruit showed no visible internal or external symptoms of BER. Samples were taken from the top and bottom pericarp of healthy fruit. In BER-affected fruit, three samples were taken: unaffected top pericarp tissue, unaffected bottom pericarp tissue adjacent to BER symptoms, and bottom pericarp tissue exhibiting BER symptoms (Figure 5.1b). These tissues were designated Top, Bottom, and BER tissue.



b Tissue sampling location

Healthy Fruit	BER-affected Fruit	Designation
Healthy top pericarp tissue	Healthy top pericarp tissue	"Top"
Healthy bottom pericarp tissue	Healthy bottom pericarp tissue, adjacent to BER-affected tissue	"Bottom"
	BER affected pericarp tissue	"BER tissue"

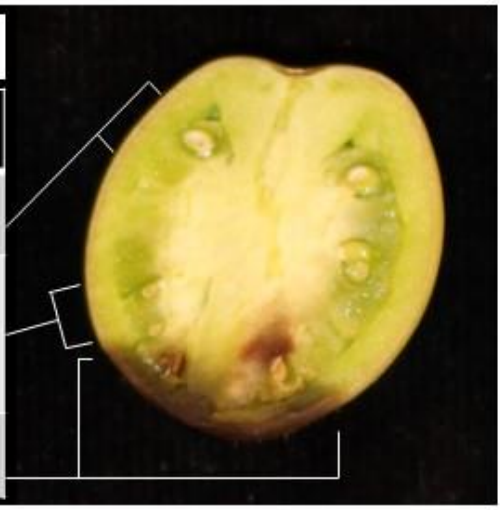


Figure 5.1: Blossom-end rot (BER) development and tissue sampling. Fruit were harvested 21 days after pollination and weighed an average of 18g. a) Healthy and BER-affected fruit in *Solanum lycopersicum* (var. HM 4885) with progressive severity from left to right. b) Top and bottom pericarp tissue was sampled from healthy fruit which showed no visual BER symptoms. Samples were taken from the top, bottom, and BER-affected pericarp tissue of BER-affected fruit.

H_2O_2 concentration

H_2O_2 concentration was measured using the potassium iodide method previously published by Junglee et al. (2014), with similar results in healthy fruit. H_2O_2 content of healthy, moderately affected, and severely affected fruit is reported in Figure 5.2. The highest H_2O_2 content was exhibited by bottom tissue in severely BER-affected fruit. H_2O_2 content in BER-affected tissue of severely and moderately affected fruits were not significantly different, but were higher than that of top and bottom tissues of healthy fruit. Bottom tissue in severely BER-affected fruit, which was visually unaffected by BER, exhibited elevated peroxide content compared to the top and bottom of healthy fruit.

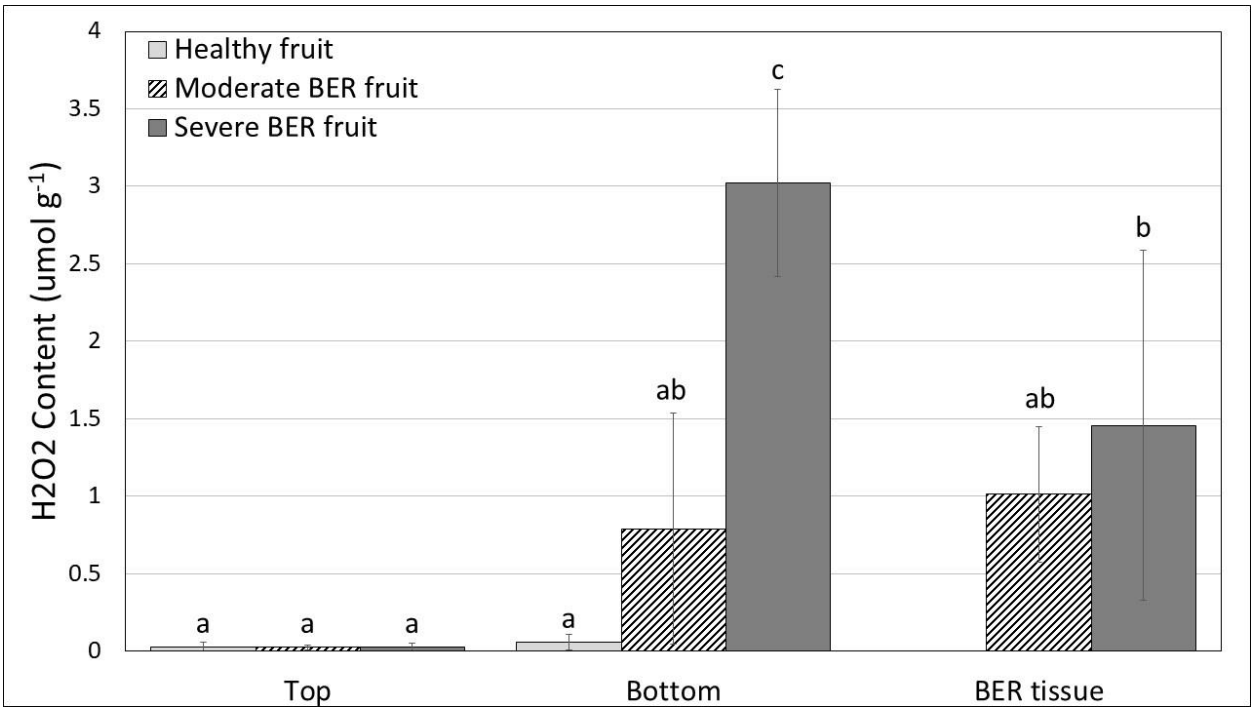


Figure 5.2: H_2O_2 content in healthy, moderately blossom-end rot (BER)-affected, and severely BER-affected fruit. Bar height indicates the mean of 4 samples from different fruit. Each bar is the mean of 4 replicates, and bars with the same letter were not statistically different ($p>0.05$). Error bars indicate the standard deviation.

Enzyme activity

Enzyme activity measurements were carried out on extracts of healthy and severely affected fruit. The bottom tissue of healthy fruit exhibited the highest catalase activity, which was 3-fold higher than that of the comparable bottom tissue in BER-affected fruit (Figure 5.3). Catalase activity in top tissue of healthy and BER-affected fruit was not significantly different, and there was no significant difference between the 3 tissue locations in BER-affected fruit.

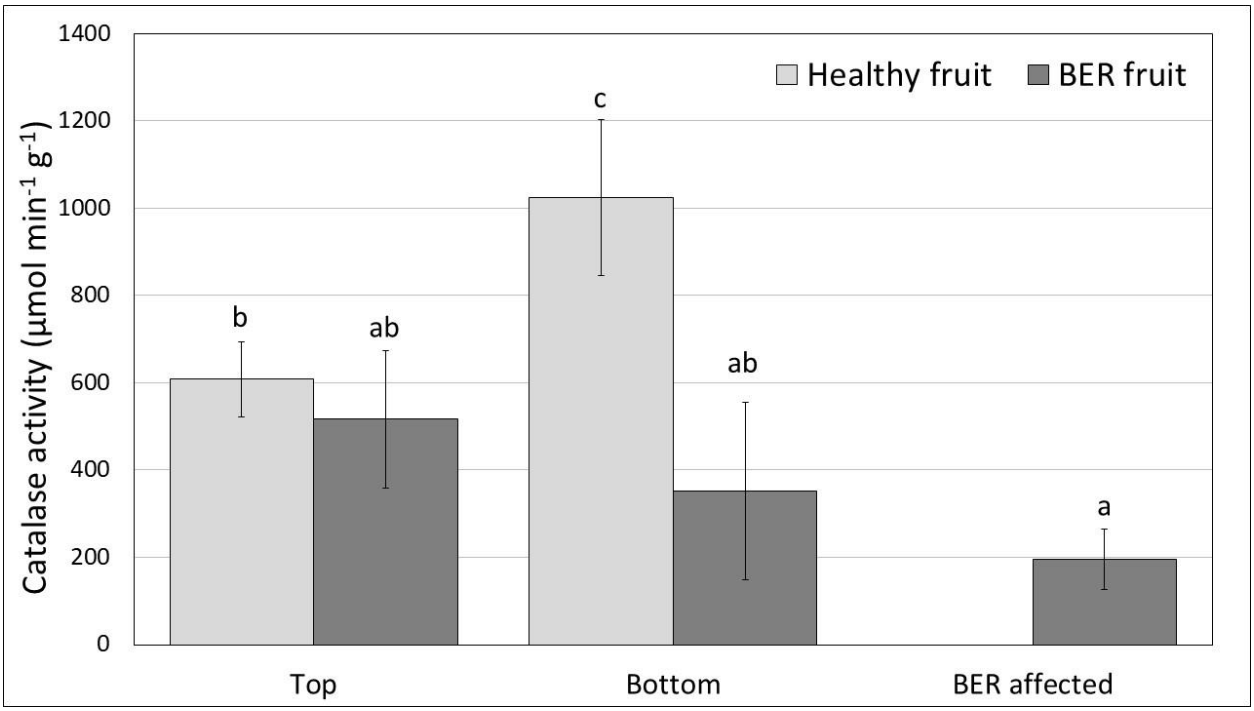


Figure 5.3: Catalase activity in healthy and severely blossom-end rot (BER)-affected fruit. Each bar is the mean of 4 replicates from different fruit, and bars with the same letter were not statistically different ($p>0.05$). Error bars indicate the standard deviation.

Pyrogallol peroxidase activity was used to measure the substrate affinity of cellular peroxidases for aromatic compounds using a phenolic substrate. The top and bottom of healthy and affected fruit were not significantly different in pyrogallol peroxidase activity (Figure 5.4a). However, pyrogallol peroxidase activity was 5.3-fold higher in BER-affected tissue than in the adjacent bottom tissue of BER-affected tomatoes and 16.5-fold higher than that of the top tissue of BER-affected fruit.

Ferulic acid was used to measure peroxidase substrate affinity to a monolignol analog. Ferulic acid peroxidase activity was highest in bottom and BER tissue of affected fruit (Figure 5.4b). Bottom tissue in healthy fruit and top tissue in BER-affected fruit had significantly lower ferulic acid peroxidase activity than the bottom and BER tissue of affected fruit.

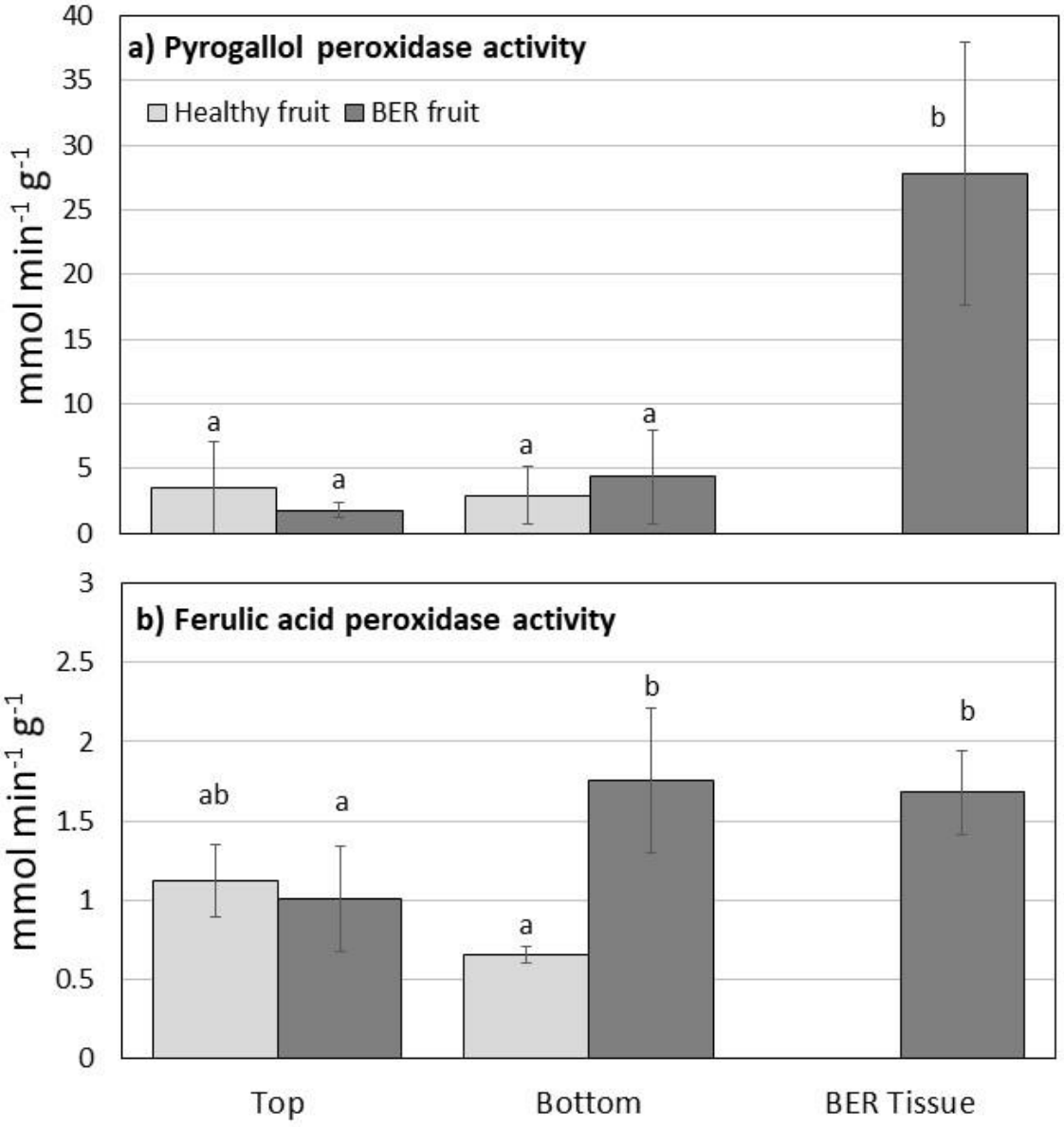


Figure 5.4: a) Peroxidase activity measured using pyrogallol as the electron donating substrate for healthy and severely blossom-end rot (BER)-affected fruit. Each bar is the mean of 4 replicates, and bars with the same letter were not statistically different ($p<0.05$). b) Peroxidase activity measured using ferulic acid as the electron donating substrate for healthy and severely blossom-end rot (BER)-affected fruit. Each bar is the mean of 4 replicates, and bars with the same letter were not statistically different ($p>0.05$). Error bars indicate the standard deviation.

