EFFECTS OF PLANT GROWTH REGULATORS AND WEATHER ON BITTER PIT INCIDENCE IN ‘HONEYCRISP’ APPLE

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ABSTRACT

EFFECTS OF PLANT GROWTH REGULATORS AND WEATHER ON BITTER PIT INCIDENCE IN 'HONEYCRISP' APPLE

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Bitter pit is a physiological disorder of apple linked with Ca deficiency and characterized by dark lesions on the surface of fruit. The commercially important cultivar 'Honeycrisp' is highly susceptible to the disorder. In-planta Ca transport occurs exclusively in the xylem; therefore, Ca deficiency in fruit is primarily the result of gradual xylem dysfunction. Because auxin promotes xylogenesis, we hypothesized that early-season applications of both native and synthetic auxins and ABA would improve xylem longevity and functionality resulting in higher Ca concentrations in fruit and reduced bitter pit. Using several experimental approaches, we demonstrate that auxins and ABA significantly prolong xylem function and markedly reduce bitter pit incidence. A multiple regression model was also developed to correlate temporal climatic data with bitter pit incidence over multiple years and disparate sites throughout Michigan. Collectively, results suggest potential novel uses of plant growth regulators to mitigate bitter pit.

Dedicated to
My parents who provided the stability that allowed me to take risks,
And to Claire who edited every word
And formatted every page of this thesis,
And who has led me to reconsider my position on stories with happy endings.

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KEY TO ABBREVIATIONS

DAFB: Days after full bloom

WAFB: Weeks after full bloom

GA: Gibberellic acid

IAA: Indole-3-acetic acid

NAA: 1-naphthaleneacetic acid

ABA: Abscisic acid

PGR: Plant growth regulator

Ca: Calcium

Mg: Magnesium

K: Potassium

B: Boron

N: Nitrogen

P: Phosphorus

S: Sulfur

CHAPTER 1. Introduction

1.1. STATEMENT OF THE PROBLEM

The United States apple industry produced 243.8 million bushels of fruit in 2021 (USApple, 2021). ‘Honeycrisp’ ranks third among cultivars in annual production at 31 million bushels, having grown in popularity to surpass ‘Fuji’ and ‘Granny Smith’. The surge in popularity of ‘Honeycrisp’ in the last 15 years has been driven by consumer preference for its unique combination of internal textural and flavor attributes and external characteristics (large fruit size) (Luby and Bedford, 1992). As a result, ‘Honeycrisp’ is the most profitable of apple cultivars grown in the United States (Gallardo et al., 2015). Apple producers appreciate the cultivar’s cold hardiness, tolerance to apple scab, and long postharvest storage life (Watkins and Nock, 2012). ‘Honeycrisp’ was bred in 1960 at the University of Minnesota. Through genetic analysis, the maternal and paternal parents of ‘Honeycrisp’ are two University of Minnesota varieties, ‘Keepsake’ (‘Malinda’ x ‘Northern Spy’) and ‘MN1627’ (‘Duchess of Oldenburg’ x ‘Golden Delicious’), respectively (Howard et al., 2017). A relatively recent and ongoing consumer phenomenon, ‘Honeycrisp’ is not without its downsides; ‘Honeycrisp’ trees are prone to biennial bearing and have low vegetative vigor. The combination of large fruit size and short pedicels results in premature fruit drop and the fruit themselves can be poorly colored and are highly susceptible to bruising and several internal disorders including soft scald, soggy breakdown, and most importantly, bitter pit (Gallardo et al., 2015). The focus of this thesis will be the tendency of ‘Honeycrisp’ to suffer from the latter disorder.

Among commercial cultivars, ‘Honeycrisp’ is especially susceptible to bitter pit with over 20% of fruit being affected in a typical year and up to 50% of fruit developing symptoms, especially when harvested from young trees (Rosenberger et al., 2004). Because bitter pit detracts from both appearance and taste, there is no fresh market for affected fruit. While bitter pit has been linked with calcium (Ca) deficiency, applications of Ca to fruit do not prevent bitter pit entirely (Wills et al., 1976). Over 150 years of investigation has led to considerable progress in understanding causes and factors associated with bitter pit disorder of apple, yet appreciable gains in managing bitter pit have been elusive. Areas of inquiry that may advance our understanding are Ca transport and the effects of phytohormones on Ca and mineral balance.

1.2. A HISTORY OF BITTER PIT

1.2.1. Description and Early Identification

Corking disorders have been a burden to fruit producers since the beginning of commercial fruit production. Interest in understanding these conditions coincides with the rapid growth of widespread commercial fruit production. Initial scientific descriptions of the disorder first appeared in Germany in the mid-1800s. Jäger seems to have been the first to describe the condition now known as bitter pit, noting sunken pits appearing in stored fruit which manifested alongside corky tissue beneath the fruit surface (Jäger, 1869). Jäger blamed excessive transpiration in the tissue as an explanation for the desiccated tissue beneath the pits.

In the next decade, Sorauer prepared a more detailed characterization of the disorder, describing the lesions produced as brown to black spots extending 0.5 to 1.5 mm into the flesh (Sorauer, 1879). He noted the lesions never expanded to cover the entire surface, and seemingly healthy fruit developed the disorder in storage. Sorauer describes an even earlier identification of the disorder by Fries, but it is impossible to say if this is the same condition Sorauer describes or a related disorder due to difficulties identifying separate conditions at this time. Sorauer's observations led him to propose a name for the condition—"Stippichwerden der Äpfel"—which is translated as a speckling or dotting of apple fruit. Sorauer also described a worsening of the condition in soft or loose-fleshed cultivars and less severe in firmer cultivars. Jones (1898) would expand on this description, noting that the lesions typically ranged in diameter from 2 to 10 mm and extended “into the flesh for a distance about equal to its diameter.” Jones’s description attributed a hemispherical shape to the lesions, each originating from a single point in the outer cortex just beneath the fruit surface. Notably, early researchers also observed that bitter pit lesions always occurred at the terminations of vascular bundles (Jones, 1898; Brooks, 1908).

1.2.2. Initial Attributions

Sorauer’s observations led him to blame the fungus *Spilocaea pomi*. *Spilocaea pomi*, now known as *Ventura inaequalis*, vectors the pervasive condition known as apple scab. Indeed, Frank (1880) attributed the disorder to a sterile variety of the scab-causing fungus. Reichelt (1884) also attributed the condition to fungal colonization, but instead attributed it to a fungus from the genus *Sychytrium*, a collection of fungi now known to be responsible for warts and galls in a variety of agricultural crops, but not apple. The condition was widely believed to be fungal in origin into the 1890s when Wortmann (1892) and Zschokke (1897) demonstrated the physiological nature of the disorder, a period coinciding with the use of fungicides to treat apple scab that facilitated separation from bitter pit. Indeed, Wortmann found no fungal mycelium in the broken epidermis of affected apples.

Like Jäger thirty years prior, Wortmann attributed the condition to excessive transpiration. Wortmann also noted that pitting-susceptible cultivars had more lenticels and a thinner-walled epidermis. Because the appearance of pits was prevented by rapidly drying fruit, Wortmann connected the disorder to the rate at which water was conducted from deep within the fruit to replace water lost to transpiration and considered acids within dying cells responsible for spreading necroses. Zschokke also explored transpiration and the epidermis regarding “Stippen.” He noted the pits were far more common on the calyx end of the fruit rather than the stem end and quantified the ratio of lenticels between the two ends of the fruit—7:4 in the heavily affected cultivar ‘Baldwin’ and 5:3 in the similarly sensitive ‘Northern Spy’. Zschokke linked the number of lenticels to pitting susceptibility of fruit because he believed they, rather than stomata, facilitated most fruit gas exchange.

As the fledgling Australian apple industry increased its exports in the 1890s, Nathan A. Cobb was tasked with describing the condition observed in fruit transported over weeks to European markets. It was in this description that Cobb coined the name for the disorder in use today—“bitter pit.” In North America, the commercial importance of highly susceptible cultivars like ‘Baldwin’, ‘Northern Spy’, ‘York Imperial’ and ‘Rhode Island Greening’ encouraged a better understanding of the disorder. ‘Baldwin’, specifically, was so badly affected that the disorder was sometimes considered to be a separate condition referred to as “Baldwin spot” or “the brown spot of Baldwins” (Brooks, 1908).

1.2.3. Differentiation from Other Corking Disorders

Because a variety of corking disorders present myriad and overlapping symptoms across nearly all cultivars, early confusion abounded in attempts to distinguish conditions known variously as “Baldwin spot”, “York spot”, “dry rot”, “brown spot”, “fruit spot”, “fruit pit”, “drought spot”, “blotchy cork”, and “cork spot” (Brooks, 1908; Faust and Shear, 1968). Inaccurate definitions plagued the early attempts to differentiate these disorders. Early on, bitter pit was differentiated from related disorders by its potential to appear both on the tree and in storage, the extension of the cortical lesions to the fruit surface, and that bitter pit became worse in storage (McAlpine, 1912; Faust and Shear, 1968). MacArthur (1940) observed microscopic differences in cells affected by the disorders she categorized as “internal cork”, “corky core”, “drought spot or superficial cork”, and “bitter pit.”

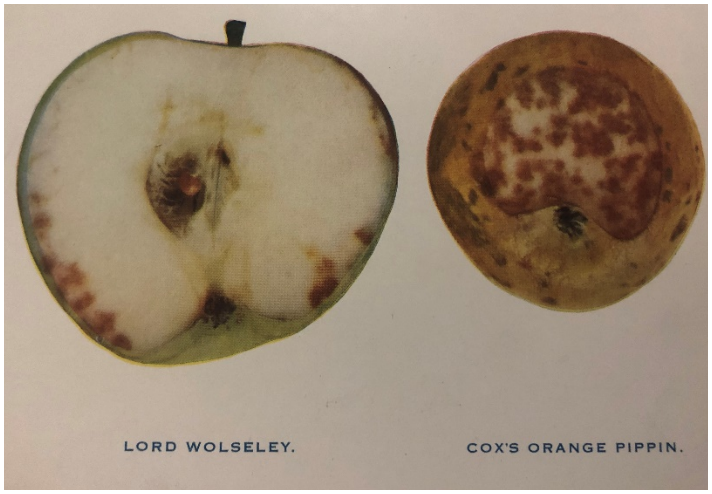

Figure 1: Cross-sections of apple fruit affected with bitter pit (McAlpine, 1912)

1.2.4. Attribution to Mineral Status

In 1912, McAlpine, while still unsure of the exact cause of bitter pit, suspected an association with minerals. He noted that “where the soil and subsoil is calcareous, the most susceptible apples are comparatively free from bitter pit”. McAlpine suggested the addition of lime to orchard soils to mitigate the condition and described a difference in the ash contents of pitted and healthy fruits. Similarly, as early as 1918, growers noticed higher bitter pit incidence in apples irrigated with Epsom salts, $MgSO_4$ (Rose, 1943). While studying the differences in ash mineral content between healthy and bitter pit affected ‘Fallawater’ and ‘Stark’ apples, DeLong (1936) was perhaps the first to notice a significant Ca deficiency in pitted fruit. The difference was not dramatic—0.071g CaO in healthy fruit vs. 0.052g CaO in pitted fruit, on average—but, among minerals, only Ca varied in concentration between the two groups of fruit.

A variety of experiments followed as researchers attempted to better understand the implications of minerals on bitter pit incidence. Garman and Mathis (1956) produced an extensive review of these mineral analyses, noting the apparently conflicting information attributing bitter pit to either excess magnesium (Mg) or Ca deficiency. Garman and Mathis ultimately ascribed bitter pit to a deviation in the optimal ratio of Mg, and possibly potassium (K), to Ca within the fruit. Their research showed that when Mg exceeded Ca, bitter pit developed. The authors described a deficiency limit of 2:1 Ca:Mg for total prevention. This ratio was never approached in the bitter pit resistant cultivars ‘McIntosh’, ‘Winesap’, ‘Stayman’ (‘Winesap’ x unknown), and ‘Delicious’. To the contrary, the ratio was frequently exceeded in ‘Northern Spy’ and ‘Baldwin’, two cultivars that were already replaced by less susceptible cultivars by 1956.

Widespread orchard trials based on grower observations noted bitter pit was increased by common horticultural techniques like ringing branches, nitrogen (N) fertilization, heavy pruning, and late irrigation (Garman and Mathis, 1956; Faust and Shear, 1968). While some of these practices are obsolete in the management of modern high-density orchards, N fertilization is common.

1.2.5. Understanding Consequences and Mechanism of Calcium Deficiency

The precise nature of *how* bitter pit lesions develop in fruit has proven elusive to researchers. Cooper and Bangerth (1976) suggested the role of Ca in bitter pit was a result of its function in cell membranes. The authors noted concurrent research on fruit in storage by Bangerth and Dilley (1971) that observed Ca’s effect in reduced respiration, delayed ripening, and increased fruit firmness, and extended these experiments to address bitter pit (a condition that frequently appears in storage). Cooper and Bangerth (1976) found that because of the cross-linkages formed between Ca and pectin, an increase in Ca decreased tissue permeability, thus highlighting the important role of Ca in cell membrane integrity. Treatment with Mg caused Mg to replace Ca in membranes, increasing permeability. This study led to the current model of bitter pit formation resulting from a loss in membrane integrity and cell collapse.

Improving Ca content of fruit, however, has proven difficult. Bangerth (1979) proposed that bitter pit may result from deficiencies in the uptake of Ca, not soil Ca levels since soil Ca typically exceeds apple tree demand. Although bitter pit can be induced by growing trees in Ca-depleted media (Ferguson and Watkins, 1989), container culture in combination with nutrient withholding confirmed the association of bitter pit with localized Ca deficiency and not total tree deficiency (Martin et al., 1962). Ca concentrations vary markedly within a tree, decreasing with height and at branch apices (Saure, 2005). Even within individual fruit, total Ca is not a sufficiently robust predictor of bitter pit incidence (Perring and Pearson, 1986). Plots of bitter pit incidence and Ca concentration in fruit have been described as “wedge-shaped” with some fruit remaining healthy despite low Ca concentrations (Perring, 1986; Ferguson and Watkins, 1989). Recently, bitter pit has been connected to apoplastic Ca pools, indicating that bitter pit is 1) a localized Ca deficiency and 2) that transport is an important regulatory contributor to bitter pit incidence (de Freitas et al., 2010; Falchi et al., 2017). This is consistent with the fact that Ca is unique among the macronutrients (N, P, K, Mg, Ca, S) *in planta* as it is exclusively xylem-mobile, with only trace amounts found in living phloem tissue (Ferguson et al., 1979).

The discovery that Ca is immobile in the phloem and transported in the xylem exclusively led to the current understanding of xylem functionality being critical in bitter pit prevention (Himelrick and McDuffie, 1983). The differences in susceptibility to bitter pit between cultivars is understood to be, at least partially, a result of the different rates of dysfunction of fruit xylem between cultivars (Lang, 1990). The vast temporal differences in the rate of xylem dysfunction has led to an exploration of fruit growth rate and hormone balance as primary modulators.

Recent bitter pit research has focused on the genes that confer bitter susceptibility and resistance among cultivars.

1.3. DIFFERENCES IN SUSCEPTIBILITY BY CULTIVAR

1.3.1. Bitter Pit Susceptibility by Cultivar

Susceptibility to bitter pit varies dramatically among apple cultivars. As previously mentioned, formerly popular cultivars such as ‘Northern Spy’, ‘Baldwin’, ‘Rhode Island Greening’, and ‘York Imperial’ were largely removed from cultivation, in part, due to their extreme susceptibility to bitter pit. Among modern cultivars, ‘Cortland’, ‘Braeburn’, ‘Mutsu’, ‘Jonagold’, ‘Ida Red’, ‘Catarina’, and ‘Honeycrisp’ are all noted as being especially susceptible to the disorder, while cultivars such as ‘Golden Delicious’, ‘Fuji’, ‘Delicious’, and ‘Granny Smith’ are moderately susceptible. A few cultivars such as ‘Gala’ and ‘McIntosh’ are almost entirely resistant to the disorder.

1.3.2. Bitter Pit as a Consequence of Selection

Cultivar susceptibility to bitter pit can be partially attributed to fruit size, with larger fruit being more prone to bitter pit as a result of dilution of Ca (Perring and Pearson, 1986). However, Volz et al. (2006) found that genetic grouping among cultivars explained bitter pit incidence better than total fruit Ca, suggesting inheritance of bitter pit related genes. Among the open-pollinated seedlings studied by Volz et al. (2006), bitter pit was not observed in the majority of seedlings, and those that were affected experienced bitter pit incidences of under 10%. These findings suggest that bitter pit resistance is not a rare trait but perhaps inadvertently selected for, when selecting parents or fruit with desirable fresh market attributes, and the reasons for bitter pit vulnerability and/or resistance must be better understood.

CHAPTER 2. Literature Review

2.1. CALCIUM FUNCTION IN APPLE

2.1.1. General Roles of Calcium

Ca is one of six macronutrients *in planta* alongside N, P, K, Mg, and S. Ca's status as a macronutrient is due to the variety of critical functions it performs as a stabilizing component of cell walls and membranes and as a signaling agent, among other roles. Because of the importance of these functions, Ca deficiency and/or limited allocation to specific tissues or organs is deleterious to plants culminating in a wide range of disorders among diverse species. In addition to bitter pit in apple, pear (*Pyrus spp*.), and quince (*Cydonia oblonga*), Ca deficiency/misallocation is recognized as the cause of tipburn in leafy greens such as lettuce (*Lactuca sativa*) and spinach (*Spinacia oleracea*) (Saure, 1998), blossom end rot in tomato (*Solanum lycopersicum*), pepper (*Capsicum spp.*), eggplant (*Solanum melongena*), squash (*Cucurbita pepo*), and watermelon (*Citrullus lanatus*) (Bangerth, 1973; de Freitas et al., 2011b), and leaf damage in a variety of species. As a result, exogenous Ca is applied to many crops, including apple. While Ca sprays and dips have proven beneficial in improving storage quality in apple fruit and are somewhat effective in mitigating bitter pit, they are not entirely efficacious (Ferguson and Watkins, 1983).

2.1.2. Calcium in Cell Walls

Sixty percent of intracellular Ca is located in the form of cross-linkages between pectins in cell walls (de Freitas et al., 2010). These cross-linkages determine many of the physical characteristics of plant cells, including rigidity and selectivity of membranes with physical changes in cells being associated with modification and/or solubilization of pectins (Hocking, 2016). The majority of these pectins occur in the middle lamella, the location of cell-to-cell junctions. Ca and B are unique in their suitability for forming these bonds; Ca bonding with homogalacturans and B between rhamnogalacturonan II units (Pérez-Castro et al., 2012; Funakawa and Miwa, 2015). Bangerth (1973) linked bitter pit with the replacement of Ca in these bonds by either K, Mg, or H, resulting in weak linkages that eventually leak pectins to the surrounding tissue. Pectin methylesterase expression has been found in a much higher degree in the calyx of bitter pit affected fruit, and the pectins present in pits are shorter than those found in healthy cells (Faust and Shear, 1968; Zúñiga et al., 2017). As a result, bitter pit lesions stain intensely when treated with solutions that bind to pectins; McAlpine attributed the brown color of the pits themselves to pectins (McAlpine, 1912; MacArthur, 1940). Because of the role of Ca in maintaining cell wall integrity, Ca has been firmly linked with storage quality in fruit, with Ca-deficient fruit having decreased firmness and increased susceptibility to postharvest disorders (Ferguson and Watkins, 1989).

2.1.3. Storage of Calcium

Among organelles, the vacuole is by far the largest site for Ca storage (Peiter, 2011) where the remaining 40% of Ca in apple cells was quantified (de Freitas et al., 2010). Vacuolar Ca concentration varies significantly among different plant species and cells of different tissues within the same plant. Ca transport across the tonoplast of the vacuole is mediated by Ca^{2+}-ATPase pumps and Ca^{2+}/H^+ antiporters. Higher concentrations of Ca in vacuoles has been associated with a greater expression of Ca^{2+}/H^+ antiporters, decreased apoplastic Ca, reduced plasma membrane stability, and a greater incidence of blossom end rot, a Ca-deficiency disorder in tomato (de Freitas et al., 2011). Knockout of these Ca^{2+}/H^+ antiporters in *Arabidopsis* resulted in higher apoplastic Ca (Conn et al., 2011). Apoplastic Ca is available to link with pectins to maintain cell stability. As a result, bitter pit resistance has been found to be associated with the total amount of Ca in the apoplast rather than the fruit as a whole (Turner et al., 1977; Falchi et al., 2017). De Freitas et al. (2010) determined that cystolic Ca in apple cells is extremely low (0.1-0.2 µM), and that proper cell function requires maintenance of at least 0.1 mM free Ca in the apoplastic pool. Ferguson and Watkins (1983) found that exogenously applied Ca remains in this apoplastic space between cells where it can be used to improve membrane stability.

2.1.4. Applications of Calcium in Apple Cultivation and Storage

Ca sprays in apple began in the late 1800s, via Bordeaux mixtures used to control apple scab (Lamson, 1897). As mentioned in section 1.2.2, bitter pit and apple scab had not been clearly understood as separate conditions, so these Ca-containing sprays may have also prevented bitter pit by supplying Ca in addition to their fungicidal activity. Following the linking of bitter pit to Ca deficiency, orchards were treated with Ca on a wider level, but this was typically as soil amendments. With increased understanding of Ca flow in trees, focus shifted to direct applications of Ca sprays to the fruit and leaves. Foliar c Ca sprays, however, are not a panacea for bitter pit for a variety of reasons: Ca sprays are ineffective in trees with high N loads (Ferguson and Watkins, 1989), can be negated by commonly used phosphate minerals which reduce their solubility (Turner et al., 1977), may not penetrate the canopy to contact interior fruit, and are limited in effectiveness by timing, cultivar, shading, and weather (Buti et al., 2018). When these factors are controlled for, Ca sprays are effective in reducing bitter pit incidence while remaining cost effective (Ferguson and Watkins, 1983; Bramlage, 1994). Because of the low absorption rate of Ca into fruit tissues, multiple sprays are recommended. Peryea et al. (2007) found that Ca in fruit at harvest was maximized by spraying later in the season, but also found that bitter pit was most controlled when Ca sprays started in June, perhaps due to the condition already having initiated by the time of later sprays.

Ca dips have been evaluated to varying degrees of effectiveness. Ca dips were found to mitigate blossom end rot in tomato (Lyon et al., 1942) and pepper (Miller, 1961), as well as lenticel spot, a related Ca -deficiency disorder, in ‘Jonathan’ apple (Bangerth, 1973). Bangerth (1973) found calcium chloride dips to be effective in bitter pit mitigation and protected fruit from other postharvest disorders as well. However, Turner et al. (1977) found that Ca dips were less effective in preventing bitter pit than sprays, as Ca absorption from dips was too slow. Lecithin has been found to improve the action of Ca dips, reducing bitter pit even further (Reid and Padfield, 1975). Lecithin increased the rate of Ca absorption from dips, modified external porosity, and slowed metabolism due to changes in the internal atmosphere of the fruit (higher concentration of CO_2 and lower O_2) (Sharples et al., 1979; Watkins et al., 1982).

