SPRING FOLIAR APPLICATION OF NITROGEN FERTILIZERS AND PLANT GROWTH REGULATORS TO SWEET CHERRY (*PRUNUS AVIUM*) SPUR LEAVES

By

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ABSTRACT

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The objective of this study was to investigate the effect of nitrogen fertilizers and plant hormones applied to sweet cherry (*Prunus avium* L.) spurs in early spring on spur leaf growth and morphological features. Preliminary studies (2017) with isolated spurs showed that gibberellic acid (GA_3, 30 ppm), 6-benzylaminopurine (BA, 150 ppm) and 6-benzylaminopurine + gibberellic acid (150 ppm BA + 30 ppm GA_{4+7}) increased total spur leaf area 30%, 37% and 47%, respectively. All three nitrogen fertilizer treatments showed no significant differences with the control. At the microscopic level, the leaf adaxial epidermis cell size was increased from 20% to 40% in the plant hormone treatments. Follow-up studies at the whole tree level (2018) showed that: 1) Rate Experiment. 150 ppm BA + 30 ppm GA_3 and 75 ppm BA + 15 ppm GA_3 applications increased total leaf area per spur by 59% and 55%, respectively. 2) Timing Experiment. 30 ppm GA_3 applied twice (when three emerging leaves were present and after accrual of an additional 100 Growing Degree Days) increased total leaf area by 36%. 3) Gibberellins Experiment. 30 ppm GA_3 increased total leaf area by 33%. The whole trees experiment did not affect the area of individual leaves; for the Timing and Rate Experiments, the larger total leaf area per spur was due primarily to the emergence of more leaves per spur.

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CHAPTER 1: INTRODUCTION

The impact of new sweet cherry rootstocks on modern orchards

Sweet cherry (*Prunus avium* L.) production, yield, and fruit quality has advanced worldwide over the past two decades to meet growing market demands. United States sweet cherry production in 2017 totaled 437,600 tons, ranking as the second-largest producer (after Turkey) of sweet cherries in the world. Around 75% of U.S. sweet cherry production is destined for the fresh market (NASS 2017). Developments in new rootstocks, training systems and cultivars have played very important roles in yield improvement.

In the 1960s and 1970s, several cherry rootstock breeding programs were started around the world with the main purpose of decreasing tree size, since hand-harvest of such small fruits on large trees requires extensive and inefficient labor (Hrotko and Rozpara, 2017). In rootstock breeding, the predominant source of parental materials has come from the subgenus *Cerasus* (Rehder, 1974). Major species in the parentage of rootstocks include *P. avium*, *P. cerasus*, *P. canescens* Bois, *P. fruticosa*, and *P. mahaleb* L. In addition to these four species, *P. x dawyckensis* Sealy, *P. incisa* Thunb., *P. concinna* Koehne, *P. serrulata* L., *P. subhirtella* Miq., *P. pseudocerasus*, P. *tomentosa*, and *P. serrula* also are used as rootstocks or in rootstock breeding programs (Webster and Schmidt, 1996). Some hybrids of the above species also are considered as useful materials in rootstock breeding (Kappel *et al*.,

2012). Besides the subgenus *Cerasus*, two genotypes of *P. cerasifera* Ehrh., from the subgenus *Prunophora*, have been used as interstocks or rootstocks: Adara and Myrobalan ‘R1’ (Moreno *et al*., 1996; Lang, 2006).

In the 1980s, cherry rootstock breeding objectives had been expanded beyond size control to include increased scion precocity and cropping, improved graft compatibility, uniformity in performance, cold hardiness, adaptation to a wide range of soils, and disease and pest tolerance (Perry, 1987). These breeding objectives are still of importance, although the order of priorities may vary. The ideal goal is to develop productive orchards that are “pedestrian orchards”, for which all work can be completed without the use of ladders. Although the major target is tree vigor reduction, the balance among multiple objectives needs to be considered.

Major rootstock breeding sources for scion vigor control include *P. fruticosa* and *P. cerasus*, as well as *P. canescens* (Wolfram, 1971; Trefois, 1980; Gruppe,1985; Wolfram, 1996). Several species within section *Pseudocerasus* (*P. pseudocerasus* and *P. serrulata*) might be considered as further sources, but the hardiness and drought tolerance of these hybrids in continental climates need more investigation (Cummins, 1979a, b). Additionally, from standard to medium vigor rootstocks have been found in selections of *P. mahaleb* in the last 15 years (Hrotkó, 2004; Hrotkó and Magyar, 2004; Lang, 2006; Sotirov, 2012; Barać *et al*., 2014). Also, genetic dwarf scion genotypes may, the future,

be considered as potential strategies for scion vigor control.

Rootstocks also influence branch growth habit. Wider branch angles were observed in some *P. avium* and *P. pseudocerasus* clones and *P. mahaleb* 'Magyar' (Webster and Schmidt, 1996; Hrotkó *et al*., 1999), as well as several of the Gisela series (e.g., Gi5, Gi6) of *P. cerasus* x *P. canescens* hybrids (Schaumberg and Gruppe, 1985). Conversely, scions on 'MxM 14' and 'MxM 97' (*P. avium* x *P. mahaleb* hybrids) showed narrower branch angles (Hrotkó *et al*., 1999).

Tree precocity, cropping and fruit quality of scion cultivars can be positively influenced by rootstocks. Scions on Mahaleb seedling rootstock have been shown to produce fruit 1 ~ 2 years earlier than on Mazzard rootstocks (Perry, 1987; Hrotkó, 1990; Hrotkó *et al*., 2008; Stachowiak *et al*., 2014). However, precocity, cropping and fruit quality are controlled by the combined effects of rootstock, training and pruning, tree spacing and nutrition. Therefore, the effect can of rootstock on these can only be determined in specific field trials.

Rootstock graft compatibility varies from species to species and genotype to genotype. In some cases, optimal growth practices (particularly use of virus-free plant materials) and environmental conditions reduce the occurrence of incompatible symptoms (Quero-Garcia *et al*., 2017).

For rootstock tolerance to cold climate winter conditions, *P. cerasus* and *P.*

fruticosa are considered to confer the best cold hardiness for rootstock breeding, and Mahaleb is hardier than Mazzard. Also, *P. avium* is the least hardy species within the *Eucerasus* section (Perry, 1987). In drought and heat tolerance, the most tolerant rootstocks appear to be the *P. mahaleb* selections and their hybrids (e.g., Mazzard × Mahaleb [M×M] series) (Quero-Garcia *et al*., 2017).

Rootstocks show different adaptabilities for diverse soil conditions. In general, cherry root systems are not well-adapted to poorly drained or wet soils. Specific rootstocks can be found to fit for some soil types. For example, if drought tolerance is required, Mahaleb can confer some benefits. And Mazzard is often used where poorer drainage is known to occur. In the northwest provinces of China, Mahaleb seedlings (e.g., 'Cema') have proven to be tolerant to calcareous and high pH soils (Hrotkó and Cai, 2014).

Tree nutrient content and water status can be affected by rootstock. Less calcium was observed in the leaves of trees on the dwarf rootstocks 'P-HL-A' and 'P-HL-C' than those of control trees (Sitarek *et al*., 1998). Also, higher Fe, Cu, Zn elements were found in fruit from trees on Mahaleb roots on sandy soil, and higher Fe, Cu and Cr were found in leaves in Poland (Stachowiak *et al*. 2015).

Rootstocks also can affect tree tolerance or resistance to pests and diseases. For instance, *P. mahaleb* showed less tolerance to root-knot nematodes

(*Meloidogyne incognita*) than Mazzard, but it was more resistant to root-lesion nematodes (*Pratylenchus vulnus*) than Mazzard (Webster and Schmidt, 1996; Hartmann *et al*., 2002). On the other hand, all rootstocks are sensitive to *Verticillium* spp., and there is no known source of resistance.

In modern high-density orchards, tree height has decreased by as much as 2.5~4.0 m (compare with 12~18 m mature heights of seedling sweet cherry trees in nature), and typical tree density varies from 667 to 1250 trees ha^{-1} by using size-reduction rootstocks. Consequently, contemporary sweet cherry orchards can and must yield high quality fruit, usually on a precocious, productive, dwarfing to semi-dwarfing rootstock. The relative importance of these three key characteristics depends on the orchard site and the economic requirements of different orchard operations. As labor efficiency becomes increasingly important in modern high-density orchards, scion vigor control will be in higher demand. There can be disadvantages of scion vigor control, for example, some highly efficient dwarfing rootstocks can increase the fruit-to-leaf area ratio and thereby have a negative effect on fruit size (Quero-Garcia *et al*., 2017). For this reason, new training systems are another important factor in modern pedestrian orchard development.

New sweet cherry training systems

Traditional sweet cherry training systems have complex canopies,

take years (usually 5~7) to fully develop, lack a systematic plan to renew fruiting

wood, and are difficult to clearly and specifically explain (Long *et al*., 2015). Consequently, a number of new sweet cherry tree training systems have been developed to meet modern orchard requirements. Seven major training systems have been described recently in detail (Long *et al*., 2015) and they can be divided into two parts: multiple leader canopy architectures and single leader canopy architectures.

For multiple leader systems, the Kym Green Bush (KGB) and Spanish Bush (SB) are free-standing trees that consist of multiple temporary (renewable) vertical fruiting units or multiple permanent leaders with temporary small fruiting laterals, respectively. The Upright Fruiting Offshoots (UFO) is a multiple leader narrow fruiting wall that consists of permanent horizontal cordon-like structure and multiple temporary vertical fruiting units (similar to KGB) that are fully supported by a trellis system. Also, the UFO system can be trained as a single-cordon or a dual-cordon UFO canopy (Long *et al*., 2015). The Steep Leader (SL) training system is configured similar to a Tall Spindle Axe (see below), but instead of a single leader, it has three or four closely-spaced vertical leaders instead to create a wider canopy and capture more light. Each leader mimics a one-quarter of a spindle tree canopy.

For single leader systems, the Vogel Central Leader (VCL) system is a freestanding, single leader tree with tiers of renewable fruiting scaffolds and it has a conical or pyramidal shape. The Tall Spindle Axe (TSA) is an evolution of the VCL system and is still a freestanding, single leader tree, but the canopy is

characterized by a continuous whorl of moderately vigorous lateral branches. The Super Slender Axe (SSA) is a very high-density system (up to 4,400 trees ha^{-1}) of central axis trees with very short limbs that are pruned severely every year. It fruits primarily on non-spur flower buds at the base of 1-year-old shoots, which is quite different from other systems (Long *et al*., 2015). The minimization of spurs on SSA trees means that most fruit growth (i.e., from the basal buds) is supported by the relatively large leaves typical of new shoots on well-managed trees.

Integration of tree training systems with dwarfing rootstocks must take into account anticipated fruit set, balancing leaf area with crop load, and the promotion of fruit quality suitable for the target market. Achieving a pedestrian orchard depends on the appropriate combination of dwarfing rootstocks and training systems. For example, the single leader systems can create fully-pedestrian orchards with dwarfing rootstocks or semi-pedestrian orchards with semi-dwarfing rootstocks (Long *et al*., 2015). Training systems also must be matched to cultivar growth habits. For example, non-spur type varieties such as ‘Regina’ and ‘Attika’ perform well on SB and SSA because the fruit are largely produced from the basal buds of 1-year-old shoots (Long *et al*., 2015).

New sweet cherry varieties

Early in the 20th century, sweet cherry breeding involving selection of elite parents was undertaken in the United State (USA) and Canada, followed in the middle of the century by European countries such as the United Kingdom (UK),

Russia, Ukraine, and others. The USDA cherry breeding program at Washington State University (Prosser, WA) had its first major release with the popular blush variety Rainier in 1952, and in the last 60~70 years, numerous breeding programs have been developed throughout the world to meet the increasing demands of growers for locally-adapted varieties. The release rate of new cultivars was relatively high during the period 1991~2004 (Sansavini and Lugli, 2008). In the last 30 years, breeding has progressed rapidly, partly due to the increased interest in high-density orchards and dwarfing rootstocks (Hrotko and Rozpara, 2017). Current sweet cherry breeding goals include precocity and productivity, resistance to rain-induced cracking, resistance to diseases and insects, improved fruit quality, extension of the ripening season, and resistance to environmental stress.

Modern high-density orchards require higher capital investment, and therefore a quicker return on investment, and precocity can help meet this demand. For example, ‘Sweetheart’ is very precocious even on the standard rootstocks and can be used as a standard benchmark for sweet cherry cultivar precocity (Kappel *et al*., 2012). Productivity is another extremely important need of growers. Increased yield must be balanced with fruit quality (especially fruit size) and yield (Omeg and Omeg, 2005). That is, very high yields of small poor quality fruit are uneconomical for growers. Precocity also can be highly influenced by rootstock and training system.

Rain-induced cracking is one of the most damaging problems in many cherry

growing regions of the world. In some years, the proportion of cracked fruits can be up to 90% in some susceptible sweet cherry cultivars, causing a complete economic loss (Christensen, 1996). Breeding for resistance to rain-induced cracking is a highly desirable goal, yet with little success thus far. There are very few commercial cultivars with a high and consistent tolerance to rain-induced cracking (e.g., 'Regina' and 'Fermina').

Sweet cherry trees are susceptible to a range of pests and diseases. Major diseases include powdery mildew (*Podosphaera clandestina*), brown rot (*Monilinia* spp.), leaf spot (*Blumeriella jaapii* (Rehm)), bacterial canker (*Pseudomonas* spp.), Cytospora canker (*Leucostoma* spp.), and various viruses. Key insect pests include cherry fruit fly (*Rhagoletis* spp.), spotted wing drosophila (*Drosophila suzukii*), black cherry aphid (*Myzus cerasi* Fab.), and cherry slug (*Caliroa cerasi* L.). Studies of potential genetic resistance to bacterial canker and brown rot haven't shown satisfactory results, but cultivars with different levels of tolerance have been discovered (Bargioni, 1996; Brown and Wilcox, 1989; Kappel and Sholberg, 2008; Kappel *et al*., 2012). No tolerance or resistance has been identified to cherry fruit fly or spotted wing drosophila. However, cultivars with black cherry aphid tolerance have been found in UK (Bargioni, 1996).

Large size, firmness, and sweetness are all considered to be extremely important fruit quality traits for sweet cherries (Proebsting, 1992; Christensen, 1995; Ystaas and Frøynes, 1990). Other fruit quality traits have been

considered as specific requirements for certain breeding programs, such as self-fertility, fruit skin color and juiciness, low chilling requirements, and excellent postharvest performance. For export quality fruit, the optimum fruit size is about 12 g and the minimum soluble solids content is 17~19% (Kappel *et al*., 1996).

The timing of fruit maturity is important for sweet cherry production. Fruits produced in the early or later part of the regular production season trend toward higher prices than in the mid-season (San Martino *et al*., 2008). Therefore, extension of harvest seasonality with improved early- and late-ripening cultivars is a primary objective in sweet cherry breeding across the world, especially in Europe (Piaskowski *et al*., 2018; Quero-Garcia *et al*., 2014). Currently, the benchmark earliest cultivar is ‘Burlat’, so breeding to combine early flowering with a short fruit ripening phase, without quality loss, is a major target (Quero-Garcia *et al*., 2017).