Autofluorescence, toluidine blue staining, and thioglycolic acid (TGA) lignin content

Results from TGA lignin content (Figure 5.5) indicated that BER-affected tissues were more lignified compared to the top of BER-affected fruit and the bottom tissue of healthy fruit. TGA lignin in BER-affected tissue was not significantly different, however, from the top tissue of healthy fruit or from the unaffected bottom tissue on BER-affected fruit. Toluidine blue O staining was completed on top tissue (Figure 6a) and a combination of the bottom and BER-affected tissues Figure 6b) of BER affected fruit. Healthy tissue treated with toluidine blue O, a histochemical cell wall stain, exhibited a violet coloration, indicating the presence of unlignified parenchyma cells. Conversely, staining of the BER-affected area resulted in areas of green coloration, indicating lignified tissue. However, the unaffected tissue adjacent to BER-affected tissue did not exhibit green coloration. UV autofluorescence images (Figure 6c and 6d) and quantification (Figure 6e) show similar results. Imaging of samples taken from the border of water soaking revealed a transition from distinct autofluorescing cell wall structures in the visually healthy bottom tissue to a less defined pattern in BER-affected tissue. Healthy tissue adjacent to BER tissue had patterning like that of tissue at the top of BER fruit or in healthy fruit, but with increased autofluorescence. UV autofluorescence indicated increased lignification in BER-affected tissue and BER-adjacent tissue compared with healthy fruit. Autofluorescence results showed a similar pattern to ferulic acid peroxidase results, with increased levels in BER-affected tissue and tissue adjacent to BER tissue.

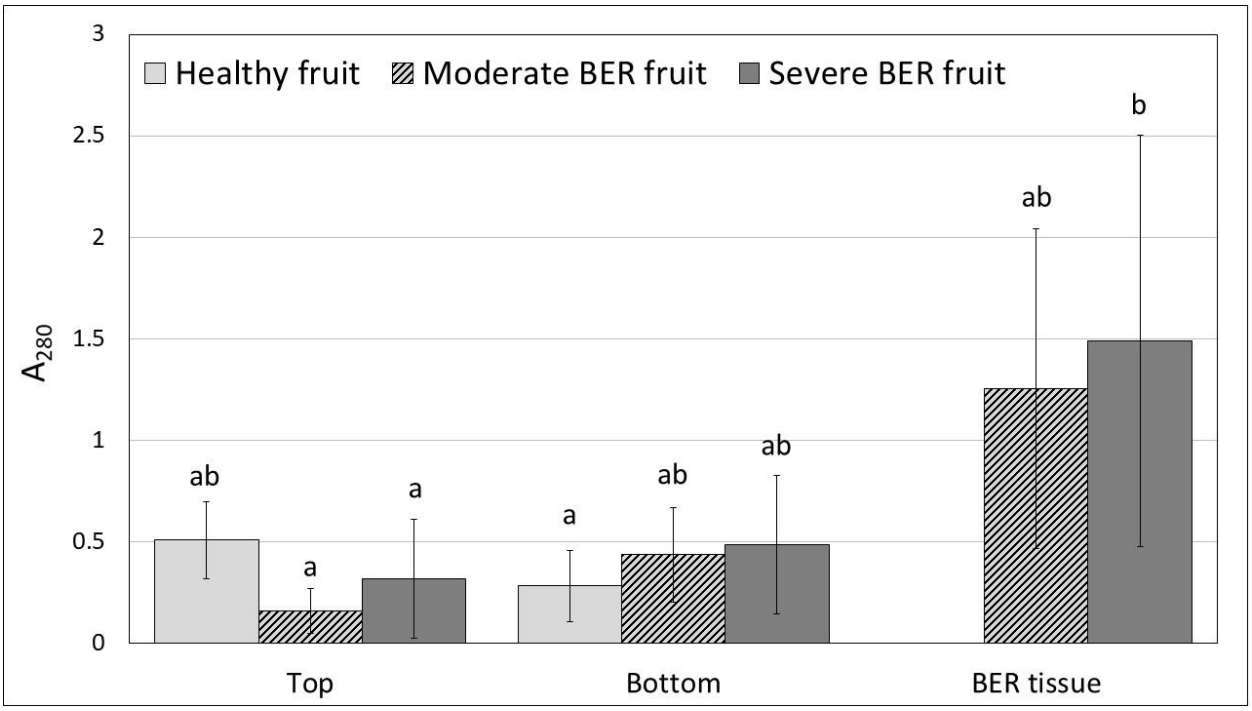


Figure 5.5: Thioglycolic acid determination of lignin content in healthy, moderately blossom-end rot (BER)-affected, and severely BER-affected fruit. Each bar is the mean of 4 replicates, and bars with the same letter were not statistically different ($p>0.05$). Error bars indicate the standard deviation.

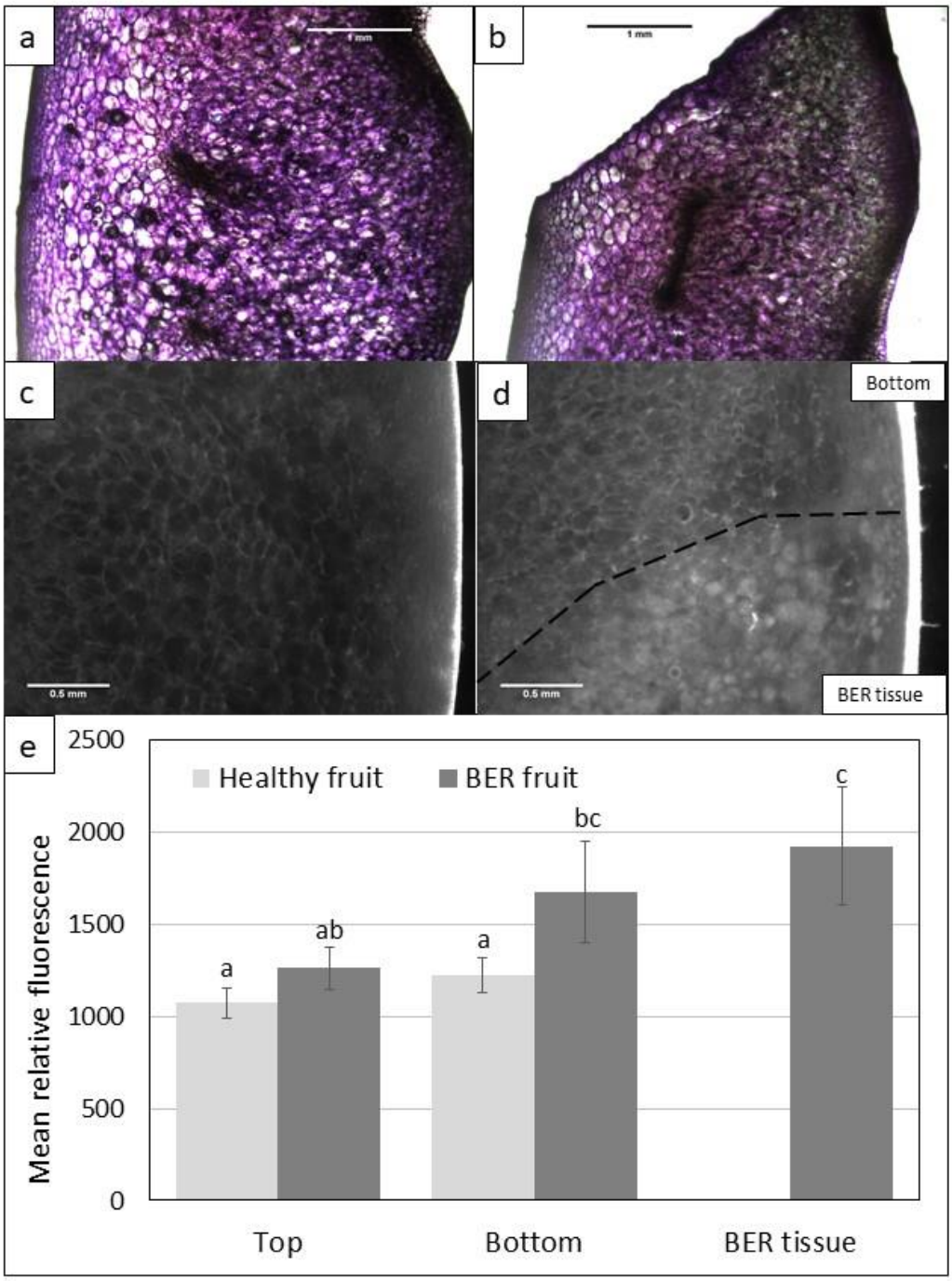


Figure 5.6 (printed in color): Transmitted and UV autofluorescence microscopy of healthy (a and c) and blossom-end-rot (BER)-affected fruit (b and d). Top (a) and bottom (b) tissue from blossom-end rot (BER)-affected fruit were stained with toluidine blue O. Purple staining indicates non-lignified tissue and blue-green staining indicates lignified tissue. Tissue

autofluorescence (357ex/447em) images of bottom tissue from healthy fruit (c) and unaffected bottom tissue and BER-affected bottom tissue from moderately BER-affected fruit (d). The approximate transition from unaffected to BER-affected tissue is indicated with a dashed black line. Mean fluorescence (e) is presented with bar height indicating the mean of 4 samples from different fruit. Bars with the same letter were not statistically different ($p>0.05$). Error bars indicate the standard deviation.

5.4 Discussion

Presence of lignin in BER-affected tissue

The potential for lignification during BER development in tomatoes and peppers has been noted previously based on the accumulation of H_2O_2 (Aktas et al., 2005; Mestre et al., 2012; Turhan et al., 2006b) and monolignols (Freitas et al., 2011; Gholipour et al., 2017), and the greatly increased activity of non-ascorbate peroxidase enzymes (Turhan et al., 2006b) in BER-affected fruit compared to healthy fruit. Results from this study are the first to demonstrate that lignification occurs during BER development, likely through a peroxidase-mediated pathway. Lignin content, assessed through three different methods, was consistently higher in BER-affected tissue compared to similarly located tissue in healthy fruit and the top tissue of BER-affected fruit. Additionally, elevated H_2O_2 content, ferulic acid peroxidase activity, and autofluorescence were observed in the visually asymptomatic bottom tissue of BER-affected fruit compared to the bottom of healthy fruit. This suggests that the lignification process starts before visual symptoms are evident.

Possible role of lignification in disrupted cell expansion and cell death

Lignification increases the elastic modulus and tensile strength of cell walls (Barros et al., 2015). BER occurs during a period of rapid cellular expansion (Ho and White, 2005), and an increase in cell wall elastic modulus may disrupt the complex coordination of cell wall relaxation and increased turgor. Reduced cell size, increased turgor, and increased cell wall elastic modulus in BER affected tissue has been reported previously (Gholipour et al., 2017). Increased membrane oxidation and electrolyte leakage is also associated with BER development (Freitas et al., 2014; Mestre et al., 2012). Increased cell wall elastic modulus from lignification may restrict cell expansion, further increase turgor forces on damaged membranes, and possibly result in plasmolysis and cell death.

Lignification as an additional effect ROS accumulation

Accumulation of ROS during BER development in tomatoes occurs through endogenous production of superoxide, and subsequently H_2O_2, accompanied by a reduction in antioxidant capacity (Mestre et al., 2012). This sequence of events has also been shown during BER development in peppers (Aktas et al., 2005; Aloni et al., 2008; Turhan et al., 2006b). The result of ROS accumulation, however, has not been determined. It has been suggested that ROS accumulation may be the cause of cell death during BER development (Mestre et al., 2012; Saure, 2014), with increased malondialdehyde content supporting this assertion (Mestre et al., 2012; Schmitz-Eiberger et al., 2002). Results from our research offer an additional effect of H_2O_2 that accumulates in BER-affected fruit. The capacity of catalase to consume H_2O_2, as measured in this study in BER-affected bottom tissue was approximately 100 μmol min^{-1} g^{-1} less than measured in bottom tissue from healthy fruit. Similar discrepancies in catalase activity between BER-affected and healthy fruit were reported previously (Mestre et al., 2012). The H_2O_2

consumption capacity of ferulic acid peroxidase was 1 mmol min^{-1} g^{-1} higher in the bottom of BER-affected fruit than in the bottom of healthy fruit, representing a significant sink for accumulated H_2O_2. As evidenced by the accumulation of malondialehyde during BER development, this increase in peroxidase activity does not fully mitigate the damaging effects of ROS accumulation. However, lignification during BER development may provide protection against water loss and microbial attack to neighboring healthy tissue. Lignification results from a complex network of cellular processes, with transcriptional upregulation of monolignol biosynthesis and H_2O_2 production through increased transcription of NADPH oxidase (Freitas et al., 2017, 2011). The possibility of regulated or programmed accumulation of H_2O_2 during BER development for the purposes of lignification should be considered.