2.2. RELEVANCE OF OTHER ELEMENTS TO BITTER PIT

2.2.1. Calcium Deficiency vs. Boron Deficiency

B is important in maintaining cell wall integrity through bonding with pectins (Fang et al., 2016), and is important in facilitating auxin transport (Quiles-Pando et al., 2019). Because of the similarities in function, B deficiency in apple manifests in a similar fashion to Ca deficiency. Early researchers struggled to differentiate bitter pit from other corking disorders such as internal cork, corky core, cork spot, blotchy pit, and drought spot, all of which have been reclassified as B deficiency disorders. One of the key differences between these disorders and bitter pit is that these conditions always occur distal to the fruit’s surface, with lesions only reaching the surface in the most severe cases (Mix, 1916). B deficiency disorders also do not worsen during postharvest storage like those associated with Ca disorders (Ferguson and Watkins, 1989).

While perhaps not playing a direct role in bitter pit lesion formation, B deficiency exacerbates bitter pit (Biggs and Peck, 2015; Fallahi, 2020) and fruit Ca has been positively associated with B concentration (Dixon et al., 1973; Fazio et al., 2017). This association has been contentious, historically, with many earlier studies contradicting an association between B and Ca (Wallace and Jones, 1941) and later studies finding the link more definitive. Korban and Swiader (1984) identified two genes that potentially conferred bitter pit resistance and noted higher B and Ca content and lower Mg and K among fruit of resistant genotypes. The mode of action by which B affects fruit Ca uptake is not clear, but B plays a role in Ca partitioning, sugar formation, and root growth, the latter of which is critical to bitter pit prevention (Atkinson, 1935; DeLong, 1936; Garman and Mathis, 1956). Unlike fruit K, which remains constant throughout the growing season, or Ca and Mg which decrease as fruit mature, B concentrations increase as fruit ripen (Cheng and Raba, 2009).

Unlike B deficiency disorders that are effectively treated by exogenous B application, bitter pit is not completely prevented by Ca sprays (Wallace and Jones, 1941). The incomplete effect of Ca sprays on bitter pit suggest bitter pit may result from the interplay of Ca with other cations.

2.2.2. Potassium and Magnesium Excess

McAlpine (1912) noted that pitted tissue contained more ash (minerals) than healthy tissue, and that this ash was more alkaline, indicating a proportionately higher concentration of cations. Among the metals, K, Ca, and Mg are present in markedly higher concentrations than other elements.

Mg deficiency can cause leaf spotting and early fruit drop, but Mg deficiency is not common in most production regions and can even be withheld (Garman and Mathis, 1956; Martin et al., 1962). Elevated levels of both Mg and K in mature fruit have been associated with higher incidence of bitter pit (Ferguson et al., 1999), and treatment of trees and their harvested fruit with Mg actually induced bitter pit (Witney et al., 1991; Amarante et al., 2005). However, labeled, exogenously applied Mg was only recovered in peel, not the cortex (Cooper and Bangerth, 1976). This finding is potentially paradoxical to the understanding of bitter pit that emerged as a condition beginning in the outer cortical tissue of fruit, rather than the peel. More recently, Baugher et al. (2017) demonstrated that peel nutrient status was a stronger predictor of bitter pit susceptibility than flesh nutrients in 'Honeycrisp'. Zúñiga et al. (2017) used Fourier transform infrared and X-ray spectrometers to demonstrate significant differences in the concentrations of Mg and K in healthy vs pitted tissue, i.e., both elements were significantly higher in pitted tissue. Askew et al. (1960) showed Mg to be present in pits at up to four times the concentration in healthy tissue. Mg is detrimental to the postharvest storage quality of apple fruit, negatively affecting firmness (Marcelle, 1995); thus, producers are encouraged to limit Mg sprays.

While Mg concentration in fruit correlates with greater incidence of bitter pit, the ratio of K/Ca in fruit tissue is a far more effective indicator of bitter pit development (Wills et al., 1976). Like Mg, high levels of K negatively affect storage characteristics in apple, but K is positively correlated with both soluble sugar, acid content of fruit, and fruit size (Garman and Mathis, 1956; Marcelle, 1995). Like Ca and Mg, K is present at its highest concentrations in the skin and core and at its lowest concentration in the outer cortex where bitter pit is believed to originate (Ferguson and Watkins, 1983). 'Honeycrisp', as a cultivar highly susceptible to bitter pit, contains higher amounts of K and lower concentrations of Ca in fruit tissue relative to 'Gala' and 'WA 38', two bitter pit resistant cultivars (Cheng and Sazo, 2018; Gomez and Kalcsits, 2020). Lightly cropped trees frequently exhibit higher levels of bitter pit from the dilution of Ca but also because fruit serve as a major K sink (Cheng and Raba, 2009). The optimal level of K in apple fruit and leaves varies with cultivar. Producers are encouraged to limit supplemental K while maintaining sufficient levels to support healthy fruit growth and development since K is mobile in soil and easily depleted (Fallahi and Mahdavi, 2020).

Mg and K are believed to initiate bitter pit in fruit by replacing Ca ions in cell membranes, resulting in a loss of rigidity and selective permeability and eventual cell death (Bangerth, 1979). The high concentrations of these ions in bitter pit lesions are likely the result of the disintegration of cell walls containing an abundance of these elements. Because K and Mg are phloem-mobile, unlike Ca, these cations accumulate in areas where xylem has become dysfunctional, leading to bitter pit lesion formation. Thus, much of the relationships among nutrients that associate with bitter pit are issues of mobility and transport.

2.2.3. Other Potential Elements of Interest

While the roles of Ca, Mg, K, and boron (B) in cell membranes and therefore bitter pit lesions are well understood, other ions play roles in other plant systems and may contribute to bitter pit susceptibility and resistance. Zinc has been positively correlated with fruit Ca uptake, potentially by encouragement of root tip growth and, therefore, soil Ca uptake. Mineral studies by Fazio et al. (2015) and Bonomelli et al. (2020) have both positively correlated fruit zinc and copper concentrations with fruit Ca. It has been suggested that zinc and copper are both able to increase apoplastic Ca by displacing Ca ions from chelation complexes, but this has not been confirmed (Ferguson and Watkins, 1989; Kullaj, 2016). It is therefore surprising that treatment with zinc sulfate has been observed to increase bitter pit incidence and decrease fruit Ca, depending on the timing of application (Bangerth, 1973). Fallahi (2020) determined Sysstem-CAL, a mineral mixture containing Ca, copper, and phosphite, was more effective in mitigating bitter pit than Ca alone.

Manganese has also been positively associated with fruit Ca and lower bitter pit incidence (Fazio et al., 2015; Bonomelli et al., 2020). Manganese may help mitigate bitter pit by moderating K influx, but this has not been confirmed. Like other minerals, manganese may be positively associated with fruit Ca not due to a causal relationship but because these minerals are transported into fruit under the same conditions as Ca, as they are also primarily xylem-mobile (Garman and Mathis, 1956). Sodium has also been associated with xylem function, and therefore fruit Ca (Raven, 1977). While iron has been positively correlated with fruit Ca (Fazio et al., 2015), it has also been positively correlated with bitter pit (Bonomelli et al., 2020). Because iron concentration in fruit decreases as fruit mature, it is possible that high iron concentrations indicate fruit were harvested too early, increasing their susceptibility to bitter pit (Garman and Mathis, 1956). Mineral studies outside of Ca, K, and Mg will be helpful in illuminating not only the roles of other minerals in bitter pit susceptibility but also the conditions which favor and disfavor other mineral accumulations alongside Ca deficiency.

2.3. THE ROLE AND FUNCTION OF XYLEM

2.3.1. Vascular Anatomy

Vascular tissue *in planta* is composed of two distinct vascular tissues, xylem and phloem. Xylem, in addition to providing structural support to the plant, stores and transports water, nutrients, and phytohormones, while phloem transports photosynthates, proteins, and mRNAs (Ye, 2002). Xylem is composed of both non-conductive cells, including xylary parenchyma cells and fibers, and conductive tracheary and vessel elements. Phloem also contains non-conductive parenchyma cells and fibers and conductive elements which are referred to as sieve tubes. In angiosperms, both tracheary elements and sieve tubes form continuous hollow columns for water transport. However, sieve tubes in phloem are composed of living cells, while tracheary elements in xylem are created from dead cells.

Within apple fruit, xylem and phloem appear in parallel within vascular bundles which run roughly in parallel to each other from the stem (proximal) end of the fruit to the calyx (distal) end. These vascular bundles can be divided into two groups based on their location and purpose: cortical and carpellary (McAlpine, 1912). The cortical or primary bundles occur on the core line surrounding the carpellary tissue and appear at the rate of two per carpal. Typically, apple fruit contain five carpels and therefore ten primary bundles. However, in instances where fruit contain four, six, or even seven carpals, eight, twelve, or fourteen primary bundles develop, respectively (McAlpine, 1912; Griffith, unpublished). These primary bundles supply nutrients to the cortical (flesh) tissue (MacDaniels, 1940). The carpellary group of bundles are composed of dorsal bundles and ventral bundles, occurring at a rate of one and two per carpal, respectively. Dorsal bundles supply the deepest portions of the cortical tissue while ventral bundles supply nutrients to growing seeds (Dražeta et al., 2004). Finally, the secondary vasculature is composed of very fine strands which branch off of the primary bundles to supply the outer cortex of the fruit. Among cultivars, a great deal of variation in the size, number, and pattern of these strands has been observed (Kraus, 1916). Among the fruits studied by Song et al. (2018) (loquat, apple, pear, Indian jujube, longan, litchi, grape, and citrus), apple was found to have the lowest development of secondary xylem and the poorest overall xylem functionality.

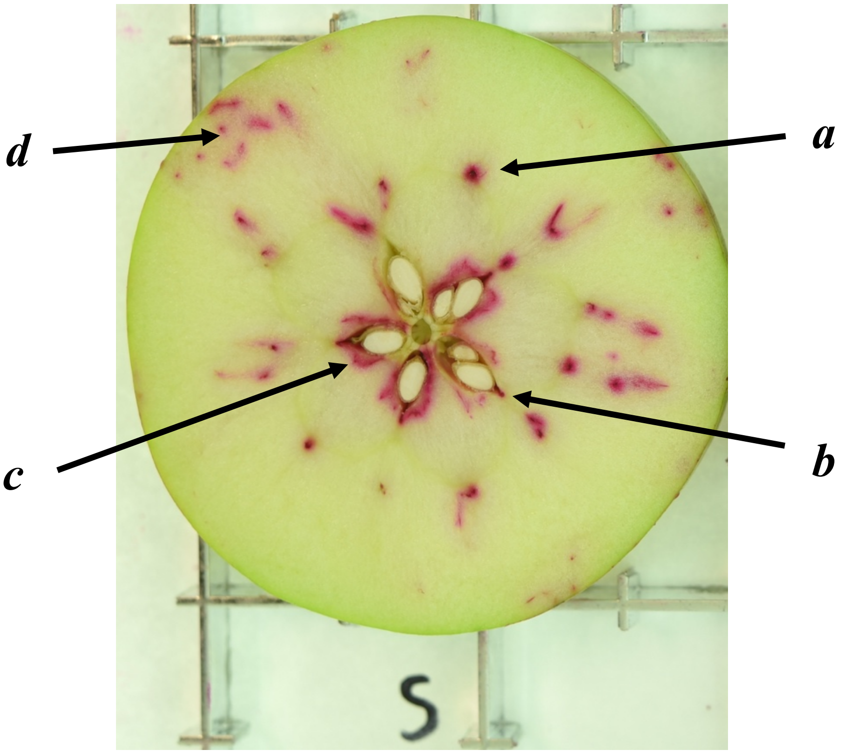

*Figure 2: 'Honeycrisp' fruit at 86 DAFB. The symbols correspond to **a**, primary bundle; **b**, dorsal bundle; **c**, ventral bundle; and **d**, secondary vasculature.*

2.3.2. Xylem Differentiation and Lignification

Xylem differentiates from meristematic cells in two phases: primary and secondary growth (Ye, 2002). When vascular tissues initially develop in the primary growth phase, xylem differentiates from procambium tissue. When plant tissues reinforce through the creation of secondary xylem (wood) in the secondary growth phase, xylem instead differentiates from vascular cambium initials. The formation of procambial cells is induced via auxin, which is transported polarly from apical meristems and developing leaves. The directional flow of auxin leads to the canalization wherein a positive feedback loop forms as auxin accumulation enhances auxin transport, which in turn facilitates auxin accumulation (Yoshimoto et al., 2016). This process determines the spatial pattern of vascular differentiation within plant tissues, leading to the mesh-like branching pattern within apple fruit. Cytokinins are also known to have a localized proxylem effect, likely due to promotion of procambial cell division (Aloni, 1987). The role of gibberellins, ethylene, and brassinosteroids in xylogenesis is less understood (Aloni, 1987; Savidge, 1996; Pesquet et al., 2005).

Following elongation, tracheary elements undergo secondary cell wall deposition, whereby the elements thicken to provide greater structural support to vessels to withstand the negative pressure generated through transpiration (Ye, 2002). The *NAC* family of transcription factors have been determined to be the regulators of xylem cell fate and secondary cell wall deposition (Johnsson et al., 2018). Gibberellins induce the expression of these transcription factors, lignifying xylem, while treatment with auxins cause these tissues to remain unlignified and flexible. While the lignification process strengthens vessels against tension, the resulting brittleness render elements more prone to dysfunction through shearing.

2.3.3. Xylem Dysfunction

Unlike phloem, xylem is observed to become gradually dysfunctional during the fruit growth period in apple (Lang, 1990) but also in *Vitis vinifera* (grape) (Düring et al., 1987; Findlay et al., 1987), *Actinidia deliciosa* (kiwifruit) (Dichio et al., 2003), *Prunus avium* L. (sweet cherry) (Grimm et al., 2017), *Prunus domestica* (European plum) (Winkler and Knoche, 2021), and *Solanum lycopersicum* (tomato) (Ho et al., 1987). Thus, water delivery to fruit is limited to phloem transport as development advances. Because functional xylem tissue is required for Ca transport into and throughout fruit, Ca concentrations become diluted with growth. In contrast, phloem-mobile ions such as Mg and K continue to accumulate in tissues and cell walls, which rely on Ca for cell integrity (Jones et al., 1983; Cheng and Sazo, 2018). Xylem tissue becomes dysfunctional in stages; fine, secondary vasculature nearest the fruit surface is the earliest to lose functionality followed by the ventral, dorsal, and primary bundles located beside the carpels, at the tips of the carpels, and on the cortex line, respectively (Dražeta et al., 2004). The rate of dysfunction of primary bundles exceeds that of dorsal bundles. Interestingly, xylem function in the pedicel was retained throughout the entire growth period implicating xylem tissue in the fruit, rather than the pedicel, as the weak link to transport (Dražeta, 2003).

The rate of xylem dysfunction is not constant among cultivars. Notably, the relative susceptibilities of various cultivars to bitter pit have been connected to the onset and rate of xylem dysfunction (Lang, 1990). The rate at which primary bundles become dysfunctional varies more widely among cultivars than either dorsal or ventral bundles, with primary bundles typically becoming completely dysfunctional at maturity (Dražeta et al., 2004). Dysfunction of primary bundles is problematic for rapidly growing fruit, as primary bundles supply nutrients to the flesh (MacDaniels, 1940). The primary bundles of bitter pit sensitive cultivars such as 'York Imperial' (Barden and Thompson, 1963), 'Braeburn' (Dražeta et al., 2004), 'Catarina' (Amarante et al., 2013), and 'Honeycrisp' (Griffith et al., unpublished) become dysfunctional significantly earlier than resistant cultivars. Primary bundles became mostly dysfunctional by 40 days after full bloom (DAFB) in the highly susceptible 'Catarina' compared to 100 DAFB in the less susceptible 'Fuji' (Amarante et al., 2013).

Regardless of the timing of dysfunction, primary bundles become dysfunctional following an exponential decay model. Consequently, nearly all Ca uptake in apple fruit occurs early in the growing season (Saure, 2005). In fact, under periods of stress, xylem dysfunction may be a programmed action to limit the backflow of water and solutes from fruit to tree (Dražeta, 2003; Winkler and Knoche, 2021). Loss of xylem functionality has been attributed to a variety of different perturbations in cells, including an increase and/or elongation of parenchyma cells which constrict xylem elements (Lang and Ryan, 1994), cessation of cambial activity by which xylem cells are generated (Dražeta, 2003), or simple stretch-induced dysfunction of xylem tissue (Findlay et al., 1987).

These suggestions may fail to account for the persistence of phloem function throughout the season as xylem becomes progressively dysfunctional. Dražeta suggests three potential pathways by which the phloem may retain its functionality:

1. Greater flexibility of sieve tube elements in phloem compared to tracheary elements in xylem (Lee, 1981)
2. The existence of a "continuous symplastic pathway through parenchyma cells that occupy the voids between the broken structures"
3. The potential for sieve tubes to be constantly differentiated to replace broken elements over the course of the season

The latter of these suggestions is possible because phloem can differentiate without the presence of xylem (the inverse is not true) and can differentiate at lower auxin levels than xylem (Aloni, 1980).

2.4. RELEVANCE OF PLANT GROWTH REGULATORS TO BITTER PIT

2.4.1. Roles of Auxin in Xylem Differentiation and Calcium Transport

Indole-3-acetic acid (IAA), the native auxin, is produced in leaf primordia, young leaves, and apple seeds alongside the intermediate indole-3-butyric acid (IBA) (Luckwill, 1953; Van der Krieken et al., 1993). Auxins produced in fruit seeds are exported basipetally from the fruit. While moving outward, auxin begins a canalization process by which an accumulation of auxin causes more auxin to accumulate in a positive feedback loop (Yoshimoto et al., 2016). The channels created for auxin transport become larger, eventually resulting in the observable vasculature in apple fruit. The initiation of xylem from meristematic procambial or cambial cells is mediated by auxins (Yoshimoto et al., 2016; Johnsson et al., 2018).

IAA has been linked to Ca levels within fruit (Cutting and Bower, 1989; Sorce et al., 2011). Sorce et al. (2011) found free IAA decreases from pollination to 70 DAFB until nearly all IAA becomes tied up in ester form. This free IAA could be the initiator of xylem maintenance through the initiation of procambial cells (Miqueloto et al., 2014). Overall fruit auxin levels could help explain why xylem vessels become dysfunctional as fruit grow larger while phloem retains its functionality as phloem differentiates separately from xylem and its differentiation requires lower auxin levels (Aloni, 1980; Aloni and Barnett, 1996; Dražeta, 2003).

Van Stuivenberg (1950) believed bitter pit resulted from B deficiency and therefore sprayed ‘Notaris’ apple trees with both B and IAA, the production of which he connected to sufficient B levels within fruit. Van Stuivenberg found midseason (late June—mid July) sprays of either 0.5% borax or 20-30 ppm IAA to have a dramatic effect on bitter pit, reducing it from 34-42% in the control to 4% in the borax treatment and 7% in the case of 20 ppm IAA. Because of the tremendous promise of these results, Mulder (1951) attempted to replicate the study the following year but could not achieve the same results. However, Mulder acknowledges that the bitter pit incidence in the control was dramatically lower in the 1950 season (just 5-7%), leading him to wonder if any treatments could have positive effects when natural incidence is so low. Mulder also suggested differences in the regions, such as weather and soil, may have affected the efficacy of these treatments.

Curiously, Mulder replicated Van Stuivenberg’s experiments with the synthetic auxin, NAA, rather than the native auxin used in the original study. In fact, the treatment which produced the highest bitter pit incidence was an NAA treatment, increasing bitter pit to 22%. Because NAA is synthetic, optimal concentrations for uses such as root formation are lower when using NAA compared to IBA or IAA, both native auxins. De Klerk et al. (1997) found the optimal concentrations for these three compounds to be 3 μM, 10 μM, and 30 μM, respectively, for adventitious root formation; above these concentrations, inhibition occurs. Perhaps by replicating Van Stuivenberg's experiment in a different region, in a year with dramatically lower bitter pit, and replacing the primary spray agent with a potentially toxic synthetic replacement, Mulder would have found it impossible to confirm Van Stuivenberg's results. Mulder's inability to confirm these findings prevented the adoption of auxin sprays to combat bitter pit.

Auxin, in the form of the synthetic NAA, is currently used in apple cultivation to manage crop load and fruit size through thinning, promotion of return bloom, and prevention of pre-harvest fruit drop and is sold under the product names Fruitone L® (Valent BioSciences U.S.A. LLC, Libertyville, IL) and PoMaxa® (Valent BioSciences U.S.A. LLC).

2.4.2. Gibberellins Increase Bitter Pit

While auxins have been shown to differentiate xylem and impart flexiblility on existing xylem, GAs have been shown to lignify xylem, causing xylem to become more prone to dysfunction (Johnsson et al., 2018). GAs reach their highest concentration at 63 DAFB in the apple cultivars studied by Luckwill et al. (1969). This period corresponds to the stage of fastest apple fruit xylem dysfunction (Dražeta et al., 2004). If bitter pit is understood to result from xylem dysfunction and a corresponding limit of Ca transport, high levels of biologically active GAs may be an exacerbating factor to bitter pit incidence.

Though at the time unaware of the role of GAs in xylem dysfunction, in 1996 Saure hypothesized that bitter pit was primarily a result of supraoptimal levels of GAs and GA-like compounds (GALCs). Saure noted research which found fruit Ca levels to be poorly correlated with bitter pit incidence within single fruit samples, even if fruit Ca appeared to be a good indicator of bitter pit incidence of bulk fruit samples (Fallahi et al., 1988, Marcelle 1990). Saure's fundamental objection to the designation of Ca deficiency as the causal factor of bitter pit incidence was that bitter pit is not guaranteed even in fruit with extremely low Ca concentrations (Stahly and Benson, 1976; Vang-Petersen, 1980; Perring, 1986). This paradox had led other researchers to the conclusion that bitter pit may not be principally caused by Ca deficiency (Shear, 1972; Drake et al., 1974; Marini and Barden, 1982; Faust, 1989). Saure synthesized these findings with other observations of bitter pit incidence to infer that bitter pit may be principally caused by an excess of GA activity, with Ca deficiency as a symptom of that excess.

Saure observed that both vigorous root growth (Richards and Rowe, 1977) and vigorous shoot growth (Saure, 1981, 1992) were correlated with increased bitter pit susceptibility of fruit, and later work by Saure (2005) confirmed the effect vigorous vegetative growth has on reducing Ca translocation to fruit. Saure postulate that root growth, which was causal of bitter pit given the dependence on shoot growth (Richards and Rowe, 1977; Kramer and Kozlowski, 1979). Saure referenced the view of Brooks and Fisher (1918) who stated that "large apples are bitter pit susceptible not because they are large but because of the conditions which made them large." Saure believed these conditions to result from an excess of GA produced by vigorously growing roots (Torrey, 1976) as well as excessive pruning (Grochowska et al., 1984).