The main environment stress risks, beyond rain-cracking, for sweet cherries include winter injury, spring frosts, and heat stress. Increasingly, sweet cherries have been planted at the margins of traditional production areas to meet growing worldwide market demands, and that is where new cultivars resistant to winter injury are primarily needed. ‘Windsor’, ‘Black Eagle’, ‘Vic’, ‘Kristin’ and ‘Hudson’ were considered as cold hardy by Bargioni (1996). Another cultivar breeding objective is to improve tolerance to spring frosts that can kill flower buds. Currently, the most effective breeding method to address this one of

indirect prevention, that is, avoidance of high frost risks by using of late flowering parental lines (a widely expressed character in the *Prunus cerasus* germplasm) (Dondini *et al*., 2018). Also, heat stress can affect the formation of fruit doubles and spurs the following year, but studies to determine the genetics of heat stress resistance have not yet been initiated (Quero-Garcia *et al*., 2017).

The new sweet cherry rootstocks, training systems and cultivars have played similar essential roles in the expansion of cherry production capacity over the past two decades. Sweet cherry trees trained to high density production systems on dwarfing rootstocks have more regular and larger yields, larger fruit, smaller tree size, easier worker access, and especially earlier fruiting (Long *et al*., 2015). Thus, the future trend for sweet cherry growers is to adopt improved sweet cherry genetic materials that are integrated into new, efficient training systems to develop sustainable, competitive and pedestrian orchards.

The importance of sweet cherry spur leaves

The majority of the fruit typically produced on sweet cherry trees arises from spurs on wood that is two or more years old, with a lesser proportion produced at the base of shoots that grew the previous season. The fruiting spurs are predominately positioned on growth that is 3 to 7 years old, decreasing in productivity as wood ages and/or becomes heavily shaded. Floral initiation occurs within spur axillary buds in late spring and early summer of the year

preceding flowering and fruiting. A typical fruiting spur consists of one vegetative bud, which usually develops 6~8 spur leaves, and 0~10 flower buds, each of which can develop 1~4 fruits. On wood two or more years old, the number of flower buds per spur typically increases from base to apex of the branch's sections of annual growth (Lang, 2005). For the seven modern training systems noted above, two (KGB, UFO) bear fruit primarily on spurs, one (SSA) bears fruit primarily on the base of one-year-old branches, and four (SB, VCL, TSA, SL) bear fruit in varying proportions on a mix of both types, generally with the majority on spurs (Long *et al*., 2015). Since the majority of the yield on most mature trees occurs on fruiting spurs, several studies have focused on the factors controlling spur development and flowering. Both traits can vary by cultivar (Kramer, 1985; Maguylo *et al*., 2004), rootstock (Gruppe, 1985; Schaumberg and Gruppe, 1985; Maguylo *et al*., 2004) and training systems, as well as branch orientation (Kramer, 1985; Lauri *et al*., 1998).

Further investigation of sweet cherry fruiting physiology has differentiated all the leaves on the tree canopy into three general populations: leaves on new (current year) shoot growth, spur leaves on the one-year-old section of shoots (without fruit buds in the spring of leaf emergence) and spur leaves on two-year and older sections of shoots with fruit buds (Ayala and Lang, 2005). Both types of spur leaves have a quite different growth period from that of shoot leaves: spur leaves develop in a very short time in spring, within 3~5 weeks after bud break, whereas formation of shoot leaves continues as new shoots elongate,

generally up to 10 weeks after bud break (Flore,1996; Ayala, 2004). Sweet cherry fruit development draws heavily upon the photosynthates supplied by the leaves on the same spur where the fruit is attached (Ayala and Lang, 2005; Ayala and Lang, 2018). However, fruit is known as a priority sink, especially during stages I and III, and leaves from neighboring non-fruiting spurs and long terminal shoots also become important sources of photosynthates for the growing fruit as early as stage I (Loescher *et al*., 1985; Roper *et al*., 1987; Roper and Loescher, 1987; Toldam-Andersen, 1998; Ayala and Lang, 2005; Ayala and Lang, 2018). Using ^{13}C to track photosynthate partitioning from selected leaf populations, Ayala and Lang (2005) demonstrated that the more distant the ^{13}C source, the lower the amount of ^{13}C that was detected in fruit. In general, for a 3-year-old section of branch with no lateral shoots, 75 to 90% of the carbon supplied to spur fruit during development originated in the two spur leaf populations (Ayala and Lang, 2018).

Research on manipulation of canopy leaf to fruit ratio (L:F) with ‘Lapins’/‘Gisela 5’ sweet cherry trees established different L:F ratios of 0.7:1, 2:1 and 3:1, and found that high L:F ratios significantly resulted in the darkest fruit color, higher fruit mass and higher total soluble solids content (Usenik *et al*., 2010). Besides the influence of fruit quality, L:F ratio also affected the fruit ripening process; fruit maturity was delayed by a low L:F ratio. Also, Whiting and Lang (2004) reported that a leaf area to fruit ratio (LA: F) around 210 cm^2 per fruit is needed to achieve adequate fruit size and sugar for good quality fresh market sweet

cherries.

Furthermore, spur leaves tend to be smaller than new shoot leaves. Therefore, in spur-bearing training systems, a better understanding of factors that affect spur leaf development, as a key determinant of photosynthetic capacity to supply fruit growth, may provide cherry growers with important management tools for optimizing fruit growth potential.

Leaf light interception and photosynthetic capacity

In addition to the environmental factors that are limiting to photosynthesis, including light intensity, carbon dioxide concentration and temperature, photosynthesis is determined by leaf photosynthetic capacity. Leaf photosynthetic capacity is a measure of the maximum rate of carbon fixation during photosynthesis. For individual leaves, photosynthetic capacity is determined by physical features (particularly leaf area, leaf thickness and stomatal properties) and biochemical features (particularly leaf nitrogen concentration per unit area) (Boardman 1977; Field and Mooney, 1986; Schulze *et al*., 1994; Hirose *et al*., 1997).

Stomatal density and conductance play a key role in the regulation of gas exchange and leaf CO_2 assimilation rate, therefore having an important influence on leaf photosynthetic capacity. A high-yielding rice variety, Habataki, showed higher leaf photosynthetic capacity because of its higher stomatal

conductance (Adachi *et al*., 2011). Similarly, with the overexpression of *StNF-YB3.1*, a gene which promotes ABA-mediated stomatal closure, potato exhibited a reduction in photosynthetic capacity (Xuanyuan *et al*., 2017). Regarding stomatal density, Tanaka *et al*. (2013) reported that the stomatal density of soybean from 18 lines had positive correlations with their photosynthetic capacity. Additionally, Tanaka *et al*. (2013) conducted further research on *Arabidopsis* mutants with STOMAGEN-overexpressing and -silencing lines, and indicated the significant positive correlation between stomatal density and leaf photosynthetic capacity.

Leaf thickness is associated with the mesophyll conductance to CO_2 which is an essential component of photosynthetic capacity. Previous study found a negative relationship between leaf thickness and mesophyll diffusion conductance (Flexas *et al*., 2008). The explanation is that CO_2 diffuses through leaf mesophyll via intercellular air spaces, cell wall and the intracellular liquid pathway, and all of these are strongly affected by the leaf thickness (Evans and Von Caemmerer, 1996). Mesophyll transport resistance was reported to be a limiting factor in leaf photosynthesis in the middle of the last century (Gaastra, 1959). Since then, more detailed positive relationships between mesophyll conductance and photosynthetic capacity have been found (Galmés *et al*., 2014a; Peguero-Pina *et al*., 2016a; Tosens *et al*., 2016).

Within a species, it is not surprising to find the strong positive correlation

between photosynthetic capacity and leaf nitrogen content, because the proteins of the Calvin cycle and thylakoids represent the majority of leaf nitrogen (Evans 1989; Walcroft *et al*. 1997). Evans and Seemann (1989) found that nearly half of leaf nitrogen is allocated to the photosynthetic apparatus. The enzyme Rubisco is involved in the first major step of carbon fixation and requires a large amount (15 ~ 30%) of leaf nitrogen (Evans, 1989; Kellomäki and Wang, 1997; Evans and Seemann 1989). Rubisco content per unit area and maximum rate of Rubisco carboxylation increased as the nitrogen content per unit leaf area increased (Warren *et al*., 2003b). Thylakoids are the site of the light dependent portion of photosynthesis. Evans (1989) showed that higher rates of oxygen evolution (the oxidation of water during oxygenic photosynthesis) was related to a greater amount of thylakoid nitrogen per unit of chlorophyll. In a study of deciduous hardwood and evergreen coniferous tree species, Reich *et al*. (1995) found that both two groups showed a positive correlation of photosynthetic capacity and leaf nitrogen content. However, the deciduous broad-leaved group (r^2=0.75) had a greater slope (photosynthetic capacity – nitrogen content) than the evergreen coniferous group (r^2=0.59).

Potential phytohormone treatments for increasing leaf photosynthetic capacity

The gibberellins (GAs) are an important group of phytohormones which exert various effects on promotion and regulation of plant growth. These can affect stem elongation, seed germination, dormancy, flowering, sex expression,

enzyme induction, and leaf and fruit senescence (Hedden and Thomas, 2012). The application of GAs to stems produces a remarkable increase in cell division in the subapical meristem (Sachs *et al*. 1960). The occurrence of rapid growth resulted from both the greater number of cells formed and an increased elongation of the individual cells. Several studies reported similar results, showing that GAs are associated with leaf expansion via their direct effects on cell division and expansion as well as via integration of internal and external stimuli (Yoshida *et al*., 2014; Davie`re and Achard, 2016). Nagel *et al*. (2001) conducted research on mutant tomatoes with reduced gibberellin synthesis, and found a decrease in leaf photosynthetic capacity. The treatment of *Plantago major* with GA increased leaf photosynthetic capacity (Dijkstra and Kuiper, 1989; Dijkstra *et al*., 1990). Similarly, with spray applications of uniconazole (an inhibitor of GA biosynthesis) and GAs on *Polygonum cuspidatum*, Sugiura *et al*. (2014) found that GA increased leaf photosynthetic capacity, whereas uniconazole showed the opposite result.

In fruit crop production, GAs can be used to improve early stages of plant development, such as stimulation of adventitious rooting of cuttings, termination of seed dormancy, and promotion of lateral branch development. Direct application of GAs to cuttings were found to have a negative effect on rooting (Hansen, 1988). However, Ford *et al*., (2002) found that pre-treatment with GA_3 on ‘Stella’, ‘F12/1’ and ‘Charger’ sweet cherries increased the rooting percentage of cuttings subsequently taken from the treated plants by 80% or

more compared to the control group. Furthermore, GA_3 pre-treatment increased the number of roots per rooted cutting. Both seed germination percentage and rate for ‘Lambert’ sweet cherry was improved to 47.1% and 1.9 seed d^{-1}, respectively, with additional 500 ppm GA_3 applications after seed washing compared to seed washing only (Javanmard *et al*., 2014). Also, 500 ppm GA_3 treatment after 120 days of stratification for Mazzard cherry seeds without the seed coat resulted in a germination percentage of 79.7% which was 29% higher than the control group (Çetinbaş and Koyuncu, 2006). Three treatments (GA_3, GA_4, and GA_{4+7} at 5000 ppm) were applied to 1-year-old shoots of 'Skeena'/Mazzard sweet cherry trees at green-tip stage, and all treatments showed similar positive effects for stimulation of branching from lateral buds (Elfving *et al*., 2011).

Additionally, GAs can elicit numerous effects on mature fruit trees, including termination of bud dormancy, floral formation, prevention or delay of flowering, and fruit development, ripening, quality and increasing yield. Applications of GA_3 and GA_{4+7} (100 ppm) to mature ‘Bing’/‘Gisela 1’ trees at the end of both stage I and II of fruit development reduced the number of reproductive buds per spur by 67% the next spring (Lenahan *et al*.,2006). Also, applications of 200 ppm GA_3 and GA_{4+7} at both dates delayed flowering the following spring by 4~5 days. Similar research was conducted by Engin *et al*. (2014) on mature ‘0900 Ziraat’/‘Gisela 5’ trees. GA_3 (25 ppm, 50 ppm and 100 ppm) was applied at two growth stages (early flowering and beginning of fruit development) and caused

the number of flowers per bud to decrease in the dormant season in the comparison with control. Also, the number of flowers per bud was correlated negatively with GA_3 concentration. Dissection of flower buds showed that differentiation of the floral primordia (sepal, petal, stamen and pistil) was considerably slower after GA_3 applications (Engin *et al*., 2014).

Applications of GA_3 at 15~30 ppm at the beginning of stage III of fruit development has become a standard practice due to a well-documented delay in fruit maturation, and increased fruit size and firmness at maturity (Proebsting *et al*., 1973; Facteau *et al*., 1985; Looney, 1996; Kappel and MacDonald, 2002; Zhang and Whiting, 2011). But in some studies, larger fruit size was not found with GA_3 applications. GA_3 applied at straw color development on 'Bing' sweet cherry trees showed similar fruit size with untreated controls (Clayton and Biasi, 2003). Furthermore, GA can affect soluble solids content (SSC) and fruit color, which are also considered as important factors in fruit quality. Fruit color and SSC development were delayed by GA_3 applications at 25 ppm applied 1 week before harvest in 'Lapins'/'Mazzard', but not in 'Regina'/'Gisela 6' (Dong *et al*., 2018). 'Bing' fruits treated with 50 and 100 ppm GA_3 had 7% and 12% higher soluble solids content, respectively (Lenahan *et al*., 2006).

In addition to the effects mentioned above, preharvest treatment with GAs can influence the postharvest quality of sweet cherry fruits. Fruits of 'Aksehir Napolyon' treated with GA_3 (10, 20 and 30 ppm) showed less loss in fruit

firmness, delayed stem discoloration and maintained fruit color brightness after four weeks of cold storage (Özkaya et al., 2006). Also, 'Lapins' fruit with GA_3 applications (25, 50 and 100 ppm) at pit hardening had significantly higher firmness compared to the control group after four weeks of storage (Einhorn *et al*., 2013). Stem discoloration after cold storage was reduced by 25 ppm GA_3 treatment.

The cytokinin 6-benzylaminopurine (6-BA) is a growth regulator that promotes cell division and has positive effects on leaf photosynthesis. Foliar treatment with 6-BA to sugar beet, pea, meadow fescue and reed fescue increased leaf net photosynthetic rate and Rubisco activity (Chernyad'ev, 1994). The effect of 6-BA on *Vicia faba* leaf structure was a stimulation of mesophyll cell elongation and it increased the amount of photosynthetic pigments (Ron'zhina, 2003). According to Harvey's *et al*. (1974) research on detached cucumber cotyledons incubated in buffer, cotyledons in buffer containing 6-BA had greater photosynthetic enzyme activities. In *Epipremnum aureum,* 6-BA sprays increased net photosynthesis as well as leaf thickness (Di-Benedetto *et al*., 2015a).