Cellular events leading to ROS accumulation: coincidence or choreography

As was recently well described by Hagassou et al. (2019), causal relationships in BER development at the cellular level remain largely unknown. As a result, the intertwined effects of calcium deficiency and ROS accumulation during BER development are unknown. What is compellingly clear, yet only lightly discussed in literature, is the coordination of cellular events leading to ROS accumulation during BER development. Aktas et al. (2005) characterized the production of superoxide during BER development in peppers through an NAD(P)H oxidase dependent pathway. These results were supported by the transcriptional analysis of de Freitas et al. (2017) Superoxide dismutase activity is also increased during BER development (Mestre et al., 2012), leading to the accumulation of H_2O_2.

Concurrently, antioxidant systems are undermined by the reduction in activity of key enzymes and the highly coordinated depletion of critical antioxidant compounds. While peroxidase activity is often associated with antioxidant functionality, it is important to consider the effect of

substrate specificity and electron donor availability when evaluating the function of peroxidases in a biological system. Substantial increases in peroxidase activity in BER-affected tissue compared to healthy fruit have been reported when an aromatic substrate has been used as an electron donor (Schmitz-Eiberger et al., 2002). Peroxidase activity in BER-affected fruit measured using ascorbate is less than that of healthy fruit (Mestre et al., 2012). Conversely, ascorbate oxidase activity and expression is increased in BER-affected fruit compared to healthy fruit (Aloni et al., 2008; Freitas et al., 2017). Though increased ascorbate oxidase activity should decrease the reduced ascorbate concentrations in a tissue, there have been varying reports of ascorbate concentrations and redox states in BER-affected fruit compared to healthy fruit. One study found decreased ascorbate concentrations in BER-affected peppers compared with healthy fruit (Aloni et al., 2008), while another found higher reduced and oxidized ascorbate concentrations in BER-affected tomato fruit compared to healthy, with a similar overall ratio of reduced to oxidized (Mestre et al., 2012). These inconsistencies may be explained by the increased activity of dehydroascorbate reductases, which regenerate ascorbate from oxidized dehydroascorbate (Mestre et al., 2012). While this may seem contrary to the trend of decreased antioxidant potential in BER-affected fruit, the substrates required for ascorbate regeneration by dehydroascorbate reductases are glutathione and NADPH. These reducing agents are key components in the plant antioxidant system. Glutathione content and glutathione reductase activity are both decreased in BER-affected fruit compared to healthy fruit (Mestre et al., 2012). One study reported increased levels of proteins involved in the pentose phosphate pathway, which recycles $NADP^+$ to NADPH, in BER-affected tomatoes compared to healthy (Casado-Vela et al., 2005). However, substrates for the pentose phosphate pathway are formed through the action of sucrose synthase, acid invertase, hexokinase, and fructokinase. The activities of

these key enzymes have been reported as reduced in BER-affected peppers compared to healthy fruit (Turhan et al., 2006a), suggesting that the regeneration of NADPH may be limited prior to the pentose phosphate pathway.

Taken together, these cellular trends represent an efficient and coordinated reduction in antioxidant capacity. Ascorbate is oxidized to dehydroascorbate by ascorbate oxidase. Dehydroascorbate is then reduced to ascorbate, completing the cycle through the oxidation of glutathione or NADPH. Once glutathione and NADPH pools are depleted, ascorbate is then oxidized by ascorbate oxidase. This, coupled with a reduction in catalase activity, results in a significant reduction in antioxidant potential.

NADPH oxidase is upregulated at the transcription level in BER-affected fruit compared to healthy (Freitas et al., 2017), suggesting that the increased production of ROS in BER fruit is a regulated process. Similarly, ascorbate oxidase is upregulated at the transcript level, suggesting that depletion of antioxidants in BER fruits is at least a partially regulated process. A transcriptome investigation of BER fruit compared to healthy found that monolignol biosynthesis is upregulated in BER fruit through an upregulation of the phenylpropanoid pathway (Freitas et al., 2011). Analysis of the transcriptional data from this study shows varied upregulation and downregulation of different peroxidase genes, suggesting that the substrate specificity of peroxidases in BER fruit is regulated at the transcriptional level. This evidence together suggests that the ROS accumulation and lignification observed in BER fruit may be a controlled and concerted process by the cell.

Physiological differences by tissue location

In addition to making comparisons between healthy fruit and BER-affected fruit, this paper investigated the effect of pericarp location on BER associated processes. Our results showed that the top pericarp tissue in BER-affected fruit is very different from the visually unaffected bottom tissue or the BER-affected tissue of BER-affected fruit. Conversely, top tissue of BER-affected fruit was not significantly different from bottom or top tissue of healthy fruit in H_2O_2 content, pyrogallol and ferulic acid peroxidases, TGA lignin, or autofluorescence measurements of lignin content. For catalase activity, however, it was significantly lower in top tissue than bottom tissue of healthy fruit. While the cause and importance of this trend is not evident from the results presented here, the difference in catalase activities between healthy top and bottom tissue may be due to differing maturities, stress levels, or expansion rates in top and bottom pericarp tissues. These results show that blossom-end rot is a highly localized event, as the name suggests. Sampling methods and the resulting comparisons should be designed accordingly. Sampling of the top tissue for the study of BER can be used to provide a secondary control from the same fruit as tissue developing BER.

5.5 Conclusions

Accumulation of ROS during BER development has been well documented, though the results of this accumulation have not been well defined. Our results showed that one effect of the accumulation of H_2O_2 during BER development is lignification of BER-affected tissues. The lignification process is initiated prior to the emergence of visual BER symptoms in tissues adjacent to BER-affected tissue. However, the top pericarp tissue of BER-affected fruit closely resembles that of healthy fruit, suggesting that BER-associated processes are limited only to the

bottom of BER-affected fruits. Lignification may explain reports of increased cell wall elastic modulus and disrupted cellular expansion in the bottom of BER-affected fruit.

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Appendix A: Effect of pre and postharvest treatments on bitter pit development in apple

Summary

The following appendix contains results from experiments designed to test the effectiveness of pre and postharvest application of phytohormones on bitter pit (BP) development during apple postharvest storage. Applications of phytohormone treatments, whether applied preharvest or postharvest, were not effective in inhibiting or significantly reducing BP development. Quality measures were not greatly affected by postharvest treatments, and phytotoxicity was not observed. Early season preharvest treatment of apple trees with prohexadione, a gibberellic acid biosynthesis inhibitor, visibly reduced shoot growth and reduced fruit acidity after storage. Although BP incidence was not significantly lower than in fruit from control trees, early season treatment with prohexadione had the lowest mean BP incidence of any preharvest treatment and the additional potential benefits of reducing shoot growth and fruit titratable acidity. Postharvest water dip increased BP incidence by 7 to 14% compared to undipped fruit. This trend was consistent over all three years and could have significant implications in an industry where water is extensively used for washing, treatment application, and fruit movement during packing. Inclusion of 1% $CaCl_2$ in the dip solution reduced BP to the level of the undipped fruit, and the inclusion of the surfactant Tween, in addition to $CaCl_2$, further reduced BP incidence. Apoplastic calcium was similar between all treatments, with the exception of significantly higher apoplastic calcium in fruit treated with the combination of $CaCl_2$ and Tween. A separate experiment determined that 58 mg of surface material per fruit was removed during a 5-minute dip in water. An additional 5 mg could be removed mechanically with a cloth after dipping and drying. Analysis of the dipping water showed that 0.07 to 0.11 mg calcium per fruit was recovered in the dipping solution, equating to a mean of 0.5 µg gfw^{-1}. Similarly, apoplastic calcium means for

undipped fruit were between 0.5 and 0.8 µg gfw^{-1} higher than water dipped fruit. This demonstrates that postharvest water dips remove calcium from the fruit surface resulting in less apoplastic calcium, and this may be the cause of increased BP in water dipped fruit compared to undipped fruit.

Similar trends in ethylene production were observed in 2019 and 2020, with undipped fruit having higher means than water dipped fruit, and calcium dipped fruit having lower ethylene production. Ethylene production was higher in fruit showing BP than healthy fruit, and ethylene production prior to pit formation was significantly correlated with subsequent BP development. However, ethylene and 1-methylcyclopropene treatments did not significantly affect BP incidence. This suggests that increased ethylene production is not a causative factor in BP development, but rather an associated effect of the pit development process. Measurement of ethylene production during storage may be useful for the prediction of subsequent BP incidence.

Dichlorofluorescein diacetate analysis indicated that reactive oxygen species were more prevalent in pits and the surrounding tissue. Electrolyte leakage was highest in healthy, water dipped fruit and lowest in Tween and Tween plus calcium treatments. Means for peroxidase and catalase activity were higher in BP-affected fruit compared to healthy fruit, though differences were only significant in 2018 catalase analysis. Ascorbic acid and total ascorbate were significantly lower in BP affected water dipped fruit compared to healthy calcium dipped fruit.

These results are not fully consistent with trends observed in the development of blossom-end rot of tomatoes and peppers, also thought to be related to calcium deficiency. Both blossom-end rot and BP affected fruit exhibit increased reactive oxygen species content and decreased ascorbic acid content compared to healthy fruit. However, an increase in catalase activity was observed in BP fruit compared to healthy fruit, while BER was associated with a decrease in catalase

activity. Similarly, blossom-end rot was associated with an increase in electrolyte leakage, while BP affected fruit had decreased electrolyte leakage compared to healthy fruit. These results suggest that conclusions from the study of one calcium deficiency disorder may not be fully applicable to similar disorders in other fruit.

Harvest and treatments

Granny smith apples were harvested from the same commercial orchard each year, which consisted of approximately 5-year old trees. Fruit were sorted to select blemish free apples of the same approximate size. Apples were treated (see Table A.1.1) with a 5 min dip that included a short agitation at 2.5 min. Apples were packed in cardboard boxes with 2 ventilation holes and stored at 20°C, >90% RH.

Table A1.1: Experimental design of postharvest treatments for BP apple research in 2018, 2019, and 2020.

Year	Reps	Days of storage	Treatment	Treatment abbreviation
2018	4	75	No dip	ND
			Water and polysorbate tween 20	TW
			Water, 0.5% polysorbate tween 20, and 300ppm abscisic acid	ABA 300ppm
			Water, 0.5% polysorbate tween 20, and 500ppm abscisic acid	ABA 500ppm
			Water, 0.5% polysorbate tween 20, and 300ppm Prohexadione-Calcium	PH 300ppm
			Water, 0.5% polysorbate tween 20, and 500ppm Prohexadione-Calcium	PH 500ppm
2019	4	68	No dip	ND
			Water	Water
			Water and 0.5% polysorbate tween 20	Tw
			Water, 0.5% polysorbate tween 20, and 1% $CaCl_2$	$CaCl_2$
			Water, 0.5% polysorbate tween 20, 1% $CaCl_2$, and 500ppm ABA	ABA 500ppm
			Water, 0.5% polysorbate tween 20, 1% $CaCl_2$, and 500ppm PH	PH 500ppm
2020	5	68	No dip	ND
			Water	Water
			Water and 1% $CaCl_2$	$CaCl_2$
			No dip and 250 ppb 1-MCP gas, 24 h at 20°C	No Dip, 1-MCP
			Water dip and 50ppm ethylene gas, 24 h at 20°C	Water Dip, Ethylene

Bitter pit evaluation

All evaluations were carried out with treatment labels coded to eliminate potential bias. Apples were visually evaluated for external BP symptoms after 75, 68, and 68 days for 2018, 2019, and 2020 experiments, respectively. Skin was removed to confirm BP symptoms as needed.

