THE EFFECT OF SPRING FREEZE ON BLOOM QUALITIES IN PECANS

By

AMANDEEP KAUR

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Punjab Agricultural University

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Thesis Approved:

Lu Zhang

Thesis Adviser

Niels Maness

Charles Graham

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Abstract:

The spring low temperature mainly targets developing buds, new leaves, reproductive organs, which are comprised of soft and fresh tissues, resulting in tremendous economic losses. Pecan is an economically important nut crop of the United States. This research was aimed to study the impact of spring freeze on pecan buds/flowers during the spring season. For this objective, different pecan cultivars were studied under artificial low-temperature conditions provided by freeze chambers as well as after the naturally occurred spring low-temperature (on April 21, 2021) in the Cimarron Valley Research Station (97° 02'13" W 35°58'55" N), Perkins, OK. For the freeze chamber experiment, three pecan cultivar/rootstock combinations i.e., Pawnee/Peruque, Kanza/Giles, and Maramec/Colby were observed after 10 treatments: 5 temperatures (-6°C, -2°C, 0°C, 2°C, and 4°C) each for 2 durations (4 and 8 hours). For the natural spring low-temperature experiments, 5 cultivar/rootstock combinations i.e., Pawnee/Peruque, Kanza/Giles, Kanza/Mount, Kanza/Colby, and Maramec/Colby were evaluated. The visual observation of damage in different pecan cultivar/rootstock combinations showed the same trend in both chamber and field low temperature for example in both cases Maramec/Colby showed minimum injury to buds/flowers/new leaves. Further, different methods such as FDA, H_2O_2, Baker's procedure were tested to check the qualities of the staminate and pistillate flowers after low-temperature treatments. The carbohydrate content from bark and wood tissues was analyzed in both of the experiments. Significant differences were observed in sugars and starch content before and after the freeze event as well as between low-temperature treated samples. In the natural spring event, the bark sugars were significantly increased after the freeze event. In the case of low-temperature treatments, it declines in most of the branches. This suggests the role and variation in carbohydrates utilization and translocation during low-temperature conditions. The gibberellins content was analyzed using the ELISA kit. Overall, this research provides information about different aspects related to spring freeze conditions in pecans.

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CHAPTER I

LITERATURE REVIEW

PECANS AND FREEZE

Pecan, *Carya illinoinensis* (Wangenh.) K. Koch, is a member of the *Juglandaceae* family which is also known as the walnut family (Fayek et al., 2008). It is a commercially valuable native American nut crop grown in the United States (Thompson and Conner, 2012). The US produced approximately 302 million pounds of pecan in 2020 which was 18 % higher than 2019 pecan production. The top pecan producing states were Georgia (142 million pounds), New Mexico (77 million pounds), Texas (45.4 million pounds), Arizona (30.5 million pounds), and Oklahoma (7.45 million pounds) (USDA 2021). Pecans are also produced in other countries including Mexico, Australia, China, South Africa, Canada, Brazil, etc., (Cao et al., 2019).

Pecan nuts are commercially important for their nutritional and medicinal values as they are rich in fats (primary oils), carbohydrates, proteins, calcium, phosphorus, magnesium, vitamins (A, B, and E), etc., (Fayek et al., 2008). Pecans are woody perennial with a relatively long vegetative growth period which takes from 4 to 12 years to produce the first nut crop depending on pecan cultivar and cultural practice (Han et al., 2018). Pecan tree can be a native/seedling (propagated from a seed without grafting or budding) or improved variety/cultivar (asexually propagated by grafting or budding of scion onto a rootstock).

Pecan is monoecious tree that produces male (staminate or catkins) and female (pistillate) inflorescences on different parts of the same tree (Cowell, 2015). The catkins are produced from primary compound buds of one-year-old branch, whereas pistillate flowers are developed from the terminal bud of the current season's shoot. The female inflorescence is a star-shaped terminal raceme (consists of 4-5 flowers) (Andersen and Crocker, 2019). Pecan produces a large number of staminate to pistillate flowers per branch.

Pecan are heterodichogamous as pistillate and staminate flowers mature at different time during flowering seasons. Protandry or type I (exhibited by 'Pawnee', 'Peruque', 'Giles', 'Oconee', 'Caddo', and 'Desirable') are protandrous cultivars which sheds mature pollen before stigmas of the female flowers are receptive. Protogyny or type II (exhibited by 'Kanza', 'Maramec', 'Kiowa', 'Lakota', and 'Colby') are protogynous cultivars in which stigmas of the female flowers are receptive before mature pollen shed (Kuden et al 2013; Andersen and Crocker, 2019; Carroll and Smith, 2017). Pecans are cross-pollinated to ensure maximum nut formation due to asynchronous flowering. Pecan cultivars of the opposing dichogamous type should be within 150 feet of one another for successful wind-borne cross-pollination. Another characteristic of pecans is alternate bearing (AB) due to variation in year-to-year pistilllate flower formation and subsequent nut production (Wood, 2011). The pistillate and staminate flowering variability due to heterodichogamy does not damage the trees but may negatively impact production and quality of nutmeat if pollen is not available in adequate quantities at the time pistillate flowers become receptive (Wood, 2011).

Freeze

Freeze damage is an important limiting factor that affects horticultural crop production and it is a common concern experienced in commercial pecan production regions of the US (Cade, 2001). Freeze injury occurs at temperatures (Sparks et al., 1976) below 0 °C (32 °F), while

chilling injury occurs at temperature above freezing point. Freeze injuries in pecan may occur in the autumn prior to cold-acclimation (Smith et al., 1993; Cade, 2001), in winter when tree are in dormant stage (Wood, 1986) or in the spring season (Malstrom et al., 1982) during and after the process of bud differentiation has started. In pecans, damage on different tree parts has been observed at low temperatures of -10 °C and -2 °C in autumn (Wood and Reilly, 2001; Smith, 2002), -12 °C and -3 °C in winter (Cannell and Smith, 1986; Smith et al., 1993; Malstrom et al., 1982), -15 °C in later winter (Wood, 1986), and -5 °C in spring season (Wood and Reilly, 2001). The damage due to autumn freeze occurs before trees harden off and may be fully foliated in some cases. The primary trigger of freeze damage during this time is generally the non-freezing daytime temperatures followed by rapid drop to below freezing temperature at night (Cade, 2001). Autumn freezes can induce early defoliation as well as canopy, branch, and root death which becomes noticeable only after bud break in the next spring season (Smith, 2002). The typical signs of winter injury are death and browning of the cambium, inner bark, and phloem, as well as cracking and browning of the rootstock phloem and inner bark, bud break delay in the following spring season (Sparks et al., 1976). The fall freeze exposure can trigger early bud break, exposing buds and flowers to early spring freeze injuries (Cade, 2001). Sparks and Payne (1978) suggested late spring freezes are the most damaging to nut production as it is occurring more often in recent years and inflict injuries to pecan buds and flowers.