6-BA can be integrated into orchard production systems for such effects as initiation and growth of new shoots, control of canopy formation and fruit thinning. It increases biosynthesis of nucleic acids and mitotic activity in bud apices. Treatments of 6-BA+GA_{4+7} at 100, 250, 500, 750 and 1000 ppm were

applied to one-year-old ‘0900 Ziraat’/‘Mahaleb’ sweet cherry trees. All treatments significantly increased tree diameter compared to the control, and the 500 ppm 6-BA+GA_{4+7} treatment was most effective for stimulating feathering of ‘0900 Ziraat’/‘Mahaleb’ sweet cherry trees (Koyuncu and Yildirim, 2008). Moghadam and Zamanipour (2012) investigated the influence of 6-BA on one-year-old ‘Siah Mashhad’/‘Mahaleb’ and ‘Dovomras’/‘ Mahaleb’ trees. All the treatments with 200, 400, or 600 ppm 6-BA applied in mid-June (when scion shoots were 60~65 cm tall) increased the number of lateral shoots compared to the control. Fruit thinning with 6-BA in mature apple trees can result in larger fruit size (Greene, 1989a; Elfving, 1989; Greene and Autio, 1989) and increased return bloom the following year (Williams and Stahly, 1969; Stembridge and Morrell, 1972; Unrath, 1974).

Potential nutritional treatments for increasing leaf photosynthetic capacity

Nitrogen supply has been strongly and positively correlated (r^2=0.97) with corn leaf nitrogen content (Bennett *et al*., 1953). In addition to the obvious effect on increasing leaf nitrogen content, nitrogen fertilization can influence leaf photosynthetic capacity. Knops and Reinhart (2000) conducted research on three grass species in Minnesota with long-term nitrogen fertilization and found that leaf photosynthetic capacity increased as the level of nitrogen fertilization increased. On sandy soil of West Australia, the greatest leaf photosynthetic capacity of wheat occurred only with additional nitrogen inputs (Asseng *et al*.,

2003). In maize, higher specific leaf area was related to higher nitrogen supply (Amanullah *et al*., 2007). There also are many studies on woody plants. Cork oak seedlings with two-month nitrogen fertilization significantly increased leaf photosynthetic capacity cmpared to the control group (Kachout *et al*. 2017). Treatment of *Handroanthus impetiginosus,* a tree species for restoration of degraded areas, with different levels of nitrogen fertilization resulted in a positive linear correlation of leaf photosynthetic capacity and nitrogen supply (Leite *et al*., 2017).

Nitrogen supply plays a vital role in the growth and productivity of fruit trees (Titus and Kang, 1982). Fruit tree growth during early spring is supported by remobilization of stored nitrogen instead of current-year root uptake, so applications of nitrogen in the fall can be considered as one possible method to improve nitrogen reserves and subsequent early spring growth (Millard, 1996). Foliar application of urea in October to ‘Hedelfinger’/‘Gisela 5’ sweet cherry trees increased the storage nitrogen levels in flower buds (up to 40%), shoot apices (up to 20%) and bark (up to 29%) (Ouzounis and Lang, 2011). Since spur leaf growth in the spring draws upon storage nitrogen levels, spur leaf size and total leaf area per spur were increased in the next spring by up to 24%. With autumn foliar application of urea to apricot trees, fruit yield was increased by 26.1%, and the abortive flower ratio decreased by 27.1% compared with the control group (Karlidag *et al*., 2017). Moreover, numerous other studies support that autumn sprays of urea significantly increase yield in

various fruit species (Sanchez *et al*., 1990; Khemira *et al*., 1998; Cheng *et al*., 2004; Ebert, 2009).

Both soil and foliar fertilizer applications are common in orchard management. Compared to soil application, foliar application can produce a more direct effect on the plant because the nutrient ions can be absorbed by the leaf immediately. Foliar application is applied most effectively taken up during dawn and dusk, especially in hot weather, because during those times, stomata are open to take up nutrients and the drying time is relatively long (resulting in a longer effective time for absorption). Also, rainy weather needs to be avoided to prevent washing off of the foliar spray.

Objectives

Given the importance of spur leaves to sweet cherry fruit growth potential and the lack of any previous studies on potential orchard management strategies in the spring that might increase spur leaf size during the 3-4 week window of spur leaf growth after budbreak, the objective of this project was to examine potential foliar applications that might increase spur leaf size. A preliminary study was undertaken in 2017 to determine spur leaf growth and quality responses following foliar application of several nitrogen fertilizers and phytohormones, including cytokinin (BA), gibberellins (GA_3), or the mix of cytokinin and gibberellins (BA+GA_{4+7}) applied at different concentrations to mature ‘Sam’ sweet cherry trees. Subsequently, follow-up studies were conducted in 2018 to

further investigate application parameters and potential fruit effects for foliar spring treatments of GA_3, GA_{4+7}, or BA combinations on spur leaf responses of mature ‘GG’, ‘DD’, ‘EE’, and ‘Burgundy Pearl’ sweet cherry trees.

CHAPTER 2: FOLIAR TREATMENT STUDIES AT THE SPUR LEVEL (2017)

Materials and methods

Before the foliar application experiment in the field, one preliminary study was undertaken in the growth chamber to determine the appropriate spur leaf growth measuring interval based on Growing Degree Days (GDD, base temperature 7°C and calculated by equation: GDD = Average daily temperature - Base temperature = $[T_{max} + T_{min}] / 2$ - Base temperature). Two spurs were chosen randomly from one potted two-year-old sweet cherry tree grown in the growth chamber at 25°C under a 16:8 hour day:night photoperiod. Leaf area measurements were begun when the first leaf began unfolding and measures were taken at time intervals of about 24 hours (30 GDD). The measuring method involved drawing an outline of the leaves, which was scanned into a computer for counting of pixels in Photoshop (*Adobe Photoshop CS*., Berkeley, CA) and quantifying the leaf area by pixel number. This pixel counting method provided extremely accurate for leaf area measurements, providing detailed data for determining a measuring interval. According to the data, most detectable differences between successive leaf area values were less than 100 mm^2 (1 cm^2), which is quite difficult to detect by the length and width measuring method (Demirsoy and Lang, 2010) in the field (Figure 1). Consequently, a leaf size measuring interval of 60 GDD was determined to be the most appropriate interval for future field measurements.

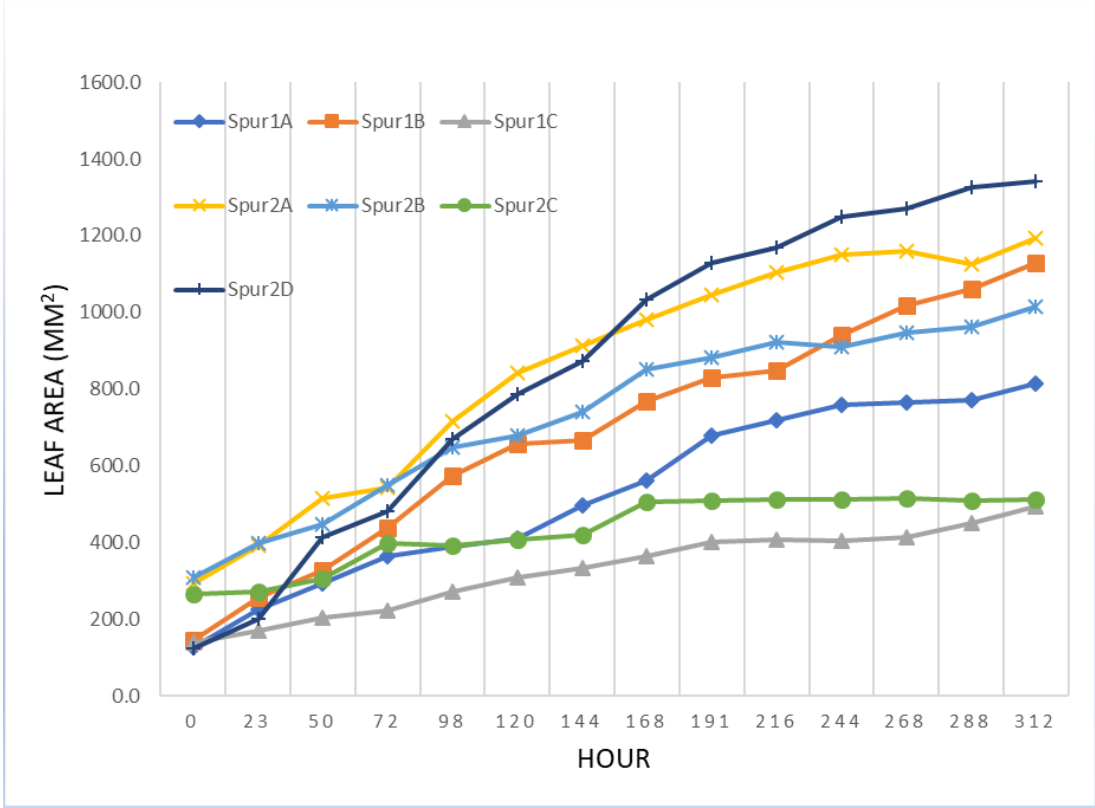

Figure 1. Spur leaf area growth curves over 312 hours as determined by 24 hour measuring intervals (30 GDD).

The study to determine spur leaf growth, leaf thickness and adaxial epidermis cell size responses following foliar application of nitrogen fertilizers and phytohormones was initiated at bud break on sweet cherry trees at the Michigan State University (MSU) Horticulture Teaching and Research Center (HTRC, aka the Hort Farm) near East Lansing, Michigan. To clearly differentiate each leaf, markings were painted (BEHR MARQUEE (Eggshell Enamel Low Odor Interior Paint) Behr Paint Co., Santa Ana, Calif.) on the abaxial leaf surface to differentiate each leaf of one spur. However, even though this paint marking had previously been used successfully on mature sweet cherry leaves (T. Wilkinson, personal communication), it was phytotoxic on newly emerging

leaves, resulting in severe leaf injury including burned holes and shrinkage. Since this severe damage affected natural leaf growth, this initial experiment was abandoned.

Consequently, the experiment was moved north to orchards less developed phenologically. The treatments were conducted at the MSU Northwest Michigan Horticultural Research Center near Traverse City, Michigan, using mature ‘Sam’ sweet cherry trees on Mazzard rootstocks. All experimental trees were cultivated with standard orchard practices (i.e., dormant and spring pruning, timely pesticide applications, irrigation, weed control as needed) in the years before imposition of the treatments. Each trial was arranged in a randomized complete block design with five single-spur replications. Spurs were selected for uniformity of flower bud number, attached branch diameter and height from ground. There were seven foliar treatments (aqueous solutions using distilled water and no additional surfactant): T1 = 0.5% urea; T2 = 150 ppm 6-BA (Maxcel); T3 = 30 ppm GA_3 (ProGibb); T4 = distilled water as a control; T5 = 2.0% calcium nitrate; T6 = 1.7% potassium nitrate; and T7 = 150 ppm 6-BA plus 30 ppm GA_{4+7}. All applications were applied to individual spurs before sunrise by using a hand-held trigger-pump sprayer until run off. The spray application dates (May 10 for 30 spurs, May 15 for an additional 5 spurs) were determined by spur leaf growth stage, that is, at least three spur leaves were eligible for measurement at the time of treatment, usually between full-bloom and petal fall stage. Paper cones with a narrow opening at the base were

used to shield each spur during treatment to protect the other spurs from contamination. The experimental design was completely randomized and individual spurs were used as a treatment unit, replicated five times.

All the spurs were chosen within the canopy at a similar height (around 1.2 to 1.5 m from the ground) and both the flower bud number per spur and the diameter of branches were recorded. Before the treatments were applied, each spur leaf length and width were measured, and a small triangular section of the leaf margin was collected for microscopy (see Chapter 3). The leaf length and width were determined with a measurement tape, recording the width of the intact half of the leaf from the midrib to the margin, opposite the half of the leaf that had been sampled for leaf margin tissue. Leaf area was estimated following the procedure of Demirsoy and Lang (2010). After the treatment dates, additional tissue samples were collected from the same side as the initial sample, and area measurements were conducted every ~60 GDD. When the leaf length and width data did not change for three continuous dates, leaf growth was considered to be finished.

All data were analyzed in R 3.5.3 (R Core Team, 2018) with mean separation using the Wilcox test for individual leaf size, ANOVA and Tukey test for leaf shape (ratio of length to width) and the area of the five largest leaves.

Results

There were several unexpected outcomes encountered with this experiment. The first was phytotoxicity caused by calcium nitrate, which was manifested by young leaf marginal burning and cupping. Consequently, this treatment and data were discarded for the analysis and further discussion. The second was leaf damage due to feeding by oblique banded leafrollers; data was not collected from any of these damaged samples. Finally, the 150 ppm 6-BA plus 30 ppm GA_{4+7} treatment stimulated 60% of the spurs to elongate into shoots in early June (around 20 days after the foliar application). Thus, the extra leaves on the shoots were not included in the spur leaf measurements.

Several of the treatments resulted in larger average leaf areas (Table 1). Most effective were the plant hormones, 6-BA, BA+GA_{4+7} and GA_3, which promoted average leaf areas that were 37%, 47% and 30% larger than the control leaves, respectively, all of which were significantly different from the control. However, the nitrogen treatments resulted in average leaf areas not significantly different from the control.

Table 1. Average 'Sam' sweet cherry spur leaf area at maturity following treatment at leaf emergence with 0.5% urea (Urea), 1.7% KNO_3 (KNO_3), 150 ppm 6-BA (BA), 30 ppm GA_3 (GA_3), 150 ppm 6-BA + 30 ppm GA_{4+7} (BA&GA_{4+7}).

Treatments	Control	Urea	KNO_3	BA	GA_3	BA&GA_{4+7}
Leaf area (cm^2)	34.2	36.3	39.7	47	44.3	50.5
Wilcox test		0.3348 NS	0.1333 NS	0.0129 *	0.0294 *	0.0182 *

P-values of Wilcox test are significantly (*) or not significantly (NS) different from the

control at a = 0.05.

Fully expanded leaf shape (ratio of length to width) was compared and there was no significant difference between any of the treatments and the control (Figure 2), suggesting that none of the treatments (even the gibberellins) caused differential leaf cell division or elongation.