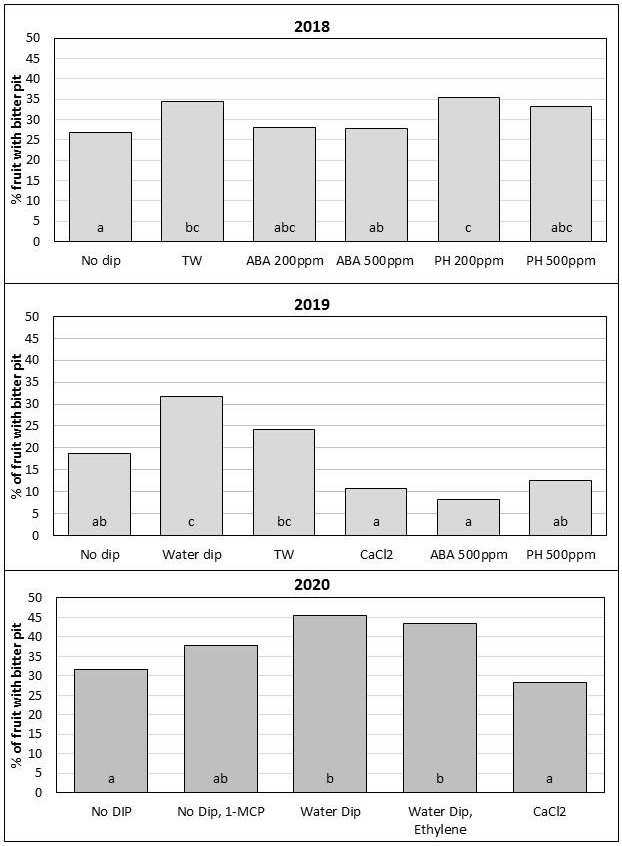

Figure A1.1: Effect of postharvest treatments on bitter pit development over 3 years of experimentation. Bars indicate the mean of 4 replications for 2018 and 2019, and 5 replications for 2020. Within each graph, bars with the same letter were not significantly different (p≥0.05). TW= 0.5% polysorbate tween 20, ABA=abscisic acid, PH=prohexadione-calcium, 1-MCP=1-methylcyclopropene, CaCl2= 1% calcium chloride.

Bitter pit was significantly increased by water dips in 2019 and 2020, and in TW dips in 2018 compared to undipped fruit in each year. Phytohormone treatments did not significantly affect bitter pit in 2018. It was hypothesized that water dips removed preharvest calcium treatments, thus increasing bitter pit. To counteract this, 1% $CaCl_2$ was added as a control and also to the phytohormone treatments in 2019. $CaCl_2$ treatment significantly reduced bitter pit, but phytohormones did not have a significant effect compared to the $CaCl_2$ control. In 2020, 1-MCP

and ethylene treatments were added to investigate the effects of differences in ethylene production observed in 2019. However, BP incidence in ethylene treated water dipped fruit and 1-MCP treated undipped fruit were not significantly different from water dipped fruit and undipped fruit. $CaCl_2$ treatment reduced BP incidence compared to water dipped fruit in 2020.

Preharvest phytohormone treatment effects on bitter pit

Abscisic acid was sprayed weekly for 4 weeks ending 45 days before harvest. Early application of prohexadione (300ppm) was carried out as two sprays spaced 14 days apart starting approximately two weeks after full bloom. Late application of prohexadione (300ppm) included 2 applications 7 days apart starting approximately 10 weeks after full bloom. Treatments were applied in the late morning using a backpack sprayer at a rate of 3.79 L per tree.

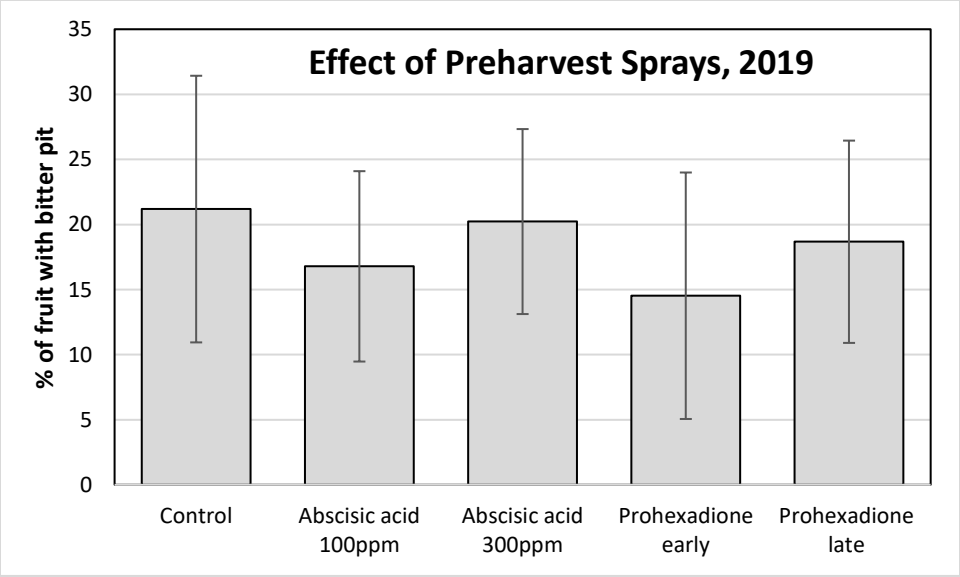

Figure A1.2: Effect of preharvest treatments of abscisic acid and prohexadione on BP development. Bars indicate the mean of 3 blocks, with 3 trees per block. Error bars indicate the standard deviation of the mean. Means were not significantly different ($p \geq 0.05$).

Phytohormone preharvest sprays did not have a significant effect on BP incidence compared to unsprayed trees. Reduced shoot growth was clearly visible in trees treated with the early application of prohexadione, a GA biosynthesis inhibitor.

Titratable acidity, pH, total soluble solids, and firmness

For experiments with postharvest treatment, firmness was measured on a total of 10 fruit per replication. Firmness measurements were taken on opposite sides of the fruit after the peel was removed. Resistance to penetration was measured using a Fruit Texture Analyzer (Güss, Strand, South Africa) with an 11mm probe and a 5mm penetration depth. For titratable acidity (TA), pH, and total soluble solids (TSS), longitudinal wedges were sliced from 5 fruit, chopped, and juiced as a combined sample. This was repeated with a second group of 5 fruit from the same replicate. Measurements from the two juices from each replicate were analyzed and the measurements were averaged for the final replicate value. Titratable acidity and pH were measured using a TIM 850 automatic titration system (Radiometer, Copenhagen, Denmark). Total soluble solids (TSS) were measured using a temperature compensated refractometer (Reichert, Depew, N.Y., U.S.A.)**.**

Table A1.2: Quality evaluations for 2018, 2019 and 2020 experiments

2018 Postharvest									
Treatment	Level	TA[z] %	± SD	pH	± SD	TSS[y] %	± SD	Firmness (kg)	± SD
Control	No dip	0.65	0.05	3.26	0.03	12.19	0.42	7.44	0.59
Control	Water dip, 0.5% tween	0.67	0.05	3.24	0.04	12.84	0.47	7.76	0.62
Prohexadione-calcium	200ppm	0.69	0.05	3.25	0.02	12.09	0.47	7.65	0.53
Prohexadione-calcium	500ppm	0.62	0.04	3.27	0.01	12.64	0.42	7.78	0.63
Abscisic acid	200ppm	0.62	0.04	3.28	0.01	11.91	0.49	7.23	0.73
Abscisic acid	500ppm	0.69	0.06	3.25	0.03	12.11	0.25	7.96	0.65
2019									

Preharvest									
Treatment	Level/timing	TA %	± SD	pH	± SD	TSS %	± SD	Firmness (kg)	± SD
Control	No spray	0.72	0.02	3.36	0.01	12.04	0.68	8.17	0.48
Abscisic acid	100ppm	0.72	0.14	3.37	0.03	11.33	0.53	8.27	0.55
Abscisic acid	300ppm	0.75	0.07	3.37	0.02	10.93	0.81	7.98	0.32
Prohexadione-calcium	2 w after full bloom	0.60	0.07	3.44	0.03	11.81	0.32	8.41	0.50
Prohexadione-calcium	10 w after full bloom	0.79	0.07	3.32	0.04	11.68	0.36	8.37	0.42
Postharvest									
Treatment	Level	TA %	± SD	pH	± SD	TSS %	± SD	Firmness (kg)	± SD
Control	No Dip	0.70	0.05	3.40	0.01	12.35	0.73	7.70	0.19
Control	Water dip	0.79	0.04	3.31	0.02	12.13	0.49	7.78	0.15
Control	0.5% tween	0.72	0.03	3.35	0.03	11.91	0.21	7.76	0.10
$CaCl_2$	1%, 0.5% tween	0.74	0.01	3.31	0.02	12.24	0.33	7.73	0.11
Abscisic acid	500ppm, 0.5% tween, 1% $CaCl_2$	0.80	0.05	3.32	0.01	12.01	0.55	7.71	0.19
Prohexadione-calcium	500ppm, 0.5% tween, 1% $CaCl_2$	0.77	0.03	3.31	0.02	12.33	0.33	7.67	0.15
2020 Postharvest									
Treatment	Level	TA	± SD	pH	± SD	TSS %	± SD	Firmness (kg)	± SD
Control	No dip	0.78	0.05	3.18	0.01	11.67	0.35	7.43	0.17
Control	Water dip	0.79	0.10	3.17	0.03	12.07	0.63	7.37	0.23
$CaCl_2$	1%	0.76	0.02	3.17	0.01	12.61	0.22	7.46	0.11
Water dip, ethylene	50ppm	0.79	0.04	3.16	0.02	12.24	0.35	7.40	0.19
No dip, 1-methylcyclopropene	250ppb	0.84	0.03	3.17	0.01	11.90	0.52	7.13	0.16

[z]TA=titratable acidity, [y]TSS=total soluble solids

Generally, there were no notable effects of the postharvest treatments on fruit quality. There were no consistent trends in the effect of water dips on titratable acidity, pH, total soluble solids, or firmness after storage compared to undipped fruit. In 2020 experiments, 1-MCP treatment reduced firmness and increased TA compared to undipped fruit. Preharvest application of prohexadione reduced titratable acidity and increased pH measurements compared to control fruit.

Ethylene production, 2019

Ethylene was measured by of sealing 1 fruit from each of the 4 replicates per treatment in a jar. This was repeated with 2 additional jars for a total of 3 jars per replicate. Fruit remained at 0°C throughout measurement and between measurements. The seal time was 12 hours for 14, 21, and 28 day measurements, and was reduced to 6 hours for later measurements to avoid overaccumulation of ethylene. Headspace samples (10mL) were injected into a gas chromatograph (Model AGC Series 400, Hach-Carle Co., USA) for quantification.

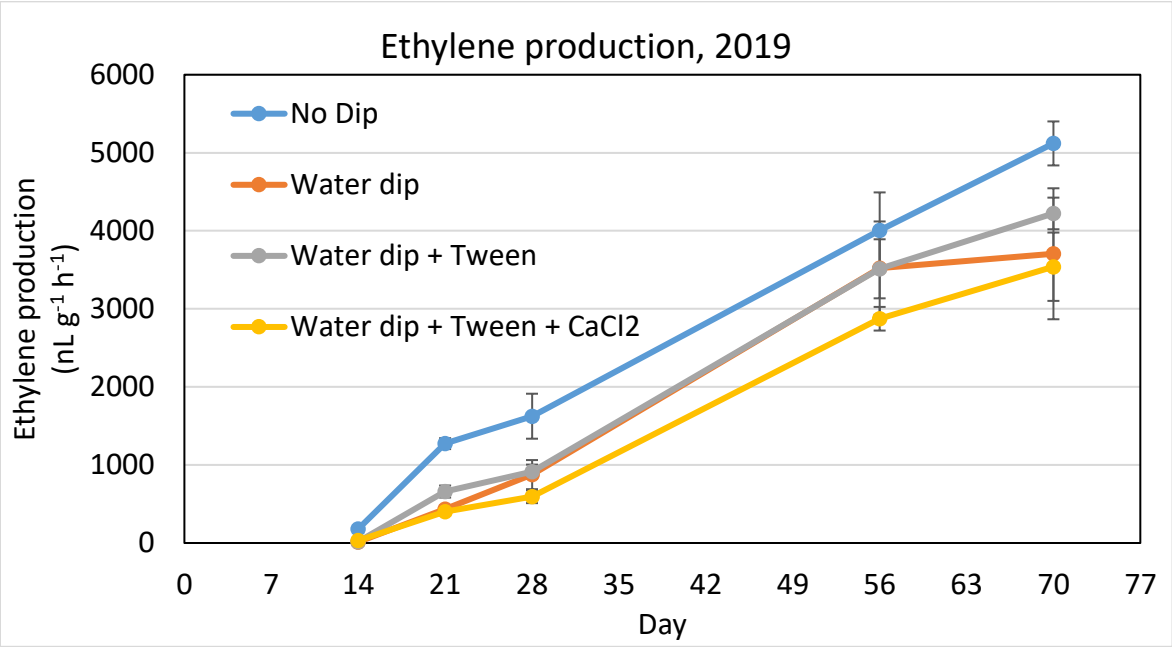

Figure A1.3: Comparison of apple ethylene production between treatments during storage at 0°C in 2019. Each point is a mean of 3 reps, each with 4 fruit. Error bars indicate the standard error of the mean. Tween= 0.05% polysorbate tween 20, CaCl2= 1% calcium chloride

Ethylene production was highest in undipped fruit, with the largest differences observed 21 and 28 days after treatment. Calcium treatment slightly reduced ethylene production compared to water dipped and Tween dipped controls.

Ethylene production in bitter pit affected and healthy undipped fruit

Two 3.79 L jars containing either 5 BP affected fruit or 5 healthy fruit (all undipped fruit) were prepared for each of 4 replications. Jars were sealed for 5-6 hours and the headspace gas was analyzed using a gas chromatograph (Model AGC Series 400, Hach-Carle Co., USA).