Spring freeze

During the spring season, pecans initiate their vegetative growth by breaking bud dormancy and producing new leaves and flowers (Han et al., 2018). During spring time, the external factors, especially temperature, control ecodormancy (imposed dormancy, a state of dormancy when growth is suppressed by external environmental factors) release and bud growth (Hentschel, 2020). The water content of the buds increase at this time, enhancing the buds sensitivity to freezing conditions (Melke, 2015). At this time, the cambium is also quite

susceptible to freezing temperatures (Sparks and Payne, 1978; Cade, 2001). As pecan only flowers once a year, spring bud development is crucial for productivity; with freezing injury during bud break and flowering reducing nut yield. In a warm spring, bud-break occurs earlier, while bud-break is delayed in a cold spring. But once the temperature starts to increase, bud break should occur quickly and early (Wells, 2015). Spring low temperature damage is usually limited to newly emerging shoots and followed by regrowth from secondary and tertiary buds. This can result in a considerable reduction in crop production as secondary and tertiary buds have lesser crop potential than primary buds (Cade, 2001).

PECAN FLOWERS

Pecan buds

There are two types of buds in pecans; mixed and compound. The terminal buds are mixed buds with reproductive (female flowers cluster) as well as vegetative tissue (shoots and leaves). This bud does not contain any male flowers. In case the terminal mixed bud dies over the winter, it leaves a lateral bud to serve as the distil (most terminal) bud. The majority of pecan buds are compound buds, which have several buds within compound buds. In pecans, generally there are three compound buds at each node on a shoot, primary (the biggest one), secondary (smaller one), and tertiary bud (the smallest one) (Figure 1.) (http://pecan.okstate.edu/html/introduction/id_2.htm ; Wetzstein and Sparks, 1986). The pecan compound bud consists of two catkin buds (with three catkins per catkin bud) as well as a central mixed bud. Further, the central mixed bud consists of two extra catkins groups (with three catkins in each group), shoot, leaves, and a female flower. If the primary buds are damaged or killed by low temperature, the secondary buds have the potential to produce staminate and pistillate flowers, however the potential to produce normal flowers is less than that of primary buds (Stein, 2003). After a freeze on April 13, during which temperature was -5.5 °C for several hours,

staminate flowers were produced by 38 % of primary buds, 16 % of secondary buds, and 3 % of tertiary buds. In case of pistillate flowers, 8.6 % of primary and 2.6 % of secondary buds produced pistillate flowers, and no pistillate flower formation from tertiary buds (Malstrom et al., 1982).

Secondary buds/abnormal flowering

In pecan, the pistillate flower formation from secondary buds after a spring freeze killed the primary buds has been reported. During normal flowering conditions, the female flowers developed from terminal apex and catkins from lateral buds (from one-year-old branch). But during abnormal flowering, catkins develop from the terminal apex of female flowers (Sparks, 1992; Wetzstein and Sparks, 1986; Woodroof and Woodroof, 1930). The degree of abnormality in flowering varied from a few catkins (staminate flowers) at the pistillate inflorescence apex with pistillate flower cluster to the complete replacement of pistillate flowers by the staminate inflorescence (Sparks, 1992; Wetzstein and Sparks, 1986; Woodroof and Woodroof, 1930). Sparks (1992) investigated the hypothesis that abnormal flowering is caused by unseasonal low temperature conditions near or during pecan bud break time. 'Desirable' was particularly sensitive to environmental fluctuations that cause abnormal flower development (Sparks, 1992). It has been suggested that spring freeze, not a genetic instability, caused the abnormal flowering (Sparks, 1992). In both Alabama and Georgia, a late spring freeze produced the same results; abnormal flower formation from secondary buds' development after low temperature (Cole and Hunter, 1965; Hagler, 1956). Similarly, after a late spring freeze, 'Desirable' pecan trees developed pistillate flowers from secondary buds (Wells, 2008). However, approximately 50 days after flowering, the quantity of fruiting terminals and cluster size dropped by 27% and 69%, respectively, with the majority of the aborted flowers being abnormal. This indicates the flower drop related to reduction of pistillate flowers on freeze damaged 'Desirable' trees were caused by damaged flowers instead of inadequate pollination and fertilization (Wells, 2008).

Pecan male and female flower

The staminate (male) flower is comprised of two components: an anther and a filament. The pollen grains (also called male gametophyte) is found in the anther. The pistilate (female) flower includes three parts: stigma, style, and ovary. The ovule containing the egg or female gametophyte, is located inside the ovary. The anther releases pollen grains at maturity, with the pollen grains being carried by the wind to the stigmatic surface. If the staminate flower is receptive, the pollen will adhere to the stigmatic surface and germinate to form a pollen tube through which male gamete reach to fuse and fertilize the egg. In trees, pollen-pistil interaction regulates the progamic phase, which is the phase of pollen tube growth through pistil from pollination to fertilization. The temperature variations will significantly impact each phase involved in the progamic phase including stigma receptivity, pollen tube germination and growth, and ovule degeneration (Hedhly, 2011).

Some studies have reported on the influence of temperature on the complex pollen–pistil interaction and its susceptibility to temperature changes. In sweet cherries, a 2.8 °C increase in the mean temperature from 13.3 °C - 16.1 °C, decreased stigma activity in the three consecutive steps; 1) the ability to support penetration of pollen tubes into the transmitting tissue of stigma surface declines, 2) the pollen grain germination slows, and 3) the adhesion of pollen grains to the stigma fails to occur (Hedhly et al., 2003). The impact of temperature on stigmatic receptivity has been reported in other plant species including peach, cherimoya, and sweet cherry (Hedhly et al., 2005; Lora et al., 2011; Zhang et al., 2018).

Ovule degeneration in response to low temperature exposure has been investigated in plum, sweet cherry, sour cherry, and citrus cultivars (Beppu, et al., 2001; Zhang et al., 2018; Postweiler, et al., 1985; Montalt et al., 2019). The damage to the ovary and whole pistillate flower due to spring low temperature/ frost was observed in almond, apple, and cherry (Hosseinpour et

al., 2018; Longstroth, 2021; Rodrigo, 2000). The ovarian locule is particularly sensitive to freezing; with the injury frequently characterized by cell wall thickening, lack of meristematic activity and destruction of the vascular tissues (Rodrigo, 2000).