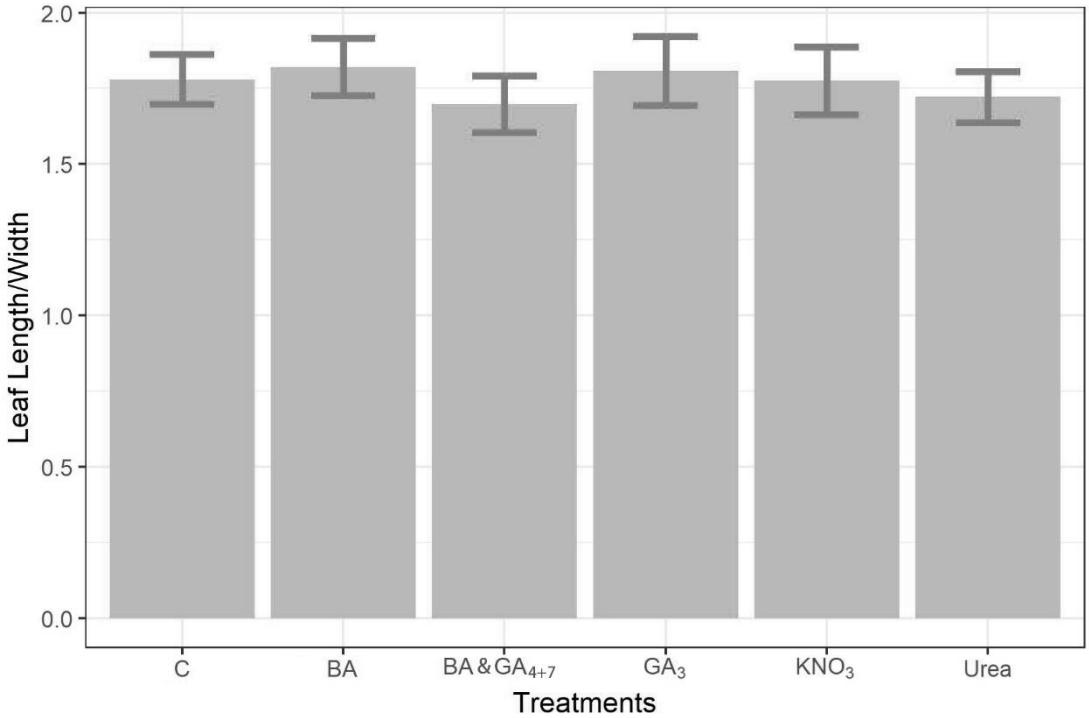

Figure 2. Comparison of 'Sam' sweet cherry spur leaf shape (length/width ratio) following nitrogen and phytohormone foliar treatments (0.5% urea [Urea], 1.7% potassium nitrate [KNO_3], 150 ppm 6-BA [BA], 30 ppm GA_3 [GA_3], 150 ppm 6-BA + 30 ppm GA_{4+7} [BA&GA_{4+7}]) applied during leaf emergence. All the bars represent standard error.

In addition to the final leaf size comparison, the leaf growth process from first measurement to full expansion of each emerging leaf was examined with repeated measures to create growth curves. For each spur, the leaf area data were arranged from largest to smallest, and the average growth curves for the

five largest leaves are depicted for each treatment with different colors (Figure 3; red, orange, yellow, green, blue, from largest to smallest). For the hormone treatments (6-BA, BA+GA_{4+7} and GA_3), the largest leaves were 170%, 270% and 200% larger in area than the fifth largest leaf, respectively. The differences in average area between the largest and fifth largest leaves for the potassium nitrate and urea treatments were 130% and 220%, respectively. This wide variation in leaf size within each spur is expected, as the earliest 3~5 leaves to emerge often include both the smallest and largest leaves and the leaves that emerge later always rank in the middle; in fact, the area of individual leaves within the control spurs varied by 200%. The 150 ppm 6-BA plus 30 ppm GA_{4+7} treatment might have increased the area of the largest leaves more than that of the smallest leaves, and the 1.7% potassium nitrate treatment may have increased the area of the relatively small leaves more than the large leaves.

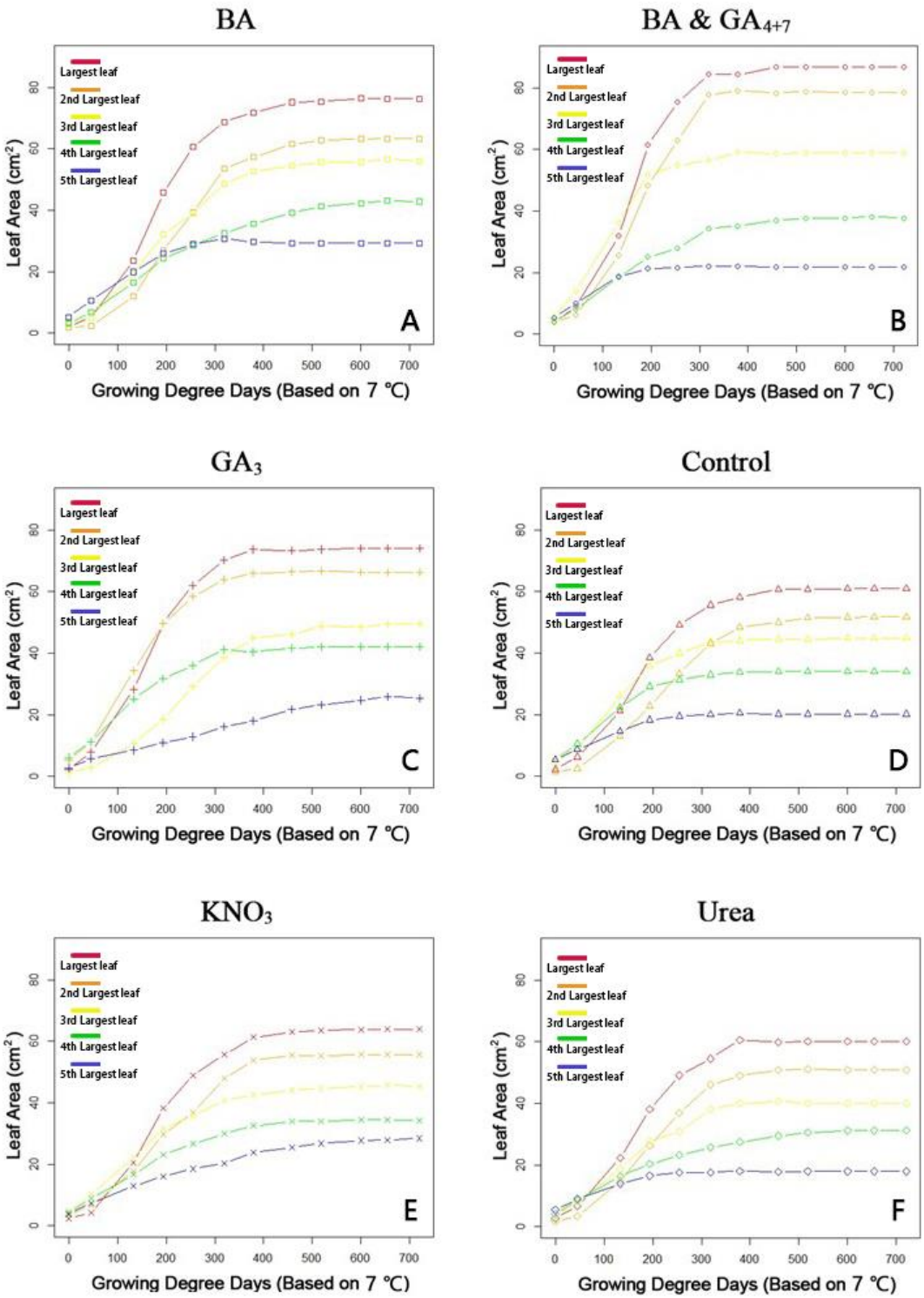

Figure 3. Average 'Sam' sweet cherry spur leaf growth curves following treatment with (A) 150 ppm 6-BA [BA], (B)150 ppm 6-BA + 30 ppm GA_{4+7} [BA&GA_{4+7}]), (C) 30 ppm GA_3 [GA_3], (D) Control), (E) 1.7% potassium nitrate [KNO_3], and (F) 0.5% urea [Urea]. Data are means of five replications.

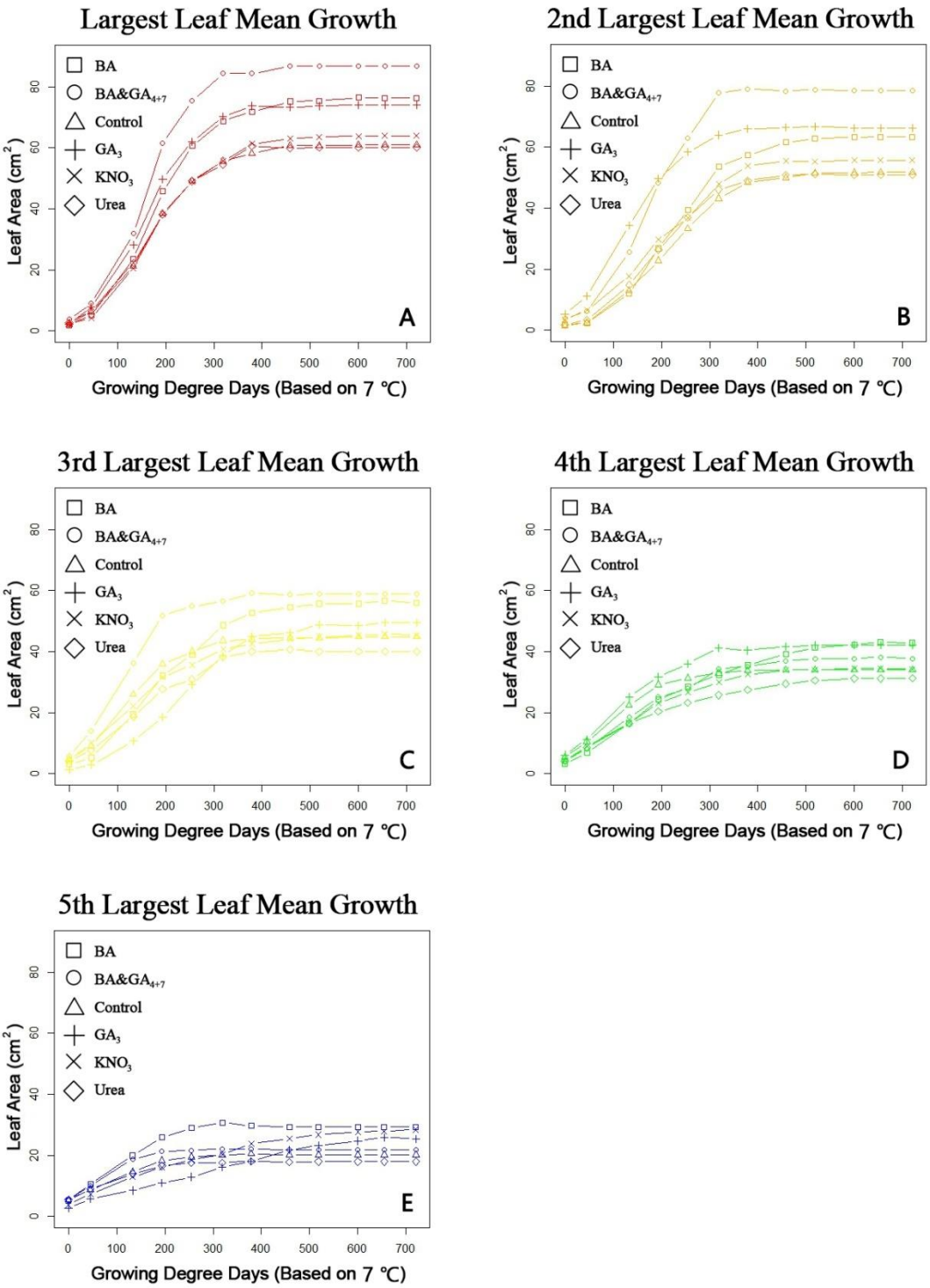

Figure 4. Combined mean growth curves of 'Sam' sweet cherry spur leaves by leaf size ranking: largest leaf (A), 2nd largest leaf (B), 3rd largest leaf (C), 4th largest leaf (D) and 5th largest leaf (E), following nitrogen and phytohormone foliar treatments (0.5% urea [Urea], 1.7% potassium nitrate [KNO_3], 150 ppm 6-BA [BA], 30 ppm GA_3 [GA_3], 150 ppm 6-BA + 30 ppm GA_{4+7} [BA&GA_{4+7}]) applied during leaf emergence. Data are means of five replications.

Since spur leaf area varied widely within a spur, the comparative effects of each of the treatments were depicted with separate graphs for each leaf size class within the spur (Figure 4). The curves in Figure 4 (A) and (B) showed a consistent result for the effect of the treatments on the two largest leaves within each spur. The 6-BA+GA_{4+7} treatment promoted about 50% larger leaf area for the two largest leaves compared to the control, and both the 6-BA and GA_3 treatments promoted about 20% larger leaf area for those leaves compared to control. However, the differences in area of the two largest leaves following the nitrogen fertilizer treatments were quite small in comparison with the control. The effect of all treatments diminished as leaf size class decreased. The curves in Figure 4 (C) for the 3rd largest leaf show that the 6-BA+GA_{4+7} and 6-BA treatments promoted larger leaf area than the GA_3 and nitrogen fertilizer treatments, which were only slightly larger (<10%) than the control. The average area of the fourth and fifth largest leaves are depicted in Figure 4 (D) and (E), with the best promotion by 6-BA treatment, followed by the 6-BA+GA_{4+7}, GA_3 and nitrogen fertilizer treatments in the comparison with the control. The GA_3 treatment resulted in fourth and fifth largest spur leaves that were second and third best in promotion of leaf area. For the nitrogen treatments, only potassium nitrate increased leaf size, and then only of the fifth largest leaf (by 30%) compared to the control (Figure 4E).

The five largest leaves in each spur were grouped by final leaf size for each

treatment for further statistical analysis (Figure 5). For the largest and second largest leaves, the 6-BA+GA_{4+7} treatment was the only treatment that differed significantly from the control. The three smaller leaves, however, did not exhibit significant differences within their leaf size classes among the treatments and the control.