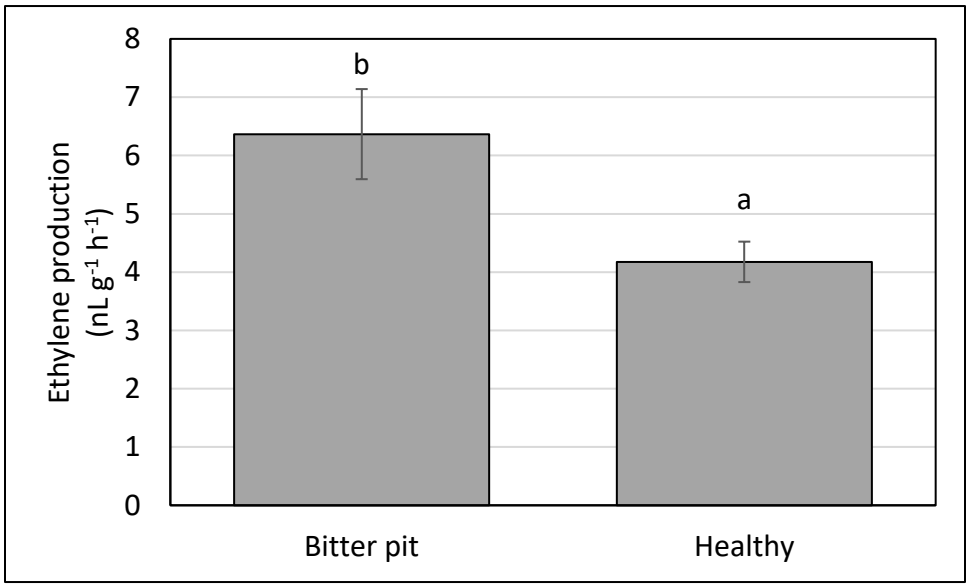

Figure A1.4: Ethylene production in bitter pit affected undipped fruit compared to healthy fruit after 68 days of 0°C storage in 2019 experiments. Measurements were taken at 0°C. Each bar indicates the mean of 4 replications, each containing 5 fruit. Error bars indicate the standard deviation of the mean. Means were significantly different (p=0.002).

Ethylene production was significantly higher in bitter pit affected fruit.

Ethylene production, 2020

Ethylene was measured by sealing 4 fruit from the same replicate in a 3.79 L jar at 0°C. This was repeated with each replicate for a total of 5 replicates per treatment. The seal time ranged from 8 hours in early measurements to 2 hours in later measurements to avoid overaccumulation of

ethylene. Headspace samples (10mL) were injected into a gas chromatograph (Model AGC Series 400, Hach-Carle Co., USA) for quantification.

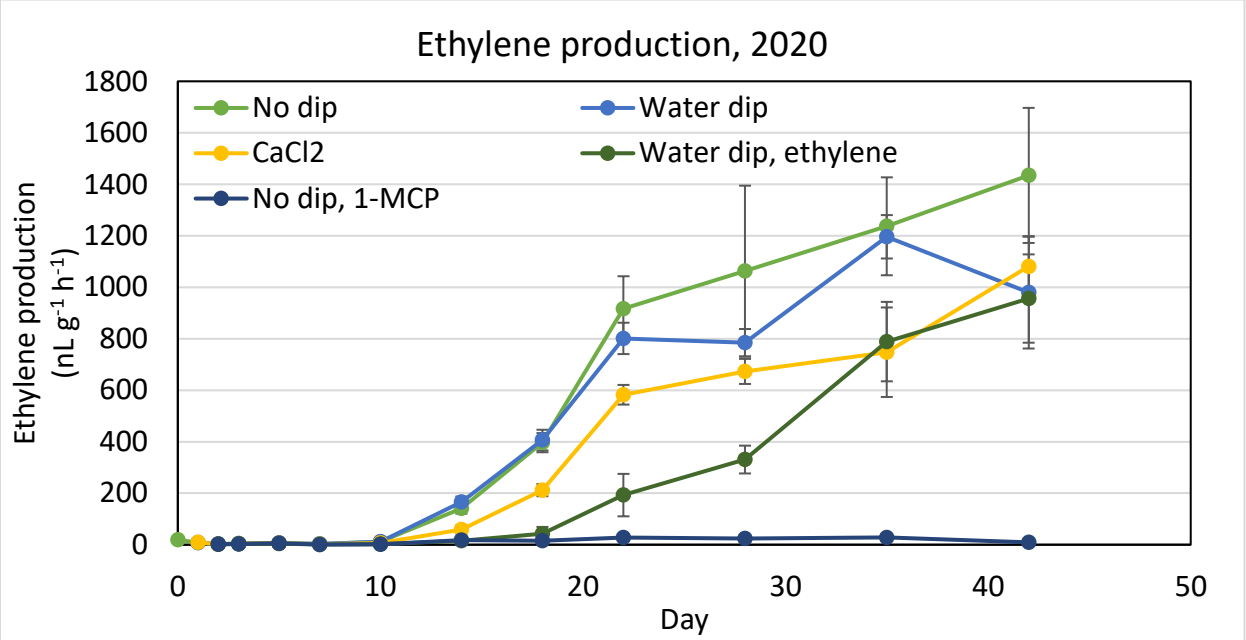

Figure A1.5: Comparison of apple ethylene production between treatments during storage at 0°C in 2020. Each point represents the mean of 5 replicates and error bars indicate the standard error of the mean. CaCl2= 1% calcium chloride, 1-MCP=1-methylcyclopropene

Results in 2020 were similar to those of 2019, although differences between undipped and dipped fruit were lesser in magnitude. Undipped fruit had the highest ethylene production starting at 22 days after treatment. Calcium treatment reduced ethylene production. Surprisingly, ethylene treated fruit had reduced ethylene production during storage compared to undipped, water dipped, and calcium dipped fruit. This may be an effect of the separate treatment conditions or separate storage. Ethylene and 1-MCP treatments were carried out at 20°C for 24 hours, while no dip, water, and calcium treated fruit were placed directly into 0°C storage during the same 24-hour period. This may have acted as a short condition period between harvest and

0°C storage. Additionally, ethylene and 1-MCP treated fruit were stored in a separate 0°C cold room for two weeks after treatment to prevent contamination of other treatments with residual gas from treatments. While both cold rooms were set to 0°C, differences in door opening, ventilation, and other products stored in the rooms may have produced slightly differing storage conditions. While fruit were well ventilated before being placed in the 0°C storage room, cross contamination of the ethylene treated fruit with residual 1-MCP may have caused the delay in increase in ethylene production in ethylene treated fruit. 1-MCP treated fruit produced very little ethylene over the course of the project.

Apoplastic calcium concentration

Apoplastic calcium was measured by cutting 2 discs of cortex tissue from 3 fruit (6 total), rinsing with water, and shaking in 10mL of 0.35M mannitol for 30 minutes. This was repeated on a second set of 3 fruit from the same replicate and the solutions were combined for a total of 20mL. Solutions were analyzed for calcium content after microwave acid/hydrogen peroxide digestion using inductively coupled plasma atomic emission spectroscopy at the UC Davis Analytical Lab (Davis, CA, USA). For 2019, 4 replicates per treatment were analyzed. For 2020, 5 replicates per treatment were analyzed.

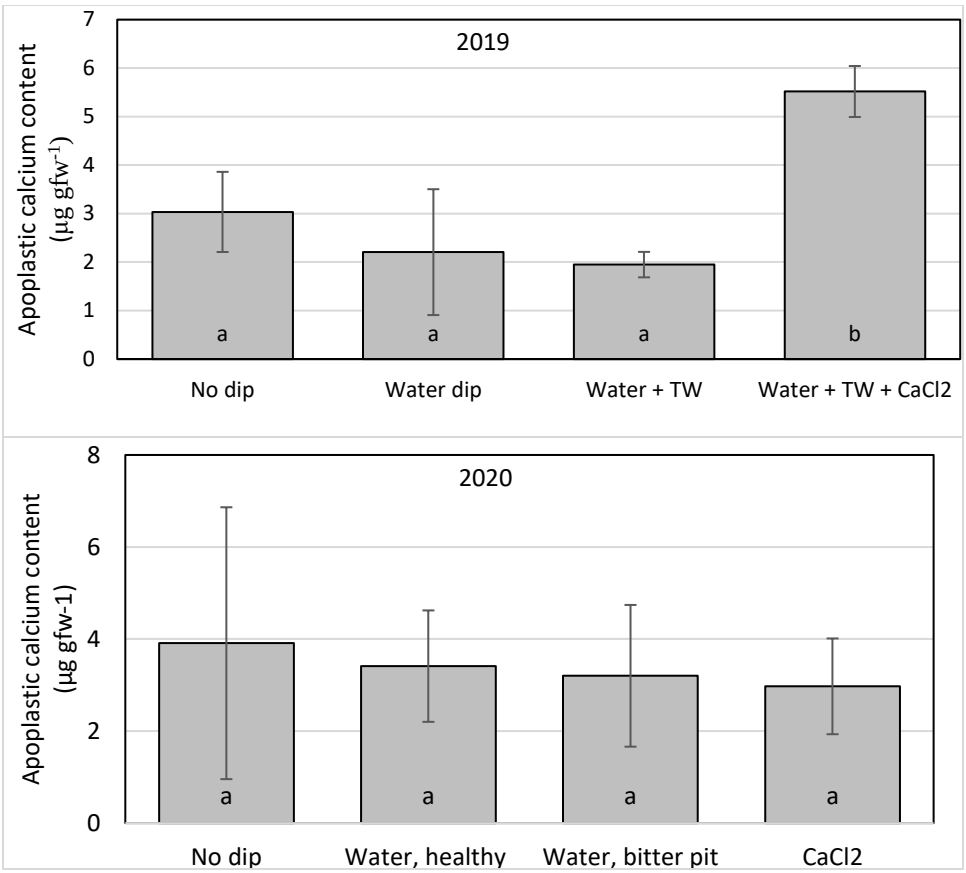

Figure A1.6: Effect of treatment on apoplastic calcium content. Each bar represents the mean of 4 or 5 replicates in 2019 or 2020, respectively. Error bars indicate the standard deviation of the mean. TW= 0.5% polysorbate tween 20, CaCl2 = 1% calcium chloride

Results from 2019 indicate increased apoplastic calcium in the calcium + tween treatment. This trend was not observed in 2020 when tween was not included with the calcium treatment.

Peroxidase activity

Peroxidase activity was measured in apples in 2018 and 2019 after 75 and 68 days of cold storage. Though BP was observed in all treatments, only healthy apples were analyzed for water dip, Tween, and calcium treatment. Both healthy and BP affected apples were analyzed from the no dip treatment. Cortex tissue was ground and mixed with 100mM potassium phosphate buffer, pH 6, and 5% polyvinyl polypyrrolidone, at a rate of 0.2g mL^{-1}. This was centrifuged for 15

minutes at 15,000g. The combination of 70 µL of ultrapure water, 3.33 µL of 100 mM phosphate buffer, 10.66 µL of pyrogallol, 10.66 µL of enzyme extract, and 5.33 µL 0.5% H_2O_2 made up a total reaction volume of 100µL. The reaction was started with the addition of H_2O_2. The change in absorbance at 420 nm was measured.

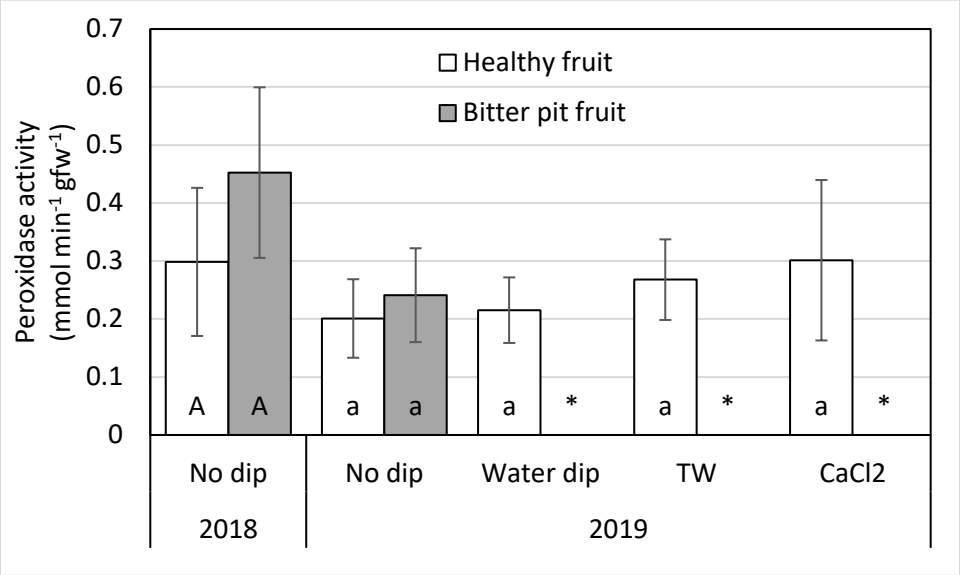

*Figure A1.7: Effect of treatment on peroxidase activity after storage in apple fruit with and without bitter pit. Bars represent the mean of 4 replicates and the error bars indicate the standard deviation of the mean. Bars with the same letter within each year were not significantly different (p≥0.05). Bitter pit was observed in all treatments, but only healthy fruit were used for water dip, TW, and CaCl2 treatments. Healthy and bitter pit fruit were analyzed from the no dip treatment. TW= 0.5% polysorbate tween 20, CaCl2= 1% calcium chloride, * = bitter pit fruit were not analyzed from this treatment*

Peroxidase activity means were higher in no dip BP fruit compared to no dip healthy fruit in 2019 and 2020, although differences were not significant. Water, Tween, and calcium treatments did not have a significant effect on peroxidase activity.