Low temperatures may reduce pollen germination and the tube growth rate, limiting fertilization (Hedhly et al., 2003). The influence of temperature on pollen performance varies among species and cultivars. For instance, pistachio pollen germination and pollen tube growth were both severely impaired following 7 °C exposure (Acar and Kakani, 2010). The lowest pollen germination and pollen tube growth rates in apricot and sweet cherry were observed at 5 °C, while pear pollen germination was reduced at temperatures below 15 °C (Pirlak, 2002; Vasilakakis and Porlingis, 1985). Temperature is a factor in filament length after the tight cluster stage in sweet cherry (Montalt et al., 2019).

Pollen performance can be used to identify genotypes resistant to low and high temperatures (Acar and Kakani, 2010). The optimum temperature for pollen performance varies from one species to another. The effect of spring frost on pollen viability of almonds is highly variable and is dependent on both the freezing stress and the plant genotype/tissue. The pollen germination in different almond cultivars after frost treatment has been studied in relation to boron presence and absence (Moheb et al., 2016). Researchers observed limited pollen growth after -3 °C treatment in the absence of boric acid (Moheb et al., 2016). The range and optimum temperatures for effective pollen germination and pollen tube growth has been studied in other fruit species; papaya, mango (*Mangifera indica*), and sweet cherry (Cohen et al., 1989; Pirlak, 2002; Sukhvibul et al., 2000).

Stigma viability

The pistilate flower organs have a special system that helps with pollen grains competition and distinguishes between pollen at various stages. In pecan, the stigma has large

cone-shapes surface with a small diameter and round surface of papillae cells which makes it well suited for wind pollination by increasing pollen collection efficiency (Sparks, 2005). The stigma surface becomes more prominent in a mature pecan female flower. At maturity, the stigmatic surface varies in color, from green (in 'Kanza', and 'Maramec') to a dark red (in 'Pawnee'). The size, shape, and color of the stigma surface varies distinctly with pecan cultivars (Wetzstein and Sparks, 1986).

The stigma receptivity refers to the ability of the stigma to facilitate pollen adherence and germination. Early or delayed stigma maturation may interfere with limiting the effective pollination period (EPP); the time period during which pollination is effective to produce a fruit. Stigma receptivity has practical applications in agriculture as it limits floral receptivity which decreases the EPP and thus fruit formation and yield (Sanzol and Herrero, 2001).

Mature receptive stigmas ready for pollination are characterized by higher levels of activity of enzymes such as peroxidases, esterases, alcohol dehydrogenases (McInnis et al., 2006; Gupta et al., 2015; Souza et al., 2016). The time period of stigma receptivity varied from few hours to several days (Ferreira et al., 2021). Pecans show two type of dichogamy, which causes pollen shed and pistil receptivity to occur at different times within a tree. In pecans, overlapping period of pistil receptivity and pollen shed varied greatly among and within cultivars, general ranging from 0-8 days (Byford, 2005; Smith and Rombbrg, 1940; Worley et al., 1992). The most common test used for measuring stigma receptivity/viability involves measuring peroxidase activity of the stigma (McInnis et al., 2006). The peroxidase test is based on amount or intensity of oxygen bubbles released by peroxidase enzyme reacting with hydrogen peroxide water (Gupta et al., 2015). Whereas in the alcohol dehydrogenase test, the application of Baker's solution to the stigma produces a violet stain (Gupta et al., 2015).

Ovule viability

In fruit crops, the ovule viability is one of the most important factors that directly affect the EPP and fertilization success (Cerović et al., 2000). The presence or absence of callose (a polysaccharide) deposition ad lignification of hypostase cell walls indicates ovule viability or nonviability (Dumas and Knox, 1983). In response to stress conditions, callose is frequently synthesized in the cells and can be seen as initial sign of ovule abortion and early stage embryo senescence. It can be observed using aniline blue staining and fluorescence microscope (Jiang et al., 2019).

Pollen viability

Pollen viability is a term used to describe the pollen grain's capacity to perform its duties of delivering sperm cells to the embryo sac after compatible pollination (Shivanna et al., 1991). Pollen qualities can be evaluated using different approaches; in vivo, in vitro pollen germination and pollen tube growth, histochemical, and impedance flow cytometry (IFC) (Abdul-Baki, 1992; Heidmann et al., 2016).

In vivo method includes applying pollen grains to emasculated flowers stigmas and counting the number of pollen tubes in crushed styles or number of seeds in the mature fruit. These procedures are time-consuming, making them impractical for testing large number of samples (Abdul-Baki, 1992). In vitro method, germinability and pollen tube growth determined after germinating pollens on artificial media. This approach takes substantially less time than in vivo approach and can be used to screen a large number of samples (Abdul-Baki, 1992). However, pollen viability or performance in vitro is influenced by number of parameters such as optimization of germination medium components (boric acid, calcium, sucrose, etc.), pH, temperature, adequate pollen amount and maturity stage (Abdul-Baki, 1992; Conner, 2011; Wang et al., 2021). Recently developed, IFC is a versatile lab-on-a-chip technique that allows quick and label-free pollen grain analysis. In this technique, pollen grains in suspension flow into a

microchannel, where an alternating electric field is applied. Each pollen grain changes the measured impedance signal depending on its dielectric properties (such as cell membrane polarity, capacitance). The difference between viable and non-viable pollen is observed by measuring the variation in electric impedance value of the suspension buffer. This technique is more efficient and provides the opportunity to test a large number of pollen grains (Ascari et al., 2020). Even though this approach is fast compared to other methods, to test pollen qualities, the instruments and equipment facilities required (such as Amphasys AG) for this procedure are relatively expensive.

The histochemical procedures are based on either the ability of pollen grain's vegetative cell to stain certain components of that cell or specific enzyme activity. These methods require a short time to test pollen viability (Abdul-Baki, 1992). Fluorescein Diacetate (FDA) is a vital staining dye that is hydrolyzed by cellular esterases, resulting in fluorescein buildup and easy detection (Colombo et al., 2017). This approach distinguishes bright green viable pollen grains from dead pollen grains (Colombo et al., 2017). This test determines the presence of active esterases from the pollen cytoplasm and the intactness of the plasma membrane (Novara et al., 2017).

Pollen vigor might be affected by heat exposure, as it increased the time taken in the pollen tube to geminate and reach the ovary (Shivanna et al., 1991).

CARBOHYRATES AND HORMONES

Carbohydrates

Carbohydrates are stored in two different forms, soluble and insoluble. They are retained as insoluble starch in trees, particularly roots (Tromp, 1983). However, in other plant parts, soluble carbohydrates are stored as sorbitol (a sugar alcohol), a key plant component along with fructose, glucose, and sucrose (Tromp, 1983). The seasonal fluctuations in carbohydrates (sugars

and starch) has been investigated in many tree species. Almost all temperate deciduous tree species are characterized by high carbohydrate reserves in late autumn and winter, which are later used during spring growth, before gradual accumulation again during the summer and early autumn (Da Silva et al., 2014; Smith and Waugh, 1938).