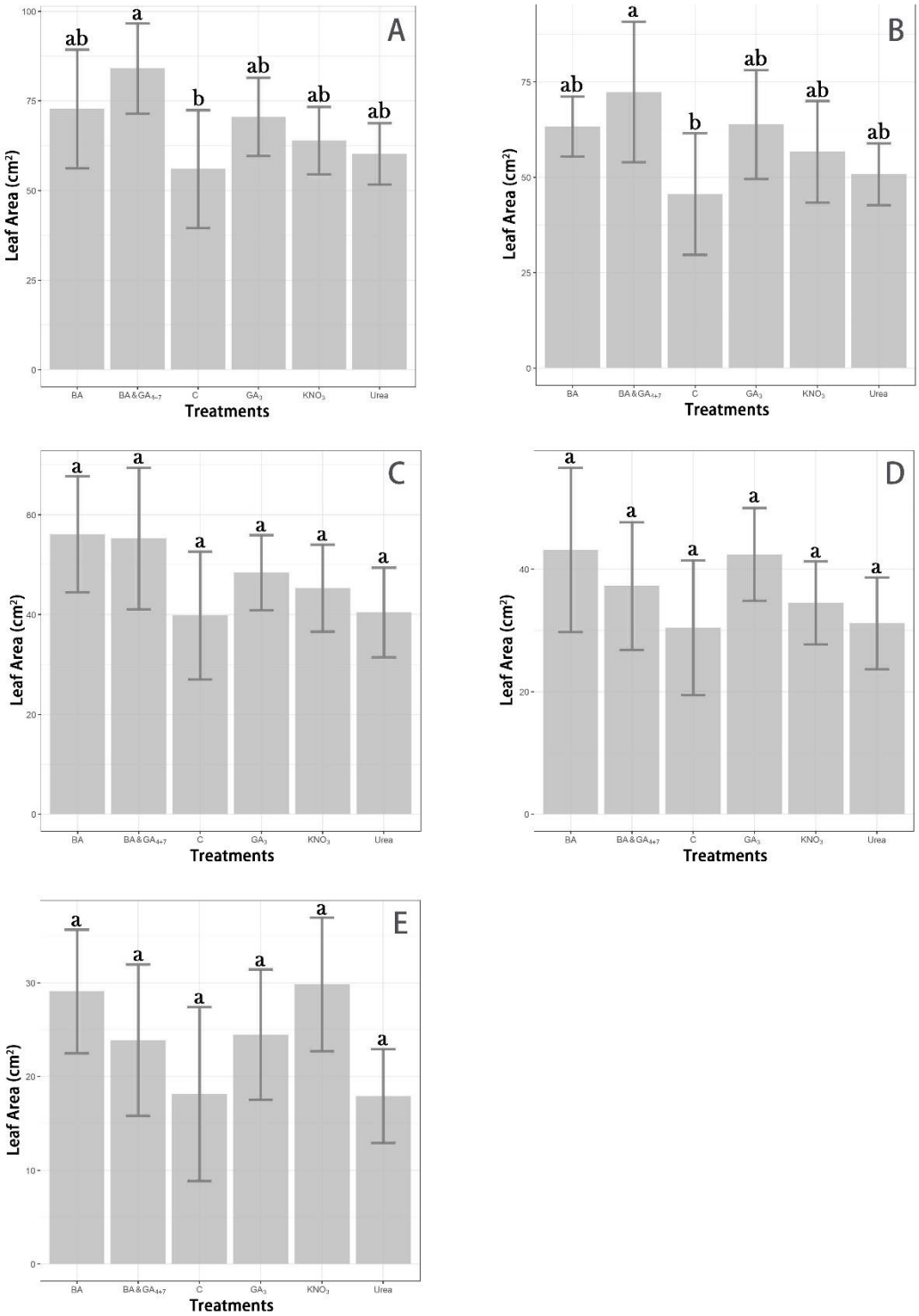

Figure 5. Statistical results of multiple comparison of area of the largest leaf (A), 2nd largest leaf (B), 3rd largest leaf (C), 4th largest leaf (D) and 5th largest leaf (E) following nitrogen and phytohormone foliar treatments (0.5% urea [Urea], 1.7% potassium nitrate [KNO_3], 150 ppm 6-BA [BA], 30 ppm GA_3 [GA_3], 150 ppm 6-BA + 30 ppm GA_{4+7} [BA&GA_{4+7}]) applied during leaf emergence. All the bars represent standard error.

Discussion

The nitrogen fertilizer treatments did not differ significantly in spur leaf area compared with the control, indicating that nitrogen fertilizer treatments applied between full bloom and petal fall have little influence on sweet cherry spur leaf growth. It is well-known that initial vegetative growth in temperate fruit trees depends on storage reserves (including carbon and nitrogen) accumulated during the previous season (Keller and Loescher, 1989; McCammant, 1988; Neilsen *et al*., 1997). Ouzounis and Lang (2011) found that nitrogen imported into sweet cherry spurs in the spring preceded bud swell. Grassi *et al*. (2002) and Millard *et al*. (2006) indicated that nitrogen remobilization in sweet cherry started immediately after bud break based on the observation of sharply increased sap concentrations of both glutamine and asparagine amides which are responsible for nitrogen remobilization. Nitrogen in leaves derived from root uptake was not found until three weeks after bud break. According to our spur leaf growth data, the rapid growth phase for the earliest 3~5 leaves is around 300 GDD (three weeks). Therefore, our results imply that in the first three weeks, those new spur leaves preferentially use storage nitrogen from previous season and foliar applications of nitrogen (in the forms used in this study) during this period are unable to provide additional nitrogenous resources for leaf growth. Viewed another way, this may suggest that nitrogen

supply was not a limiting factor for spur leaf growth in the trees used for this experiment.

However, the fact that the plant growth regulator treatments significantly increased the size of some spur leaves suggests that while nitrogen may not have been a limiting factor, plant growth regulators may be able to increase spur leaf demand for or response to storage reserves. Thus, larger spur leaves occurred after application of 6-BA, 6-BA+GA_{4+7} and GA_3 treatments. Although this is promising, the effects must be repeated, application parameters refined, and effective application rates studied further for potential unanticipated side effects. For example, Engin *et al*. (2014) reported that 30 ppm GA_3 applied at full bloom affected flower bud initiation, with floral differentiation in the summer delayed and the flower number per bud decreased in the following year. In highly productive cultivars, this may be a desirable effect, whereas in cultivars of low productivity or in growing regions with a high risk of spring frost, this may be an undesirable effect.

CHAPTER 3: FOLIAR TREATMENT STUDIES AT THE MICROSCOPIC LEVEL (2017)

Material and Methods

The triangular leaf margin tissue samples were cut with razor blades (sections varied from 0.5 cm^2 to 2 cm^2, relative to sampled leaf size) and stored in FAA (10% formaldehyde [37%], 5% acetic acid [100%], 50% ethanol [95%], 35% distilled water) solution immediately after cutting. After immersion in the FAA solution for at least 24 h, the samples were dehydrated with a 50%, 75%, 90%, 95%, 100% alcohol series and infiltrated with 50% alcohol/50% n-Butanol, then 25% alcohol/75% n-Butanol, and finally 100% n-Butanol; each step required at least 1 h of immersion (Ruzin, 1999). The infiltrated samples were observed under a Nikon H600L Upright Microscope at 200X magnification to measure the adaxial epidermis cell size. The mean cell size was calculated by counting the cell numbers in a quantified area (100 µm x 100 µm) instead of measuring the length and width of each cell. For each treatment sample, the average cell size was calculated from four randomly chosen zones (Figure 6A).

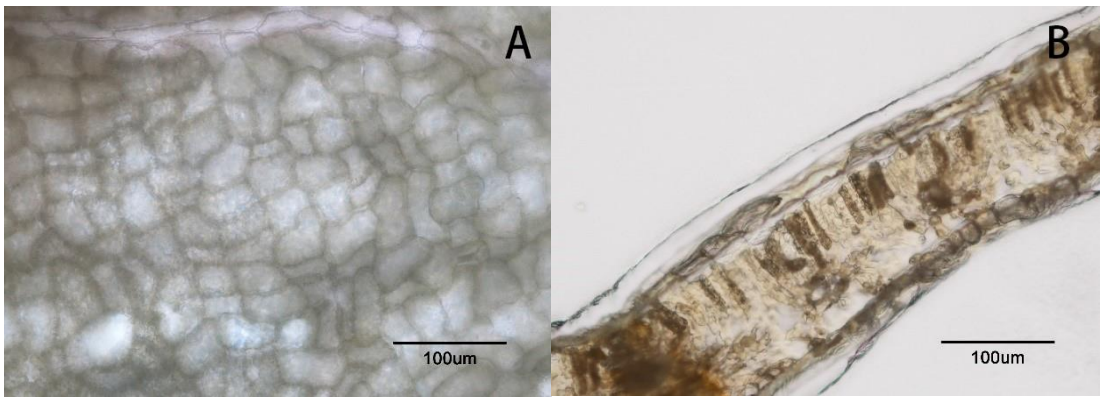

Figure 6. Examples of sweet cherry spur leaf A) adaxial epidermis cell and B) leaf cross-section.

Additionally, the thickness of whole leaf and mesophyll tissue was determined by measuring cross-sections of 174 spur leaf samples under the Nikon H600L Upright Microscope at 200X magnification (Figure 6B). The leaf cross-sections were obtained by using a Leica CM1850 Cryostat at the MSU Center for Advanced Microscopy. The difference between a regular microtome and the cryostat is the medium and temperature. The leaf samples were immersed in the medium (10.24% polyvinyl alcohol, 4.26% polyethylene glycol and 85.5% nonreactive) on the cold plate at -20°C. The medium would be liquid under room temperature and freezes at -10°C. For sectioning, the leaf sample was oriented perpendicular to the plate. After the medium had been frozen, the leaf cross-sections were obtained as with a regular microtome except in at -20°C (Dey, 2018).

All data were analyzed in R 3.5.3 (R Core Team, 2018) with mean separation using the ANOVA and Tukey test.

Results

Larger cells were observed in all of the plant growth regulator treatments (Figure 7). The BA+GA_{4+7}, 6-BA and GA_3 treatments resulted in 39%, 22% and 26% larger epidermis cells, respectively, compared to the control. The epidermis cells in the BA+GA_{4+7} treatment were significantly larger than the other two phytohormone treatments, which were significantly larger than the nitrogen or control treatments.

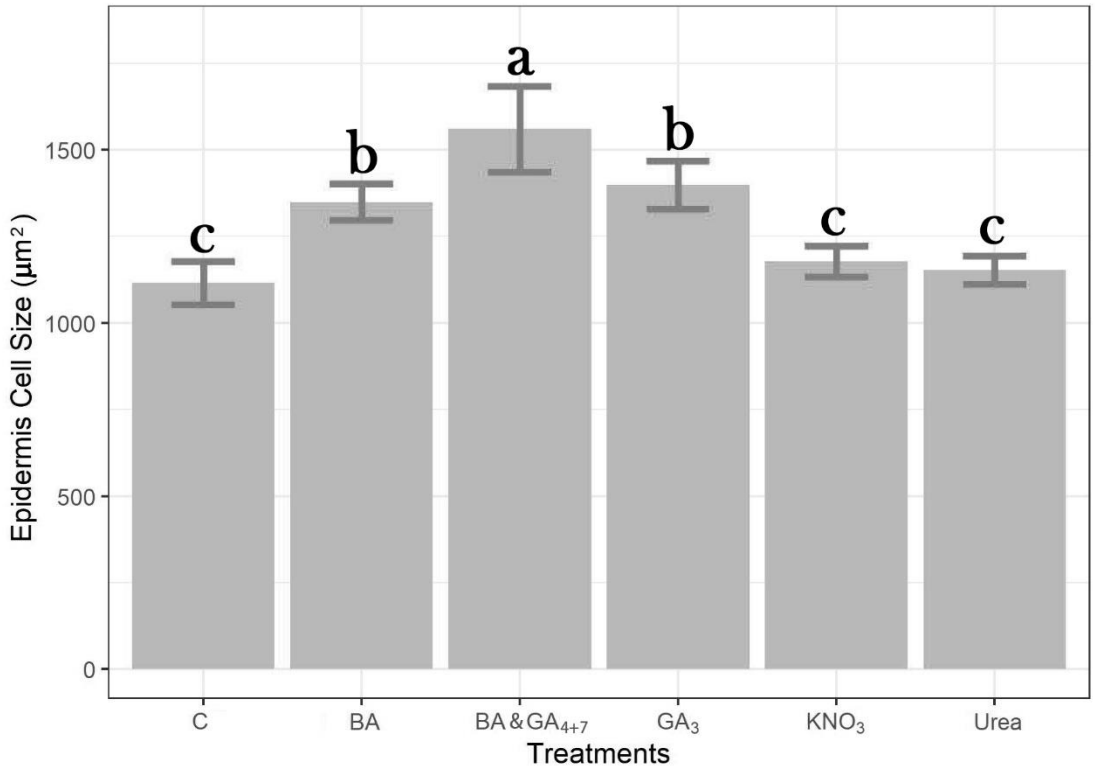

Figure 7. Comparison of mature 'Sam' sweet cherry leaf adaxial epidermis cell size following phytohormone and nitrogen foliar treatments (0.5% urea [Urea], 1.7% potassium nitrate [KNO_3], 150 ppm 6-BA [BA], 30 ppm GA_3 [GA_3], 150 ppm 6-BA + 30 ppm GA_{4+7} [BA&GA_{4+7}]) during leaf emergence. All the bars represent standard error.

In addition to greater leaf area and epidermal cell size, leaf thickness is another key parameter for leaf quality. The average leaf thickness of each group was

similar (Figure 8), with all treatments exhibiting less than 10% differences in comparison with the control, none of which were significant.

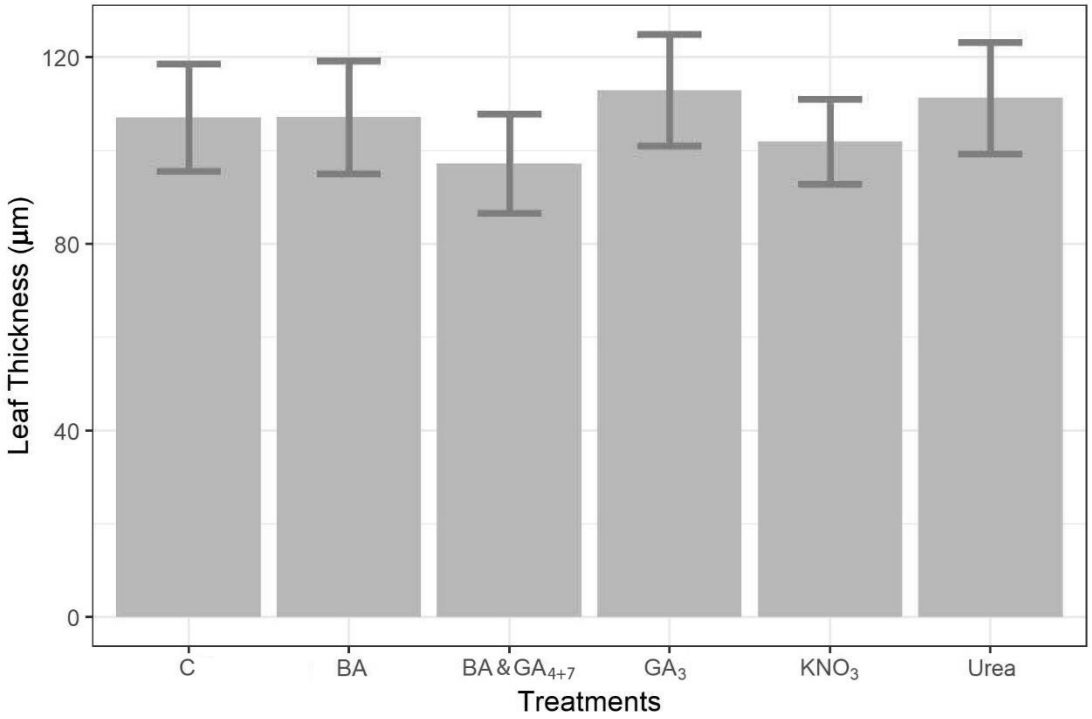

Figure 8. Comparison of mature 'Sam' sweet cherry leaf cross-sectional thickness following phytohormone and nitrogen foliar treatments (0.5% urea [Urea], 1.7% potassium nitrate [KNO_3], 150 ppm 6-BA [BA], 30 ppm GA_3 [GA_3], 150 ppm 6-BA + 30 ppm GA_{4+7} [BA&GA_{4+7}]) during leaf emergence. All the bars represent standard error.