Saure believed GAs caused bitter pit by interfering with Ca translocation to fruit (Bangerth, 1973; Bramlage et al., 1990), exacerbating Ca deficiency symptoms while increasing demand for Ca (Leh, 1963; Granger and Looney, 1983), and increasing permeability of cell membranes, causing them to lose their selectivity and making them more susceptible to water stress (Jones, 1973). Additionally, applications of GA to young apple fruit have been shown to induce parthenocarpy, and parthenocarpic fruit have been found to be Ca deficient (Bramlage et al., 1990; Buccheri and Di Vaio, 2004). Paclobutrazol (Luo et al., 1989) and daminozide (Tsur and Gutmacher, 1988) inhibit the synthesis or action of GA, and have been found to decrease the K/Ca ratio in fruit and reduce bitter pit (Martin et al., 1968; Naumann, 1971; Greene, 1986; Sharples and Johnson, 1986). Ethephon, a precursor of ethylene which also antagonizes GA, has also been found to reduce bitter pit, but it has not been shown to affect fruit Ca levels (Prinja, 1990). Ethylene and ABA, another GA antagonist, increase in concentration as fruit mature, potentially explaining why fruit which are picked early are more likely to be affected by bitter pit in storage (McGlasson et al., 1978; Brady, 1987; Ferguson and Watkins, 1989). Finally, Saure observed that russeted fruits rarely developed bitter pit (Hilkenbaumer and Reinken, 1959) and russeted clones of apple cultivars contained fewer GALCs than normal clones (Eccher, 1978), again suggesting GALCs may be to blame for bitter pit incidence.

Successive research in tomato corroborate Saure’s conclusions. GAs in tomato have been shown to increase the expression of genes coding for Ca transporters which sequester Ca into the vacuole (de Freitas et al., 2010; Falchi et al., 2017). Because vacuolar Ca is relatively immobile, overexpression of these genes results in a decrease of the apoplastic Ca used to reinforce cells and prevent their collapse (Conn et al., 2011; Hocking et al., 2016; Falchi et al., 2017; Paiva, 2019). In tomato, treatments of plants with GAs increased the incidence of blossom end rot by 35% and caused cell membranes to leak (de Freitas and Mitcham, 2012). GAs in tomato had the effect of increasing the expression of CAX and Ca-ATPase genes, decreasing concentrations of apoplastic Ca (Hocking et al., 2016). Repeated treatments of plants with GAs increased the BER incidence to 100%. However, treating tomato plants with prohexadione calcium, a GA biosynthesis inhibitor, eliminated blossom end rot (de Freitas et al. 2011c). Donahue et al. (2018) found applications of prohexadione calcium, sold under the trade name Apogee®, reduced vegetative growth and significantly reduced bitter pit when applied at the full pink stage of development but significantly increased bitter pit when applied at petal fall timings or later; these results seem contradictory to the GA associated mechanisms described previously.

GAs (as $GA_4 + GA_7$) are currently used in apple cultivation in combination with 6-benzyladenine (6-BA) to mitigate fruit drop following frost events, to improve fruit shape, to control russeting, and to promote branching in young trees under the product name Promalin® (Valent BioSciences U.S.A. LLC).

2.4.3. Abscisic Acid, Transpiration, and Calcium

ABA is produced from mevalonic acid in roots and mature leaves and, like auxin, also in growing seeds (Davies, 2010). The transition in fruit from growth via cell division to growth via cell enlargement is linked to an increase in seed ABA concentrations (Finkelstein, 2013). ABA accumulation in seeds occurs in two stages with the first accumulation resulting from import of maternal ABA from leaves and roots and the second accumulation the result of ABA synthesized by the seed as fruit near maturity, allowing for the induction of dormancy (Karssen, et al., 1983). A primary function of ABA is regulation of transpiration. Because leaf tissue transpires at higher rates than fruit tissue, Ca is normally preferentially delivered to leaves rather than fruit (de Freitas et al., 2011). Apple cultivars which experience higher leaf transpiration relative to fruit transpiration are more likely to suffer from Ca deficiency, especially in the calyx end of fruit, and higher (K+Mg)/Ca ratios (Gomez and Kalcsits, 2020). Application of ABA triggers stomatal closure, resulting in a closing of the transpirational gap between fruit and leaf tissues. As a result, there is a higher uptake of Ca by fruit tissue and decreased uptake by leaves (Hocking et al., 2016). De Freitas et al. (2013) found nightly export of water and therefore Ca, from tomato fruit not treated with ABA. Leaves from ABA-treated tomato plants were observed to accumulate half the Ca of control plants, and fruit from treated plants contained ten times the Ca of control fruit (de Freitas et al., 2011b). Similarly, Falchi et al. (2017) found spraying apple fruit with ABA increased Ca allocation in fruit and specifically increased the Ca concentration in the apoplast. This finding is significant as low levels of apoplastic Ca have been observed consistently in fruit affected with Ca deficiency disorder symptoms. Preliminary research by Angmo et al. (2022) recently showed whole-tree ABA sprays increased Ca concentration most in the calyx of fruit, where bitter pit tends to initiate.

Dipping growing tomato fruit in an ABA solution early in the growing season eliminated blossom end rot and also increased fruit growth rate and sugar content in fruit (de Freitas et al., 2013). ABA regulates several genes governing Ca -portioning across membranes, often in opposition to GAs (McGlasson et al., 1978; Brady, 1987; Dodd et al., 2010; Falchi et al., 2017). In *Vitis vinifera*, ABA was found to cause faster maturation of fruit because of its role in upregulating genes coding for hexose transporters (Murcia et al., 2016). As a water-stress hormone, ABA is also known to decrease the resistance of roots to water uptake, another mode of action of increasing Ca uptake (Bangerth, 1979). ABA also inhibits shoot growth, allowing for more Ca to end up in fruit rather than vegetative tissue (Davies, 2010). More direct evidence of a regulatory role of ABA on vascular transport has been demonstrated as an increase in the number of functional vascular bundles in the distal end of both tomato and apple fruit, potentially allowing for the uptake of more Ca (de Freitas et al., 2011; Angmo et al., 2022). The mechanism by which ABA increases xylem functionality is poorly understood. Similar to many other functions of ABA, this activity may result from antagonism of the action of GAs as these phytohormones frequently act in opposition to one another (Saure, 1998). The relationship between GAs and ABA will require future research to be better understood. More research is also necessary to determine the timing of ABA production by seeds in apple fruit, as this timing might be ideal for exogenous ABA applications.

ABA is currently used in apple cultivation as a thinning agent in the form of ProTone®, a product also labeled for use as a defoliant, as a coloring agent in grape, and as a bud break suppression agent in coffee.

2.4.4. Balancing of Cytokinins

Cytokinins are similar to GAs in that they are synthesized in the roots and transported throughout the plant where they encourage growth (Torrey, 1976). Because cytokinins encourage vegetative growth and increase Ca translocation to shoots, treatment with cytokinin results in less Ca translocation to fruit, greater K/Ca ratios, and increased incidence of Ca deficiency disorders (Richards and Rowe, 1977; Saure, 1996; de Freitas and Mitcham, 2012). However, like auxins, cytokinins are also produced in seeds and promote xylem differentiation, allowing for Ca transport within fruit (Aloni, 1987; Pesquet et al., 2005). Excessive application of cytokinins to plants may result in increased Ca deficiency symptoms in fruit, but cytokinin is necessary for Ca transport.

6-benzyladenine, a cytokinin produced under the name MaxCel (Valent BioSciences, LLC), is currently labeled for use in apple production to enhance fruit size, as a thinning agent, and to promote branching of young trees.

2.4.5. Stress Hormones

Ca functions as a second messenger in response to stress (Verma et al., 2016). Because bitter pit is linked to Ca deficiency, genes responsible for stress regulation have been studied for their role in Ca deficiency disorders (Mao et al., 2021). In addition to ABA and cytokinins, other stress hormones *in-planta* include ethylene, jasmonates, and salicylic acid.

Ripening of fruit is governed via the action of ethylene. Early bitter pit research determined bitter pit lesions to be areas of accelerated ripening with an increase of localized ethylene serving as one of the first indicators of bitter pit lesion development, (Faust and Shear, 1969) and later research confirmed fruit with lower Ca produced more ethylene (Ferguson, 1984). A negative relationship between the responses of Ca and ethylene has been suggested (de Freitas and Mitcham, 2012). Ethylene increases cellular respiration and membrane permeability through translocation of the enzyme phospholipase D (Aghdam, 2012). However, bitter pit resistance has been correlated to increased internal ethylene (Marini et al., 2020). This correlation may be the result of ethylene's antagonism of GA (Pearce et al., 1991). The timing of the action of ethylene appears to be important. Ethylene decreased bitter pit incidence when applied to fruit before harvest (Schumacher and Fankhauser, 1972; Pfammater and Dessimoz, 1974) and increased bitter pit incidence when applied to harvested fruit (Lötze et al., 2010).

Jasmonates are involved with callus tissue formation, seed germination, flowering, primary root growth, tissue senescence, and aid in prevention of damage from various stresses (Aslam et al., 2021). Through the influence of jasmonates, plant cells respond to abiotic stress by increasing cytoplasmic Ca via efflux of Ca stored in the vacuole. Because vacuolar Ca is not useful in preventing Ca deficiency disorders, jasmonates might play a role in bitter pit prevention. However, methyl jasmonate was found to increase bitter pit and calyx cracking in 'Fuji' apple fruit, especially in late season applications (Rudell et al. 2005). Because Ca, Mg, and K levels were not significantly affected by the use of methyl jasmonate, the hormone may play a different, unclear role in bitter pit.

Treatment with methyl jasmonate has been observed to increase ABA concentration in apple fruit, but not sweet cherry (Kondo et al., 2000). Methyl jasmonate has also been found to increase titratable acidity and delay starch degradation in 'Fuji' (Ozturk et al., 2015). N-propyl dihydrojasmonate (PDJ), a JA derivative, is used in Japan to improve quality and reddening of apple (Kondo, 2010).

Salicylic acid is involved with plant growth, ion uptake and transport, photosynthetic rate, membrane permeability, and transpiration—nearly all subjects of interest in bitter pit resistance (Kazemi, 2014). Ca deficiency is connected to stress resistance *in-planta*, and salicylic acid has been found to improve stress response (de Freitas and Mitcham, 2012). Kazemi (2014) found salicylic acid was effective in increasing both vegetative and fruit growth in tomato while reducing blossom end rot. A combination of methyl jasmonate and salicylic acid reduced blossom end rot in tomato while increasing fruit size, yield, soluble solids, acidity, and ascorbic acid. Salicylic acid's role in Ca deficiency disorders like bitter pit and blossom end rot may be because of the reduction in ethylene and an inhibition of cell wall membrane degrading enzymes. Because of these effects, dilute whole-tree salicylic acid sprays were found to reduce softening in 'Red Delicious' apples in storage (Hadian-Deljou et al., 2017). While salicylic acid improves the various conditions tangential to bitter pit development, salicylic acid has not yet been studied directly for the reduction of bitter pit.

2.5. OTHER FACTORS TO BITTER PIT INCIDENCE

2.5.1. Seed Status

Seeds are critical to the development of healthy fruit as sources of hormone production and macronutrient sinks. In apple, the presence of at least two full seeds was required for an increase in fruit Ca, and seed count was a good predictor of bitter pit within trees (Brookfield et al., 1996;

Broom et al., 1998). Flat, non-developed seeds, likely due to aborting embryos, were negatively correlated with fruit Ca (Brookfield et al., 1996). Buccheri and Di Vaio (2004) found parthenocarpic fruit had lower levels of fruit Ca and increased asynchrony (i.e., misshapenness increased with decreasing seed count). Increasing seed count resulted in firmer fruit with improved storage characteristics. Increasing seed count may also affect pulp acidity due to its role in increased fruit Ca, but this relationship has only been established definitively in the cultivar ‘Annurca Rossa del Sud’ (Buccheri and Di Vaio, 2004). However, seed number did not influence the N, K, or Mg content of fruit tissue. Seed count at harvest varies between cultivars. A definitive link between the typical mature seed count of cultivars at harvest and their bitter pit susceptibility has not been established.

There is a genetic component to seed content, but the number of fully developed, fertilized seeds in harvested fruit depends largely on factors related to pollinator and pollinizer. The effectiveness of pollinator (generally honeybee) activity depends mainly on density per unit land area, temperature and light conditions. The flowering time of pollinizers (cultivars or species) needs to overlap with the key cultivar and have compatible alleles to facilitate pollination (Buccheri and Di Vaio, 2004). Simply planting compatible trees more closely together significantly increases seed count, though this is due to pollinator activity since wind dissemination of apple pollen is purportedly minimum (Westwood, 1993). Likewise, increasing the distance to pollinizers resulted in fruit with decreased seed count, increased misshapenness, and lower Ca, as well as higher levels of bitter pit and lenticel blotch, a similar postharvest disorder.

2.5.2. Vegetative Effects

Because Ca is exclusively xylem mobile, fruit must rely on transpiration for uptake of Ca. However, because leaves transpire at a greater rate than fruit, significantly more Ca is taken up by leaves than fruit. In fact, studies have shown the partitioning to be 85% of Ca in new growth ending up in leaves while only 13-14% ends up in fruit (Warner, 2014). Warner speculates that in regions with higher evapotranspirative demand, the difference could be even greater. Carne (1927) hypothesized that bitter pit was initiated by excessive transpiration, but Smock (1941) was among the first to suggest transpirational disparity between leaves and growing fruit as the primary driver of bitter pit. Smock described these differences in terms of osmotic values, noting the osmotic potential of leaves was significantly higher than fruit. In periods of extreme water stress, water, and therefore Ca, was moved from fruit to leaves. Ca allocated to leaves remains in leaves until harvest and is not reallocated to fruit (Mulder, 1951). Malone et al. (2002) found xylem flow could be reversed through heating of leaves, however, allowing leaf Ca to be returned to fruit. Smock further proved his theory of bitter pit relating to osmotic differences in fruit and leaves by ringing branches. By disrupting nutrient flow to and from the leaves, larger leaves were promoted, and bitter pit was promoted (Mulder, 1951). Smock also induced bitter pit by ringing the pedicel of growing fruit, further lowering their osmotic potential.

The effects of osmotic potential are also clear in shaded fruit versus fruit growing in full sunshine. Shading has been found to increase bitter pit by lowering the osmotic value in both leaves and fruit and increasing fruit size (and therefore Ca dilution) by reducing evaporation from fruit (Mulder, 1951). Many successive studies have confirmed shaded fruit are more likely to develop bitter pit despite containing increased Ca concentrations (Wallace and Jones, 1941; Bangerth, 1973; Witney et al., 1991; de Freitas et al., 2013; Kalcsits, 2019), though Wallace and

Jones attributed the negative effects of shading to B deficiency, as B transport is influenced by light (Van Stuivenberg, 1950).

Vegetative shoots also compete with growing fruit for Ca. Therefore, control of vegetative growth in fruit-bearing trees is important in bitter pit prevention (Cutting and Bower, 1989; Buti et al., 2018). N fertilization must be carefully considered. If N is applied early in the growing season, the growth of the entire tree is supported, including reproductive tissue (Warner, 2014). However, applications from May onward support the growth of vegetative shoots almost exclusively (Baugher et al. 2017). Similarly, excessive shoot growth, and therefore, bitter pit, occurs under condition of excessive irrigation (Cheng and Sazo, 2018). Pinching off apical meristems at 24 DAFB reduced BP, but pinching earlier had no effect (Donahue et al., 2018). Donahue et al. (2018) also found that prohexadione calcium applied at the pink bloom stage reduced shoot extension and also reduced bitter pit incidence. Shoots may also play a supportive role in bitter pit incidence as potent sources of gibberellins. Saure (2005) found that while vigorous vegetative shoot growth is indeed correlated with lower fruit Ca, larger bourse shoots on spurs increased the Ca content of adjacent fruit.

2.5.3. Soil Conditions

Typically, the total Ca in fruit tissues originates in the soil before being taken up by the roots. As early as 1912, McAlpine noted that fruit grown in “calcareous soils” were more often than not pit-free. Ca is most readily available to roots when soil pH is between 6.5 and 7. In more acidic soils, the addition of lime has been shown to reduce bitter pit by up to 75% (Wang et al., 2005). While the addition of Ca as lime to soils led to a nonstatistical increase in fruit Ca, the benefit of liming soil is likely via an effect on pH (Ferguson and Watkins, 1989; de Freitas and Mitcham, 2012). Indeed, the amount of Ca in most soils is more than sufficient for the needs of apple trees

(Bangerth, 1979). Bitter pit can, however, be forced by growing trees in an extremely low Ca soil (Ferguson and Watkins, 1989). As previously mentioned, soil K and Mg levels are also important to bitter pit incidence; an excess of these minerals can exacerbate symptoms (Cheng and Sazo, 2018). Soils deficient in B can compound the effects of Ca deficiency as well (Garman and Mathis, 1956), but soils with supraoptimal B levels can be deleterious to fruit development (Peryea, 2004).

Soil texture and water holding capacity can also affect fruit Ca uptake indirectly. While dry soils inhibit the ability of roots to capture Ca, excessively wet soils promote the uptake of too much K, limiting the amount of Ca in fruit tissue (Cheng and Sazo, 2018). Excessively wet soils can also increase bitter pit by promoting the growth of vigorous vegetative shoots which compete with fruits for Ca and/or encourage the growth of large fruits which contain less Ca per gram than small fruits because of dilution (Buti et al., 2018; Cheng and Sazo, 2018). For these reasons, trees grown on sand (without regulated irrigation) and heavy clay may be prone to bitter pit.

2.5.4. Rootstock Effects

Just as fruit of different cultivars range in levels of susceptibility to bitter pit, the rootstocks chosen for cultivation each confer a different level of susceptibility to scions. In the absence of foliar Ca, Ca in the fruit must first be taken up from the soil by the roots. Each rootstock has a different capacity for mineral exchange, and each has varying compatibilities with different cultivars. In the case of ‘Honeycrisp’, CG.6976, CG.4002, G.16, G.214, and M.7 delivered more Ca than average to fruit while fruit Ca was lower for fruit grown on trees with CG.4013, M9Nic29, and G.11 rootstocks (Fazio et al., 2015). Fazio et al. also found fruit Ca concentrations to be closely correlated with fruit iron and B levels. Fruit Mg levels were found to be highest for fruit grown on G.210 rootstock and lowest for fruit grown on G.969 and B.9. Fruit K was also found to be highest in fruit grown on G.210 and lowest in fruit grown on G.935 and C.G. 5087. Because bitter pit can be described as a result as a low (K+Mg):Ca ratio, the ideal rootstock for preventing bitter pit could be a rootstock which maximizes Ca uptake while minimizing K and Mg. Indeed, Donahue et al. (2021) described a significantly lower bitter pit incidence in fruit grown on B.9, a rootstock which is poor at uptake of Mg, compared to M.26.

Rootstocks also affect bitter pit incidence in their impact on overall tree vigor. Rootstock selection varies based on the cultivar, soil conditions, and climate as each impact vigor. Vigorous shoots create competition for developing fruit for the limited Ca taken up by roots (Buti et al., 2018). McAlpine (1912) noted that fruit grown on 'Northern Spy' rootstock were more susceptible to bitter pit because of the vigor conferred by the roots. B.9 is widely regarded as the best overall performing rootstock on bitter pit incidence (Phil Schwallier, personal communication).

Finally, rootstocks impact the length of the growing period of apple fruit. Mulder (1951) refers to unpublished work by a Dr. T. Van Hiele, that demonstrated an effect of rootstock selection on bitter pit of Cox's Orange Pippin fruit. Van Hiele found that the rootstocks which led to shorter developmental periods and earlier ripening fruit exacerbated bitter pit compared to rootstocks which promoted a longer growing season. While rootstocks that conferred a longer developmental period caused some bitter pit to become visible while on the tree, this effect was drastically less than the effect that season-shortening rootstocks had on bitter pit incidence during storage.

2.5.5. Storage Conditions

Bitter pit was originally recognized as a postharvest/storage disorder as the majority of affected fruit develop visible symptoms while in storage (Sorauer, 1879). Bitter pit incidence increases in storage up to two months after harvest (Telias et al., 2006). Smock (1941) found bitter pit incidence was greater in storage conditions favoring high transpiration, such as low humidity, high temperature, and free air movement. Higher fruit Ca levels help maintain the quality of stored fruit such as firmness, and the first applications of Ca to apple fruit were to improve storage characteristics (Buccheri and Di Vaio, 2004; Ferguson and Watkins, 1989). Mg, however, has been found to be detrimental to the quality of stored fruit (Marcelle, 1995).

The effect of storage conditions on the appearance of bitter pit lesions is in part because ions continue to move throughout fruit even after harvest. While in storage, Ca is gradually transferred from the core of the fruit into the cortical tissue and peel (Perring and Pearson, 1986; Perring and Pearson, 1987). This movement of Ca into the cortical tissue during storage could be beneficial as bitter pit develops in the outer cortex, but Mg and K increase in these tissues as well (Ferguson and Watkins, 1983). The movement of these minerals in storage is driven by transpiration. Therefore, rapidly transferring fruit to low temperature and high humidity storage reduced bitter pit (Faust and Shear, 1968). However, lenticel spot, a similar condition, is exacerbated by high humidity in storage (Richmond and Dewey 1969). Smock (1941) found that dipping apples in wax after harvest and prior to storage reduced transpiration and thereby bitter pit.

2.5.6. Climate and Weather

Bitter pit information related to climatic variables and/or weather is incomplete and often paradoxical. Climate and weather likely contribute to bitter pit incidence due to the extremely variable incidence of bitter pit from year to year (Jemrić et al., 2016). Leaves transpire at markedly higher rates than fruit, resulting in more Ca delivery to foliage than fruit (de Freitas et al., 2011). Indeed, hot and dry weather has been shown to exacerbate bitter pit (Biggs and Peck, 2015). Under particularly dry, and potentially stressful, conditions, Ca has been shown to move out of fruit along water potential gradients in the tree (Wilkinson, 1968). Saure (2005) found low soil temperature to be correlated with increased Ca in both fruit and leaves. Soil salinity, root pruning, root restriction, low soil N, and lower average amounts of water all resulted in increased Ca as well, but these factors could also be attributed to reduced vegetative growth. Because a difference in transpiration rates between leaves and fruit is responsible for tissue differences in Ca concentrations, conditions which reduce transpiration increase the relative amount of Ca in fruit, conditions such as lower temperatures and higher humidity (Ferguson and Watkins, 1989). There is reason to believe weather has variable effects on bitter pit incidence relative to phenology. Because of more rapid fruit growth in the cell expansion rather than cell division phase, hot, dry weather in July and August has been linked to increased bitter pit incidence because of high fruit demand for Ca (Ferguson and Watkins, 1989). Because of the high demand for Ca during this phase of growth, foliar Ca sprays are recommended in the late summer. However, calcium chloride can become corrosive under hot and dry conditions (Biggs and Peck, 2015; Fallahi, 2020). Ford (1979) found lower daytime temperatures in the early stages of fruit growth reduced Ca concentrations in fruit, mainly by increasing the final fruit weight, but also by slightly reducing Ca input. Finally, very early research indicated that damp and/or foggy conditions in the final weeks before harvest caused lesions to develop rapidly on fruit while still on the tree (Brooks, 1908).

As the effects of weather and climate on bitter pit development are better understood, eventually growers may be able to predict years predisposed to higher bitter pit incidence and deploy a variety of tools to increase fruit Ca while potentially saving money by withholding these measures in years predicted to have low bitter pit occurrence.

CHAPTER 3. The Effect of Fruit, Leaf, and Pedicel Applications of Auxins on Bitter Pit Incidence of Apple Fruit

3.1. INTRODUCTION

While phloem tissue remains functional throughout the seasonal development and growth period of apple fruit, xylem gradually becomes dysfunctional (Lang, 1990). Bitter pit susceptibility of cultivars is linked to the rate of xylem dysfunction in primary bundles of fruit (Lang and Ryan, 1994). Xylem tissue is differentiated through the action of auxin and cytokinin, among other factors (Pesquet et al., 2005); thus, it is plausible that methods to auxin in fruit could prolong the functionality of vascular bundles, resulting in improved Ca transport that ultimately mitigates bitter pit incidence.