Carbohydrates, especially soluble sugars, play essential roles during flowering as they are the primary source of energy. Fluctuation in levels of soluble sugar affect flower induction and development. Sugar could potentially serve as communication molecule between buds and leaves (Fan et al., 2016). In *Arabidopsis thaliana*, activation of *FLOWERING LOCUS T* (*FT*) gene by CONSTANS (CO) protein in response to inductive photoperiod during spring required activity by trehalose-6-phosphate synthase 1 (TPS1) as well as high carbohydrate content as a physiological signal (Cho et al., 2018). Barnet and Mielke (1981) proposed two separate theories, the "carbohydrate theory" and the "phytohormone theory", which proposed the role of carbohydrates and hormones, respectively, in pecan flowering. These theories were later redefined by Wood et al. (2004), proposing that regulation of flowering in pecan is a two-step process, with the first step relying on hormones produced by the fruit and shoots (including foliage), and the second step relying on the size of the dormant season's available carbohydrate reserves at bud break. Wood (2011) later revised this theory to include a third factor. In his three-phase theory, the first stage is regulated by florigen (flowering hormone which controls flowering) with the flowering locus T (FT) protein acting as a long-distance signal. The second and third phases are then regulated by hormones and carbohydrates.

Carbohydrate metabolism, as an energy source, has an important role in the floral induction and flowering processes of plants (Chen et al., 2018). The carbohydrate content has been suggested as an important limiting factor for flower formation in fruit crops (Goldschmidt et al., 1985). A high rate of sugar import is required to meet the respiratory demand of floral tissues, and indeed, high rates of respiration have commonly been documented in floral organs (Borghi

and Fernie, 2017). As a high sugar content surrounding buds is a prerequisite for successful bud break and growth resumption, it is widely accepted that budburst requires rapid and efficient mobilization of carbohydrates at the whole tree level (Zwieniecki and Lampinen, 2015; Simões et al., 2014). During the spring, soluble carbohydrates increase in the xylem sap of walnut, maple, grapevine, willow and pear (Ito et al., 2012; Wong et al., 2003), suggesting the xylem plays a critical role in the translocation of these metabolites during bud break and development. In walnut (*Juglans sp*.), for example, the starch-degrading enzyme activity and co-transport of sucrose in parenchyma cells coincides with bud break and growth initiation (Bonhomme et al., 2010). The reduction of sugars and starch in storage organs can be linked to the increase in soluble sugars in buds, which supports bud break as well as flowering (Simões et al., 2014). The carbohydrate (starch, sucrose, etc.,) levels in roots, shoots, branches, wood, and leaves of various trees such as pistachio (*Pistacia vera* L), chestnut (*Castanea sativa*), pear, orange, peach, apple, pecan, olive, and walnut have been studied with varying degrees of linkage to flowering (Smith and Waugh, 1938; Spann et al., 2008; Zwieniecki and Lampinen, 2015).

Carbohydrates can be used to enhance cold hardiness or to support metabolic processes (Morin et al., 2007). In peach, after low temperature and short day photoperiod, changes in protein abundances related to carbohydrate metabolism were observed, which could be linked to accumulation of sugars as potent cryoprotects (Renaut et al., 2008). Sugars are known to protect cells and membranes (Levitt, 1980). The soluble sugars play a role in vitrification, which protected plants from freezing damage and embryos from desiccation injury (Gusta et al., 1996). The processes that underpin the relationship between cold hardiness and carbohydrate concentration are still unclear (Morin et al., 2007). Understanding the physiological processes underlying low temperature resistance will provide more robust predictions than simply understanding the empirical relationships between environmental conditions and frost risk.

Plant Hormones

The plant hormones are organic compounds which control growth and development of plants at low concentrations. The major plant hormones are auxin, cytokinins (CTK), gibberellins (GA), ethylene, and abscisic acid (ABA). Additional phytohormones thought to be involved in a variety of plant physiological processes include jasmonic acid (JA), salicylic acid (SA), and brassinosteroid (BR) (Wang and Irving, 2011). Plant hormones produced by the plant are known as phytohormones/endogenous/natural plant hormones, while human-made or synthetic compounds known as bio-regulators, plant growth regulators (PGRs) (Gangwar et al., 2014). Synthetic PGRs regulate plant growth and development by mimicking the activity of natural plant hormones. For example, indole-3-acetic acid (IAA), the most abundant form of auxin, indole-3-butyric acid (IBA), and 4-chloroindole-3-acetic acid (4-CL-IAA) are natural auxins, while naphthalene-1-acetic acid (1-NAA), and 2,4-dichlorophenoxyacetic acid (2,4-D), are synthetic auxins. Similarly, zeatin, is the most common naturally occurring CTK, while kinetin is a synthetic analog (Gaspar et al., 1996). The external application of growth regulators can enhance or inhibit the actions of specific plant hormones.

Auxin, the first plant growth hormone to be discovered, regulates various plant processes including apical dominance, stem elongation, formation of lateral and adventitious roots, cell division and expansion, floral bud development, and fruit development (Taiz and Zeiger, 2002; Porfírio et al., 2016). Auxin is a key component in the development of flower primordia, without auxins the plant cannot form normal flowers (Okada et al., 1991). The CTKs are involved in the regulation of growth and differentiation, including cell division, apical dominance, nutrient metabolism, chloroplast development, senescence, flowering, nodulation, and circadian rhythms (Roitsch and Ehneß, 2000; Gangwar et al., 2014). One of CTK's major roles in flowering is delaying senescence. They also play a role in cell differentiation in the floral meristem, influencing the activity of the floral meristem (Gangwar et al., 2014).

ABA is known as ‘stress hormone”, for its roles in response to stress conditions. ABA plays major roles in seed and bud dormancy and responses to water stress by regulating stomatal closure (Wang and Irving, 2011). Ethylene is a gaseous hormone that regulates fruit ripening and processes related to leaf and flower senescence, leaf and fruit abscission, and floral transition (Campos-Rivero et al., 2017; Taiz and Zeiger, 2002; Achard et al., 2007). SA is a phenol hormone involves in flowering transition and timing, thermogenesis, and systematic resistance to plant pathogens (Wang and Irving, 2011; Campos-Rivero et al., 2017; Wada et al., 2010). JA can inhibit germination in non-dormant seed, stimulate germination of dormant seeds, inhibit root growth and tuber formation. It is also related to sterile flower organ formation, senescence, and plant defense metabolism in response to mechanical or biotic injuries (Wang and Irving, 2011; Campos-Rivero et al., 2017). BA are low molecular steroid hormones involved in regulation of cell expansion and division, tissue differentiation, seed germination, reproductive development, and stress resistance (Du et al., 2020; Porfírio et al., 2016; Wang and Irving, 2011).