Catalase activity

Catalase activity was measured in 2018 and 2019 on apples after 75 and 68 days of storage. Though BP was observed in all treatments, only healthy apples were analyzed for water dip, Tween, and calcium treatment. Both healthy and BP affected apples were analyzed from the no dip treatment. Catalase was extracted in the same manner as for peroxidase. The enzyme extract (8.33µL) was added to 241µL of a mixture containing 0.036% H_2O_2 in 100mM potassium phosphate buffer, pH 6, and left for 20 minutes to react. The addition of 500µL of 1M potassium iodide and 250µL 0.1% trichloroacetic acid (TCA) stopped the reaction. This was further incubated for 10 min. Sample blanks were assayed by substituting ultrapure water for potassium iodide. An initial H_2O_2 concentration was determined. Absorbance measurements at 350nm were compared to a standard curve ranging from 1µM to 1mM H_2O_2. Enzyme extracts were prepared from a mixture of cortex tissue from 5 apples per replication, with 4 replications per treatments.

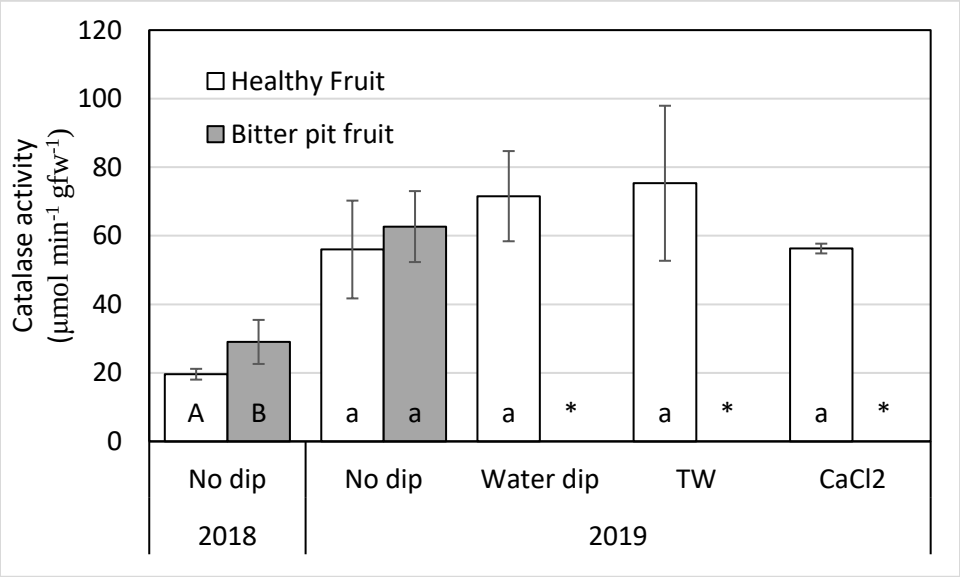

Figure A1.8: Effect of treatment on catalase activity after storage in apples with and without bitter pit. Bars represent the mean of 4 replicates and the error bars indicate the standard

*deviation of the mean Bitter pit was observed in all treatments, but only healthy fruit were used for water dip, TW, and CaCl2 treatments. Healthy and bitter pit fruit were analyzed from the no dip treatment. Bars with the same letter within each year were not significantly different (p≥0.05). TW= 0.5% polysorbate tween 20, CaCl2= 1% calcium chloride, * = bitter pit fruit were not analyzed from this treatment*

Results from 2018 indicated a significant increase in catalase activity in BP fruit compared to healthy fruit. A similar trend was observed in 2019, although differences were not significant. Water treatment and water + tween treatment exhibited higher mean catalase activity compared to undipped and calcium treated fruit, although differences were not significant.

Dichlorofluorescein diacetate fluorescence microscopy

Reactive oxygen species were estimated in apple tissues using a dichlorofluorescein diacetate (DCFDA) analysis (Macarisin et al., 2007; Sabban-Amin et al., 2011). Four apple slices (500μm) consisting of skin and cortex tissue were made from the pitted tissue of 3 BP affected fruit. These slices included both the pitted area and the pit adjacent tissue. Additionally, 4 slices from the healthy tissue of the same 3 BP affected fruit were collected. Four slices were also collected from the blossom-end of 3 healthy fruit. Each slice was incubated in 25mM DCFDA in phosphate buffer saline for 15 minutes, then blotted dry and placed on a glass slide with 50μL phosphate buffer saline. Slices were imaged using the 4X objective and GFP filter of an EVOS FL Auto fluorescence microscope (Thermo Fisher, Waltham, MA, USA). The mean fluorescence intensity was measured using ImageJ software on the cortex tissue, excluding the skin. In pitted slices, the mean fluorescence was measured in the pit-adjacent tissue and pit tissue.

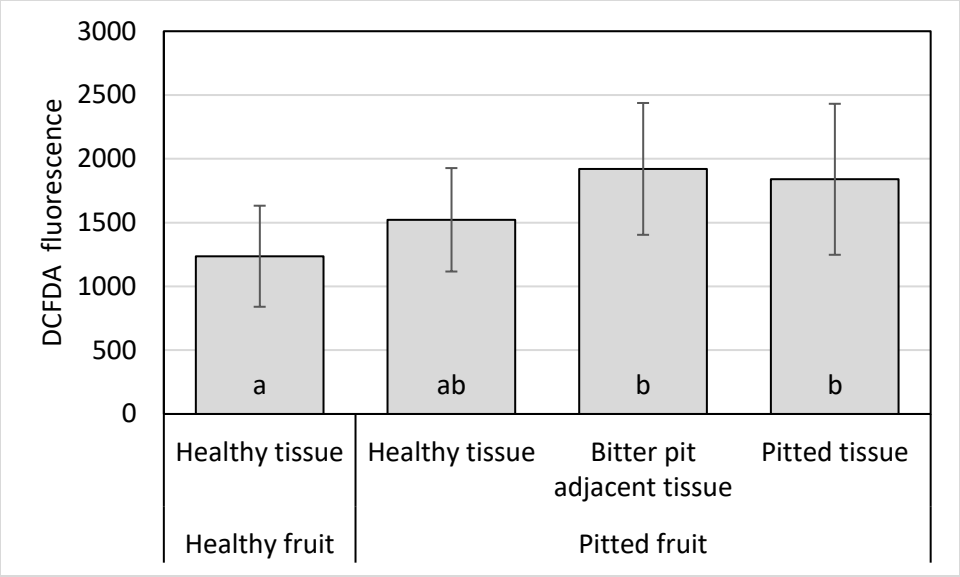

Figure A1.9: Reactive oxygen species in bitter pit affected and healthy apple fruit tissue as measured using dichlorofluorescein. Each bar indicates the mean of 3 fruit, with 4 slices per fruit. Error bars indicate the standard deviation of the mean. Bars with the same letter were not significantly different (p≥0.05).

Results indicated significantly increased fluorescence, and thus reactive oxygen species, in pits and the pit adjacent tissue compared to healthy tissue of healthy fruit. Healthy tissue from pitted fruit was not significantly different from healthy tissue from healthy fruit or from pitted and adjacent tissue from pitted apples.

Electrolyte leakage

Electrolyte leakage was measured on 2 slices from 3 fruit (total of 6 slices) from each of 4 reps of each treatment. Though BP was observed in all treatments, only healthy apples were analyzed for no dip, Tween, and calcium treatment. Both healthy and BP affected apples were analyzed from the water dip treatment. These 6 slices were submerged with agitation in 10mL of 0.35M mannitol. The conductivity was measured at 1, 1.5, and 2 hours. After freezing and thawing 3

times, the total conductivity was measured. The average rate of increase in conductivity was calculated as a percent of total conductivity.

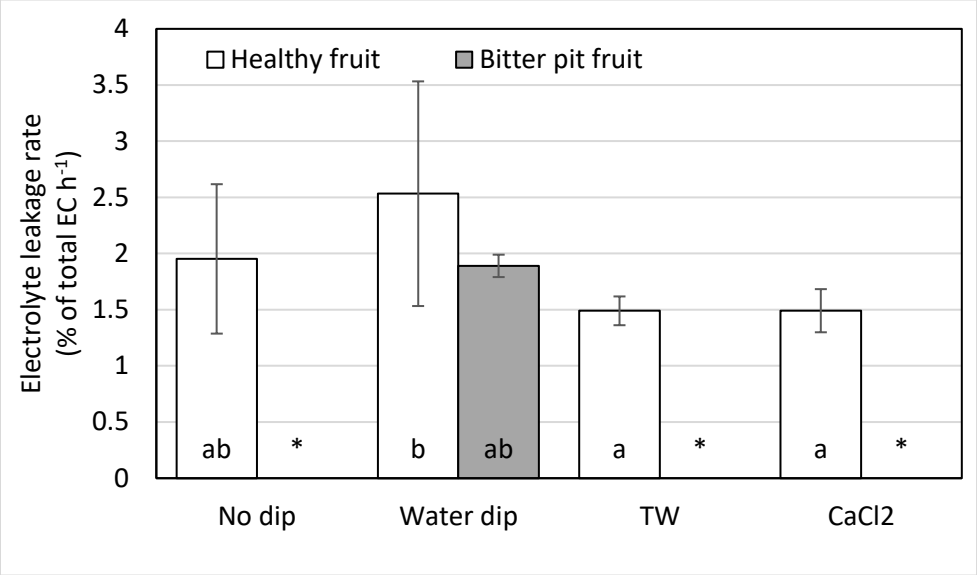

*Figure A1.10: Effect of treatment and presence of bitter pit on electrolyte leakage of apple tissue. Each bar represents the mean of 4 replications and error bars represent the standard deviation of the mean. Bitter pit was observed in all treatments, but only healthy fruit were used from the no dip, TW, and CaCl2 treatments. Healthy and bitter pit fruit were analyzed from the water dip treatment. Bars with the same letter are not significantly different (p≥0.05). TW= 0.5% polysorbate tween 20, CaCl2= 1% calcium chloride, * = bitter pit fruit were not analyzed from this treatment*

Electrolyte leakage was significantly higher in healthy, water dipped fruit compared to apples dipped in tween or $CaCl_2$ + tween. As a nonionic surfactant, tween may affect membrane integrity, thus explaining the reduction in electrolyte leakage.

Ascorbic acid and glutathione evaluation

Ascorbic acid (AA), total ascorbic acid, dehydroascorbic acid (DHA), glutathione (GSH), total glutathione, and oxidized glutathione (GSSG) were measured using reverse phase high performance liquid chromatography. Apple cortex samples were ground in liquid nitrogen and vortexed with 6% metaphosphoric acid containing 2 mM EDTA and 1% insoluble PVPP. The extracts were centrifuged at 20,000g for 12 min at 4°C and the supernatant was filtered through a 0.45µm syringe filter. Filtered samples were injected (5µL) into an Agilent 1200 HPLC. The column was an Agilent eclipse XDB-C18 5um 4.6x150mm and the mobile phase was 400 µL/L o-phosphoric acid, pH of 2.5, and 0.1mM EDTA. The flow rate was 0.8 mL min^{-1} and a gradient of 0-30-0% acetonitrile was run from 12-15-18 minutes, respectively. The total run time was 25 minutes. Samples were analyzed at 243nm and 197nm for AA and GSH, respectively, with retention times of approximately 2.9 and 4.3 min. A standard of 1mM AA and 1mM GSH in 3% metaphosphoric acid, stabilized with 200mM dithiothreitol, was used for quantification. Total AA and total GSH was measured by mixing 250µL of sample with 125µL of 200mM dithiothreitol in 400mM tris base. The reaction was stopped after 15min with the addition of 125µL of 8.5% phosphoric acid. This mixture was analyzed using the same HPLC method for total AA and total GSH measurements. DHA was calculated by subtracting the AA from total AA. GSSG was calculated by subtracting the GSH from total GSH.

Table A1.3: Effect of treatment and bitter pit on reduced ascorbic acid (AA), total ascorbic acid, dehydroascorbic acid (DHA), reduced glutathione (GSH), total glutathione, and oxidized glutathione (GSSG).