Nearly all commercial applications of plant growth regulators (PGRs) target leaves of whole canopies in either dilute or concentrated sprays. Leaf penetration of PGRs requires that the a.i. dissolves into the lipid phase of the cuticle and diffuses across the cuticle, desorbs into the aqueous phase of cell walls of epidermal cells and is taken up in the lipid bilayer of the plasm membrane to facilitate transport via the symplast (Knoche and Petracek, 2014). In the case of nutrient sprays such as Ca, efficacy is related to direct contact and penetration through the cuticle of fruit (Wills et al., 1976). Because auxins have not previously been studied for their effect on bitter pit, the transport and action of auxins on xylem development within fruit is unknown. The basipetal, polar nature of auxin transport from fruit suggests potential need for fruit contact in bitter pit mitigation. Previous studies have showed that auxin produced in apple fruit is exported through the pedicel and helps differentiate pedicel xylem (Dražeta et al., 2004), and auxins applied to the surface of banana fruit infiltrate the peel, establish a gradient, and eventually travel to the fruit center (Vendrell, 1970). TIBA, an auxin transport inhibitor, significantly increases bitter pit incidence in apple when applied as a whole-tree spray (Stahly and Benson, 1970; Bangerth, 1979; Bukovac, unpublished).

The objective of this experiment was to determine the effects of rate and placement of native (IAA) and synthetic (NAA) auxins and TIBA alone and in combination on vascular bundle functionality and bitter pit incidence of ‘Honeycrisp’ apple. A secondary objective tested the effect of IAA applications on ¼ fruit in order to identify localized responses to the factors above. We hypothesized that higher concentrations of auxin would increase vascular functionality and therefore decrease bitter pit incidence in fruit when applied to either the pedicel or the fruit surface, while no effect on fruit would be observed when the compounds were applied to the leaves. We further hypothesized that TIBA, when applied alone, would have the opposite effect, decreasing vascular bundle functionality and increasing bitter pit incidence.

Experiments took place in the same block of ‘Honeycrisp’ trees at Michigan State University’s Clarksville Research Center (CRC) in Clarksville, MI (42.873° latitude, -85.258° longitude).

3.2. MATERIALS AND METHODS

3.2.1. Tree Selection and Preparation for Lanolin Applications

5th leaf ‘Honeycrisp’ trees on Geneva 11 rootstock were selected based on trunk diameter (between 9 and 13 cm, measured 20 cm above the graft union) and bloom uniformity. From these trees, limbs were then selected for uniformity based on limb diameter (between 8 and 12 mm). At full bloom (May 20th, 2020), fruiting spurs were reduced to a maximum of three per limb, and all flowers were removed from excess spurs. Each spur was then thinned to a king bloom fruitlet at 12 mm fruitlet diameter. These methods were imposed to ensure consistency among treatment limbs and to limit effects of high sink strength from reducing bitter pit incidence. Following

June drop, each group of 18 consecutive fruit on trees received a treatment from the following list, and each treatment was replicated 5 times for a total of 1,260 fruit:

Table 1: Treatments used in lanolin paste pedicel applications.

Compound	Rate	TIBA (30 ppm)
Control (No Auxins)	-	No
IAA	1 mg·g^{-1}	No
IAA	5 mg·g^{-1}	No
IAA	10 mg·g^{-1}	No
NAA	1 mg·g^{-1}	No
NAA	5 mg·g^{-1}	No
NAA	10 mg·g^{-1}	No
Control (No Auxins)	-	Yes
IAA	1 mg·g^{-1}	Yes
IAA	5 mg·g^{-1}	Yes
IAA	10 mg·g^{-1}	Yes
NAA	1 mg·g^{-1}	Yes
NAA	5 mg·g^{-1}	Yes
NAA	10 mg·g^{-1}	Yes

3.2.2. Tree Selection and Preparation for Direct Fruit and Leaf Applications

In 2021, the experimental approach was altered. 6th leaf 'Honeycrisp' trees on Geneva 11 rootstock were selected on criteria described above. At full bloom (May 4th, 2021), hand thinning gauges (Equilifruit; Maîtrise de la Fructification - Concepts et Techniques (MAFCOT) group of the Institut National de la Recherche Agronomique (INRA), Montpellier, France) were used to improve crop load consistency among limbs by fixing the number of spurs per limb to roughly 4 per limb cross sectional area, 66% of the value indicated on the hand thinning gauge, to encourage large fruit. At 7 days after full bloom (DAFB), the remaining clusters were hand thinned to the strongest three lateral blooms, and all king blooms were removed due to the high mortality observed in king flowers following episodic spring frost events. In each of the successive weeks, the weakest lateral bloom was removed until only the strongest single lateral remained. At this point, 20 DAFB, 1,400 spurs were divided equally and assigned at random to each of the following seven treatments: 90 spurs received auxin treatments to a quarter of the fruit surface, 90 received auxin treatments to the spur leaves, and an additional 20 received auxin treatments to the entire fruit surface:

Table 2: Treatments applied as aqueous solution to fruit surfaces and spur leaves. Successive applications were made at 30, 45, and 60 DAFB to 5

Compound	Rate
Control	-
IAA	1 ppm
IAA	10 ppm
IAA	100 ppm
NAA	1 ppm
NAA	10 ppm
TIBA	30 ppm

3.2.3. Preparation and Application of Lanolin Treatments

Auxins and/or TIBA were applied to apple fruit pedicels in the form of a lanolin paste. Fourteen lanolin paste mixtures were created by combining 100 microliters of distilled water with 2.75 g anhydrous lanolin (Sigma-Aldrich, St. Louis, MO) and 450 µliters of Tween 80 (Sigma-Aldrich, St. Louis, MO). The finely powdered active ingredients (IAA or NAA) (both, Sigma-Aldrich, St. Louis, MO) were then added to each of these mixtures; 33, 16.5 and 3.3 mg for 10, 5 and 1 $mg \cdot g^{-1}$ treatments, respectively. For combination treatments receiving TIBA (Sigma-Aldrich, St. Louis, MO), 0.01 mg was added to create a Yes concentration. The mixtures were then stirred vigorously with a spatula for 15 s.

At 30, 45, and 60 DAFB, 30-40 mg of lanolin paste was collected on a laboratory spatula rolling a 1 mm-thick dollop of the paste up to the flat sides of the spatula (Fig. 3). The lanolin paste was uniformly spread over the surface of each pedicel, with care taken to avoid the fruit.

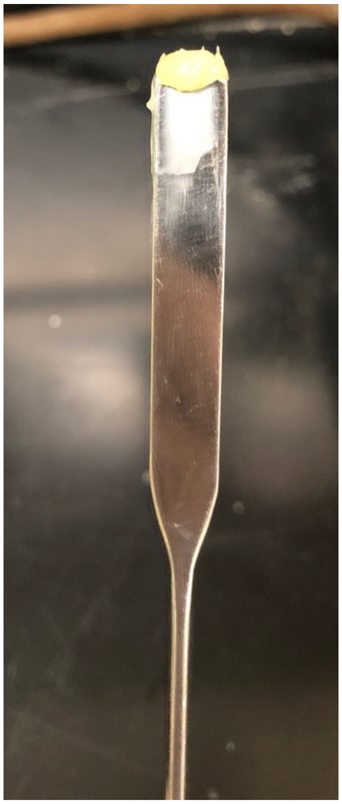

Figure 3: Laboratory spatula with 30-40 mg of auxin-infused lanolin paste

Figure 4: Hormone-infused lanolin paste applied to apple pedicel at 30 DAFB

Beginning three weeks before harvest and continuing each of the following weeks, fruit were scored for bitter pit incidence while still on the tree using a 0-3 scale (Fig. 5) Beaudry and Yildiz-Ocal, personal communication).

Figure 5: Bitter pit rating scale developed by Beaudry and Yildiz-Ocal. Apples ranked from 0 (no bitter pit, left) to 3 (extensive bitter pit, right).

3.2.4. Preparation and Application of Auxin and/or TIBA Solutions to Leaves or Fruit

Auxins or TIBA were applied to the surface of fruit or leaves in the form of a water-based solution. These solutions were created by mixing the active ingredient with 0.1% Tween 80 and diluted with distilled water to the appropriate treatment concentration. For spurs whose fruit received a direct application of the compounds, solutions were applied using cotton swabs in a vertical motion perpendicular to either, a permanent marker line previously applied to a quarter of the fruit’s equator. For spurs that received foliar applications, solutions were applied to both the adaxial and abaxial sides of all primary and bourse leaves.

Figure 6: Demonstration of application of auxin solution to one-quarter of a fruit surface (30 DAFB)

3.2.5. Harvest, Staining, and Analysis of Selected Spurs

Whole spurs were harvested on 81 and 123 DAFB in 2020 and 85, 106, and 134 DAFB in 2021. Whole spurs were severed with pruners where they originated from limbs. Spurs were harvested in the early morning and placed in sealed sandwich bags to limit transpiration. Spurs were placed in a cooler on ice and immediately transported to the laboratory. Fruit were separated from spurs by cutting 1 mm below the base of the pedicel while submerged in water to prevent formation of emboli and placed on a grid so that fruit were suspended above 1% w/w acid fuchsin solution but pedicels were submerged as per the procedure described by Dražeta et al. (2004). A fan was used to disrupt the formation of a boundary layer and to encourage fruit transpiration and uptake of the dye. Fruit remained in the acid fuchsin for 8 h to allow for maximum uptake of dye. Following the dyeing procedure, fruit were weighed on a balance (manufacturer info), scored for bitter pit, and then sliced in 5 mm thick longitudinal slices with a vegetable chopper (Nemco, model number 56750-4, Hicksville, OH). Equatorial discs were then selected from the calyx (distal) and stem (proximal) ends of each fruit, with the exception of lanolin-treated fruit harvested at 81 DAFB which were too small to accommodate multiple sectioning and thus were sliced once at the equator. Slices were then placed in a light box and imaged with a Nikon D750 camera (Nikon, model number 1543, Tokyo, Japan). Images were then scored for the number of primary and dorsal vascular bundles dyed with acid fuchsin; stained bundles were considered to be functional. The maximum number of primary and dorsal bundles per fruit is 10 and 5, respectively. Ventral bundles were not assessed due to their near total dysfunction by mid-season (Dražeta et al., 2004). In the fruit treated over a quarter of their surface, the number of functional bundles within the treated quarter was scored separately, as was fruit asymmetry. Apples were also assessed for ripeness by staining with a 10% w/w, 2% KI solution for starch in 2020 and by scoring background color in 2021 using a subjective 1-5 scale to indicate ripeness, with a "1" signifying the lowest level of "greenness" and the highest degree of maturity.

Remaining fruit from the lanolin trials were placed in controlled atmosphere storage (3% CO_2, 0°C, 100% RH) for three months before being scored for bitter pit as described previously, with the calyx peeled to ensure that no additional pits were developing beneath the surface.

Remaining fruit from the direct fruit/leaf trials were then stored for two weeks at room temperature (20°C) to simulate three months of commercial low-temperature storage in a controlled atmosphere environment based on unpublished data (Al Shoffe, unpublished).

Following storage, these fruit were scored for bitter pit, with bitter pit scored separately for treated vs. untreated portions of fruit treated with solution over one quarter of their surface.

3.3 RESULTS

3.3.1. Effects of Lanolin Treatments on Fruit Mass

NAA treatments were phytotoxic to fruit, as evidenced by reduced fruit mass, premature maturation and early abscission (Fig. 7). Toxicity increased with increasing concentration of NAA. Detrimental effects of NAA application at 30 DAFB were visible by 45 DAFB, i.e., the time of the second treatment, concomitant with fruit already reddening and displaying necrotic spots where the NAA mixture was applied to the fruit's surface. While IAA treatments did not induce premature fruit maturity, IAA rate was generally negatively related to fruit mass. TIBA did not affect fruit weight.

Figure 7: Apple fruit (45 DAFB) prematurely reddening following treatment with NAA. Necrosis is visible where NAA mixture made contact with fruit.

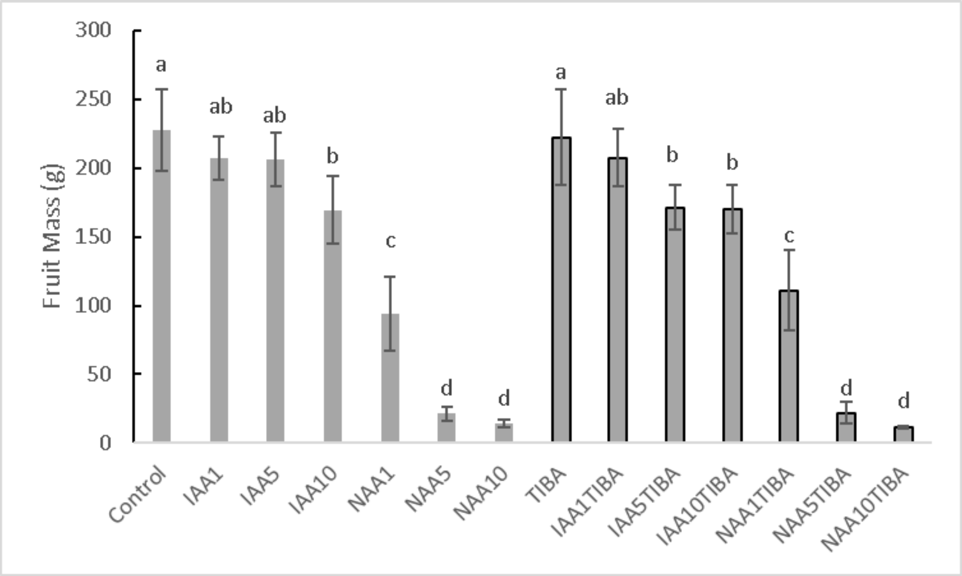

Figure 8: The effect of auxin and/or TIBA embedded in lanolin paste and applied to fruitlet pedicels on fruit mass at harvest. Successive applications were made at 30, 45, and 60 DAFB. Data are means of five unbalanced reps. Error bars are ± 1 SE of the mean.

3.3.2. Effects of IAA and TIBA Lanolin Treatments on Xylem Functionality and Bitter Pit Incidence

Because of the toxicity effects of NAA, xylem functionality of these fruit were not compared to control, IAA, and/or TIBA treated fruit. Among control fruit, an average of 7.1 primary bundles out of a maximum 10 and 2.2 dorsal bundles out of a possible 5 were functional at the fruit's equator when examined at 81 DAFB (Table 3). IAA and TIBA had no significant effect on either of the vascular bundles.

Among control fruit assessed for xylem functionality at harvest, an average of 0.76 and 0 primary and dorsal bundles were functional in the stem-end, respectively, and 1 and 0 primary and dorsal bundles in the calyx end of the fruit, respectively. In both the stem and calyx end of the fruit, both primary and dorsal bundle functionality were generally increased by treatment with IAA, but these increases were not statistically significant (Table 3). Because of the early senescence of fruit treated with NAA, xylem functionality at harvest was not considered.

Treatment with TIBA had no significant effect on xylem functionality but combination treatments generally increased staining when compared to TIBA alone (Table 3).

Among all treatments, notwithstanding NAA treatments due to toxicity), bitter pit incidence increased during the month preceding harvest (93 to 123 DAFB) (data not shown). Overall, bitter pit incidence of control fruit was low for 'Honeycrisp' with only 16% incidence and an average bitter pit rating of 0.159 on the 0-3 scale. Large variation between replicates precluded statistical significance among treatments.

Table 3: The effect of auxin and/or TIBA embedded in lanolin paste and applied to fruitlet pedicels on fruit mass at harvest. Successive applications were made at 30, 45, and 60 DAFB. Data are means of five unbalanced reps. P-values were calculated using one-way ANOVA.

Treatment		Equatorial Primary Bundles (no.)	Equatorial Dorsal Bundles	Stem Primary Bundles	Stem Dorsal Bundles	Calyx Primary	Calyx Dorsal	Bitter Pit Incidence
Compound	Rate (ppm)							
UTC	0	7.1	2.2	0.8	0	1.0	0	0.16
IAA	1	5.6	1.5	1.0	0.5	1.2	0.2	0.15
	5	6.8	2.6	0.7	0.2	1.7	0.1	0.30
	10	7.6	2.9	2.6	0.9	2.6	0.7	0.21
TIBA	30	7.0	2.4	0.7	0	1.2	0	0.34
IAA+TIBA	1+30	7.3	2.0	0.8	0.3	1.0	0.3	0.16
	5+30	8.0	2.6	1.6	0.6	2.0	0.2	0.22
	10+30	6.6	2.6	1.6	0.9	1.7	0.8	0.33
P-value		0.647	0.541	0.299	0.187	0.172	0.066	0.954

3.3.3. Effects of Quarter-Fruit and Leaf Auxin and TIBA Treatments on Xylem Functionality

No treatments produced significant differences in xylem functionality relative to the control (Fig. 9). However, quarter-fruit treatment generally resulted in greater xylem functionality compared to fruit from spurs with treated leaves. 1 ppm IAA applied to quarter-fruit resulted in the greatest number of functional primary and dorsal bundles in the stem end of the fruit with an average of 0.32 primary bundles and 0.21 dorsal bundles remaining functional at harvest compared to 0.18 and 0.05, respectively, in control fruit. While 10 ppm IAA also resulted in improved xylem functionality when applied to quarter-fruit surface compared to control fruit, 100 ppm IAA resulted in fewer functional primary and dorsal bundles in both ends of the fruit relative to the control.

Control fruit were observed to contain 2.2 functional primary bundles and 0.55 functional dorsal bundles in the stem and 0.6 functional primary bundles and 0.2 functional dorsal bundles in the calyx at harvest. In the stem end of fruit, all treatments produced a numerical decrease in the number of functional vascular bundles at harvest. The greatest amount of dysfunction of primary bundles in the stem end of fruit was observed in fruit with leaves treated with TIBA, which caused a reduction to just 0.85 functional primary bundles, on average. TIBA also decreased the number of functional primary bundles in the calyx end of fruit, but this decrease was also observed 1 ppm and 10 ppm IAA treatments. NAA at 1 and 10 ppm made no statistically significant differences in xylem functionality. All treatments either decreased or did not affect the number of functional dorsal bundles in both ends of the fruit, but statistically significant decreases were only observed in the stem end of fruit. Overall, the number of functional vascular bundles was significantly decreased in the calyx end of the fruit rather than the stem end.

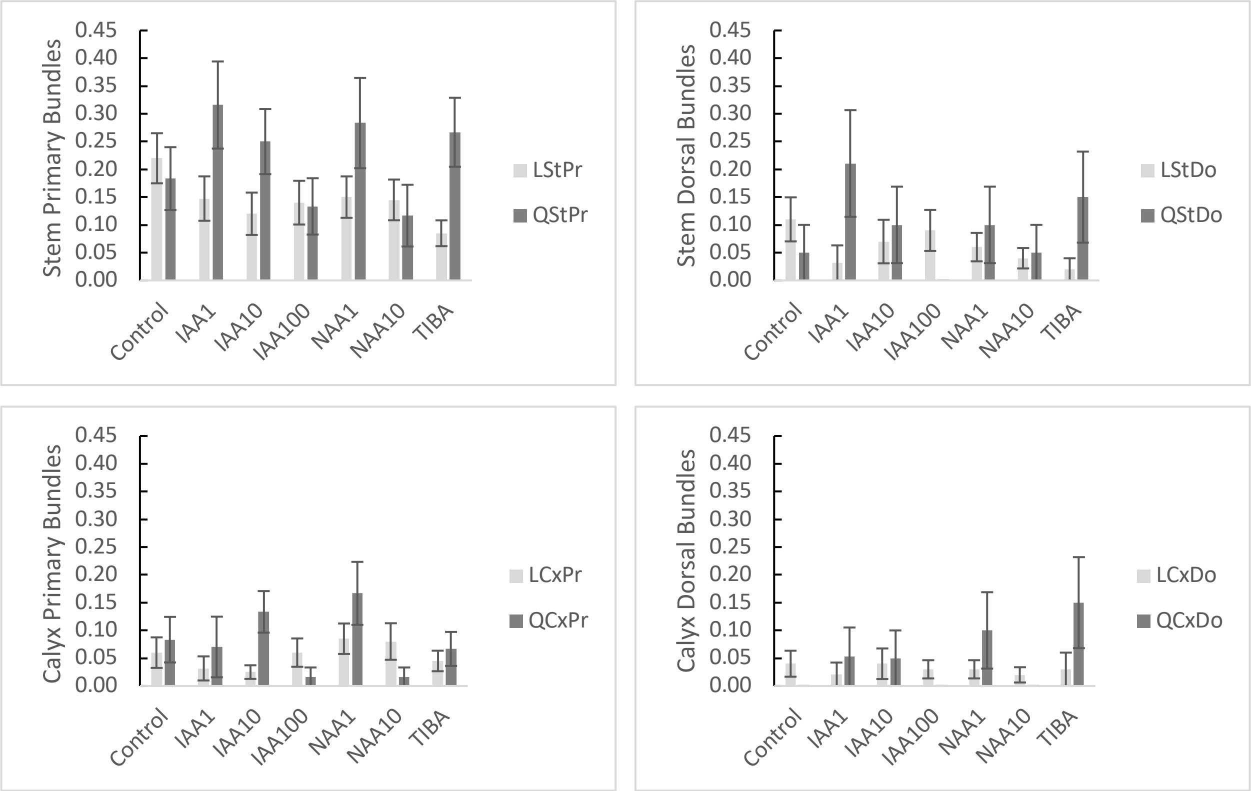

Figure 9: The effect of auxin and/or TIBA applied as solution to fruit (Q) or leaves (L) on the number of acid fuchsin stained (functional) primary bundles in the stem (top left) and calyx (bottom left) and dorsal bundles in the stem (top right) and calyx regions of mature 'Honeycrisp' fruit (bottom right). Successive applications were made at 30, 45, and 60 DAFB. Data are means of five unbalanced reps. Error bars represent ± 1 SE of the mean.

3.3.4. Effects of Quarter-Fruit Auxin and TIBA Treatments on Average Bitter Pit Rating

Treatment of a quarter of the fruit surface did not produce any significant differences in average bitter pit rating relative to the rest of the fruit's surface or the control. However, treatment of leaves with TIBA resulted in a significant increase in average bitter pit incidence relative to all other treatments, especially the control (1.59 vs. 0.38, respectively). The significance of the effect of TIBA when applied to leaves rather than the surface of fruit suggests TIBA may be poorly absorbed by the fruit surface and could potentially mobile when applied to leaves. More research is necessary to explain TIBA transport.