Gibberellins

GAs are a large family of tetracyclic diterpinoid (more than 125 are known) plant growth substances, that are defined by their chemical structure (Taiz and Zeiger, 2002). The structural feature of all gibberellins have in common is the ent-kaurene ring structure (Taiz and Zeiger, 2002). The gibberellins are numbered as gibberellin AX (GAX), where X is a number in the order of their discovery such as GA_1, GA_2, GA_3 ($_{GA3}$ are identical as gibberellic A) (Taiz and Zeiger, 2002). GA synthesis requires the activity of gibberellin dioxygenases enzymes which consist of two biosynthetic enzymes GA20ox (Gibberellic acid-20-oxidase) and GA3ox (Gibberellic acid-20-oxidase), and an inactivating GA2ox enzyme (Gibberellic acid-20-oxidase), the most important sites of regulation in the GA pathway (Dijkstra et al., 2008).

GA promotes various growth and development processes in plants including seed germination, growth through elongation, leaf expansion, floral initiation, floral organ and fruit development (Matsuoka, 2003; Taiz and Zeiger, 2002). The GAs regulate the development and fertility of flowers by suppressing the function of the DELLA proteins (Cheng et al., 2004). Generally, DELLA proteins inhibit plant growth and GA receptors such as GID1 (GIBBERELLIN INSENSITIVE DWARF1) enhance the degradation of the transcriptional regulators of the DELLA proteins (Murase et al., 2008). Flowering defects results from a loss of function of any component of GA biosynthesis and signaling (Wilson et al., 1992). For example, the GA1 gene encodes an ent-kaurene synthetase enzyme in the first step of GA biosynthesis. Gibberellin-insensitive1-3 (GA 1-3) mutants which are deficient in GA1 gene either never flower or delay flowering during short-day conditions (Sun and Kamiya, 1994; Wilson et al., 1992).

GA_3 and GA_4 are major bioactive forms of GA that promotes flowering in Arabidopsis (Dhar et al., 2019), while in higher plants GA_1 and GA_4 are major bioactive forms of GA (Colebrook et al., 2014). In *Arabidopsis*, GA promote flowering by activating genes encoding the floral integrators *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*), *LEAFY* (*LFY*), and *FLOWERING LOCUS T* (*FT*) in the inflorescence and floral meristems, and in leaves, respectively (Mutasa-Göttgens and Hedden, 2009). A study on *Castanea henryi* suggested that GA, CTK, and ABA have important roles during sex differentiation, whereas the involvement of IAA does not appear to be important (Fan et al., 2017). Their results also indicated that GA and ABA are more involved in male flower development (stamen and anther development), while CTK is more active in female flower development (pistil primordium induction). The hormone CTK is considered a “female hormone” because it exerts significant control of female flower development (Fan et al., 2017). The molecular studies also suggested the involvement of GA in male flower development (Huang et al., 2003).

In many woody perennials GAs inhibit flowering, for example in apple, Jatropha (Bangerth, 2006; Fan et al., 2016; Mutasa-Göttgens and Hedden, 2009). During floral transition in Jatropha, the expression levels of GA biosynthesis genes Jatropha GA 3-oxidase 3 (JcGA3ox3) and GA receptor genes, Jatropha GA-INSENSITIVE DWARF 1C (JcGID1C) were decreased, while a GA catabolism gene, Jatropha GA 2-oxidase 8 (JcGA2ox8), was increased indicating that GA inhibits floral initiation (Li et al., 2018). In pecans, exogenous application of GAs inhibited flowering as its treatment reduced the number of flowering shoots and female flowers per cluster (Wood, 2011). On the other hand, in cashew, treatment of GA_3 led to peak flowering 4 weeks earlier during cool temperatures and therefore might be beneficial in promoting flowering as it led to flower initiation and development (Aliyu et al., 2011). Therefore, there is a complex relationship between GAs and flowering in tree crops.

The plant growth restriction has been linked to reduced GA concentration and signaling in response to various stresses such as cold, salt, and osmotic stress (Colebrook et al., 2014). Low temperature (2 °C, 7 °C) and GA both accelerated plant bolting and flowering through inducing the GA content in the shoot apices of Chinese cabbage (Song et al., 2019). Low temperature, results in increase in GAs and GA inducible gene in *Arabidopsis thaliana* seeds (Yamauchi et al., 2004). On the other hand, in fruit tree, it has also been demonstrated that low temperature can reduce endogenous GAs in fruit species such as citrus and mango (Potchanasin et al., 2009). The relationship between GA signaling and cold stress tolerance is still not fully understood (Colebrook et al., 2014). However, GA is clearly involved in a wide range of responses to both mild and severe abiotic stress, and a better understanding of the role of GA signaling in these responses would be a significant step towards better understanding and improving growth and stress responses of plants under adverse environments (Colebrook et al., 2014).

Accurate plant hormone determination required development of highly sensitive and efficient analytical plant hormone analysis techniques, as the effectiveness of analysis is limited

due to their extremely low concentrations or trace amount in plants (usually ng/g or μg/g). Other factors such as plant hormones are unstable and extremely sensitive to environmental change including temperature, humidity, and light. For example, GAs are highly sensitive to pH and temperature (Wang et al., 2020). Several methods have been developed for quantification/ detection of plant hormones such as bioassays, immunoassays, electroanalysis, and especially chromatographic methods, such as gas chromatography, capillary electrophoresis, and high-performance liquid chromatography coupled with different detectors (Daie and Wyse, 1982; Dhar et al., 2019; Du et al., 2012). Immunoassays based on detection of antibody-antigen interaction, includes radioimmunoassay, enzyme-linked immunosorbent assays (ELISAs), avidin–biotin amplified ELISA, scintillation proximity assays, and immunocytochemical techniques (Du et al., 2012). Different types of ELISA have been used for quantification of various plant hormones including ABA, BR, GA, IAA, ZR, CTK (Daie and Wyse, 1982; Du et al., 2012; Fan et al., 2016; Pradko et al., 2015; Sousa et al., 2011), because it is a comparatively simple procedure, with less expensive equipment required with commercially available hormones kits.

RESEARCH GOALS

The long-term goal of this research was to observe the effect of spring freeze on pecan bud and bloom qualities, i.e., male and female flowers during different growth stages, and to study if there is any correlation in changes in carbohydrates and gibberellins with low temperature.