	AA	±SD	Total ascorbic acid	±SD	DHA	±SD	% AA of total AA[ɨ]	±SD	GSH	±SD	Total glutathione	±SD	GSSG	±SD	% GSH of total glutathione[«]	±SD
No dip	39.66*^ab	18.36	41.83ab	17.89	2.16a	1.34	92.28a	9.71	106.73a	21.18	135.47a	19.07	28.74a	9.03	78.48a	7.09
Water dip, healthy	40.73ab	12.25	41.54ab	12.04	0.81a	0.57	97.85a	1.73	111.61a	5.37	133.43a	7.20	21.82a	2.42	83.68a	1.26
Water dip, BP	20.09a	10.86	23.62a	8.23	3.52a	3.95	81.65a	21.04	95.25a	22.73	117.54a	21.91	22.28a	1.19	80.39a	4.49
CaCl2 dip, 1%	58.46b	15.95	60.13b	16.70	1.68a	1.55	60.13a	16.70	112.14a	12.33	134.55a	11.43	22.42a	1.40	83.20a	2.24

[z] all values are in µmol gfw^{-1} and are a mean of 5 replications

[y] values followed by the same letter within each measurement are not significantly different ($p \geq 0.05$)

[y] "% AA of total AA" indicates the percent of reduced ascorbic acid compared to the total reduced and oxidized ascorbic acid

[x] "% GSH of total GSH" indicates the percent of reduced glutathione compared to the total reduced and oxidized glutathione

Reduced AA and total ascorbic acid contents were significantly higher in calcium treated fruit compared to pitted fruit from the water dip treatment. Means for healthy water dipped fruit and healthy undipped fruit were higher than pitted, water dipped fruit, although differences were not significant. A similar but smaller trend was also observed in GSH and total glutathione.

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Appendix B: Comparison of in situ and extractive methods for measuring apoplastic calcium content

Summary

Apoplastic calcium is integral in cell wall, plasma membrane, and signaling functions. Additionally, maintenance of apoplastic calcium has been shown to be associated with reduced calcium deficiency disorders, such as bitter pit in apples and blossom-end rot in tomatoes. However, apoplastic calcium is difficult, time consuming, and expensive to measure. Cells must remain intact and unfrozen, as any disruption of the cellular membranes can contaminate the apoplastic solution and thus affect measurements. Extraction of apoplastic calcium from the tissue requires either submerging or infiltrating the tissue in an isotonic solution and analyzing the solution to determine the calcium concentration. This appendix presents results from a new fluorescence microscopy method for determining apoplastic calcium concentrations in fruit tissue using the fluorophore Fluo-4 cell impermeant pentasodium salt. A standard curve (figure A2.1) of calcium chloride produced a linear response between 0.1 to 10 μM calcium chloride, with reduced response per amount of calcium added between 10 μM and 100 μM. To test the effect of including a buffer, two approximately isotonic solutions were tested, only one of which included a buffer. Fluorescence response was higher in 0.2 M mannitol with 100mM potassium phosphate buffer, pH 6, compared to 0.35 M mannitol (figure A2.2). One possible explanation is the effect of pH on fluorescence response. Inclusion of a buffer ensures that the pH of analysis is consistent between samples. Because the pH of the apples used in this study was 3.16, the difference in fluorescence responses may be due to pH effects. To test the effect of pH on fluorescence response, a variety of buffers and pH values were used to analyze water and calcium treated apples. Results (Figure A2.3) showed that increasing pH increased fluorescence

and differentiation between water and calcium treated fruit. Comparisons to an extraction-based method (figure A2.4) indicated poor correlation between methods in individual apples, but improved results when evaluating the effects of treatments as a whole. This was supported by results from postharvest storage experiments in 2019, but not 2020 (figure A2.5). Results from 2020 experiments showed a low range of calcium concentrations and very little difference between treatments, likely resulting in the low correlation between the two methods. Overall, this method works best in higher pH solutions, is faster and less expensive than the extraction-based method, and has less variation than the extraction-based method. However, correlation was poor between this fluorescence method and the extraction-based method, particularly when comparing individual samples within a treatment and when the range of concentrations measured was small. Because measuring apoplastic calcium requires preserving the separation between the cytoplasmic constituents and the apoplast constituents, using an approximately isotonic solution is critical. Highly hypertonic or hypotonic solutions could induce an efflux or influx of water, respectively. This would, at minimum, affect the concentration of calcium in the apoplast, and possibly disrupt the plasma membrane. Weight loss measurements (figure A2.6) indicated that 0.35 M mannitol or 0.2 M mannitol with 100mM potassium phosphate buffer, pH 6, had similar osmotic effects for granny smith apples after 4 months of storage. The solution of 0.3M mannitol was the optimal dissolution solution for limiting weight change. However, the data shown in Figure A2.2 indicates that including a buffer is critical for fluorescence response. The limited weight change induced by the solution of 0.2M mannitol with 100mM phosphate buffer makes it a better option.

Fluo-4 pentasodium salt fluorescence response with increasing calcium chloride concentrations

Fluo-4 pentasodium salt, cell impermeable, was purchased from Invitrogen (Thermo Fisher Scientific, Massachusetts, USA). A stock solution of 1mM was prepared in double deionized water and working solutions were made at a concentration of 1μM, following the manufacturer's instructions. A standard curve using calcium chloride with concentrations of 0.01μM, 0.1μM, 1μM, 10μM, and 100μM was prepared with a final concentration of 1μM Fluo-4 pentasodium salt, 0.2M mannitol, and 100mM potassium phosphate buffer pH 6. Three replicates were analyzed at each concentration. These were measured in a 96 well plate using a Biotek multimode H1 plate reader (Biotek, Winooski VT, USA), with an excitation wavelength of 470nm and emission of 535nm.

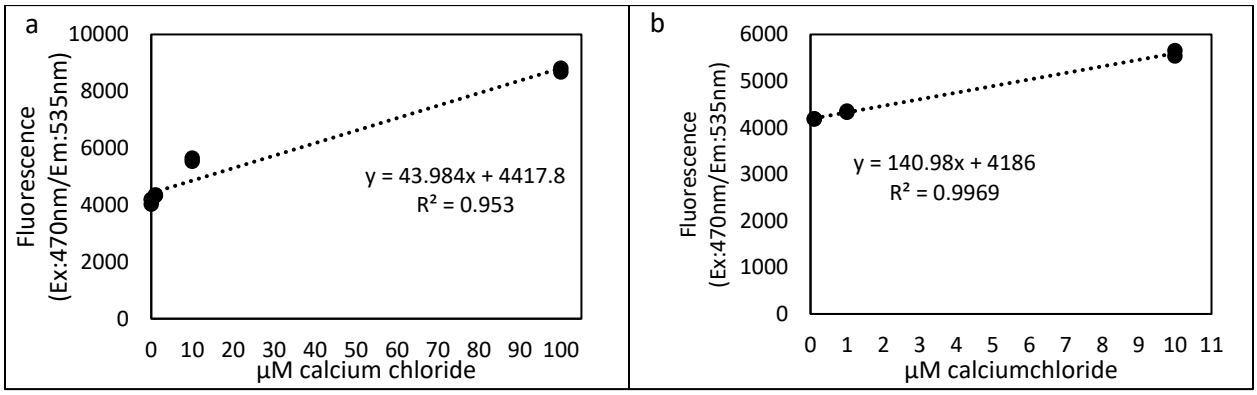

Figure A2.1: Fluo-4 fluorescence response curve for 0.01μM to 100μM (a) and 0.1μM to 10μM (b) calcium chloride.

The fluorescence response curve of Fluo-4 in 0.2 M mannitol and 100 mM phosphate buffer, pH 6, indicated a linear trend between 0.1 μM $CaCl_2$ to 10 μM $CaCl_2$. Response at 0.01 μM $CaCl_2$

followed the linear trend from 10 μM to 0.1 μM, but variation between replicates increased. The linear regression was less accurate when including 100μM measurements, possibly due to saturation of the fluorophore. Based on these results, this fluorophore shows promise for in vitro calcium measurements.

Effect of buffer inclusion on fluorescence response

Solutions of 1uM Fluo-4 cell pentasodium salt were made in 0.2M mannitol with 100mM potassium phosphate, pH 6, and 0.35M mannitol. Apple treatment groups included water treated and calcium treated fruit. Water treated fruit were dipped for 5 minutes after harvest in deionized water and stored at 0°C. Calcium treated fruit were treated with a 5 minute dip in 1% calcium chloride at harvest, and a second 5 minute dip in 1% calcium chloride and 0.5% polysorbate tween 20 after 3 months of 0°C storage. Apples were analyzed after 4 months of storage. Slices of apple tissue were cut from the bottom of the fruit. To make each 500 μm slice, a subsection of apple (approximately 1cm × 1cm × 4 cm) was placed in a graduated handheld microtome and the protruding section was removed to create a flat surface. Vertical incisions were made to create a 5 mm x 3 mm section that included the skin, perpendicular to the movement of the microtome. Slices of 500 μm thickness were made by hand using a razor. From each fruit, six slices were prepared, and two were treated with the 1μM fluo-4, 0.2M mannitol, 100mM phosphate buffer solution. As a control, one slice was treated with 0.2M mannitol, 100mM phosphate buffer solution. Two additional slices were treated with 1μM fluo-4, 0.35M mannitol solution. The final slice was treated with 0.35M mannitol solution as a control. All slices were imaged using an Evos FL auto microscope with the GFP filter (Ex:422nm/Em:525nm). Images were analyzed using Imagej software. The mean relative fluorescence was measured in the cortex tissue, excluding the highly fluorescent skin. From each three-slice set, the mean relative fluorescence

of the control slice was subtracted from the average of the two Fluo-4 treated slices for the final mean relative fluorescence value.

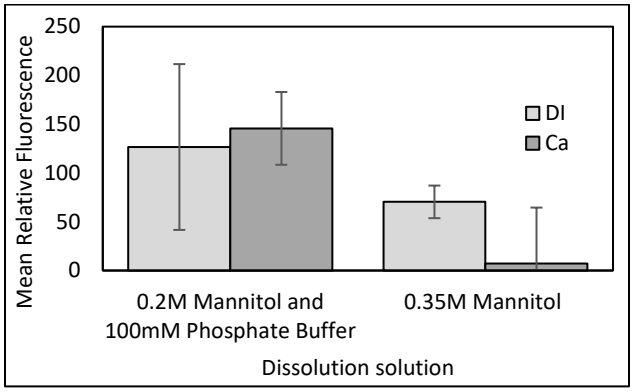

Figure A2.2: Effect of buffer inclusion on fluorescence response. Either 0.2M mannitol with 100mM potassium phosphate buffer, pH 6, or 0.35 mannitol was used to prepare a 1μM fluo-4 impermeant solution. Bars indicate the mean of measurements of tissue taken from 3 fruit treated at harvest with either water (DI) or at harvest with 1% calcium chloride and again 15 weeks after harvest with 1% calcium chloride and 0.5% polysorbate tween 20 (Ca). Error bars indicate the standard deviation of the mean.

Using 0.2 M mannitol with 100 mM potassium phosphate buffer, pH 6, as a carrier solution for fluo-4 impermeant produced a better fluorescence response for fluorescence microscopy measurements compared to a similar osmotic solution without a buffer (0.3 M mannitol). Juice from the apples used in this study had a pH of 3.31. Figure A2.3 indicates that increasing pH improves fluorescence response, suggesting that the results from figure A2.3 were at least in part a result of pH effects on fluorescence response.

Effect of dissolution buffer on fluorescence response

Increasing pH within the same buffer system (acetate buffer pH 3.6 and 5.7) drastically increased the fluorescence response. Use of potassium phosphate buffer improved differentiation between treatments. Increasing to pH 8 using tris base further improved separation and fluorescence response.

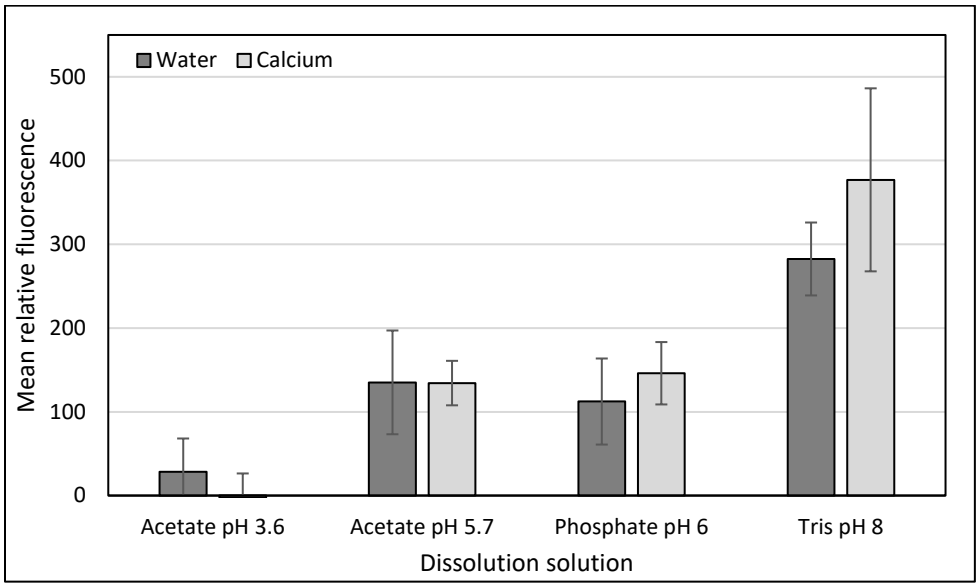

Figure A2.3: Effect of dissolution buffer on fluorescence response. Each bar indicates the mean of 4 fruit, with error bars indicating the standard deviation of the mean.

To determine the effect of buffer and pH on fluorescence response, slices from 4 calcium treated apples and 4 water treated apples were analyzed as stated above with varying buffers. Buffers at a concentration of 0.1 M included potassium acetate buffer pH 3.6, potassium acetate buffer pH 5.7, potassium phosphate buffer pH 6, and Tris base pH 8, and were prepared in a 0.2 M mannitol solution.