Discussion

All plant hormone treatments increased leaf area by promoting larger cell size. Leaf thickness was not affected. Cell elongation is the combination of two events, including biochemical and biophysical processes such as water absorption and cell expansion (Taiz, 1984; Cosgrove, 1986). Cell wall extensibility plays an important role in cell expansion and it is probably controlled by the orientation of both cellulose microfibrils and the cell wall matrix. The orientation of cellulose microfibrils can be regulated by gibberellins

(Shibaoka, 1994). Also, GAs have been shown to stimulate cell elongation by altering the rheological properties of the cell wall (Jones, 1983). Calculations of the different increased ratios of leaf area and epidermis cell size infer that epidermis cell numbers increased by 12%, 3% and 3% for the 6-BA, BA+GA_{4+7} and GA_3 treatments, respectively. The higher cell numbers for the 6-BA treatment may be due to an acceleration of cell division by regulation of the cell cycle, including activating RNA synthesis, stimulating protein synthesis and the activities of some enzymes (Kulaeva, 1980; Kulaeva *et al*., 1996). Although the 6-BA+GA_{4+7} treatment had the same concentration of 6-BA, the leaf cell division may have been lower due to the 60% of those spurs that exhibited shoot elongation.

CHAPTER 4: FOLIAR TREATMENT STUDIES AT THE WHOLE TREE LEVEL (2018)

Based on the 2017 results of the nitrogen fertilizer treatments and plant growth regulator treatments, the nitrogen fertilizer applications were omitted for additional study in 2018. The main objectives of the 2018 experiments were to examine application parameters for the promising plant hormone treatments and to treat entire trees in the orchard rather than individual spurs, as well as to determine whether the hormone treatments had any adverse effects on fruiting or fruit quality. Among the phytohormone application parameters studied were the timings (from bud break to fully expanded leaves), rates of BA, GA_3 and GA_{4+7}, and a comparison of GA_3 and GA_{4+7}. Since the largest 3 leaves showed the greatest response to plant growth regulator treatments in 2017, the question of whether repeated applications would extend these growth promotive effects to additional, later-developing leaves (such as those that emerge around 100 GDD later) is of particular interest.

Materials and methods

All experiments in 2018 were conducted at the MSU HTRC near East Lansing, Michigan. Treatments were applied to nine-year-old trees of ‘GG’/Gi5, ‘DD’/Gi5, ‘EE’/Gi5, and ‘Burgundy Pearl’/Gi5 sweet cherry. All experimental trees were cultivated with standard cultural practices (i.e., dormant and spring pruning,

irrigation, weed control, and bird netting), with the exception of pesticide applications which were minimal due to limited tractor access (the plot was originally grown organically under plastic-covered high tunnels, but organic and high tunnel management had ceased several years earlier).

Timing experiment. To study the effect of phytohormone application timing, a block of 'Burgundy Pearl'/Gi5 trees was selected, and whole trees were treated with foliar applications of 150 ppm 6-BA (Maxcel), 30 ppm GA_3 (ProGibb), and 150 ppm 6-BA + 30 ppm GA_3. The experimental design was completely randomized with seven replications. For each application, sprays were applied either when most of the spurs had one to three leaves emerging (May 6th) or at 100 GDD (base 7.2°C) later (May 10th), alone (single applications) or in combination (double applications) (Table 2). A tank sprayer with a handgun was used to apply the spray treatments to run-off in the early morning. Control trees were left unsprayed.

Table 2. Timing for foliar growth regulator treatments applied to 'Burgundy Pearl'/Gi5 sweet cherry trees during Spring 2018.

Treatment / Application Timing	Untreated	150 ppm 6-BA + 30 ppm GA_{4+7}	150 ppm 6-BA	30 ppm GA_3
3 leaves		√	√	√
100 GDD		√	√	√
3 leaves+100 GDD		√	√	√
Control	√			

Rate Experiment. To study the effect of phytohormone application rates, a block

of ‘DD’/Gi5 and ‘EE’/Gi5 sweet cherry trees were selected, and whole trees were treated with foliar applications of three plant growth regulators at different concentrations: 6-BA (75 ppm, 150 ppm and 300 ppm), GA_3 (15 ppm, 30 ppm, 60 ppm), and combination of 6-BA and gibberellins (6-BA+ GA_{4+7}) (75 ppm+15 ppm, 150 ppm+30 ppm, 300 ppm+60 ppm). All treatments were applied in the morning when most of the spurs had one to three leaves emerging, using a tank sprayer. The experimental design was completely randomized with eight replicatations and control trees were left unsprayed.

Gibberellins experiment. To study the effect of gibberellin type, treatments were applied to a plot of ‘GG’/Gi5 sweet cherry trees in a completely randomized design with five replications. Foliar applications of 30 ppm GA_3 (ProGibb), 30 ppm GA_{4+7} (ProVide), and 30 ppm 6-BA+GA_{4+7} (Promalin) were applied when most of the spurs had one to three leaves emerging. Treatments were applied in the morning with a tank sprayer.

Before treatment, four spurs were selected for measurements from each tree and marked with flags. All sampled spurs were at a similar canopy height (between 1.25 m and 1.75 m) and in different quadrants of the canopy (north, south, west and east). After fruit harvest, the leaf area of the marked spurs was estimated by measuring length and width according to the method of Demirsoy and Lang (2010). In late June, cherries were harvested from all of the experimental trees. Besides the whole tree yield data, the weight and size of 25

fruits from each tree were recorded. Due to the rapid spread of a severe brown rot fungus infection, other fruit qualities such as the firmness and brix were not measured.

In the cases where spurs elongated into shoots, data were not collected since these became marked as “shoots”. Treatment data were analyzed in three fields: total leaf area per spur, individual leaf area and leaf number per spur. The data were analyzed using SAS software (SAS Institute Inc., Cary, NC). A linear mixed model was fitted by GLIMMIX procedure with tree effects and spur effects treated as random effects according to the experiment design. Multiple comparisons of all treatments with Tukey contrasts were conducted in the above linear mixed model.

Results

Timing experiment. The 30 ppm GA_3 treatment applied twice resulted in the largest total leaf area per spur, which was significantly 36% larger than the control (Figure 9). It was also significantly more than the 150 ppm 6-BA and 30 ppm GA_3 treatments that were only applied at the second timing, resulting in 46% and 51% larger total leaf area than those two treatments, respectively. Also, the 150 ppm 6-BA+30 ppm GA_{4+7} treatment applied twice was significantly different from the single 150 ppm 6-BA and 30 ppm GA_3 treatments. There were no significant differences in mean individual leaf area between the phytohormone treatments and the control (Figure 10).

The results for mean leaf number per spur were similar to those for total leaf area per spur (Figure 11). Treatment of 30 ppm GA_3 applied twice had the greatest significant difference from the control, resulting in 33% more leaves per spur. Also, there were significant differences between 30 ppm GA_3 applied twice with the 150 ppm 6-BA and 30 ppm GA_3 treatments that were only applied at the second timing.

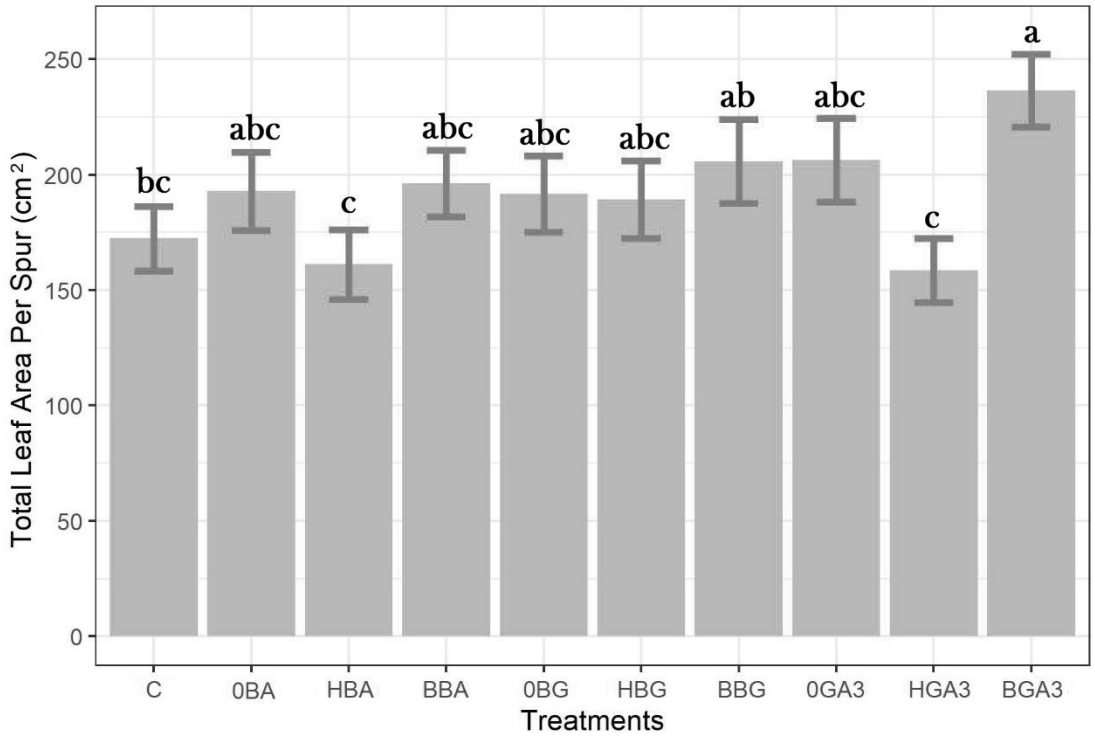

Figure 9. The total leaf area per spur at harvest for the phytohormone timing experiment with 'Burgundy Pearl'/Gi5 sweet cherry trees. 0BA, 0BG and 0GA3 represent 6-BA (150 ppm), 6-BA (150 ppm) plus GA_{4+7} (30 ppm) and GA_3 (30 ppm), respectively, applied May 6. BBA, BBG and BGA3 represent the same rates for 6-BA, 6-BA+GA_{4+7} and GA_3, respectively, applied twice, on May 6 and May 10. HBA, HBG and HGA represent the same rates for 6-BA, 6-BA+GA_{4+7} and GA_3, respectively, applied only on May 10. All the bars represent standard error.

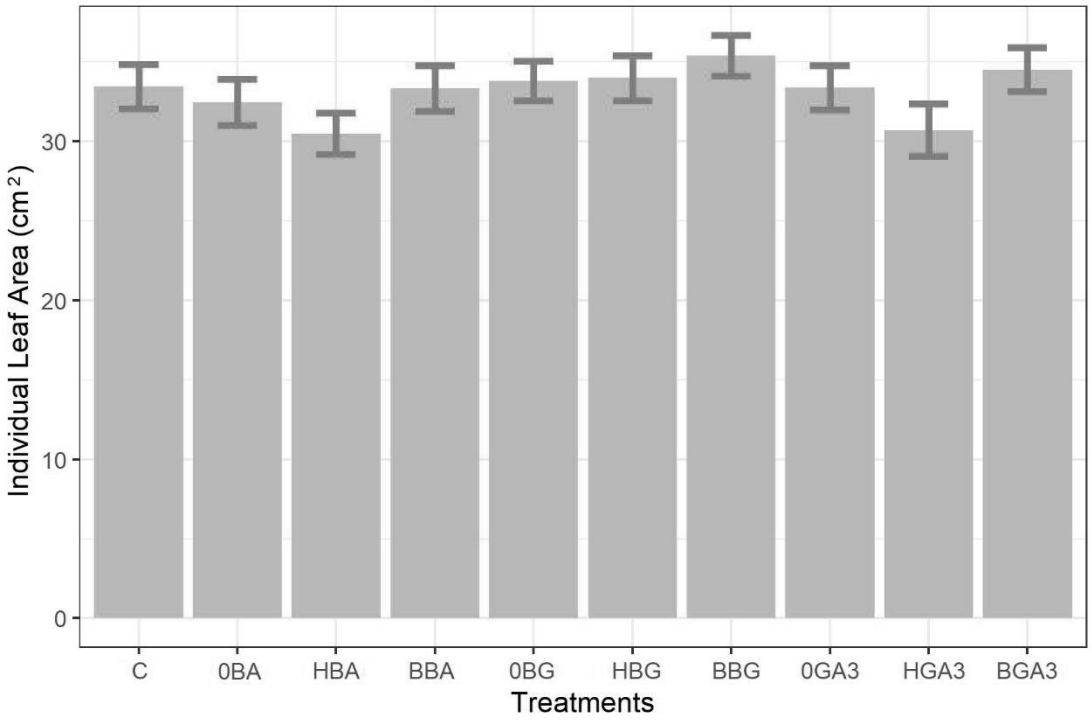

Figure 10. The average spur single leaf area at harvest for the phytohormone timing experiment with 'Burgundy Pearl'/Gi5 sweet cherry trees. 0BA, 0BG and 0GA3 represent 6-BA (150 ppm), 6-BA (150 ppm) plus GA_{4+7} (30 ppm) and GA_3 (30 ppm), respectively, applied May 6. BBA, BBG and BGA3 represent the same rates for 6-BA, 6-BA+GA_{4+7} and GA_3, respectively, applied twice, on May 6 and May 10. HBA, HBG and HGA represent the same rates for 6-BA, 6-BA+GA_{4+7} and GA_3, respectively, applied only on May 10. All the bars represent standard error.