The objectives of this research were as follows:

- To find the threshold temperatures and tolerance range of pecan buds and flowers to spring freeze.
- To investigate the variation in carbohydrate levels after different low temperature treatments and damage of different pecan cultivars.

- To investigate the changes in Gibberellin levels after low temperature treatments in different pecan cultivars.
- To evaluate different pecan cultivars for their extent of damage to new leaves and male flowers (catkins) by spring freeze in the field environment on April 21, 2021.

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Figures

Figure 1. Multiple buds (primary, secondary, and tertiary) on a node.

CHAPTER II

INFLUENCE OF LOW TEMPERATURE ON VARIOUS DEVELOPMENT STAGES OF PECAN BUD AND FLOWER

ABSTRACT

Pecan (*Carya illinoensis*), an economically important deciduous tree, is well known for its medicinal and nutritional properties and pleasant flavor. Environmental variables have a significant impact on pecan bud, flower, and nut development. Spring freeze can cause severe injuries to pecan bud and flower growth and development. The aim of this study was to observe how various low temperatures can affect pecan buds/flowers at different stages in several pecan cultivars. For this experiment, five pecan cultivar/rootstock combinations; Pawnee/Peruque (PP), Kanza/Giles (KG), Kanza/Mount (KM), Kanza/Colby (KC), and Maramec/Colby (MC), were used grown at the Cimarron Valley Research Station, Perkins, Oklahoma. Branch samples at three different growth stages, i.e., outer bud scale shed, one week after bud break, and early bloom stages were collected from three PP, MC, and KG. A total of 8 treatments: 4 temperatures (-2°C, 0°C, 2°C, and 4°C) each for 2 durations (4 and 8 hours) were given to branch samples by using a Conviron E8 freezing unit, and one set of samples was kept as an untreated control. Shoots were transferred to the growth chambers after the temperature treatment and were provided with average spring temperature and humidity conditions based on 10 years of historical

spring climate data. After 2-3 weeks, branch samples from all temperature treatments were visually observed. The extent of damage was measured in the form of survived and healthy buds, formation of healthy leaves, and formation of healthy flowers. Further male and female flower qualities such as stigma viability, pollen viability, were observed using dissecting and fluorescent microscopy. The carbohydrate and gibberellins content was analyzed from the treated branch samples using anthrone reagent and ELISA method, respectively. From the visual observations, differences in damage among the cultivars, low temperature treatments, and growth stages was observed. Furthermore, significant differences in sugars and starch levels after low temperature treatments and control samples were seen in pecan bark and wood. In case of gibberellins content, there was no significant differences among cultivars and low temperature treatments except PP (-2 °C treatment).

INTRODUCTION

The US produced approximately 302 million pounds of pecan in 2020 which was18 % higher than 2019 pecan production. The top pecan producing states were Georgia (142 million pounds), New Mexico (77 million pounds), Texas (45.4 million pounds), Arizona (30.5 million pounds), and Oklahoma (7.45 million pounds) (USDA, 2021). Temperature is one of the major factors that affects fruit set during the bloom stage (Sanzol and Herrero, 2001). A spring low temperature weather event may affect bud and flower growth and development and thus can cause tremendous economic losses (Warmund et al., 2008). For example, the economic loss for agricultural crops after the 2007 eastern US spring freeze was up to $112 million, with fruit crop loss of $86 million (Ma et al., 2019; Warmund et al., 2008). In 2018, late spring freeze was one of the major reasons for the overall pecan industry's large production drop: the production within the state of Oklahoma was only 64.3% (9,000,000 lbs) of the previous year's production (14,000,000lbs) (USDA, 2018). As April 2018 freeze in Oklahoma damaged approximately 70 percent of the pecan crop (Estimate from pecan grower's meetings).

Pecan is a member of the *Juglandaceae* family, native to North America (Thompson and Conner, 2012). Pecans are monoecious, heterodichogamous woody perennial nut trees commercially important for their nutritional and medicinal values (Andersen and Crocker, 2019; Carroll, and Smith, 2017; Cowell, 2015; Fayek et al., 2008). The damage in pecans can be due to different type of freezes such as autumn freeze, fall freeze, or spring freeze, with all having been reported corresponding to different growth stages. Spring freeze which occurs during the bud break and bloom time can have a damaging impact of nut yield. The success of fertilization is highly dependent on pistil-pollen interactions (Sutyemez, 2011). The stigma viability is a critical factor in the success of fertilization and it varies greatly between plant species (Sutyemez, 2011). Low temperature affects pollen tube growth, while it can extend ovule viability. However, extremely low temperature can reduce effective pollination period (EPP) if ovule longevity is not enough to match slower pollen tube growth (Sanzol and Herrero, 2001).

Several reports included visual observations of damage by spring freeze in pecans (Graham, 2020; Malstrom, et al., 1982). Determining critical temperatures for damage are necessary to evaluate production losses (Matzneller et al., 2016). These critical low temperature experiments for different growth stages in different trees have been conducted using controlled chamber experiments such as in different *Prunus* species, peach (Matzneller et al., 2016; Miranda et al., 2005; Szalay et al., 2018), and to select frost resistant genotypes in Persian walnut and hazelnut (Panahi et al., 2021; Baldwin, 2009). The critical temperatures derived from controlled chamber studies should have an indicative value when deciding whether to activate frost protection systems in the orchard. Field observations are required to validate chamber based critical temperatures to make them more credible (Matzneller et al., 2016).

In peach trees, electrolyte leakage, sugars, starch, and proline content has been studied in correlation to freeze damage in controlled temperature treatments (Yun et al., 2014). In most of the studies it is the proline and electrolyte leakage that has been studied in relation to freeze

damage. Carbohydrate metabolism, has important role in floral induction and flowering processes of plants (Chen et al., 2018). Carbohydrates can be used to enhance cold hardiness or to support metabolic processes (Morin et al., 2007). Gibberellins (GAs) are key hormones that regulate bud dormancy (Liu and Sherif, 2019). Low temperature exposure results in an increases in both GAs and the GA inducible gene in *Arabidopsis thaliana* seeds (Yamauchi et al., 2004). On the other hand, low temperature can reduce endogenous GAs in the fruit species such as citrus and mango (Potchanasin et al., 2009). The relationship between GA signaling and stress tolerance (such as cold stress) is still not fully understood (Colebrook et al., 2014). Elucidation of the role of carbohydrates and GAs in the low temperature responses of pecans would be a significant step towards better understanding and improving growth and stress responses under adverse environments.

In this study, different pecan cultivars were observed after various low temperature treatments. Carbohydrate and gibberellin content was measured from branches and leaves (respectively) and male and female flower qualities were assessed after low temperature exposures. One aim of this study was to determine critical temperatures at which freeze damage begins for different pecan cultivars under controlled conditions at three different bud/flower growth stages and further evaluate variation in carbohydrate and GA content.