Comparison of apoplastic calcium measured using fluorescence microscopy or diffusive extraction

Fluo-4 analysis was carried out as described above using 0.2 M mannitol and 100 mM potassium phosphate buffer, pH 6. Apoplastic calcium analysis through diffusive extraction was measured by cutting 12 discs from each fruit, rinsing with water, draining thoroughly, and shaking in 10mL of 0.35 M mannitol for 30 minutes. The collected solutions were analyzed for calcium content after microwave acid/hydrogen peroxide digestion using inductively coupled plasma atomic emission spectroscopy at the UC Davis Analytical Lab (Davis, CA, USA).

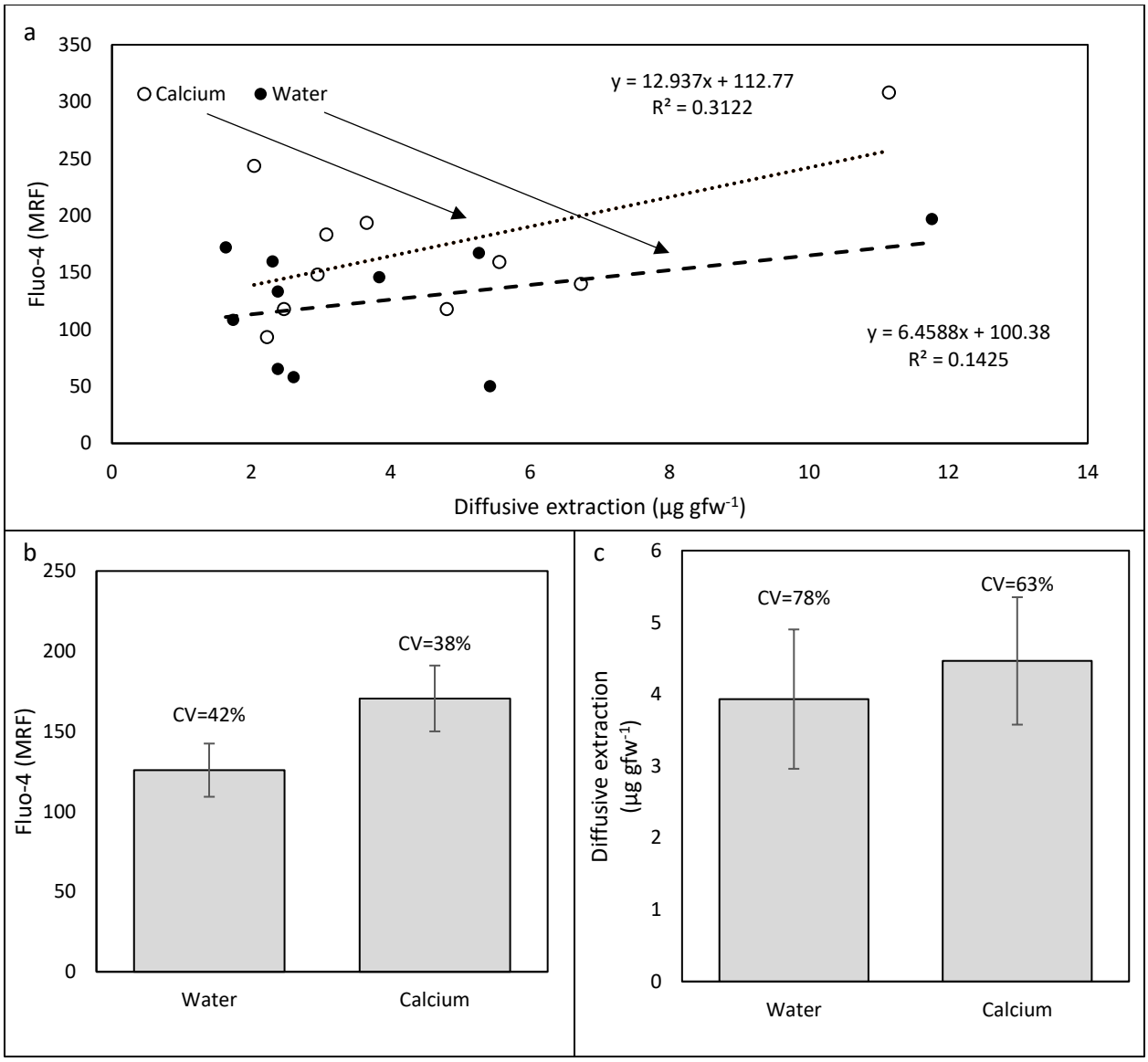

Figure A2.4: Comparison of individual fruit apoplastic calcium between fluor-4 cell impermeant microscopy and diffusive extraction with subsequent inductively coupled atomic emission spectroscopy. For the direct comparison (a), each point indicates one fruit analyzed using both methods. Pearson's correlation between the two methods for all 20 fruit was 0.47. For comparison of treatment means and variation (b, c), each bar indicates the mean of 10 fruit. Error bars indicate the standard error of the mean and the coefficient of variation (CV) was calculated dividing the standard deviation by the mean and multiplying by 100%. MRF=mean relative fluorescence

Though correlation between measurements from Fluo-4 testing and the diffusive extraction method was significant, the correlation was generally low with a Pearson's correlation coefficient of 0.47. When evaluating results at the treatment level, fluo-4 analysis exhibited better differentiation between treatments and lower coefficients of variation within both treatments compared to the extraction method. Calcium treatments had higher means in both treatments, though the difference in means was not significant using either treatment.

Comparison of fluo-4 and diffusive extraction apoplastic calcium measurement of treatment groups

Postharvest treatments were carried out on the day after harvest as 5-minute dips with a gentle agitation for 2.5 minutes. In 2019, 4 replicates of 80 apples per replicate were treated with, deionized (DI) water, DI water + 0.5% polysorbate tween 20, and DI water + 0.5% polysorbate tween 20 + 1% calcium chloride. An undipped control was also included. For 2020, treatment groups included healthy undipped fruit, healthy fruit treated with DI water, bitter pit fruit treated with DI water, and healthy fruit treated with a 1% $CaCl_2$ solution.

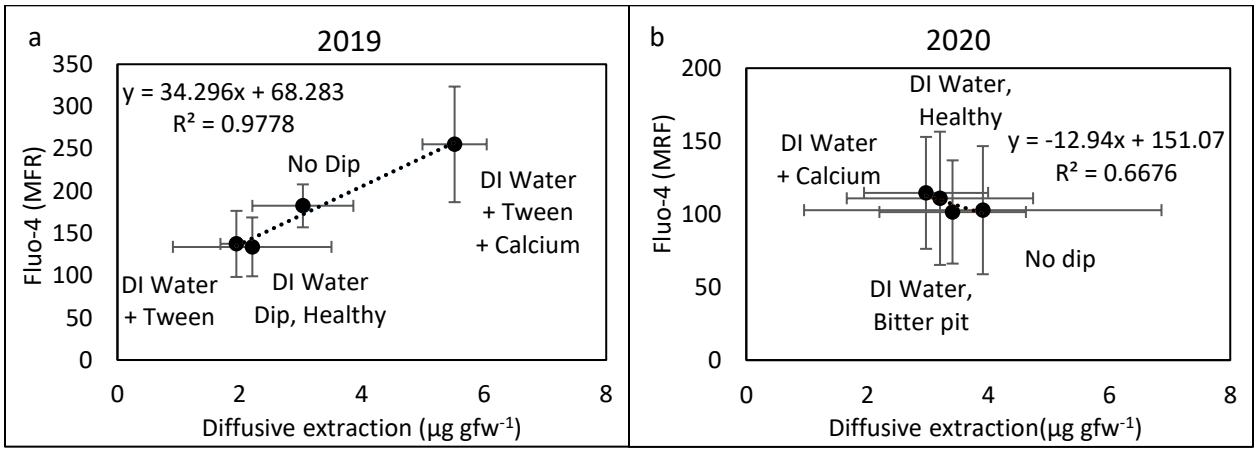

Figure A2.5: Comparison of treatment group apoplastic calcium analysis methods fluo-4 cell impermeant microscopy and diffusive extraction with subsequent inductively coupled atomic

emission spectroscopy during 2019 (a) and 2020 (b) apple postharvest experiments. During the 2019 and 2020 seasons, 4 and 5 replications per treatment were used. DI= deionized

Comparisons of treatment means between measurement methods indicated strong positive correlation in 2019, but weak negative correlation in 2020. One possible explanation is the difference between years in the range of measurements. In 2019, diffusive extraction measurements ranged from 1.9 to 5.5 μg gfw^{-1}, while 2020 measurements ranged from 3.0 to 3.9 μg gfw^{-1}. Based on these results, the use of either of these methods is optimum when large differences between treatments is expected.

Effect of mannitol concentration of weight change over time

Cylinders with a diameter of 13.5 mm were cut from a Granny Smith apple from the DI water treatment group and two discs (approximately 2mm thick) consisting of only cortical tissue were made from each cylinder, starting 2mm under the skin. Discs were rinsed briefly in ultrapure water to remove intracellular material released by cutting and blotted dry on filter paper. Two randomly selected discs were weighed and placed in a Petri dish. Solutions of 0M mannitol, 0.35M mannitol, 0.65M mannitol, and 0.72M mannitol were prepared in ultrapure water. Additionally, a solution of 0.2M mannitol and 100mM phosphate buffer was prepared. These solutions (10mL) were applied to three sets of 2 discs and incubated with shaking. At 60, 120, and 240 minutes, each set of 2 discs was blotted dry, weighed, and re-submerged in a fresh 10mL of solution.

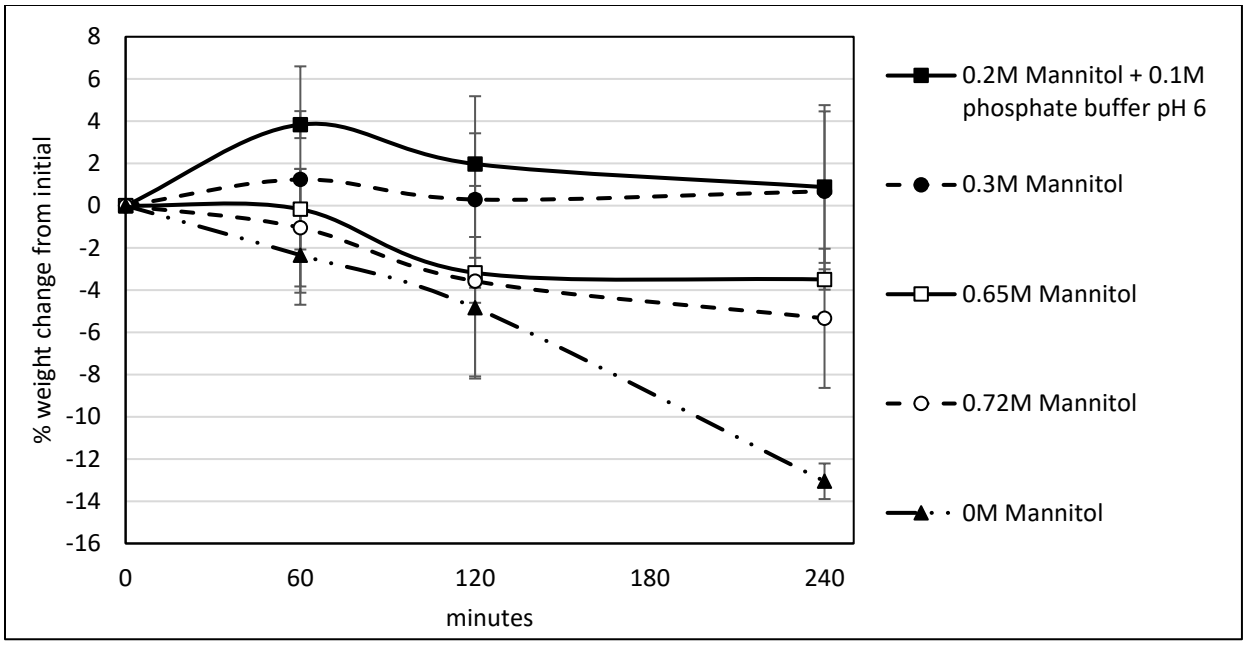

Figure A2.6: Effect of immersion solution on weight loss of Granny Smith apples. Each point indicates the mean of 3 replicates with error bars indicating the standard deviation of the mean.

The initial increase in weight was highest in samples submerged in 0.2 M mannitol with 100mM phosphate buffer. Discs submerged in 0.3 M mannitol had a lower initial weight gain, but a similar final weight gain compared to 0.2 M mannitol with 100 mM phosphate buffer. Solutions of 0 M, 0.65 M, and 0.72 M mannitol did not exhibit an initial increase in weight at 60 minutes, though an increase may have occurred before the 60-minute measurement. The drastic decrease in weight for 0 M mannitol may be due to osmotic shock and disruption of the normal function of the cellular compartmentation. A similar trend was reported in tomatoes by Saltveit (Saltveit, 2002), though results from that study exhibited an initial increase followed by a continuous decrease in weight loss. Solutions of 0.65 M mannitol and 0.72 M mannitol were previously reported as isotonic solutions for apples. However, results presented here indicate that 0.65 M and 0.72 M mannitol solutions were hypertonic.