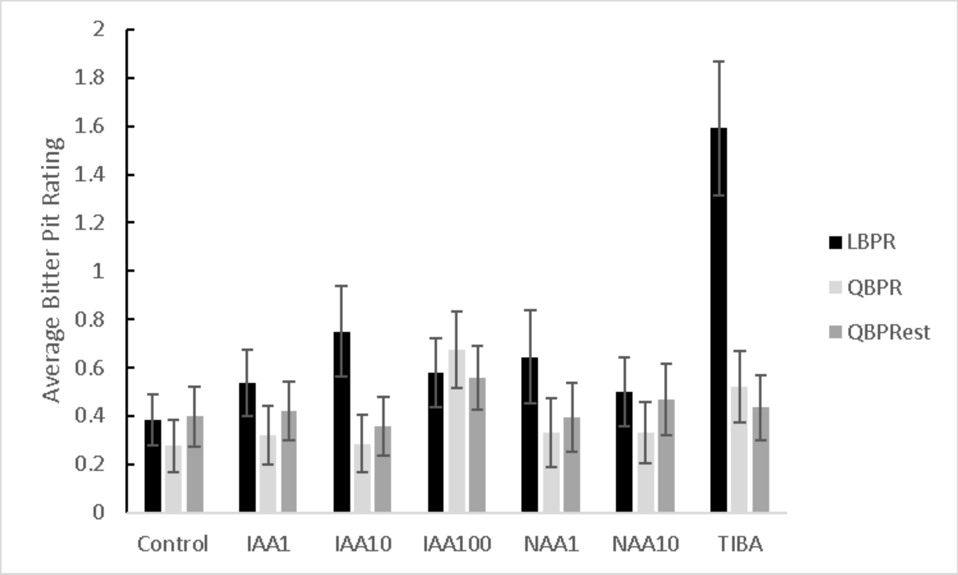

Figure 10: The effect of auxin and/or TIBA applied as solution to fruit or leaves on the average bitter pit rating of mature 'Honeycrisp' fruit. "LBPR" refers to the bitter pit rating of fruit where spur leaves were treated. "QBPR" refers to the bitter pit rating of the treated portion (one quarter of the fruit surface) while "QBPRest" refers to the bitter pit rating of the untreated surface of the same fruit. Successive applications were made at 30, 45, and 60 DAFB. Data are means of five unbalanced reps. Error bars represent ± 1 SE of the mean.

3.4. DISCUSSION

3.4.1. Auxin Toxicity

Auxin concentrations chosen for lanolin applications to pedicels were excessive and proved toxic, as evidenced by adverse effects on fruit mass and early senescence of treated fruit (Fig. 8). Though the concentrations of IAA and NAA used were the same, NAA treatments were significantly more toxic than IAA treatments. NAA is more stable in plant systems than IAA. The longer residence time of NAA has been attributed to the fact that while the native IAA is both conjugated and oxidized to remove it from activity, the synthetic NAA can only be conjugated by plant cells (de Klerk et al. 1997). As a result, while NAA and IAA were both found to be beneficial in the promotion of adventitious root generation in apple tissue culture, the optimal concentration of NAA was found to be 10% of the optimal concentration of IAA.

Supraoptimal concentrations of both compounds were found to inhibit root growth. This is consistent with the results of lanolin treatment, as both compounds were found to decrease fruit mass with increasing concentrations of auxin (Fig. 8). Thus, in 2021 we altered the auxin rates based on research demonstrating a 10:1 difference in relative auxin power in adventitious root growth between NAA and IAA (de Klerk et al., 1997).

3.4.2. Auxin Transport Directionality

Auxins and/or TIBA were applied to either the pedicel, the fruit surface, or spur leaves to characterize auxin transport and action on vascular differentiation. Auxin and/or TIBA treatments did not significantly affect xylem functionality or bitter pit when applied to apple fruit pedicels (Table 3). While GAs, cytokinins, and auxins have been shown to affect fruit characteristics when applied to fruit pedicels, through their effect on pedicel differentiation (Dražeta et al., 2004b; Matsumoto et al., 2018), those applications were made earlier in the development period of pedicels than those in the current study. Pedicels have been shown to be differentiated by 28 DAFB, which precedes our first application date. Because auxin is exported from the fruit and pedicels were fully differentiated by the time of application, pedicel treatments may not have affected the fruit characteristics assessed in this experiment.

Auxin treatments did not locally increase xylem functionality or mitigate bitter pit when applied to a portion of the fruit surface (Fig. 9 and 10). While auxins have been determined to penetrate the cuticle and epidermis of the fruit and establish a gradient in the cortex (Vendrell, 1970), it is possible that the concentrations of auxins used were not sufficient to establish a significant gradient within the studied apple fruit. When applied to ‘Bartlett’ pear, IAA penetration was determined to be low with internal concentration of IAA reaching only one-twentieth of the treatment concentration (Frenkel and Dyck, 1973). Because auxins were applied over only one-quarter of the surface of fruit, any penetrating auxin would be diluted as it is transported throughout the fruit, though whole-fruit applications did not produce a significant effect (data not shown). Additionally, the method of application (swabbing with auxin solution-dipped cotton swabs) may not have delivered a sufficient quantity to the fruit surface or allowed the solution to persist on the fruit surface long enough for penetration. Finally, because IAA is rapidly degraded by light, slow penetration of fruit would reduce the efficacy of treatments (Leasure et al., 2013). Foliar auxin treatments also did not improve fruit xylem functionality (Fig. 9). These results are consistent with previous experiments demonstrating that seeds are the source of auxins facilitating vascular differentiation within fruit (Bangerth et al., 1989; Buccheri and Di Vaio, 2004; Dražeta et al., 2004b); since auxin transport is basipetal and therefore is not transported into fruit from other tissues. Because TIBA had no effect when applied to the surface of fruit, but significantly increased bitter pit incidence of fruit when applied to spur leaves (Fig. 10), it could therefore be hypothesized that TIBA, unlike auxin (Homan, 1964), may be able to be translocated through the pedicel into fruit where it blocks the transport of endogenous auxin. Further research is required to confirm previous findings of auxin transport directionality and potentially explore the role of fruit-synthesized versus leaf-synthesized auxins on fruit vascular differentiation and bitter pit incidence.

3.4.3. Effect of Auxins and TIBA on Bitter Pit Incidence

Auxins did not mitigate bitter pit when applied to the pedicel, fruit surface, or spur leaves (Table 3, Fig. 10). TIBA treatment of spur leaves increased average bitter pit rating of associated fruit from 0.55 in the control to 2.06 (Fig. 10). This result is not surprising given the effects of TIBA sprays described in previous literature (Stahly and Benson, 1970; Bangerth, 1979), but it surprising considering the inverse was not true, i.e., auxin treatments of leaves did not improve fruit vascular function or mitigate bitter pit. These results suggest a unique set of circumstances are created by sprays as opposed to direct treatment of tissues. Perhaps sprays are more efficacious through a greater contact or slower treatment evaporation time. Further experimentation is necessary to determine the necessary placement of compounds for bitter pit mitigation and to determine why whole-tree sprays are more efficacious.

CHAPTER 4. The Effect of Exogenous, Whole Canopy Applications of Plant Growth Regulators on Bitter Pit Incidence

4.1. INTRODUCTION

While phloem remains functional throughout the growth period, xylem gradually becomes dysfunctional in apple fruit (Lang, 1990). Bitter pit susceptibility of cultivars has been linked to the rate of xylem dysfunction in primary bundles in fruit (Lang and Ryan, 1994). Xylem tissue is differentiated through the action of auxin and cytokinin, among other factors (Pesquet et al., 2005); thus, hormone concentrations are implicated in promoting vascular bundle functionality and Ca transport and concentration within the fruit that may reduce the incidence of bitter pit. Though the mechanism is not yet fully understood, ABA has also been shown to increase vascular bundle functionality in tomato fruit (de Freitas et al., 2011), and preliminary work suggests it may play a similar role in apple (Angmo et al., 2022). ABA has the additional effect of reducing stomatal conductance and transpiration, which is purported to improve Ca relations in fruit given the strong effect of transpiration on Ca transport (Falchi et al., 2017).

NAA is a synthetic auxin already widely used by commercial apple producers to thin flowers and developing fruitlets and to prevent premature fruit drop prior to or during the harvest season. Penetration and activity of NAA sprays are influenced by temperature and light conditions (Black et al., 1995). Because NAA is a synthetic auxin, plants do not possess a pathway to oxidize it, leading to longer residence times in plant tissues when compared to the native auxin, IAA (Smulders et al., 1990). As a result, treatment with NAA can result in phytotoxicity and "pygmy" fruit (Jones et al., 1991). However, because NAA is currently used in apple cultivation, it is a compound with which growers are already familiar in contrast to the native IAA, which lacks a commercial label and may be cost prohibitive to formulate and commercialize. ABA has been formulated and labeled for use as a post-bloom apple thinning agent.

The aim of this study was to determine the effectiveness of whole-tree NAA, IAA, and ABA sprays on vascular bundle functionality and bitter pit incidence of 'Honeycrisp' apple fruit. Trees were treated with PGR sprays, and fruit were sampled throughout the season and assessed for vascular bundle functionality and bitter pit incidence. We hypothesized that NAA, IAA, and ABA sprays would each be efficacious in mitigating bitter pit by promoting vascular bundle longevity. From a practical perspective, if NAA or ABA were proven effective, growers could readily adopt a program for bitter pit management. If IAA was proven effective, potential for a use label could be explored.

4.2. MATERIALS AND METHODS

4.2.1. Tree Selection and Preparation

Fifty 6th leaf 'Honeycrisp' trees on Geneva 11 rootstock were selected based on trunk diameter (between 9 and 13 cm, measured 20 cm above the graft union) and bloom uniformity. Guard trees were maintained between treatment trees to avoid contamination via drift. Five single-tree replicates were assigned to each of the following treatments (Table 4) with colored ribbons:

Table 4: Plant growth regulator treatments applied in 2021. Treatments were applied as whole tree sprays via a pressurized handgun at 30, 45, and 60 DAFB to 5 replicates consisting of one tree each.

Compound	Rate	Form Used
Control	-	-
IAA	5 ppm	Pure Solid
IAA	10 ppm	Pure Solid
IAA	20 ppm	Pure Solid
NAA	5 ppm	Fruitone L®
NAA	10 ppm	Fruitone L®
NAA	20 ppm	Fruitone L®

Table 4 (cont'd).

ABA	75 ppm	ProTone®
ABA	150 ppm	ProTone®
GA_3	20 ppm	Falgro®

IAA solutions were created by dissolving the pure solid (Sigma-Aldrich, St. Louis, MO) in water while NAA, ABA, and GA solutions were created by dissolving the commercial formulations (Fruitone L®, ProTone®, and Falgro®, Valent BioSciences U.S.A. LLC,) in water to the desired concentrations. Treatment concentrations of NAA and GA_3 were chosen based on physiological concentrations already used in commercial apple production for a wide range of processes including, regulation of return bloom, thinning, and fruit color promotion. Treatment solutions were composed of the active ingredient (ppm), a non-ionic surfactant (Regulaid, KALO Inc., Overland Park, KS) to promote adhesion (at 0.1% v:v), and, specifically for ABA treatments, Tri-fol® (Wilbur-Ellis Agribusiness, San Francisco, CA, U.S.A.) to buffer pH and reduce dissociation of the weak acid under high pH water. A total of 0.5 gal (2.273 L) of solution was applied to each tree to ensure uniform coverage (i.e., to drip) with the use of a pressurized handgun research sprayer. Solutions were applied early in the morning to encourage absorption (i.e., high RH) and minimize losses or drift attributed to increased wind speed later in the day. Successive treatment applications were made at 30, 45, and 60 DAFB. Treatment trees were thinned to an ideal commercial crop load through by hand thinning to commercial crop loads just prior to June drop by removing the smallest fruits (i.e., those destined to abscise).

4.2.2. Harvest and Staining

Whole spurs were harvested at 86, 107, and 136 DAFB in 2021. Whole spurs were severed with pruners at the base where the spur originates from the limb. Intact spurs were harvested in the early morning and then placed in sealed Ziploc bags to limit transpiration and immediately transported to the laboratory. Fruit were then separated from spurs with a razor 1 mm below the base of the pedicel while submerged in water to prevent emboli. Fruit were then placed on a grid with pedicels submerged in a 1% w/w acid fuchsin solution as described by Dražeta et al. (2004). A fan was used to disrupt the formation of a boundary layer at the fruit surface and encourage fruit transpiration and uptake of the dye. Fruit remained in the acid fuchsin for 8 h to allow full uptake of dye.

After 8 h, fruit were weighed, scored for bitter pit (in the case of the 136 DAFB sample date), and sectioned in 5 mm thick latitudinal discs with the use of a vegetable chopper (Nemco, Hicksville, OH, model number 56750-4). Two equatorial discs were selected, one from the calyx (distal) and one from the stem (proximal) end of the fruit. Slices were then placed in a light box and imaged with a Nikon D750 camera (Model Number 1543, Toyko, Japan). Images were later scored for the number of functional primary and dorsal vascular bundles dyed with acid fuchsin. The maximum number of primary and dorsal bundles per fruit is 10 and 5, respectively. Ventral bundles were not assessed due to their near total dysfunction by mid-season as previously shown in other cultivars (Dražeta et al., 2004).

An additional equatorial disc was sectioned from dyed fruit at the equator for nutrient analyses. One quarter of the peel of each apple was removed using a knife along with cortical tissue as described above. All tissues were combined in a paper coin envelope and dried at 60°C in a convection drying oven (VWR Oven F Air 6.3CF, Thermo Electron LED GmbH, Lanenselbold, Germany) until equilibrium mass was reached. The dried peel and fruit tissues were then ground, separately, using a ball mill. Nutrient analyses were performed using method P-4.30 in Gavlak et al. (2005) by Brookside Laboratories (New Bremen, OH) on tissue taken from fruit treated with 20 ppm IAA, 5 ppm NAA, and 150 ppm ABA.

Remaining fruit were then harvested from trees and taken to the MSU RidgeLab (Sparta, MI). Fruit were assessed for percentage color, shape and mass using the Compaq/Spectrim sorting system (TOMRA Foods, Leuven, Belgium), and were manually weighed and scored for bitter pit. These fruit were then stored at room temperature for 14 days to simulate three months of low temperature storage in a regular atmosphere environment (Al Shoffe, unpublished). Following storage, fruit were reassessed for bitter pit incidence. Fruit were then dissected and the seed content of all fruit was evaluated as the number of matured, undeveloped, and unfertilized seeds according to Elsysy et al. (2019).

Figure 11: Classification of seeds as mature/fertilized (left), undeveloped (center), and unfertilized (right). Undeveloped seeds are the result of seeds being fertilized but not developing properly (Elsysy et al., 2019).

4.3. RESULTS

4.3.1. Effects of Plant Growth Regulators on Vascular Bundle Functionality

Vascular bundles were observed to become dysfunctional as fruit matured with little functionality remaining at harvest, especially in the calyx end of fruit where bitter pit originates (data not shown). In 2021, significant differences among treatments were observed. While treatment with IAA, ABA, and GA_3 produced a numerical improvement in the number of functional bundles, these improvements were not statistically significant (Table 5). However, treatment with NAA greatly improved the number of dorsal bundles throughout the fruit and the number of primary bundles in the calyx (Table 5). NAA (10 ppm) was the most effective treatment in the promotion of vascular bundle functionality. While dorsal bundles were almost completely dysfunctional on average in control fruit (0.12 and 0.08 in the stem- calyx-end, respectively), treatment with 10 ppm NAA increased these values to 1.64 in the stem end of fruit and 1.2 in the calyx end of fruit.

Table 5: Effect of whole tree sprays on the number of acid fuchsin stained (functional) primary and dorsal bundles in the stem and calyx regions of mature 'Honeycrisp' fruit. Successive applications were made at 30, 45, and 60 DAFB. Data are means of five reps. Mean separation was performed using Tukey's HSD when significant differences among treatments were indicated by P-values < 0.05 calculated using one-way ANOVA.

Treatment	Stem Primary Bundles	Stem Dorsal Bundles	Calyx Primary Bundles	Calyx Dorsal Bundles
Control	1.88	0.12b	0.52b	0.08b
IAA @ 5 ppm	2.32	0.32b	0.6b	0.08b
IAA @ 10 ppm	1.4	0.36b	0.6b	0.28ab
IAA @ 20 ppm	1.84	0.48b	0.76ab	0.52ab
NAA @ 5 ppm	2.8	0.56b	1.72a	0.48ab
NAA @ 10 ppm	3	1.68a	1.64a	1.2a
NAA @ 20 ppm	2.16	1.00ab	0.88ab	1.0ab
ABA @ 75 ppm	2.28	0.64ab	1.04ab	0.4ab
ABA @ 150 ppm	2.12	0.92ab	1.4ab	0.8ab
GA3 @ 20 ppm	2	0.28b	0.61ab	0.17ab
P-Value	0.397	<0.001	0.026	0.015

4.3.2. Effects of Plant Growth Regulators on Bitter Pit Incidence

Overall bitter pit incidence was high in 2021, with an incidence of 33% observed in control fruit (Table 6). Control fruit were rated, on average, with a bitter pit rating of 0.72 out of a maximum severity index of 3 at harvest. Treatment with 20 ppm IAA and both concentrations of ABA significantly reduced average bitter pit incidence compared to treatment with GA_3 (Table 6). 20 ppm IAA produced the greatest effect reducing average bitter pit rating by 84.7% of UTC fruit and reducing bitter pit incidence at harvest to 7%. GA_3 treatment numerically increased bitter pit rating, though this increase did not statistically differ from the control. After a two-week storage period at room temperature, average bitter pit rating and incidence of control fruit increased to 1.06 and 45%, respectively (Table 6). The beneficial effects of all treatments were reduced during this storage period with no treatments retaining a statistically significant reduction compared to either control fruit or GA_3-treated fruit.

Table 6: Effect of whole tree sprays on average bitter pit rating and bitter pit incidence at harvest and after a 14 day postharvest storage period at room temperature. Bitter pit ratings were subjective and based on the 0-3 scale described above. Successive applications were made at 30, 45, and 60 DAFB. Data are means of five reps. Mean separation was performed using Tukey's HSD when significant differences among treatments were indicated by P-values < 0.05 calculated using one-way ANOVA.

Treatment	Average Bitter Pit Rating at Harvest	Bitter Pit Incidence at Harvest	Average Bitter Pit Rating after Storage	Bitter Pit Incidence After Storage
Control	0.72ab	0.33ab	1.06	0.45
IAA @ 5 ppm	0.27ab	0.16ab	0.40	0.20
IAA @ 10 ppm	0.26ab	0.15ab	0.47	0.26
IAA @ 20 ppm	0.11b	0.07b	0.32	0.20
NAA @ 5 ppm	0.37ab	0.18ab	0.60	0.33
NAA @ 10 ppm	0.37ab	0.18ab	0.59	0.27
NAA @ 20 ppm	0.29ab	0.15ab	0.85	0.37
ABA @ 75 ppm	0.22ab	0.11b	0.30	0.17
ABA @ 150 ppm	0.27ab	0.13b	0.56	0.31
GA_3 @ 20 ppm	1.05a	0.50a	1.24	0.64
P-Value	0.019	0.011	0.143	0.146

4.3.3. Effect of Plant Growth Regulators on Seed Count

The total number of mature seeds was increased, on average, in all fruit except those treated with 20 ppm NAA and GA_3. The greatest increase was observed in fruit treated with 10 ppm IAA while the greatest decrease was observed in fruit treated with 10 ppm NAA. Fruit treated with 20 ppm NAA had fewer mature seeds and more undeveloped seeds which were aborted as fruit matured. The unfertilized seed content was unaffected by treatment.

Table 7: Effect of whole tree sprays on the number of unfertilized, mature, and undeveloped seeds in 'Honeycrisp' fruit. Successive applications were made at 30, 45, and 60 DAFB. Data are means of five reps. Mean separation was performed using Tukey's HSD when significant differences among treatments were indicated by P-values < 0.05 calculated using one-way ANOVA.

Treatment	Unfertilized Seeds	Mature Seeds	Undeveloped Seeds
Control	1.49	6.62ab	0.14b
IAA @ 5 ppm	1.65	6.79ab	0.08b
IAA @ 10 ppm	1.59	7.33a	0.08b
IAA @ 20 ppm	1.96	7.14ab	0.06b
NAA @ 5 ppm	1.75	7.19ab	0.14b
NAA @ 10 ppm	1.55	6.80ab	0.29b
NAA @ 20 ppm	1.92	5.81b	1.11a
ABA @ 75 ppm	1.51	7.16ab	0.12b
ABA @ 150 ppm	1.71	7.21ab	0.07b
GA_3 @ 20 ppm	1.84	6.44ab	0.01b
P-Value	0.861	0.034	<0.001

4.3.4. Effect of Plant Growth Regulators on Fruit Color

Overall, treatments significantly affected the yellow and green coloration of fruit (Table 8). While neither treatment resulted in statistically significant differences compared to control fruit, 20 ppm NAA produced significantly greener fruit compared to fruit treated with 20 ppm IAA. Though not statistically significant, there existed a trend of increasing concentrations of IAA increasing the red coloration of fruit while NAA treatment increased green coloration of fruit. The increasing redness of IAA-treated fruit came at the expense of yellow, green, and pink coloration. Fruit coloration varied significantly between replicates.

Table 8: Effect of whole tree sprays on the fruit color of mature 'Honeycrisp' fruit as determined by the Compaq/Spectrim system. Successive applications were made at 30, 45, and 60 DAFB. Data are means of five reps. Mean separation was performed using Tukey's HSD when significant differences among treatments were indicated by P-values < 0.05 calculated using one-way ANOVA.

Treatment	Red (%)	Yellow (%)	Green (%)	Pink (%)
Control	62.19	2.03a	6.72ab	29.06
IAA @ 5 ppm	60.46	2.46a	6.52ab	30.56
IAA @ 10 ppm	64.39	2.29a	5.61ab	27.70
IAA @ 20 ppm	71.28	1.58a	5.07b	22.07
NAA @ 5 ppm	49.59	5.51a	5.81ab	39.09

Table 8 (cont'd)

NAA @ 10 ppm	59.54	2.30a	7.14ab	31.02
NAA @ 20 ppm	48.23	5.65a	8.52a	37.60
ABA @ 75 ppm	48.37	5.94a	6.79ab	38.91
ABA @ 150 ppm	57.78	4.07a	5.56ab	32.59
GA_3 @ 20 ppm	61.32	3.08a	7.41ab	28.19
P-Value	0.281	0.047	0.043	0.480

4.3.5. Effect of Plant Growth Regulators on Fruit Mass

Increasing NAA concentration resulted in a statistical decrease in fruit mass with 20 ppm NAA treatment reducing the mass of control fruit by ~1/3rd (Figure 12). All other treatments did not significantly deviate from control values.

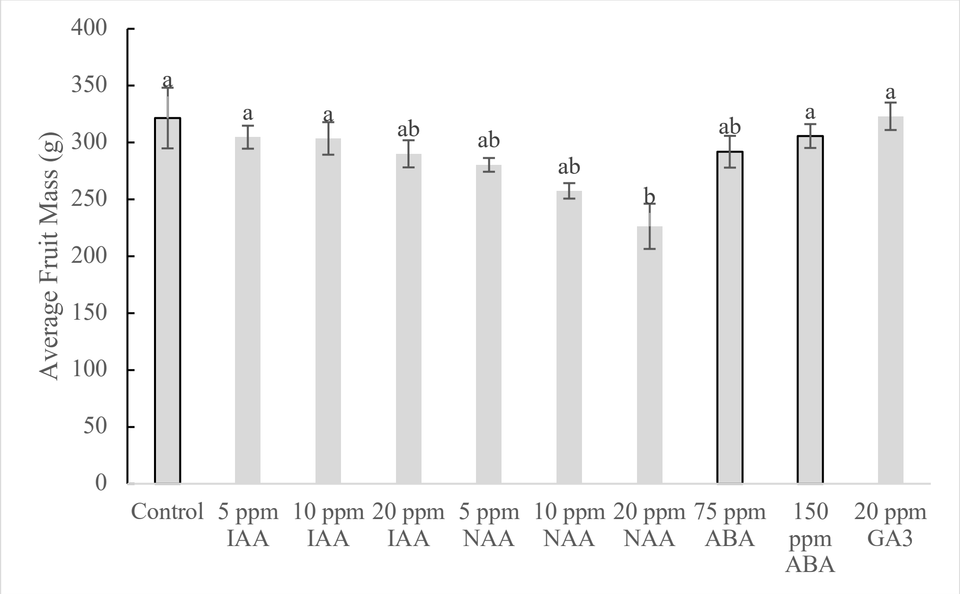

Figure 12: Effect of whole tree sprays on average fruit mass of mature 'Honeycrisp' fruit. Successive applications were made at 30, 45, and 60 DAFB. Data are means of five reps. Mean separation was performed using Tukey's HSD when significant differences among treatments were indicated by P-values < 0.05 calculated using one-way ANOVA.