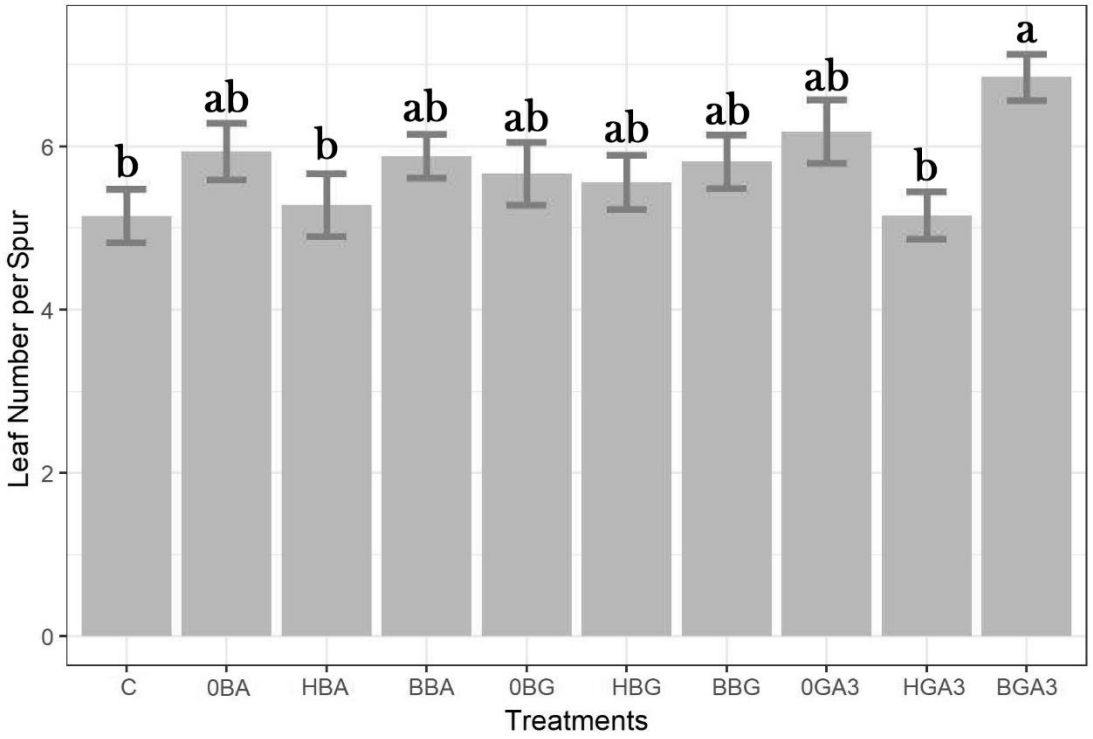

Figure 11. The mean leaf number per spur at harvest for the phytohormone timing experiment with 'Burgundy Pearl'/Gi5 sweet cherry trees. 0BA, 0BG and 0GA3 represent 6-BA (150 ppm), 6-BA (150 ppm) plus GA_{4+7} (30 ppm) and GA_3 (30 ppm), respectively, applied May 6. BBA, BBG and BGA3 represent the same rates for 6-BA, 6-BA+GA_{4+7} and GA_3, respectively, applied twice, on May 6 and May 10. HBA, HBG and HGA represent the same rates for 6-BA, 6-BA+GA_{4+7} and GA_3, respectively, applied only on May 10. All the bars represent standard error.

The 150 ppm 6-BA treatment applied at the first timing and the 30 ppm GA_3 treatment applied at the second time appeared to stimulate more spurs to elongate into shoots, but results were highly variable between replications and none of the treatments were significantly different from each other (Table 3).

Table 3. The percentage of spurs that elongated into shoots for each phytohormone treatment in the application timing experiment with 'Burgundy Pearl'/Gi5 sweet cherry trees. 0BA, 0BG and 0GA3 represent 6-BA (150 ppm), 6-BA (150 ppm) plus GA_{4+7} (30 ppm) and GA_3 (30 ppm), respectively, applied May 6. BBA, BBG and BGA3 represent the same rates for 6-BA, 6-BA+GA_{4+7} and GA_3, respectively, applied twice, on May 6 and May 10. HBA, HBG and HGA represent the same rates for 6-BA, 6-BA+GA_{4+7} and GA_3, respectively, applied only on May 10.

Treatment	0BA	0BG	0GA3	BBA	BBG	BGA3	C	HBA	HBG	HGA3
Shoot Percentage	17.9% NS	14.3% NS	14.3% NS	7.1% NS	3.6% NS	10.7% NS	7.1% NS	3.6% NS	3.6% NS	17.9% NS

P-values of ANOVA test are significantly (*) or not significantly (NS) different from the control at a = 0.05.

Rate experiment. The 75 ppm 6-BA+15 ppm GA_{4+7} and 150 ppm 6-BA+30 ppm GA_{4+7} treatments resulted in 55% and 59%, respectively, larger total leaf area per spur compared to the control, which was significant (Figure 12). The 300 ppm 6-BA treatment also differed significantly from the control, with 42% larger total leaf area per spur. The other treatments had high variability and were not statistically different from the control, even though some were also not statistically different from the best treatments, either.

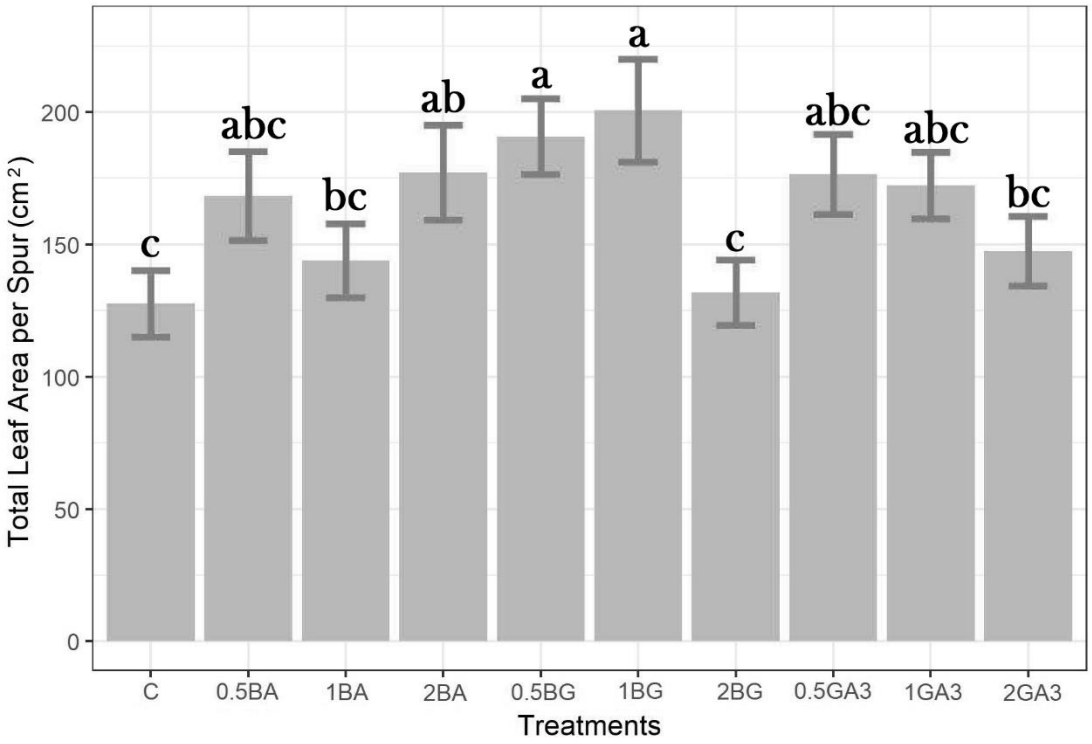

Figure 12. The total leaf area per spur at harvest for the phytohormone rate experiment with 'DD'/Gi5 and 'EE'/Gi5 sweet cherry trees, applied when most of the spurs had one to three leaves emerging. 0.5BA, 1BA and 2BA represent 6-BA at 75 ppm, 150 ppm and 300 ppm, respectively. 0.5BG, 1BG and 2BG represent 6-BA + GA_{4+7} at 75 ppm plus 15 ppm, 150 ppm plus 30 ppm and 300 ppm plus 60 ppm, respectively. 0.5GA3, 1GA3 and 2GA3 represent GA_3 at 15 ppm, 30 ppm and 60 ppm, respectively. All the bars represent standard error.

In the statistical analysis of individual spur leaf areas, there were no significant differences between any of the hormone treatments and the control (Figure 13).

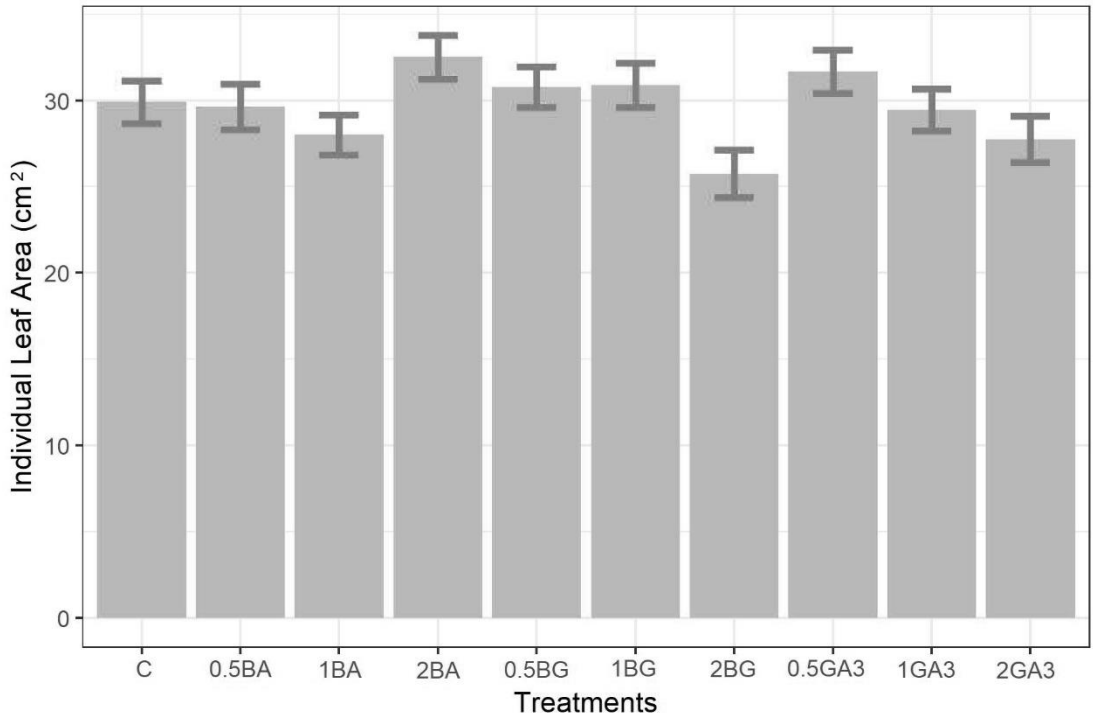

Figure 13. The mean single leaf area at harvest for the phytohormone rate experiment with 'DD'/Gi5 and 'EE'/Gi5 sweet cherry trees, applied when most of the spurs had one to three leaves emerging. 0.5BA, 1BA and 2BA represent 6-BA at 75 ppm, 150 ppm and 300 ppm, respectively. 0.5BG, 1BG and 2BG represent 6-BA + GA_{4+7} at 75 ppm plus 15 ppm, 150 ppm plus 30 ppm and 300 ppm plus 60 ppm, respectively. 0.5GA3, 1GA3 and 2GA3 represent GA_3 at 15 ppm, 30 ppm and 60 ppm, respectively. All the bars represent standard error.

Statistical analysis of the leaf number per spur showed results similar to that for total leaf area per spur (Figure 14). The 75 ppm 6-BA+15 ppm GA_{4+7} and 150 ppm 6-BA+30 ppm GA_{4+7} treatments had significantly more than the control, 44% and 52% more, respectively. The 75 ppm 6-BA group had 33% more leaves per spur than control, which was also significant, as was the 30 ppm GA_3 treatment which was 37% more. The other treatments were more variable, resulting in no significant differences from either the control or the best treatments.

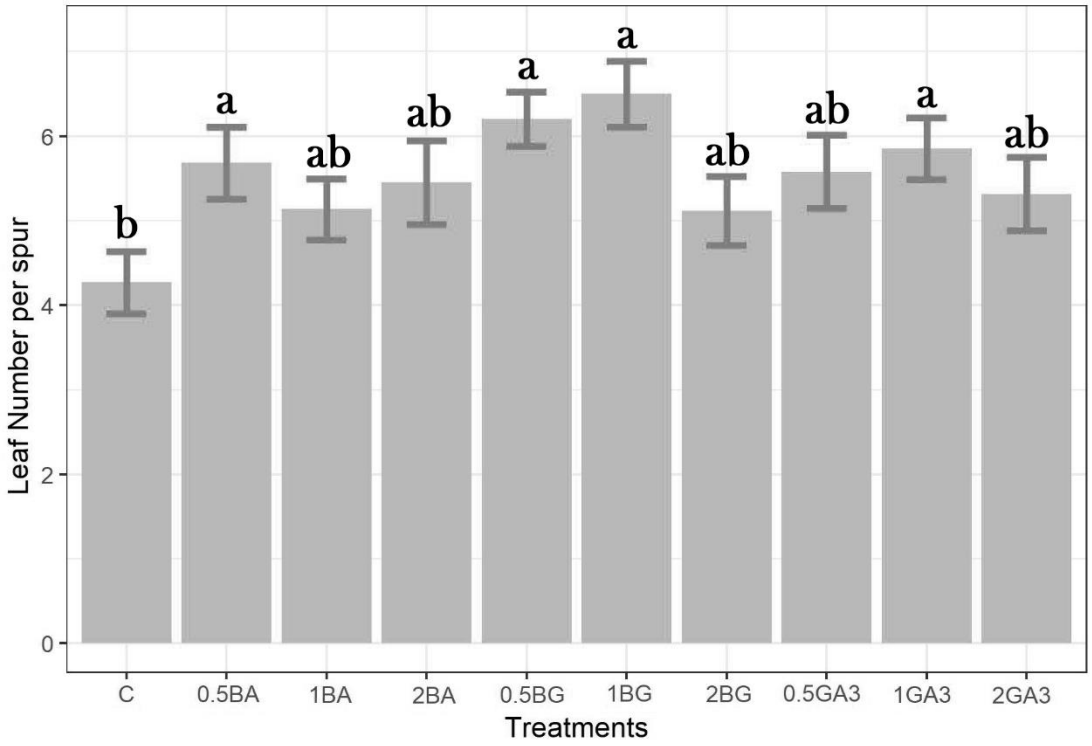

Figure 14. The mean leaf number per spur at harvest for the phytohormone rate experiment with 'DD'/Gi5 and 'EE'/Gi5 sweet cherry trees, applied when most of the spurs had one to three leaves emerging. 0.5BA, 1BA and 2BA represent 6-BA at 75 ppm, 150 ppm and 300 ppm, respectively. 0.5BG, 1BG and 2BG represent 6-BA + GA_{4+7} at 75 ppm plus 15 ppm, 150 ppm plus 30 ppm and 300 ppm plus 60 ppm, respectively. 0.5GA3, 1GA3 and 2GA3 represent GA_3 at 15 ppm, 30 ppm and 60 ppm, respectively. All the bars represent standard error.

As in the Timing experiment, it appeared that some treatments (especially 300 pm 6-BA and 300 ppm 6-BA + 60 ppm GA_{4+7}) stimulated more spurs to elongate into shoots, but due to high variability between replications, none of the treatments differed significantly from the others (Table 4).

Table 4. The percentage of spurs that elongated into shoots for each phytohormone treatment in the application rate experiment with 'DD'/Gi5 and 'EE'/Gi5 sweet cherry trees. 0.5BA, 1BA and 2BA represent 6-BA at 75 ppm, 150 ppm and 300 ppm, respectively. 0.5BG, 1BG and 2BG represent 6-BA + GA_{4+7} at 75 ppm plus 15 ppm, 150 ppm plus 30 ppm and 300 ppm plus 60 ppm, respectively. 0.5GA3, 1GA3 and 2GA3 represent GA_3 at 15 ppm, 30 ppm and 60 ppm, respectively.

Treatment	0.5BG	1BG	2BG	0.5BA	1BA	2BA	0.5GA	1GA	2GA	C
Shoot Percentage	18.75% NS	28.1% NS	31.3% NS	25.0% NS	18.8% NS	31.3% NS	25.0% NS	25.0% NS	25.0% NS	6.3% NS

P-values of ANOVA test are significantly (*) or not significantly (NS) different from the control at a = 0.05.