OBJECTIVES

- To find the damage threshold temperatures and tolerance range of pecan buds and flowers to spring freeze.
- To evaluate effect of temperature treatments on pecan bloom qualities in terms of stigma viability, pollen viability.
- To investigate the correlation between carbohydrate level and low temperature injury in different pecan cultivars.

- To investigate the correlation between plant hormone (Gibberellins) level and low temperature damage in leaf samples of different pecan cultivars.

HYPOTHESIS

- Different pecan cultivars and growth stages have different tolerance range to spring freeze
- The low temperature during bloom stage affect pecan stigma viability, and pollen viability
- Change of carbohydrate content of the pecan tissues is correlated to low temperature conditions.
- Gibberellins content in pecan leaves change in response to low temperature

MATERIAL AND METHODS

This experiment was conducted in field and laboratory facilities at Oklahoma State University. Five pecan cultivar/rootstock combinations used in the experiments were Pawnee/Peruque (PP), Kanza/Giles (KG), Kanza/Mount (KM), Kanza/Colby (KC), and Maramec/Colby (MC), grown at Cimarron Valley Research Station (97° 02'13" W 35°58'55" N), in Perkins, Oklahoma. These trees were planted in 1993 and 1994. The trees in our orchard were damaged by the freeze last year (October, 2020). We selected and marked trees of approximately equal damage (less than 50 % canopy damage) from MC, PP, and KG in order to keep uniformity in sampling. In KC and KM, we had comparatively less number of trees and almost all the trees were severely damaged so we used all the trees from these while sampling. Further, we collected the branch samples from middle part of canopy of approximately same height and growth stage. The branch samples from pecan cultivar/rootstock combinations were collected at different growth stages; stage 1 (outer bud scale shed stage), stage 2 (one week after outer bud scale shed stage), stage 3 (early bloom stage), and stage 4 (bloom stage). The branch samples of

approximately 30 cm were collected and placed immediately into water buckets to prevent wilting and kept in growth chambers until treated. In the growth chambers (Conviron, CMP3244), temperature, humidity, and light (Fluorescent and incandescent) conditions were used that mimicked spring conditions (Table 1.). The branch samples from PP, KG, and MC were treated with 5 different temperatures (-6, -2, 0, 2, and 4 °C) using a Conviron E8 Freezing Unit 2 different durations (4 and 8 hours). The branch samples from KM and KC were given one temperature treatment (-6 °C for 8 hours). During low temperature treatment, temperature of freezing unit was lowered gradually from 12 °C to treatment temperature (for example -6 °C) by decreasing 3 °C per hour. One set was kept as control in growth chambers (with conditions provided in Table 1.) without low temperature treatment. The water in all the buckets was changed twice per week. All branches were kept in growth chambers for 2-3 weeks after the temperature treatments and observed visually.

Pollen viability

The experiment was conducted in Noble Research Center (NRC) and Life Sciences East laboratories on Oklahoma State University campus in Stillwater. Catkins were collected (at bloom stage) and placed in paper box from five cultivar/rootstock combinations until pollen shed. Pollen was dried for 3-4 days and pollen samples were transferred into small glass tubes and stored in a refrigerator (4 °C) for further analysis (for few days). The fluorescein diacetate (FDA, Sigma-Aldrich, Milwaukee, WI) staining based method was used to test pollen viability after pollen was treated with different low and high temperatures (Impe et al., 2020). Pollen treatment regimens were -2 °C (for 8 hours), 35 °C (for 1 day), -20 °C (for 1 day), 35 °C (for 2 day), -20 °C (for 2 day), 45°C (for 5 day), and fresh (as control). FDA solution was prepared by dissolving 2.4 mM FDA in acetone. At time of pollen analysis, in a petri dish, few drop of sucrose solution (0.5 M, prepared separately) were added (drop by drop) to FDA solution until the solution turned milky. Pollen sample was scattered on the milky solution and mixed. Then immediately a droplet

containing pollen-FDA- solution was placed on microscope slide. Due to the bursting of the pollen, the stain faded fast in the drop. Fluorescence microscope (Nikon Eclipse 80i, Nikon, Japan) was used at blue light to observe difference in pollen fluorescence. Bright green fluorescing pollen indicate that pollens are viable (Impe et al., 2020).

Stigma viability

Different methods were used to observe the stigma viability in pecan pistillate flower to compare different temperature treatment and cultivars. At first, pistillate flowers from all five pecan cultivar/rootstock combinations were observed under dissecting microscope without using any testing or dye solution. The samples were collected from branches treated with temperature treatment (-2 ºC and 2 ºC for 4 hours and 8 hours' duration) and controls.

3% H_2O_2 (Hydrogen Peroxide)

The fresh pistillate flowers were collected from approximately 27-year-old Pawnee/Peruque, Kanza/Giles, and Maramec/Colby on different days during bloom (May 14, May 20, May 24, and May 26, 2021). Also some stigmas from Pawnee/Peruque and Kanza/Giles were collected after branches (at bloom stage) were treated with -2 ºC for 8 hours. The collected flower samples were stained with 3% H_2O_2 for 15 to 20 min at room temperature. Dissecting microscope (Nikon Inc. SMZ1000, Nikon, Japan) was used to observe air bubble formation from stigma surface. Stigma viability was evaluated by amount of bubbles emanating from stigma surface (Fang et al., 2013; Souza et al., 2016). The presence of bigger and many air bubble on stigma surface indicate the presence of more peroxidase enzyme in its surface and viability of stigma surface cells.

Baker's procedure

Principle of procedure: To detect the presence of alcohol dehydrogenase in the stigma surface cells (Dafni and Maués, 1998). The test solution was prepared with: 10 ml of 1 M phosphate buffer (pH 7.3–7.5), diluted (1part buffer to 2 parts distilled water); 5–10 mg nitroblue-tetrazolium; 6 mg of nicotinamide adenine dinucleotide; 1 ml of ethanol (95%). The stigmas were cut and placed on a large droplet of the test solution. The samples were incubated at room temperature in a petri dish for different time intervals (20 minutes, 1 hour, 2 hours, 16 hours), and observed under a dissecting microscope (Nikon Inc. SMZ1000, Japan) to locate the stained areas of stigma surface. The stained area of stigma surface indicate the stigma cells are viable or receptive while non-stained areas indicate non-viable or receptive part of stigma surface.