4.3.6. Effect of Select Treatments on Fruit Nutrients

Nutrient concentrations were examined for all fruit which were examined for xylem functionality. The most efficacious rate was compared to the control for each of the three compounds tested, based on bundle staining and bitter pit incidence data: 20 ppm IAA, 5 ppm

NAA, and 150 ppm ABA. IAA (20 ppm) produced significantly higher N, Ca, and sodium levels in peel tissue relative to the control (Table 9). NAA (5 ppm) significantly increased B in peel tissue and ABA (150 ppm) significantly increased B and sodium in the peel. In flesh tissue, N and copper were significantly increased by treatment with 5 ppm NAA, while sodium was increased by treatment with 150 ppm ABA. Mg and K, two nutrients whose concentrations have been associated with bitter pit incidence, were not significantly affected by any of the treatments tested.

Table 9: Effect of whole tree sprays on the peel nutrients of mature 'Honeycrisp' fruit. Successive applications were made at 30, 45, and 60 DAFB. Data are means of five reps. Mean separation was performed using Tukey's HSD when significant differences among treatments were indicated by P-values < 0.05 calculated using one-way ANOVA.

Peel Nutrients	N (%)	P (%)	Mg (%)	K (%)	Ca (%)	S (%)	B (ppm)	Fe (ppm)	Mn (ppm)	Cu (ppm)	Zn (ppm)	Al (ppm)	Na (ppm)
Control	0.38b	0.08	0.13	0.51	0.08b	0.03	23.04b	14.43a	5.64	2.63b	3.47	33.79	131.30b
IAA @ 20 ppm	0.51a	0.07	0.08	0.52	0.12a	0.04	27.37ab	35.29a	6.68	1.91c	3.49	3.22	193.04a
NAA @ 5 ppm	0.40b	0.09	0.08	0.57	0.10ab	0.04	35.56a	17.25a	6.91	3.66a	4.03	7.58	155.31ab
ABA @ 150 ppm	0.39b	0.09	0.08	0.56	0.11ab	0.04	26.21b	33.51a	6.89	3.38a	3.18	6.38	201.24a
P-Value	<0.001	0.299	0.425	0.821	0.036	0.419	<0.001	0.038	0.337	<0.001	0.259	0.398	<0.001

Table 10: Effect of whole tree sprays on the flesh nutrients of mature 'Honeycrisp' fruit. Samples were harvested from the outer one cm of cortical tissue. Successive applications were made at 30, 45, and 60 DAFB. Data are means of five reps. Mean separation was performed using Tukey's HSD when significant differences among treatments were indicated by P-values < 0.05 calculated using one-way ANOVA.

Fruit Nutrients	N (%)	P (%)	Mg (%)	K (%)	Ca (%)	S (%)	B (ppm)	Fe (ppm)	Mn (ppm)	Cu (ppm)	Zn (ppm)	Al (ppm)	Na (ppm)
Control	0.44b	0.05	0.10	0.51	0.05	0.02	24.72	71.7	2.06	2.59b	6.54	28.90	119.54b
IAA @ 20 ppm	1.58b	0.05	0.03	0.54	0.06	0.02	18.92	77.92	2.32	1.56b	4.49	11.32	135.20b
NAA @ 5 ppm	4.91a	0.06	0.03	0.55	0.05	0.02	25.09	11.20	2.01	4.23a	3.79	8.84	120.13b
ABA @ 150 ppm	2.13ab	0.06	0.03	0.57	0.07	0.03	19.53	63.81	2.27	2.18b	3.14	8.95	197.71a
P-Value	0.007	0.517	0.413	0.881	0.350	0.419	0.566	0.055	0.337	<0.001	0.075	0.541	<0.001

4.3.7. Effect of Plant Growth Regulators on Leaf Nutrients

In extension leaves, only copper content was significantly altered by the treatments (Table 11). 20 ppm NAA significantly increased copper relative to the control (7.18 ppm vs. 5.30 ppm). In bourse leaves (Table 12), only sodium differed significantly between treatments as 75 ppm ABA and GA$_3$ each increased sodium content compared to bourse leaves treated with 5 ppm NAA.

Table 11: Effect of whole tree sprays on the extension shoot leaf nutrients of mature 'Honeycrisp' fruit. Successive applications were made at 30, 45, and 60 DAFB. Data are means of five reps. Mean separation was performed using Tukey's HSD when significant differences among treatments were indicated by P-values < 0.05 calculated using one-way ANOVA.

Treatment Means (Ext)	N	P	K	Ca	Mg	S	B	Fe	Mn	Cu	Zn	Al	Na
Control	1.64	0.28	1.04	1.58	0.28	0.11	45.98	41.32	53.2	5.3b	13.44	19.66	45.76
IAA @ 5 ppm	1.83	0.24	0.91	1.76	0.32	0.12	47.52	49.34	52.26	5.52ab	13.4	19.34	49.32
IAA @ 10 ppm	1.61	0.43	1.28	1.88	0.29	0.12	52.7	44.2	53.28	5.6ab	12.26	21.26	43.7
IAA @ 20 ppm	1.69	0.27	0.93	1.72	0.32	0.12	47.5	44.86	55.4	5.56ab	12.88	18.66	50.24
NAA @ 5 ppm	1.75	0.34	1.22	1.97	0.35	0.13	51.72	54.28	51.28	6.32ab	14.56	23.4	35.02
NAA @ 10 ppm	1.69	0.37	1.20	1.67	0.31	0.12	48.02	44.92	54.04	5.72ab	13.62	22.4	36.12
NAA @ 20 ppm	1.85	0.38	1.23	1.84	0.31	0.12	47.68	50.14	52.76	7.18a	13.78	26.26	37.62
ABA @ 75 ppm	1.76	0.36	1.13	1.82	0.31	0.13	48.9	51.36	55.04	6.44ab	12.9	19.54	43.8
ABA @ 150 ppm	1.51	0.36	1.11	1.79	0.30	0.13	49.06	50.24	48.18	5.74ab	12.6	24.04	35.92
GA$_3$ @ 20 ppm	1.56	0.32	1.11	1.72	0.28	0.12	48.42	46.96	49.26	5.5ab	13.68	20.4	35.88
P-Value	0.681	0.052	0.052	0.94	0.397	0.334	0.530	0.149	0.999	0.024	0.826	0.397	0.854

Table 12: Effect of whole tree sprays on the bourse shoot leaf nutrients of mature 'Honeycrisp' fruit. Successive applications were made at 30, 45, and 60 DAFB. Data are means of five reps. Mean separation was performed using Tukey's HSD when significant differences among treatments were indicated by P-values < 0.05 calculated using one-way ANOVA.

Treatment Means (Bourse)	N	P	K	Ca	Mg	S	B	Fe	Mn	Cu	Zn	Al	Na
Control	1.61	0.32	1.19	1.64	0.28	0.12	50.84a	43.38	46.40	5.56	11.34	20.60	38.88ab
IAA @ 5 ppm	1.67	0.47	1.29	2.06	0.32	0.13	61.26a	47.06	51.52	6.14	15.04	24.58	70.82ab

Table 12 (cont'd)

IAA @ 10 ppm	1.75	0.24	1.01	1.85	0.33	0.12	51.98a	52.04	51.04	6.00	14.00	24.10	49.24ab
IAA @ 20 ppm	1.86	0.30	1.07	1.93	0.34	0.13	54.92a	54.02	53.18	6.62	14.90	26.74	51.46ab
NAA @ 5 ppm	1.89	0.41	1.31	2.03	0.33	0.14	60.94a	61.08	48.84	6.86	16.98	27.22	31.84b
NAA @ 10 ppm	1.56	0.38	1.16	1.72	0.32	0.12	50.38a	49.84	49.18	5.92	13.14	26.00	35.44ab
NAA @ 20 ppm	1.69	0.38	1.29	1.84	0.32	0.13	52.02a	57.96	49.18	7.20	13.78	34.56	36.80ab
ABA @ 75 ppm	1.70	0.45	1.27	1.89	0.29	0.14	56.38a	54.26	49.92	6.64	12.02	24.48	74.68a
ABA @ 150 ppm	1.59	0.42	1.19	2.20	0.34	0.14	51.64a	54.46	56.04	7.08	14.74	24.06	44.74ab
GA_3 @ 20 ppm	1.67	0.38	1.12	1.94	0.31	0.13	51.28a	50.60	54.38	5.54	12.36	21.44	75.38a
P-Value	0.305	0.07	0.345	0.398	0.461	0.081	0.011	0.206	0.992	0.270	0.060	0.293	0.002

4.4 DISCUSSION

4.4.1. Vascular Bundle Functionality

The functionality of stem and dorsal bundles in both ends of the fruit was observed to diminish as fruit matured (data not shown). In untreated fruit, an average of 8.5 primary bundles were observed to be functional in the calyx of the fruit when assessed at 86 DAFB compared to 3.7 at 107 DAFB and 0.5 at 136 DAFB at fruit maturity. Similar rates of decrease were observed in dorsal bundles. Fruit xylem was observed to be more dysfunctional in the calyx end of fruit compared to the stem end of fruit. The median untreated apple examined at harvest contained zero functional primary or dorsal bundles.

PGR treatments produced significant improvements to primary bundle functionality in the calyx and dorsal bundles at both ends of the fruit (Table 5), but responses varied among compounds and rates within compounds. While IAA was ineffective in improving bundle functionality, 10 ppm NAA resulted in the greatest effect resulting by maintaining one primary bundle and 1-1.5 dorsal bundles at harvest compared to control fruit. This effect of NAA is consistent with findings that treatment of plants with NAA caused vascular tissue to remain flexible and unlignified and therefore less prone to breakage and dysfunction (Johnsson et al., 2018). Auxins in general are known to promote xylogenesis and reduce the thickness of cell walls in vascular tissue (Aloni, 1987; Pesquet et al., 2005). GAs have been observed to do the opposite, increasing cell wall thickness and brittleness of vascular elements (Johnsson et al., 2018). However, GA was not observed to negatively impact xylem functionality in harvested fruit (Table 5).

ABA, especially 150 ppm ABA, also produced numerically more functional bundles, but this effect was not statistically significant. ABA has been shown to improve xylem longevity in tomato fruit (de Freitas et al., 2011) and recently in apple fruit (Angmo et al., 2022), but the mechanism is not yet well understood. ABA may perform a variety of roles which combine to improve xylem longevity. There is evidence in the literature to suggest ABA is also involved in the differentiation of vascular tissue as ABA has been observed to increase phloem area (Murcia et al., 2016). Xylem dysfunction in apple has been shown to be at least partly driven through constriction of tracheary elements by parenchyma cells (Lang and Ryan, 1994; Miqueloto et al., 2014).

Because phloem also differentiates in a similar fashion to xylem, it is possible that ABA is able to increase the area of xylem tissue as well, allowing for continued flow of both Ca to growing cells and auxins to maintain xylem functionality. Because ABA is also known to antagonize GAs (McGlasson et al., 1978; Brady, 1987), prevention of the lignifying effect of GAs is another potential avenue by which ABA may improve xylem functionality. The effects of ABA in the studied fruit increased with concentration but were not statistically significant, suggesting higher concentrations may be more effective. Caution must be exercised when spraying with high concentrations of ABA, however, as excess ABA can partially defoliate trees. Rates of 500 ppm have been reported to cause leaf yellowing and abscission in apple (Greene, 2012) and *Pyrus communis* L. pear (Arrington et al., 2017). ABA also reduces transpiration of apple leaves (Einhorn, unpublished data; McArtney et al., 2014) and pear (Einhorn and Arrington, 2017) and thus may improve Ca transport to fruit as proposed by Falchi et al. (2017).

4.4.2. Bitter Pit Mitigation

Average bitter pit rating of fruit at harvest was reduced by all auxin and ABA treatments relative to the control (Table 6). The greatest decrease in bitter pit rating was observed in fruit treated with 20 ppm IAA as bitter pit rating was decreased from 0.72 to 0.11 which represents an approximate reduction of 85%. The effect was diminished following two weeks of storage at room temperature as no statistically significant differences remained between treatments. We suspect that large variations between replicates (trees) and relatively low sample populations of fruit contributed to this since percent reductions from control bitter pit were roughly 50% for all treatments, with the exception of 20 ppm NAA and GA_3. While the average bitter pit rating of control fruit increased from 0.72 to 1.06 after storage, average bitter pit rating doubled in the case of most auxin and ABA treatments and tripled in fruit treated with 20 ppm NAA. However, bitter pit rating only slightly increased after storage among fruit which had been treated with GA. It is possible that the reductions in bitter pit of fruit treated with auxins or ABA may be the result of their antagonism of the action of GA on fruit membranes where GA has been shown to increase membrane permeability (Jones, 1973; Pauls et al., 1982) thereby increasing the susceptibility of fruit to bitter pit (Bangerth 1976, Stahly and Benson, 1976; Greene et al., 1982). This effect may diminish as fruit remain in storage, allowing GA to act on membranes, causing cells to collapse, while GA has had sufficient time to act in GA-treated fruit.

Bitter pit incidence of 34% was markedly higher than incidences in 2020 whereby only 3.2% of control fruit were affected (data not shown) compared to 9% in thinned trees used for lanolin experiments that year (see chapter 3). The low incidence observed in 2020 was likely partially explained by a lack of thinning that resulted in reduced fruit weight. Interestingly, in 2020 TIBA did significantly reduce the number of functional vascular bundles compared to treatments not containing TIBA. Treatment with TIBA also resulted in the greatest incidence of bitter pit, but low overall bitter pit incidence and large variability between replicates precluded any statistical significance.

Based on 2021 results, an argument could be made that each of the compounds and rates assessed herein could be near to the ideal for bitter pit mitigation, notwithstanding NAA 20 ppm or GA_3. A significant dose response was not detected for any of the PGRs evaluated (data not shown). The commercially labeled auxin, NAA, at 5 and 10 ppm had the largest effect on functional xylem in apple fruit at harvest while not significantly reducing fruit mass, mature seed content, or redness (Figure 12, Tables 8 and 7). On the other hand, 20 ppm IAA produced the greatest reduction in average bitter pit rating at harvest (Table 6) and improved fruit redness and mature seed count (Tables 7 and 8). Because the trend in vascular bundle functionality was positively associated with increasing IAA concentration, the optimal concentration of IAA may be higher. NAA has been observed to function similarly to IAA in promoting adventitious root formation (de Klerk et al., 1997). Both IAA and NAA produced deleterious effects on root formation at supraoptimal concentrations, but the optimal concentration of NAA was much lower than that of IAA (30 µM for NAA vs. 3 µM for IAA) (de Klerk et al., 1997). This difference has been attributed to the fact that, while IAA is quickly oxidized by plant tissues by auxin-oxidase (Epstein and Ludwig-Muller, 1993), NAA is not oxidized by this enzyme and can therefore only be conjugated for removal (Smulders et al., 1990). As a result, NAA is much more stable in plant tissues than IAA (Dunlap et al., 1986). NAA has also been observed to be taken up by plant tissues 6 times faster than IAA (Peeters et al., 1991).

These factors combine to explain differences observed between treatments with similar concentrations of IAA and NAA. While 20 ppm NAA produced a variety of negative effects suggesting toxicity, 10 ppm NAA was the treatment most efficacious in increasing vascular bundle functionality (Table 5), and did so without significantly reducing fruit mass, redness, or mature seed content (Figure 12, Tables 8 and 7). If the ratios of optimal concentrations found by De Klerk et al. (1997) are applicable to apple fruit vascular maintenance and bitter pit mitigation, then the lowest concentration of NAA used, 5 ppm, would represent 4x the effective auxin concentration present in fruit treated with the highest IAA concentration, 20 ppm.

While auxins are only recently being studied in relation to Ca deficiency disorders, ABA has already shown promise in mitigating BER in tomato (de Freitas et al., 2011) and bitter pit in apple (Angmo et al., 2022). Our experiments support findings that ABA treatment mitigates bitter pit incidence in apple (Table 6) as treatment with ABA reduced average bitter pit rating of fruit by roughly 65%. ABA also was observed to increase the number of functional vascular bundles in mature fruit (Table 5) and fruit peel and flesh Ca (Tables 9 and 10). While these effects were not statistically significant, they were consistent and were observed to vary with ABA rate. Further experimentation is warranted to confirm ABA’s effect on vascular bundles and fruit nutrients, but these findings are consistent with previous research.

ABA’s direct effect on transpiration through closing stomata provides a logical explanation for the observed increases in fruit and bourse leaf Ca (de Freitas et al., 2011). However, the mechanism by which ABA improves vascular bundle functionality is not yet fully understood.

This effect could be related or unrelated to ABA's action on stomata. Additional potential mechanisms of action of ABA are its antagonism of GAs (Brady, 1987) and its regulation of genes responsible for Ca allocation (Falchi et al., 2017). Mitigation of bitter pit by ABA could be the result of any of these functions or, more likely, a combination of them. Because ABA appears to have different modes of action in relation to bitter pit than auxins, the potential exists for auxins and ABA to be used together in the mitigation of bitter pit and other Ca deficiency disorders.

4.4.3. Toxicity of Increasing Auxin Concentrations

Treatment with 20 ppm NAA produced a variety of detrimental effects when compared to treatment with lower concentrations of NAA. Fruit mass was reduced by treatment with 20 ppm NAA relative to the control and increasing concentrations of NAA produced smaller fruit, though these differences were not statistically significant. Additionally, 20 ppm NAA caused fruit to be less red and more green in color than control fruit, and the number of fully developed seeds was reduced with more seeds being aborted during fruit development. These observations demonstrate the toxic effects that higher concentrations of NAA have on apple fruit development.

4.4.4. 2020 Spray Experiments

In 2020, a similar experiment was conducted, examining the effects of IAA, NAA, and ABA treatments on bitter pit mitigation in 'Honeycrisp' apple when used with or without TIBA, an auxin transport inhibitor. However, as trees were not thinned, all trees carried a supraoptimal number of low-mass fruit.

4.4.5. Fruit Nutrient Effects

20 ppm IAA, 5 ppm NAA, and 150 ppm ABA were studied for their effects on fruit nutrients in the peel and flesh relative to untreated fruit as each of these treatments were beneficial in regard to either vascular bundle functionality or bitter pit mitigation. N was shown to be strongly affected by treatment with IAA in the peel and both IAA, NAA, and ABA in the flesh (Tables 9 and 10). In the peel, an increase in N resulting from IAA treatment may be the result of rapid oxidation of IAA, producing a variety of nitrogenous metabolites as IAA contains N while NAA and ABA do not. The increase of N in flesh may be the result of increased vascular functionality, allowing more N-containing ions to be transported throughout the fruit.

While Ca was increased in both the peel and flesh by all treatments relative to the control, the increases were only significant in the peel of fruit treated with 20 ppm IAA (Table 9). While the overall increase (0.12% with IAA vs 0.08% without) was small, DeLong's (1936) initial attribution of bitter pit to Ca deficiency was the result of an observed 0.02% difference in Ca content between affected and healthy fruit, and other studies have confirmed small Ca differences are enough to cause cell necrosis and pit formation (Wilkinson, 1968; Perring and Pearson, 1986). While treatments did not produce significant effects on fruit flesh Ca content, peel Ca has been found to be a better predictor of fruit bitter pit susceptibility (Baugher et al., 2017; Marini et al., 2020). This is consistent with observations of a Ca gradient which exists within apple fruit with higher concentrations at the center of the fruit and decreasing Ca concentrations toward the peel and the knowledge that bitter pit lesions develop just below the peel in the outer cortex where Ca reaches its lowest concentration (Faust and Shear, 1968; de Freitas et al., 2010; Ferguson and Watkins, 1983).

K and Mg, two minerals known to exacerbate bitter pit when in excess relative to Ca, were not increased in either the flesh or the peel by IAA, NAA, and ABA treatments (Tables 9 and 10). This is consistent with the ability of these minerals to be transported in both the phloem and xylem while Ca is exclusively xylem-mobile (Garman and Mathis, 1956). However, these findings are not consistent with those of Falchi et al. (2017) which showed an increase in the amount of Mg allocated to fruit as a result of ABA treatment.

Like Ca, B was increased by auxin treatment in the peel but not the flesh (Tables 9 and 10). However, unlike peel Ca, peel B was most increased by treatment with 5 ppm NAA, though 20 ppm IAA did produce a numerical effect. Bitter pit resistance has been connected to higher B concentrations, and B sprays have been found to be effective in preventing bitter pit when used in combination with a synthetic auxin (Hewetson, 1965; Korban and Swiader, 1984), and B concentration has been positively correlated with Ca in fruit (Garman and Mathis, 1956; Fazio et al., 2015). B and Ca behave similarly in maintaining cell wall structure with both forming cross-linkages with pectins between cell walls (Fang et al., 2016). The increase of B as a result of NAA treatment may be a symptom of greater vascular functionality as, like Ca, B is known to be poorly-mobile in phloem tissue (Raven, 1977). The increases of B concentrations observed in the studied treatments are consistent with the increases in vascular bundle functionality caused by the treatments (Table 5) with 5 ppm NAA producing greater increases in primary bundle functionality than the other treatments considered.

Iron, manganese, copper, zinc, and to a lesser extent, sodium, are considered to be poorly mobile in xylem and therefore linked to fruit Ca concentrations and bitter pit susceptibility of fruit (Garman and Mathis, 1956; Raven, 1977; Fazio et al., 2015; Bonomelli et al., 2020). In this experiment, iron, copper, and sodium were observed to be significantly altered in fruit peels, and copper and sodium were observed to be altered in flesh tissue (Tables 9 and 10). Of these minerals, iron and sodium behaved similarly as each were increased by treatment with IAA and ABA, but not NAA while copper was increased in the peel by treatment with NAA and ABA and by NAA alone in flesh tissue. The significantly lower concentrations of these minerals in fruit compared to Ca mean that even two-fold increases in mineral content only accounts for 2 ppm difference in overall concentration change at most. However, a better understanding of the transport and function of these ions in relation to Ca and bitter pit may help to account for the significant differences observed between treatments.

Overall, useful differences were observed between treatments which demonstrate the powerful effects caused by applications of these plant growth regulators. We recognize that limited quantity of fruit may have precluded statistical separation allowing for potentially contradictory differences in the effects of NAA and IAA to be confirmed or rejected.