Gibberellins experiment. The 30 ppm GA_3 treatment was significantly different from the control, resulting in 33% more total leaf area per spur (Figure 15). The 30 ppm GA_3 and 30 ppm 6-BA + 30 ppm GA_{4+7} treatments were significantly different from the 30 ppm GA_{4+7} treatment, which had the lowest total leaf area per spur.

There was no significant difference between any of the hormone treatments and the control for comparisons of either individual leaf area (Figure 16) or leaf number per spur (Figure 17). Unlike the close relationship between total leaf area and leaf number per spur, the larger total leaf area per spur was not primarily explained as more leaves per spur. However, the treatment effects on total leaf area per spur might reflect the combined effects on leaf number and individual leaf area.

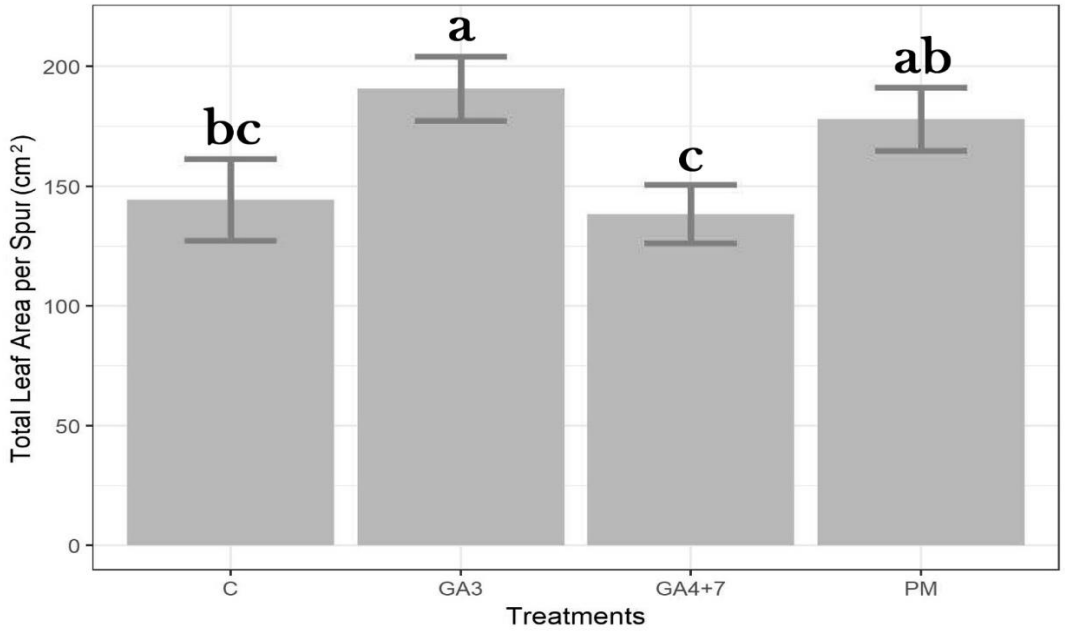

Figure 15. The total leaf area per spur at harvest for the gibberellins experiment with 'GG'/Gi5 sweet cherry trees, applied when most of the spurs had one to three leaves emerging. GA3, GA4+7, and PM represents 30 ppm GA_3, 30 ppm GA_{4+7} and 30 ppm 6-BA + 30 ppm GA_{4+7}, respectively. All the bars represent standard error.

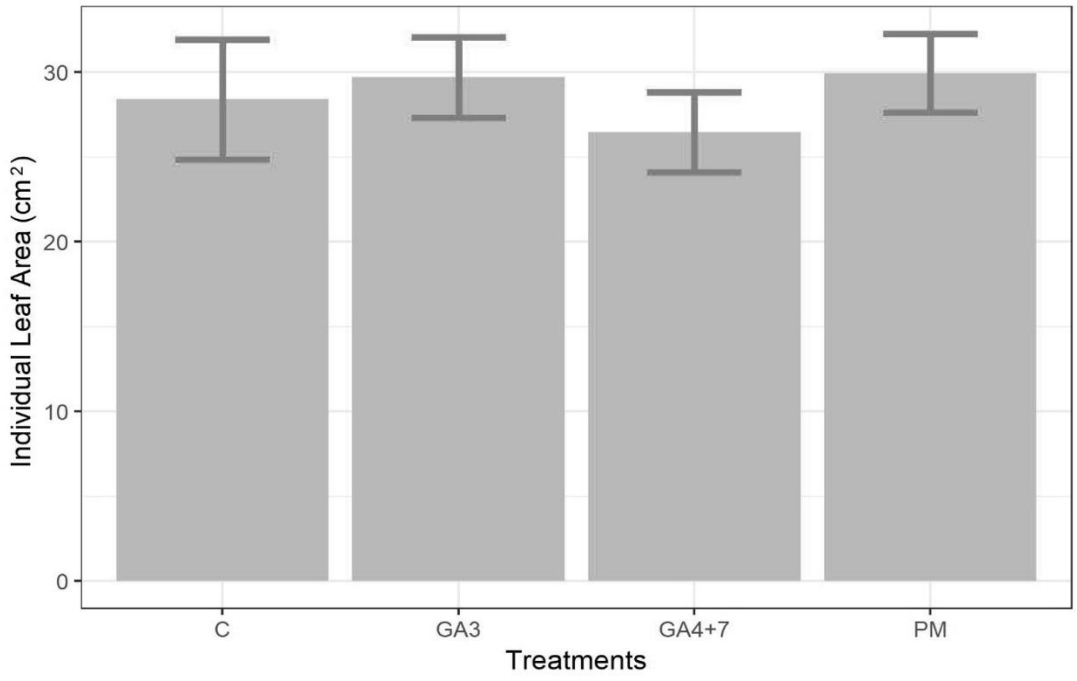

Figure 16. The mean single leaf area at harvest for the gibberellins experiment with 'GG'/Gi5 sweet cherry trees, applied when most of the spurs had one to three leaves emerging. GA3, GA4+7, and PM represents 30 ppm GA3, 30 ppm GA4+7 and 30 ppm 6-BA + 30 ppm GA4+7, respectively. All the bars represent standard error.

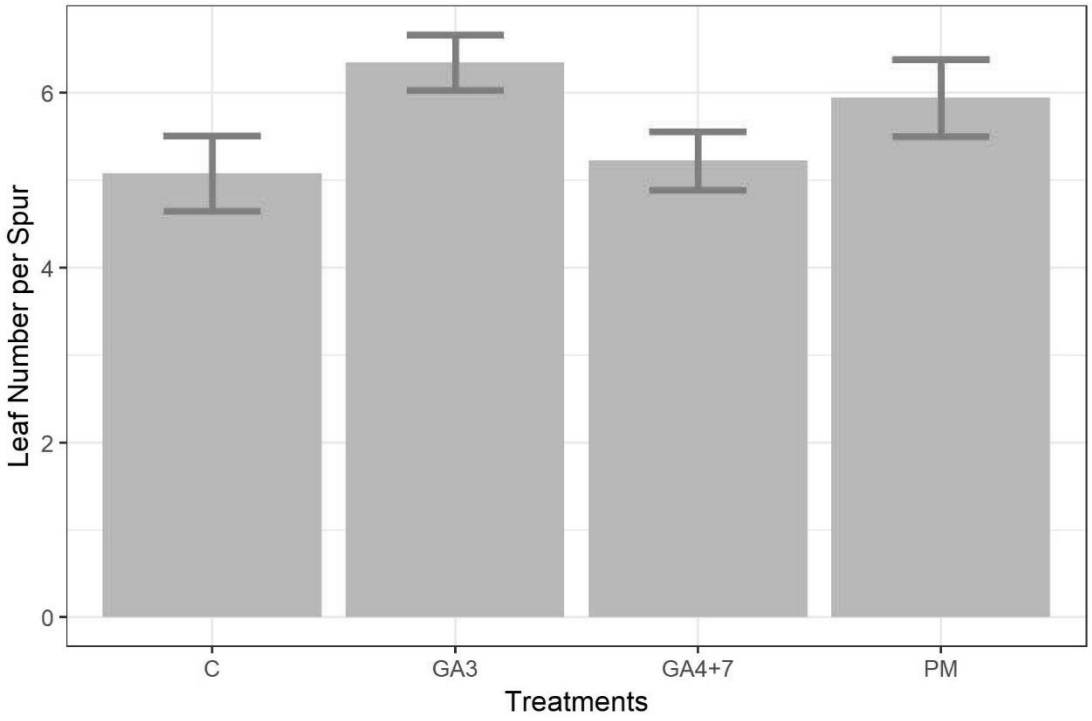

Figure 17. The mean leaf number per spur at harvest for the gibberellins experiment with 'GG'/Gi5 sweet cherry trees, applied when most of the spurs had one to three leaves emerging. GA3, GA4+7, and PM represents 30 ppm GA_3, 30 ppm GA_{4+7} and 30 ppm 6-BA + 30 ppm GA_{4+7}, respectively. All the bars represent standard error.

The stimulation by the various forms of GA for spur elongation into shoots was much lower than for the most active treatments in the Rate experiments, and none of the treatments were significantly different from the others (Table 5).

Table 5. The percentage of spurs that elongated into shoots for each phytohormone treatment in the gibberellin type experiment with 'GG'/Gi5 sweet cherry trees. GA3, GA4+7, and PM represents 30 ppm GA_3, 30 ppm GA_{4+7} and 30 ppm 6-BA + 30 ppm GA_{4+7}, respectively.

Treatment	GA3	GA4+7	PM	C
Shoot Percentage	10.7% NS	0.0% NS	3.6% NS	7.1% NS

P-values of ANOVA test are significantly (*) or not significantly (NS) different from the control at a = 0.05.

Although fruiting data (yields and fruit quality) were planned for each of the

three experiments in 2018, rampant brown rot infections affected the quality of the data and so these were discarded. Since this project was developed for a Masters degree program, the degree timeline was not sufficient to follow the spring 2019 bloom to determine whether any of the hormone treatments significantly affected flower bud initiation and differentiation in 2018.

Discussion

The whole tree applications in 2018 resulted in much more variability and less effect of the plant hormone treatments on spur leaf area. However, in three different experiments (Timing, Rate and Gibberellin types), GA_3 and 6-BA + GA_{4+7} resulted in significant differences with the control, showing a promotion of up to 50% larger total leaf area per spur. In the Timing Experiment, the first spray timing was the same time as in 2017, but none of these treatments gave similar results in 2018. Previous research on different timings of plant hormone applications to leaves was conducted on cashew (*Anacardium occidentale*) trees. Using foliar application of eight different plant growth regulators during cashew flushing, flowering and fruiting, Lakshmipathi et al. (2017) found that the largest leaf area was recorded for trees sprayed with GA_3 at 50 ppm and Ethrel at 50 ppm, regardless of timing. Even though cashew and sweet cherry trees have different leaf growth patterns, application timing should be investigated further, beyond the two timings of the present study to optimize the spur leaf size.

In both the Timing and Rate experiments, individual leaf size did not differ

significantly between treatments. Microscopic quantification of leaf thickness was not measured in the 2018 experiments. Falcioni *et al.* (2017) conducted experiments with GA_3 and paclobutrazol (PAC, a GA biosynthesis inhibitor) treatments on tobacco plants. The results found greater leaf area, thinner leaves and reduced pigment content (expressed on a leaf area basis) in GA_3-treated plants. More work needs to be conducted to determine the potential for plant growth regulators to reliably alter sweet cherry spur leaf size properties.

CHAPTER 5: CONCLUSIONS

Using isolated spurs in 2017, spring foliar applications of the plant hormones 6-BA, GA_3, and GA_{4+7} had variable but positive effects (up to 30% larger leaves) on spur leaf area, especially the BA+GA_{4+7} (150 ppm + 30 ppm) treatment. Also, this mix had a promotive effect on spur elongation into shoots, which could be useful in some training systems (e.g., TSA, SSA, VCL, SB) and undesirable in other systems (e.g., KGB, UFO. Based on light microscope analysis, all plant hormone treatments increased leaf size by promoting larger epidermal cell size. Epidermis cell numbers increased by 12%, 3% and 3% for the 6-BA, BA+GA_{4+7} and GA_3 treatments, respectively.

The 2017 and 2018 experiments shared several application timings and concentrations (30 ppm GA_3, 150 ppm 6-BA and 150 ppm BA+30 ppm GA_{4+7} applied at the stage of one to three leaves emerging), but 2018 data did not show similarly significant different results as in 2017. Possible reasons might be differences between the cultivars used, the higher temperature at application (10°C) in 2018 than in 2017 (4°C), or the different soil types between the MSU Hort Farm near East Lansing (2018) and the MSU Northwest Michigan Horticultural Research Center near Traverse City (2017).

Another possibility for the different results of 2017 and 2018 is rootstock. The experimental trees in 2017 were on vigorous rootstocks with a canopy 5 m in

height and 3 m in width. The 2018 experimental trees were on ‘Gisela 5’ dwarfing rootstock with a much smaller canopy 3 m in height and 1.5 m in width. There are several factors can influence the fruit tree vigor, and rootstock is a strong factor (Sorce *et al*., 2007), which can alter root-to-shoot and shoot-to-root chemical signaling (Gregory *et al*., 2013). Dwarfing rootstocks may influence scion growth by reducing endogenous concentrations of growth-promoting hormones (e.g., auxin, GAs, and cytokinins) and/or increasing endogenous concentrations of growth-inhibiting hormones (e.g., abscisic acid [ABA] and ethylene) at the active meristematic zones for leaf and shoot growth (Gregory *et al*., 2013). Several studies focused on peach, apple and pear dwarfing rootstocks have demonstrated that vigor reduction could be affected by deficiencies in gibberellin levels or signaling (Cristoferi and Filiti, 1981; Erez, 1984; Webster, 2004).)

Van Hooijdonk *et al*. (2011) conducted research on ‘Royal Gala’ apple scions which were grafted onto dwarfing apple rootstock (M.9) to investigate whether changes in scion architecture were explained by changes in endogenous hormones within the scion. The GA_{19} concentration in the xylem sap of the scion on M.9 was significantly lower than in the control. Even though GA_{19} is biologically inactive, it can be converted to GA_{20} by GA_{20}-oxidase. Furthermore, the GA_{20} can be converted to bioactive GA_1 by GA_3-oxidase (Yamaguchi, 2008). Hence, Van Hooijdonk *et al*. concluded that GA_{19} might be an important

root-produced signal regulating scion vigor.

Considering the overall similar and different results in 2017 and 2018, in which individual leaf area was increased in 2017, was not in 2018, and yet overall leaf area increased in some 2018 trials due to spur extension into shoots, suggests that additional research is clearly warranted and necessary before any conclusions regarding potential orchard management recommendations can be made. Only two seasons of research are rather limited with respect to plant growth regulator effects in highly variable orchard environments. However, this research project does show that 6-BA and GAs appear to be more promising than nitrogen-based early spring foliar treatments for potentially positive effects on sweet cherry spur leaf size.

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