4.4.6. Leaf Nutrient Effects

Among extension shoot leaves, only copper was observed to have been significantly affected by the PGRs evaluated (Table 11) with 20 ppm NAA increasing copper relative to untreated leaves. Because trees were considered as individual replicates, this difference may be better explained by a tree-effect rather than a treatment effect.

Nutrient status of bourse leaves was considered to be more closely connected to the nutrient status of fruits than extension leaves due to their proximity on the spur. Interestingly, bourse leaf Ca and Mg were increased, though not significantly so, by increasing rates of ABA with 150 ppm ABA producing the greatest effect (Table 12). Both the Ca and Mg content of fruit from plants treated with ABA has been shown to increase in both tomato (Falchi et al., 2017) and apple (Angmo et al., 2022). The effect of ABA in reducing the marked difference between transpiration of leaves and fruit is expected. Though Ca and Mg content of bourse leaves did not significantly differ between treatments, B and sodium were observed to be significantly affect by treatments. However, while B was observed to have been affected by treatment, treatments were not different enough to have been separated by the Tukey HSD test.

4.5. CONCLUSIONS

All studied treatments except 20 ppm NAA and GA_3 were found to be effective in reducing bitter pit both at harvest and after storage. Each of the beneficial treatments were associated on vascular functionality, 20 ppm IAA most improving fruit redness and peel Ca, and ABA producing the greatest reduction of bitter pit after the storage period.

Due to the variety of compounds and concentrations used, further research is required to determine the ideal compounds, concentrations, treatment timings, and potentially compound combinations to use in bitter pit mitigation. As each of IAA, NAA, and ABA were observed to be promising in bitter pit mitigation, each warrant further study.

CHAPTER 5. A Preliminary Model to Estimate Annual Variations in Regional-Scale Bitter Pit Incidence for the Lower Peninsula of Michigan

5.1. INTRODUCTION

5.1.1. Bitter Pit and Its Yearly Variation

The apple cultivar ‘Honeycrisp’ has grown in popularity both with consumers who favor its fresh-eating characteristics and apple growers who appreciate its cold hardiness, apple scab tolerance, and long postharvest storage life (Watkins and Nock, 2012). Popularity with consumers has led ‘Honeycrisp’ to become the most profitable of the apple cultivars grown in the United States (Gallardo et al., 2015). However, ‘Honeycrisp’ is especially susceptible to bitter pit, a physiological disorder which has been linked with Ca deficiency (DeLong, 1936; Rosenberger et al., 2004). Because of the dark, circular lesions which appear on the surface of fruit, bitter pit renders apples unable to be sold for fresh consumption. While the disorder can appear on fruit prior to harvest in some cases, the disorder typically appears following storage of fruit (Telias et al., 2006).

Bitter pit incidence is known to vary significantly from year to year (Mix, 1916; Volz et al., 2006; Fallahi and Mahdavi, 2020). This variance has been attributed to differences in yearly weather patterns. Because bitter pit lesions form as cells collapse under water stress (Jones, 1973), various researchers have inferred that bitter pit is exacerbated by hot, dry weather (Faust and Shear, 1968; Wilkinson, 1968; Ferguson and Watkins, 1989; Grégoire, 2017; Fallahi and Mahdavi, 2020). However, others have suggested cool and wet weather may hinder fruit development (Brooks, 1908; McAlpine, 1912) as nearly all Ca uptake in fruit occurs early in the growing season (Saure, 2005). However, no study has definitively linked yearly variation in bitter pit incidence with specific weather conditions.

5.1.2. Variations in Calcium Status of Maturing Apple Fruit

Just as air temperature and rainfall vary from month to month, Ca uptake and concentration in fruit varies throughout the growing season. The early period in which apple fruit rapidly take up Ca corresponds to the cell division phase of fruit growth and the period in which leaves have not yet reached their full size and transpirational capacity (Lakso and Goffinet, 2013). Because Ca uptake is driven by transpiration, it could be expected that warmer, drier, sunnier, and windier conditions early in the growing season would each increase fruit Ca concentration. While Ca intake has been shown to be rapid during the first weeks of fruit development, Ca uptake slows to almost nothing by the second month of fruit development as the xylem elements which deliver it become dysfunctional (Tromp, 1979, Dražeta et al., 2004) and leaves begin to outcompete fruit for Ca due to their higher rate of transpiration (de Freitas et al., 2013). As a result, periods of increased transpiration later in the growing season result in higher leaf Ca, not fruit Ca (Hocking et al., 2016; Gomez and Kalcsits, 2020).

Ca demand also varies, increasing during periods of hot and dry weather typically associated with the second and third months of fruit development, and these periods aggravate Ca deficiency disorders (Bangerth, 1979; Ferguson and Watkins, 1989). Periods of high evaporative demand can also result in the reversal of xylem flow causing Ca to be removed from fruit and pulled back up the branch (Lang, 1990). However, warm and wet conditions early in the growing season encourage vigorous growth of vegetative tissue which competes with fruit for Ca, potentially also causing bitter pit (Saure, 2005; Fallahi and Mahdavi, 2020).

5.1.3. Limitations of Bitter Pit Incidence Data Collection

Historically, fruit that developed bitter pit while still on the tree were simply not harvested or counted by apple growers, while packing houses did not distinguish bitter pit from other conditions which caused fruit to be ruined in storage such as lenticel breakdown, leather blotch, and soft scald. As a result, it has been difficult to quantify annual bitter pit incidence across geographic regions. The recent adoption of automated systems such as the Compaq/Spectrim system described in Chapter 4 to diagnose disorders in apple fruit has allowed for record keeping to improve dramatically in regard to annual bitter pit incidence. Additionally, this data can also be linked with the orchards which supplied the apples, allowing for observations of local effects of weather on bitter pit incidence.

5.1.4. The Previous Weather Model Created by Grégoire (2017)

A model created by Grégoire (2017) utilized bitter pit incidence data of fruit samples provided by programs operated by the Canadian Ministry of Agriculture. Bitter pit incidence data was collected after fruit had been stored for four months to allow bitter pit to fully develop. These bitter pit samples were collected from 10 different sites across Ontario and Quebec from the years 2002-2006 and 2014-2015 for a total of 30 years-sites. The model was created with days after full bloom (DAFB) as the time variable to allow weather patterns to be linked with phenological stages of apple fruit development. The growing season was divided into four 30-day periods following full bloom. 80 potential model weather variables were considered before being reduced to the 20 variables most correlated to bitter pit incidence by principal components analysis. These 20 variables considered precipitation, relative humidity, and air temperature in each of the growing periods.

Through stepwise regression, Grégoire (2017) created four models based on the weather variables from 0-30 DAFB, 0-60 DAFB, 0-90 DAFB, and bloom to harvest, with adjusted-R^2 values of 0.652, 0.877, 0.899, and 0.865, respectively. The bloom to harvest model consisted of only two parameters: the number of hours with relative humidity <50% from 0-30 DAFB, and the number of hours with air temperature >30°C from 90 DAFB to harvest. The model indicated bitter pit incidence decreased with increasing hours of relative humidity below 50% early in the season and increased with hours at high air temperature late in the season. These findings are consistent with the hypothesis that high transpiration is beneficial for Ca uptake early in fruit development but results in water stress and eventual bitter pit formation later in the season when Ca uptake has largely ceased.

5.1.5. Objectives for the Creation of a New Model

The aim of this analysis is to explore whether the effects of potential evapotranspiration during the growing season can provide an estimate of interannual variation in regional-scale bitter pit incidence. Potential evapotranspiration (PET) is defined by the American Meteorological Society as "the amount of water evaporated (both as transpiration and evaporation from the soil) from an area of continuous, uniform vegetation that covers the whole ground and that is well supplied with water." While many methods exist for the calculation of PET, Enviro-weather (and therefore the created model calculates reference evapotranspiration (ET_0) with the modified Penman-Monteith equation described by Allen et al. (1998). This is a method for the calculation of PET for a standardized, well-watered grass crop based on net radiation at the crop surface, soil heat flux, air temperature, wind speed, and vapor pressure. These weather data are collected by Enviro-weather, therefore allowing for the calculation of ET_0.

While the model created by Grégoire (2017) was successful in modeling bitter pit incidence with only two parameters, relative humidity early in the growing season and air temperature later in the growing season, the impact of these specific factors on bitter pit incidence was not explored. As bitter pit has been shown to be exacerbated by both heat stress and water stress, transpiration, a variable which considers both factors, could be expected to better explain bitter pit incidence

(Fallahi and Mahdavi, 2020). Additionally, Ca is moved within plant tissues as the result of transpirational pull (Hocking et al., 2016). Potential evapotranspiration is an acceptable substitute for actual evapotranspiration as commercial orchards supply irrigation water to replace water lost to evapotranspiration. Because of the availability of calculated PET through Enviro-weather, this data is also readily accessible by apple industry stakeholders in Michigan.

Due to the role of transpiration in both fruit Ca uptake early in the growing season and Ca demand, heat stress, and water stress later in the growing season, it is hypothesized that high ET_0 early in apple fruit development and low ET_0 late in apple fruit development will each be correlated with lower risk of bitter pit occurrence. If weather, especially early in the growing season, can be linked to the risk of bitter pit occurrence, growers can respond by heavily employing the bitter pit mitigation techniques which currently exist such as calcium chloride and prohexadione calcium sprays, and packing houses could choose to market fruit for immediate consumption or processing rather than storage. In years predicted to have low bitter pit incidence, growers could utilize techniques which potentially increase bitter pit but have other benefits such as N, Mg, and K fertigation and cytokinin and/or GA sprays (Garman and Mathis, 1956; de Freitas and Mitcham, 2012). Additionally, a better understanding of the weather factors involved with bitter pit incidence would allow for projections to be made of the future effects of climate change on bitter pit incidence.

5.2. METHODS

5.2.1. Data Collection

Bitter pit incidence data was provided by Riveridge Produce Marketing of Sparta, MI. The data consisted of random samples of 20-50 fruit collected by Riveridge from bins of ‘Honeycrisp’ fruit provided by 60 growers from 10 counties throughout the state of Michigan from 2015-2021. However, most data were collected from the west side of the state where commercial apple cultivation is concentrated. The method by which bins were filled by growers was not known to the packing house, and bitter pit incidence varied from 0-100% within samples provided by the same grower.

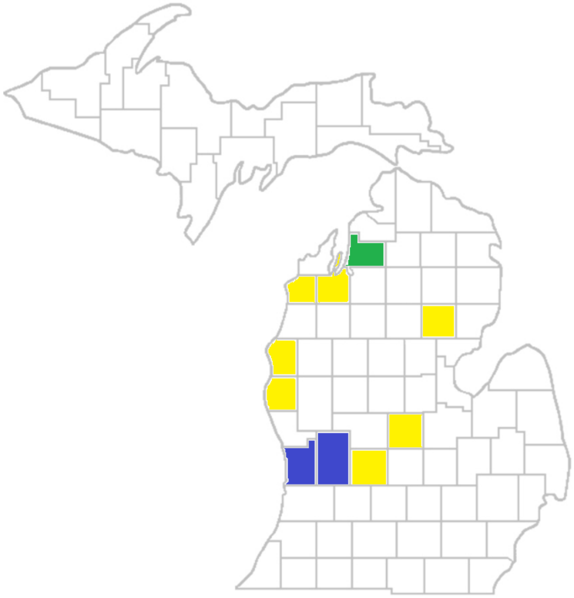

Figure 13: Map of the state of Michigan with counties marked with growers who provided fruit samples. County color corresponds to the relative number of samples provided by growers in each county over 2015-2021 with blue counties providing 50-55 samples, green counties providing 5-10 samples, and yellow counties providing <5 samples.

Observations were grouped according to geographic area corresponding to regions of roughly similar climate. Because of the independence of annual weather conditions, annual observations for a given geographic group were considered to be independent of each other. Likewise, observations for a group in a given year were considered to be independent of each other due to differences in geography. The climate of the groups, defined in Table 13, differ from each due to both latitude and distance to Lake Michigan, both of which have an effect on climate and weather patterns and therefore bloom date and fruit development. Within these five groups, bitter pit data was aggregated across all growers and samples for the given year and the median observation of bitter pit incidence (BPMed) in each year and in each region was selected. Only the region centered on Sparta, MI contained data for each of the seven years considered. As a result of the close proximity of the orchards in this group and the much higher number of samples produced in it relative to the other groups (123 samples compared to 140 total) and the fact that apple production in Michigan is centered on this region, two models were created: one for the state as whole and one for only the observations from this region.

Table 13: Median bitter pit incidence within regions for the years 2015-2021 as defined as proportion of sampled fruit displaying bitter pit symptoms. Entries marked with "-" contained no observations for that region/year.

Counties Within Region	2015 BPMed	2016 BPMed	2017 BPMed	2018 BPMed	2019 BPMed	2020 BPMed	2021 BPMed
Ottawa, Kent, Ionia	0.12	0.04	0.08	0	0.05	0	0.1
Benzie, Mason, Oceana	-	-	-	0	0.08	0.04	0.01
Grand Traverse, Antrim	-	-	-	-	0	0.04	0
Clinton	-	-	-	-	0	0.07	-
Ogemaw	-	-	-	-		0	0.02

ET_0 data was gathered using Michigan State University's Enviro-weather system which operates 89 weather stations throughout the state of Michigan and 14 in western Wisconsin. ET_0 data was calculated using the data provided by the weather station nearest to the orchard which provided the BPMed sample for a given year. In western Michigan, Enviro-weather stations were arranged in close proximity to orchards which provided samples (<5 miles). Observations associated with Ogemaw County, however, were taken from an orchard 38 miles from the nearest Enviro-weather station, limiting the applicability of weather data for that group.

Because ‘Honeycrisp’ bloom dates are not yet recorded by Michigan State Extension the way ‘Red Delicious’, ‘McIntosh’, and ‘Jonathan’ are, dates for each region and year were approximated using 400 growing degree days (GDD) using a 41°F (5°C) threshold, a number consistent with prior research (Degaetano, 2017). GDD were calculated with numerical integration of hourly data provided by Enviro-weather at mawn.geo.msu.edu. The calculated ‘Honeycrisp’ bloom dates were then compared to ‘McIntosh’ bloom dates from 2015-2021 recorded by Michigan State Extension. They consistently lagged behind ‘McIntosh’ by 0-3 days, a difference supported by MSU Extension observations (Einhorn, personal communication). The calculated ‘Honeycrisp’ full bloom dates varied substantially between the observed regions and years.

Table 14: Calculated full bloom dates for ‘Honeycrisp’ apples for each region and year. Bloom dates were calculated using numerical integration of growing degree days with a 41°F (5°C) threshold. Entries marked “-“ contained no observations for that region/year.

Counties Within Region	2015	2016	2017	2018	2019	2020	2021
Ottawa, Kent, Ionia	5/9	5/7	4/30	5/13	5/18	5/18	5/2
Benzie, Mason, Oceana	-	-	-	5/17	5/23	5/23	5/13
Grand Traverse, Antrim	-	-	-	-	5/28	5/27	5/15
Clinton	-	-	-	-	5/17	5/29	-
Ogemaw	-	-	-	-		5/24	5/8

For each year and region, average daily ET_0 was then averaged across two-week periods beginning with one DAFB and ending with 129 DAFB as ‘Honeycrisp’ fruit typically mature between 120-140 DAFB (see Chapters 4 and 5). Biweekly periods were chosen to provide a greater degree of granularity than would be achieved with monthly periods while still limiting the number of independent variables used in the model. The average daily ET$_0$ values of these nine biweekly periods (Weeks 1 and 2, 3 and 4, 5 and 6, 7 and 8, 9 and 10, 11 and 12, 13 and 14, 15 and 16, and 17 and 18) were assigned to each region and year.

5.2.2. Creation of the State-Wide Model

Because bitter pit data was only available for 2015-2021, a relatively small number of years, an exploratory approach was utilized to determine which of the biweekly periods had the greatest effect on median bitter pit incidence of a region. First, Pearson's correlation coefficient was calculated to determine the relationship between each of the daily average biweekly ET$_0$ values and median bitter pit incidence.

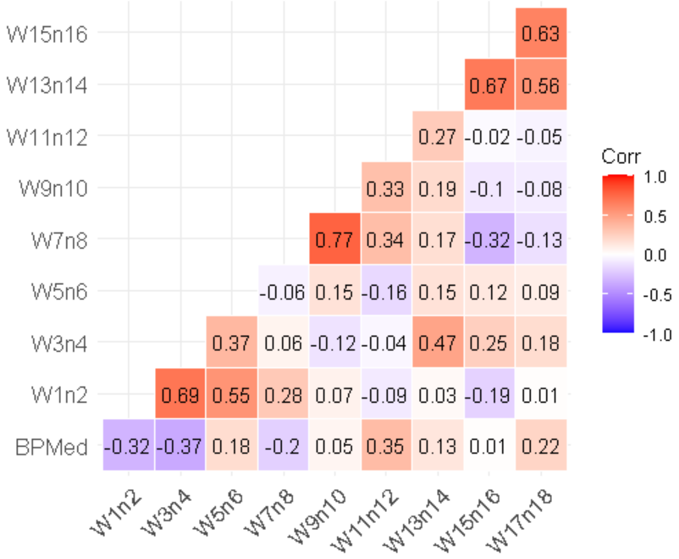

Figure 14: Correlation matrix displaying Pearson correlation coefficients to demonstrate the relationships between BPMed and the biweekly ET$_0$ values. "W1n2" corresponds to the average of the average daily ET$_0$ values for the first two weeks of apple fruit development, beginning one DAFB. Positive values denote a positive relationship between variables, while negative values denote a negative relationship.

Due to the small number of region/year observations of bitter pit, a regression model was created using only the biweekly, which showed the most correlation to BPMed, Weeks 1-2, Weeks 3-4, and Weeks 11-12. Additionally, as the ET$_0$ values of Weeks 1-2 and Weeks 3-4 were significantly correlated ($P < 0.05$), these variables were combined by averaging ET_0 values over the combined four-week period. Combining these biweekly periods also reduced the number of predictors and increased the degrees of freedom of the model. A least-squares linear regression model was created using BPMed as the dependent variable and the reference evapotranspiration of Weeks 1-4 and Weeks 11-12 as the independent variables and R's "lm" function (R Foundation, Vienna, Austria). The equation of the model is the following with positive values indicating a positive relationship with bitter pit incidence:

$$BPMed = -0.02421 - 0.02295 * Wk1234 + 0.03668 * W11n12$$

Equation 1: Linear least-squares regression of the relationship between the average ET_0 values of weeks 1-4 and weeks 11 and 12 of apple fruit development with bitter pit incidence.

5.2.3. Creation of a Kent/Ottawa/Ionia County Model

Due to the preponderance of data available for the data grouping centered on Kent, Ottawa, and Ionia Counties, the region with the greatest apple production in Michigan, a linear regression model was also created using data from only this region. The model was constructed similarly to the state-wide model by first creating a correlation matrix of the relationships between the variables.

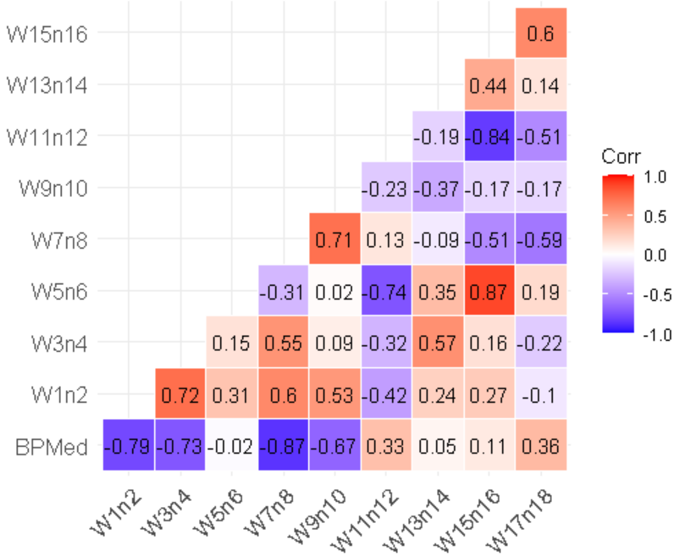

Figure 15: Correlation matrix displaying Pearson correlation coefficients to demonstrate the relationships between BPMed and the biweekly ET_0 values within the dataset gathered from orchards in Kent, Ottawa, and Ionia Counties. "W1n2" corresponds to the average of the average daily ET_0 values for the first two weeks of apple fruit development, beginning one DAFB. Positive values denote a positive relationship between variables while negative values denote a negative relationship.

Due to the small number of yearly observations of bitter pit in this dataset, a regression model was created using only the biweekly which showed the most correlation to BPMed, Weeks 1-2, Weeks 3-4, Weeks 7-8, and Weeks 9-10. As the ET_0 values of Weeks 1-2 and Weeks 3-4 were highly correlated ($P < 0.05$), these variables were combined by averaging ET_0 values over the combined four week period, as were Weeks 7-10. A least-squares linear regression model was created using BPMed as the dependent variable and the weather of Weeks 1-4 and Weeks 7-10 as the independent variable and R's "lm" function. The equation of the model is the following with positive values indicating a positive relationship with bitter pit incidence:

$$BPMed = 0.518 - 0.04756 * Wk1234 - 0.066 * W78910$$

Equation 2: Linear least-squares regression of the relationship between the average ET_0 values of weeks 1-4 and weeks 7-10 of apple fruit development with bitter pit incidence.

5.3. RESULTS

5.3.1. Analysis of the State-Wide Model

An adjusted R^2 of 0.145 and a P-value of 0.121 was obtained for the regression model based on Equation 1, indicating relatively poor prediction of bitter pit incidence values based on ET_0 values. When applied to the model, a Breusch-Pagan Test results in a P-value of 0.631, meaning the null hypothesis of the presence of heteroscedasticity cannot be rejected. Plotting residuals versus predicted bitter pit incidence indicates that a linear regression model is appropriate, but there is a slight tendency of the variance of residuals to increase at higher predicted values of bitter pit incidence.

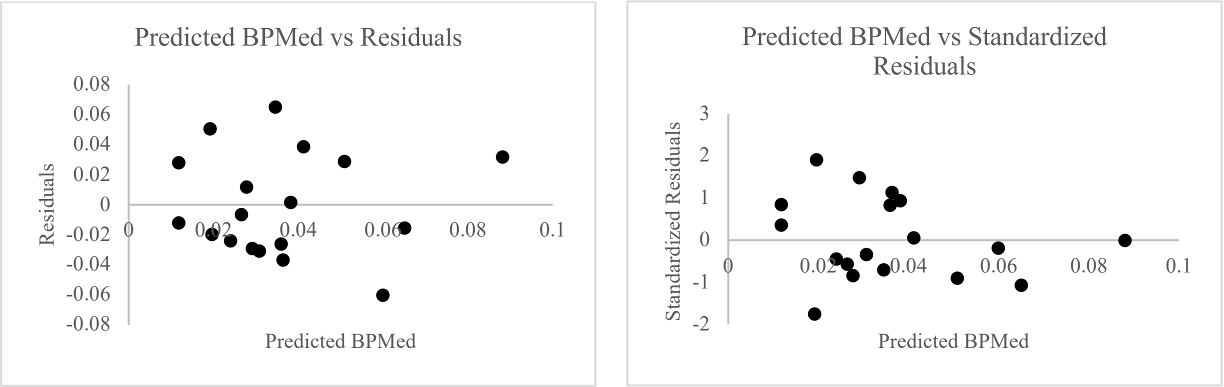

Figure 16: Plot of residuals against predicted median bitter pit incidence (left) and plot of standardized residuals against predicted median bitter pit (right) as a result of the state-wide model.

Analysis of Cook's distance of each of the observations reveals three outliers which correspond to observations from Kent/Ottawa/Ionia Counties in 2015 and 2021 and Clinton County in 2020. However, of these three outliers, only the observation from Kent/Ottawa/Ionia Counties in 2015 has high leverage on the model. This observation marks a substantially higher median bitter pit incidence than the other group/year combinations (0.12 compared to an average of 0.03 for the other observations). However, a bitter pit incidence of 12% is quite low for 'Honeycrisp' as much more bitter pit is typically observed in the cultivar (Rosenberger et al., 2004), so this observation is not unreasonable.

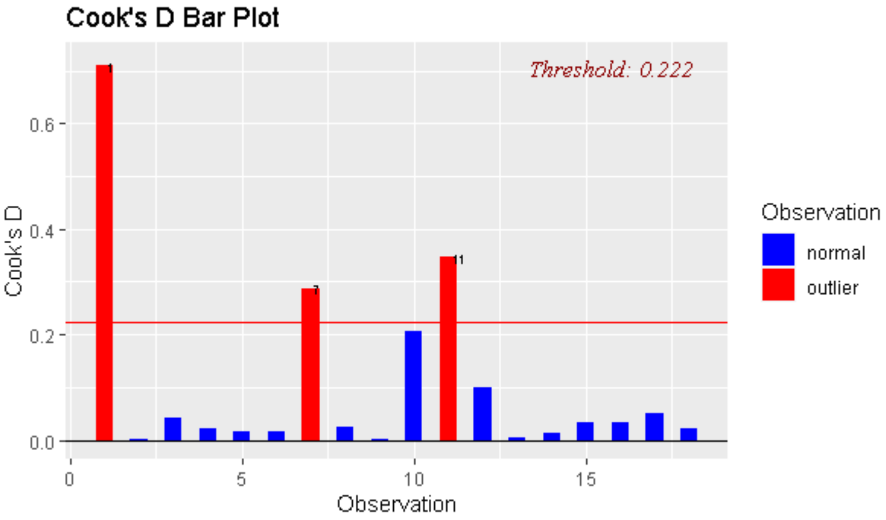

Figure 17: Plot of observations by Cook’s distance using a threshold of 0.222. Outliers are marked in red.

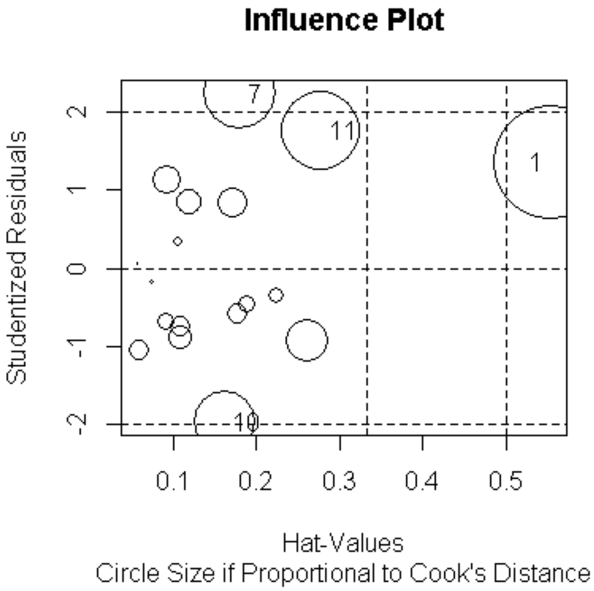

Figure 18: Plot of studentized residuals by leverage values. Size of circles is proportional to Cook’s distance.

When standardized using the “lm.beta” function in R, the coefficients of the two predictor variables become -0.35 for the Wk1234 variable and 0.33 for the Wk11n12 variable, indicating nearly equal contribution to bitter pit incidence from ET_0 in the first four weeks of apple fruit development and ET_0 in the 11th and 12th weeks of apple fruit development. However, the opposite sign of the coefficients indicate an association of increased ET_0 early in the season with decreased bitter pit incidence and an association of increased ET_0 late in the season with increased bitter pit incidence.

5.3.2. Analysis of the Kent/Ottawa/Ionia County Model

An adjusted R^2 of 0.845 and a P-value of 0.011 was obtained for the regression model based on Equation 2, indicating a relatively good prediction of bitter pit incidence values based on ET_0 values. When applied to the model, a Breusch-Pagan Test results in a P-value of 0.051. While the null hypothesis of the presence of heteroscedasticity cannot be rejected, this P-value is much lower than that of the state-wide model. Plotting residuals versus predicted bitter pit incidence indicates that a linear regression model is appropriate, but there is a slight tendency of the variance of residuals to increase at higher predicted values of bitter pit incidence.

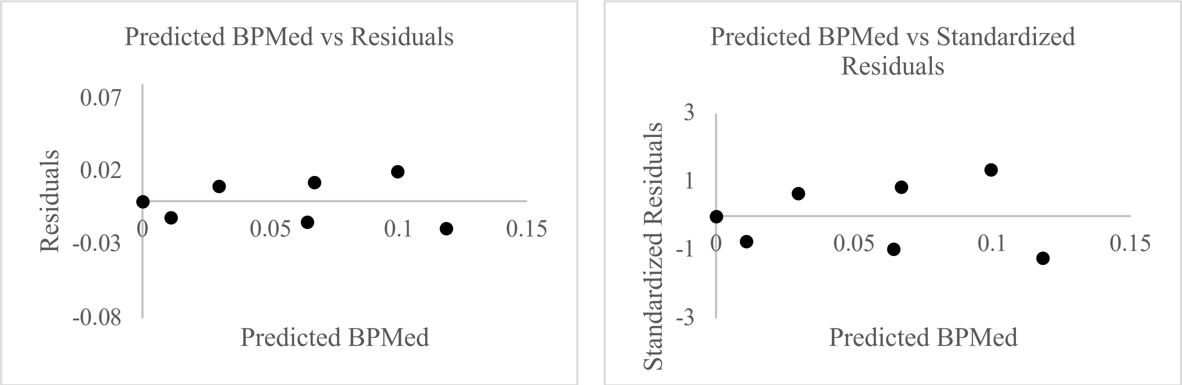

Figure 19: Plot of residuals against predicted median bitter pit incidence (left) and plot of standardized residuals against predicted median bitter pit (right) as a result of the Kent/Ottawa/Ionia County Model

Analysis of Cook's distance of each of the seven observations reveals two outliers which correspond to the years 2018 and 2020, years where the median bitter pit incidence of the samples taken from Kent, Ottawa, and Ionia Counties was 0%. Both of these observations demonstrate high leverage on the model, but the leverage of the 2020 observation was extremely high. While bitter pit incidence was observed to be low in western Michigan in both of these years (Einhorn, personal communication), the median incidence of 0 from this dataset was certainly much lower than the actual bitter pit incidence. It should also be noted that while the 2015 observation from this group was an outlier in the statewide model, it was not in the model that only considered Kent, Ottawa, and Ionia Counties.

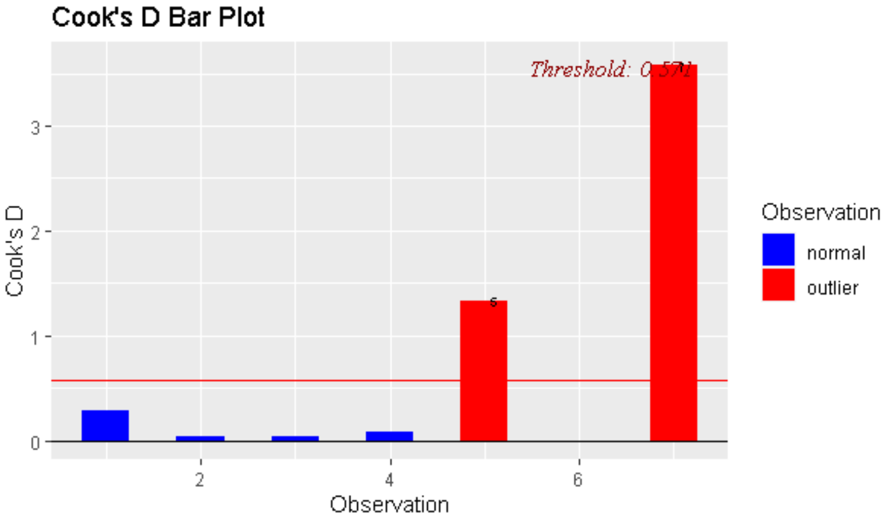

Figure 20: Plot of observations by Cook's distance using a threshold of 0.571. Outliers are marked in red.

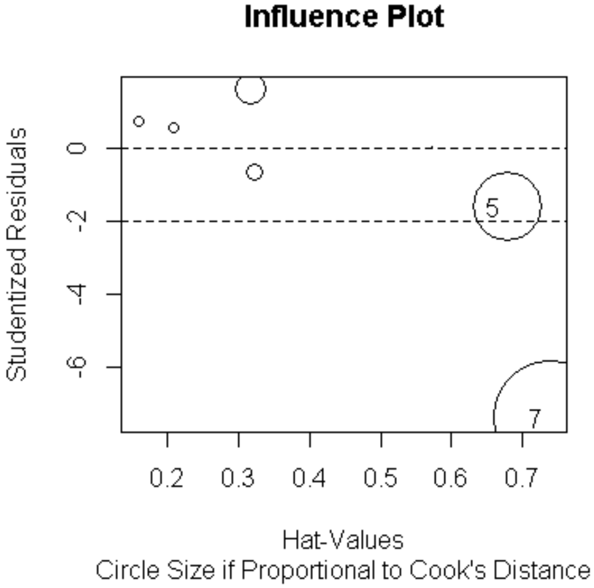

Figure 21: Plot of studentized residuals by leverage values. Size of circles is proportional to Cook's distance.

When standardized using the "lm.beta" function in R, the coefficients of the two predictor variables become -0.468 for the Wk1234 variable and -0.594 for the Wk78910 variable. These coefficients indicates ET_0 during Weeks 7-10 of apple fruit development is slightly more influential to bitter pit development than ET_0 during Weeks 1-4. Both coefficients are negative, indicating higher ET_0 during these periods results in lower bitter pit incidence according to the model.

5.4. DISCUSSION

5.4.1. Limitations of the Dataset

As more packing houses adopt sophisticated techniques of identifying and quantifying bitter pit incidence among apple fruit, annual regional bitter pit incidence datasets will improve in accuracy. The dataset considered in this experiment consisted of data from only one packing house. While 60 growers contributed fruit samples which combined to form the dataset, the vast majority of samples (124 out of 140 total observations) were from Kent, Ottawa, and Ionia Counties. While samples were theoretically chosen at random, many of the apple samples had no bitter pit at all (39 out of 140 total observations). As ‘Honeycrisp’ is highly susceptible to bitter pit, it is unlikely that 0% incidence value for a sample was representative of the total bitter pit incidence of an orchard block. Additionally, a large proportion of observations from orchards outside of Kent, Ottawa, and Ionia Counties (8 out of 24) had a bitter pit incidence of 0%, resulting in the dependent variable for 5 of 11 of these group/year being 0% median bitter pit incidence (Table 13). This is the result of the much smaller number of samples from these other regional groups compared to Kent, Ottawa, and Ionia Counties, likely due to the increased distance to the Riveridge packing facility.

Bitter pit incidences also varied significantly between growers in a similar geographic area within a given year. These differences could be the result of different degrees of bitter pit control such as Ca sprays, Mg and K fertigation, N fertilization, rootstock selection, and irrigation (Ferguson and Watkins, 1989) as none of these variables were addressed by the regression model. The wide range of bitter pit incidences among this group in a given year (in some cases varying from 0-100%) indicate the difficulty in determining the actual bitter pit incidence of a region and potential issues with sampling. It is also possible that samples could have been selected from blocks within the orchard more prone to bitter pit development due to potential differences in irrigation, soil conditions, tree age, pruning, etc. (Faust and Shear, 1968; Saure, 2005; Marini et al., 2020). Ideally, fruit samples should be selected at random from throughout the orchard instead of being grouped by block. Bitter pit incidence data was also collected by assessing fruit at harvest, even though fruit typically develop symptoms following storage (Telias et al., 2006).

While the total number of samples used to construct this model was quite high at 140 samples, this was reduced to just 18 group/year observations of median bitter pit incidence. The choice to reduce the number of samples to group/year observations was intended to preserve the independence of the predictor variables, but the regional groupings created still experienced similar weather patterns from year to year due to their geographic proximity. The model could be improved with bitter pit incidence data from sites further geographically removed from the Lower Peninsula of Michigan.

While average biweekly ET_0 as a weather variable is both convenient and incorporates many of the weather conditions likely to affect bitter pit such as solar radiation, wind speed, temperature, and humidity, the variable does not account for daily or even weekly ET_0 differences. Especially early in fruit development, apple fruit development changes rapidly over 14 day periods. While average biweekly ET_0 early in the growing season was found to be strongly correlated with median bitter pit incidence (Figures 14 and 15), it is difficult to determine exactly which phenological stage of fruit development is affected by changes in ET_0 and what relation this has to bitter pit development as fruit mature.

5.4.2. Effect of Temporal ET_0 Changes on Bitter Pit

Both the state-wide and Kent/Ottawa/Ionia County models indicated that higher average ET_0 during the first four weeks of apple fruit development resulted in lower bitter pit incidence (Equations 1 and 2). This period corresponds to the period in which apple fruit growth is driven by cell division rather than cell expansion (Lakso and Goffinet, 2013). The rate of cell division has been observed to be affected by temperature, with higher temperatures resulting in more cell division (Ferguson and Watkins, 1989). Because application of TIBA during this period has been shown to be most effective in curtailing cell division and increasing bitter pit incidence, it can be assumed that a greater degree of cell division results in fruit less susceptible to bitter pit, potentially through increased differentiation of xylem tissue and increased Ca uptake (Ferguson and Watkins, 1989; Lakso and Goffinet, 2013).

The state-wide model indicated increased average ET_0 in Weeks 11 and 12 of apple fruit development to result in increased bitter pit incidence (Equation 1). This finding is consistent with the known effects of heat/water stress on bitter pit incidence (Fallahi and Mahdavi 2020) and the model created by Grégoire (2017). However, the state-wide model was not significant. The Kent/Ottawa/Ionia model was statistically significant, indicating increased average ET_0 from Weeks 7-10 of apple fruit development resulted in decreased bitter pit incidence (Equations 1 and 2). Weeks 7-10 of apple fruit development correspond to 49-70 DAFB, the period in which the bitter pit susceptible 'Braeburn' (Dražeta et al., 2004) and 'Honeycrisp' (see Chapters 3 and 4) experience rapid xylem dysfunction. As xylem dysfunction is believed to be exacerbated by rapid fruit growth, higher ET_0 during this period could be expected to increase the susceptibility of fruit to bitter pit, contrary to the indications of the model. The time period indicated as significant to bitter pit development by this model is earlier than the late-season period (90 DAFB to harvest) determined to be influential by Grégoire (2017), but still corresponds to a period in which Ca uptake of apple fruit has essentially ceased (Tromp, 1979). While the findings of Grégoire (2017) indicate that the influence of high temperatures in the fourth and fifth months of fruit development is correlated with bitter pit incidence match the anecdotal findings by previous researchers that weather conditions in August and September contribute the most to bitter pit incidence (Brooks, 1908; Ferguson and Watkins, 1989; DeBrouwer et al., 2020), the Kent/Ottawa/Ionia model described above suggests weather in the late second and third months of fruit development is a better predictor of bitter pit incidence.

5.4.3. Improving the Models

The greatest limitation of the dataset was the dependent variable, median bitter pit incidence of samples in group each year, and how it was collected. Bitter pit incidence values for samples ranged from 0-100% even among samples taken from the same orchard. This wide range of incidences necessitated the selection of median bitter pit incidence rather than mean bitter pit incidence for a region. However, due to the large number of samples without any bitter pit, the median bitter pit incidence for one-third of all group/year combinations was 0 (Table 13). A standardized protocol for fruit sampling would allow for samples to be better representations of the blocks and orchards from which they were taken. The relatively small number of observations from outside Kent, Ottawa, and Ionia Counties also reduced the significance of the state-wide model substantially. While the Kent/Ottawa/Ionia model was much more significant than the state-wide model, it suffered from a very limited number of observations, 7, of which 2 were considered outliers by Cook's distance (Figure 21). In the absence of an increased number of observations, both the state-wide and Kent/Ottawa/Ionia models would benefit from multiple regressions wherein problematic data points are removed and estimated again using the model to determine which group/year observations are likely erroneous.

The choice to use the average of daily cumulative ET_0 values across a two week period blurs the hourly and daily changes in transpiration experienced by apple fruit. While daily cumulative ET_0 value provides a good indication of the total transpiration of apple fruit from one day to the next, it does not account for hourly variation in ET_0. Additionally, as commercial orchards are not typically irrigated during the periods of highest evapotranspiration to conserve water, the actual heat/water stress experienced by plants is not perfectly modeled by the choice of daily cumulative ET_0 as a contributor to the independent variable. Likewise, averaging ET_0 across biweekly periods has the advantage of increasing the degrees of freedom of the model, but blurs the exact phenological stages of apple fruit development which are affected by change in evapotranspiration.

5.5. CONCLUSIONS

Because weather is only a secondary factor to bitter pit incidence due to its effect on fruit growth and mineral uptake, a weather model will never be able to predict bitter pit incidence for an individual orchard in a given year. The purpose of this model was to explore the effects of changes in ET_0 at various stages of apple fruit development on bitter pit incidence at harvest. The analyses illustrate the potential for estimation of median annual bitter pit incidence within a geographic region of relatively similar weather conditions. While the state-wide model was not statistically significant, the Kent/Ottawa/Ionia model suggests that higher ET_0 in both the early and middle portions of the growing season reduces bitter pit occurrence. Increased early season transpiration may correspond to increased cell division and vascular development allowing for increased Ca uptake, but it is unknown how increased mid-season ET_0 might decrease bitter pit incidence as Ca uptake slows to a virtual stop during the second and third months of apple fruit development as fruit xylem becomes dysfunctional (Tromp, 1979, Dražeta et al., 2004).

While the models in this chapter suffered from dataset limitations, they demonstrate the potential use of models for use in bitter pit prediction based on weather factors. As bitter pit detection and record keeping technologies improve and weather-based models become more powerful, these models could be combined with biological models like those created by Marini et al. (2020) to create even more comprehensive models for bitter pit prediction. However, given the exceptionally high number of predictive variables which would be present in such a model, a large number of observations over multiple years would be necessary for its creation.

CHAPTER 6. Conclusion

6.1. OVERVIEW

The apple cultivar ‘Honeycrisp’ is popular with growers and consumers alike (Gallardo et al., 2015). However, ‘Honeycrisp’ is highly susceptible to the bitter pit, and many fruit are lost to the disorder every year. Bitter pit has been linked to Ca deficiency, but applications of Ca to fruit do not completely mitigate the disorder (Willis et al., 1976). Observations in the last 100 years have highlighted the complexity of bitter pit suggesting roles of hormones and weather in its incidence. The experiments described in this thesis were designed to help further the understanding of the cause of bitter pit lesion formation and explore potential options for mitigation of the disorder.

6.2. SUMMARY OF FINDINGS

Because bitter pit results from Ca deficiency and Ca is only mobile in xylem *in-planta*, dysfunction of xylem in fruit has linked with bitter pit incidence (Lang, 1990). Auxins contribute to the differentiation of xylem (Aloni 1980) while GAs lignify xylem, making tracheary elements more prone to dysfunction (Johnsson et al., 2018). In chapter 3, auxins, GAs, and the auxin transport inhibitor, TIBA, were applied to the surface and pedicels of fruit and to spur leaves and the effect of these applications on fruit vascular functionality and bitter pit incidence was observed. The concentrations of auxins applied to fruit pedicels were supraoptimal and toxic to the developing fruit (Figure 8). Little difference was observed between treatments in regard to vascular function or bitter pit incidence, regardless of whether they were applied to fruit or leaves (Figures 9 and 10). However, when applied to leaves, TIBA reduced vascular bundle functionality and significantly increased bitter pit of fruit, suggesting TIBA is readily absorbed by leaves and transported to fruit.

In chapter 4, auxins and ABA were applied as whole-tree sprays and their effect on fruit vasculature and bitter pit incidence was observed. 20 ppm IAA had the most significant effect, reducing average bitter pit rating of fruit by 85% relative to the control (Table 6). 75 ppm and 150 ppm ABA treatments were similarly effective. However, the significant effect of these treatments diminished after fruit were stored for 14 and reassessed. While IAA and ABA had the greatest effect in mitigating bitter pit at harvest, 10 ppm NAA produced the greatest increase in vascular functionality in mature fruit (Table 5) implying bitter pit incidence is not a function of xylem functionality alone. 20 ppm NAA reduced fruit mass (Figure 12), mature seed content (Table 7), and increased green coloration of fruit (Table 8), demonstrating toxic effects of NAA at this concentration.

Bitter pit incidence has been observed to vary annually (Fallahi and Mahdavi, 2020). This variation suggests weather affects bitter pit occurrence. In chapter 5, two regression models were created using bitter pit incidence data from samples collected by a packing house: the first model utilized data from samples taken from throughout the Lower Peninsula of Michigan while the second model used only samples collected from Kent, Ottawa, and Ionia Counties as commercial apple production in Michigan centers around this geographic region. The effect of weather was considered in the form of average biweekly ET_0 to determine the effect of variations of evapotranspiration at various phenological stages on bitter pit development. While the state-wide model was not found to be statistically significant, the Kent/Ottawa/Ionia model was determined to be significant. This model indicated higher ET_0 in Weeks 1-4 and also in Weeks 7-10 of apple fruit development results in less bitter pit occurrence in the region considered (Equation 2).

While the finding that higher ET_0 in Weeks 1-4 is consistent with previous bitter pit models and the period in which Ca uptake to fruit is assisted by increased transpiration, the same finding in Weeks 7-10 is not consistent with observations that Ca uptake of apple fruit essentially ceases by the second month of fruit development when xylem becomes dysfunctional in apple fruit (Tromp 1979, Dražeta et al., 2004). Due to issues with sampling, however, the Kent/Ottawa/Ionia model was constructed with a small number of observations and suffered from the influence of outliers (Figure 21).

6.3. FUTURE RESEARCH

The efficacy of IAA and ABA sprays demonstrated in chapter 4 suggest that both auxins and ABA may be beneficial in bitter pit mitigation. However, this experiment must be replicated to confirm these findings. The ideal concentrations of these compounds and the proper timing of their application must be determined. Additionally, the mode of action of these sprays must be determined as the most efficacious placement of application was not fully explained by the experiments described in chapter 3.

The weather model described in chapter 5, while limited by the dataset, demonstrated the potential of models for regional bitter pit incidence estimation for areas of similar climate. Improved sampling and an increased number of observations could potentially allow these models to be combined with biological models for the use of the apple industry.

Taken together, the findings of the three chapters indicate that bitter pit is more complex than its definition of a Ca-deficiency disorder would indicate. Bitter pit is a complicated physiological disorder which is also influenced by hormones and weather. Further research will be needed to connect seemingly disparate observations of contributions to bitter pit incidence.

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LITERATURE CITED

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