Carbohydrate analysis

The branch samples of three pecan cultivar/rootstock combinations; Pawnee/Peruque (PP), Maramec/Colby (MC), and Kanza/Giles (KG) kept in the growth chamber after different low temperature treatment were used for this experiment. The branch was divide into three parts; apical, middle, and basal (each approximate size 4-5 cm) from the terminal bud. Wood and bark was separated manually from all branches. Then wood and bark were chopped using pruning shears and scissors and placed in the labelled envelopes (2.25×3.5") and dried in oven (Isotemp oven Model 655F, Fisher Scientific) for 2-3 days at 75 ºC. After drying, the samples were kept at room temperature until further use. Samples were first ground into small pieces using Wiley mill (in Perkins) and placed into 2 ml Eppendorf with 4 mm bearing balls (Precision Chrome Steel G25, UXCELL, China) in it. After first grinding, then Mini-Beadbeater 96 (Biospec Products, Bartlesville, OK) in NRC laboratory was used to make fine powder of samples collected in 2 ml Eppendorf. The fine powder (25-27 mg) was used to quantify the sugar and starch content using a colorimetric method (anthrone reagent method).

Principle of anthrone reagent method

Carbohydrates are dehydrated and depolymerized by concentrated sulfuric acid (H_2SO_4) to form furfural or hydroxymethyl furfural (Katoch, 2011). Anthranol, the enol tautomer of anthrone, is the active form of the reagent, which combines with the carbohydrate furfural derivative to produce a green color in dilute solutions and a blue color in concentrated solutions, which is detected by measuring the absorbance at 620 nm. The anthrone reagent (0.1%) was prepared fresh for every use by dissolving 0.1g of anthrone in 100 mL of concentrated chilled H_2SO_4.

Extraction procedure for sugars and starch

One ml of ultra-pure (UP) water was added to powdered sample (25-27 mg) in a 1.5 ml Eppendorf tube and vortexed. Samples were incubated at 70 °C for 15 minutes and centrifuged for 10 minutes at 15000 rpm. Supernatant (50 µl) was collected to new 1.5 ml eppendorf for sugar analysis, while the pellet was used for starch analysis. For starch extraction, 1 ml ethanol was added to the pellet and centrifuged at 15000 rpm for 10 minutes after vortexing. Supernatant was discarded again, vortexed, and centrifuged after adding 1 ml water to the pellet. Supernatant was discarded again and the pellet was boiled at 100 °C for 10 minutes. After cooling the pellet at room temperature for 20 minutes, 100 µl of each of the enzymes amylo-glucosidase (700 units/ml), alpha-amylase (70 units/ml) and 500 µl of 0.2 M sodium acetate (pH 5.5) were added to the pellet. The samples were vortexed and incubated at 37 °C for 4 hours in a Roto-Therm™ Plus Incubated Rotator (H2024, Benchmark Scientific, USA). After incubation, samples were vortexed and centrifuged for 5 minutes at 15000 rpm. From the supernatant, 200 µl supernatant was transferred to new Eppendorf to use for further analysis.

Assay procedure

Standards- Stock solution of glucose (1 mg/ml) was prepared and stored in refrigerator for future use. Fresh dilutions from stock (as mentioned below) were prepared at time of analysis.

Dilutions- SD1 500 µl glucose + 500 µl water

SD2 300 µl glucose + 700 µl water

SD3 100 µl glucose + 900 µl water

SD4 30 µl glucose + 970 µl water

SD5 15µl glucose + 985 µl water

SD6 0 µl glucose + 1000 µl water

Fifty microliter of each standards and samples was added to 96 wells microplate. Anthrone solution of 150 µl was added to each well and mixed properly. The microplate was incubated for 20 minutes at 100 °C and then cooled down at room temperature for 10 minutes. A microplate reader (Epoch, Bio-TEK, Instruments Inc. Winooski, VT) was used to read the plate at 620 nm. Gen5 3.10.lnk software was used to convert absorbance readings into excel sheet.

Statistical analysis

The absorption reading was compared to a standard curve based on different standard concentrations of glucose and total glucose equivalents was expressed as mg/g DW.

The data was analyzed using PROC GLIM in Statistical Analysis System (Version 9.4; SAS Institute Inc., Cary, NC). A one-way analysis of variance (ANOVA) was performed to determine the effects of freeze on sugars and starch concentration in different cultivar/rootstock combinations, and the treatment differences were analyzed using the LSMEANS with LINES statement at $\alpha = 0.05$.

Gibberellin analysis

Five pecan cultivar/rootstock (approximately 27-year-old) combinations used in experiment were Pawnee/Peruque, Kanza/Giles, Kanza/Mount, Kanza/Colby, and Maramec/Colby, grown at Cimarron Valley Research station, in Perkins, Oklahoma. The branch samples (approximately 30 cm in length) from pecan cultivar/rootstock combinations were collected at stage 4 (bloom stage- May 15, 2021). The branch samples were collected and directly placed into water to prevent wilting and kept in growth chamber (under conditions provided in Table 1.) until treated with low temperatures. The branch samples were treated with 2 different temperatures (-2 and 2 ºC) using Conviron E8 Freezing Unit 2 for 4-hour duration. One set was kept as a control in growth chambers without low temperature treatment. The young leaves from all five pecan cultivar/rootstock combinations were collected from growth chamber conditions (Table 1.) after three-four days of low temperature treatments.

Sample collection and storage

The young leaves from all five pecan cultivar/rootstock combinations were collected in liquid nitrogen and stored at -20 ºC. Also some fresh leaves and bud samples from Pawnee/Peruque were collected one day before analysis i.e. in August. The steps described in the ELISA kit were followed for sample collection, storage, analysis, and data analysis (CEA759Ge, Enzyme-linked Immunosorbent Assay Kit for Gibberellic Acid (GA), Lifeome, Cloud-Clone Corp. USA). Samples were ground to a fine powder in liquid nitrogen using mortar and pestle. Samples were weighed before and after grinding. Samples were collected into a 2 ml Eppendorf tube after grinding and 1.5 ml extraction buffer (10 % TCA) was added. Samples were kept in extraction buffer overnight at -20 ºC and centrifuged the next day at 8000 rpm for 1 hour at 4 ºC. After discarding the supernatant, ice cold 100% acetone (1.5 ml) was added to pellet and centrifuged at 8000 rpm for 15 minutes at 4 ºC. Samples were dried using speed vacuum (Integrated SpeedVac SPD1030-115, Thermo Fisher, USA) for 30 minutes after centrifugation. Samples were kept at room temperature for 30 minutes after adding the 500 µl of lysis buffer (2.7

g urea, 0.2 g CHAPS in 5 ml distilled water), centrifuged at 8000 rpm for 15 minutes at 4 ºC and supernatant was used for the assay.

Reagent preparation and Assay procedure

The standards, Detection Reagent A, Detection Reagent B, Wash solution, TMB substrate, and pre-coated 96 well plate was provided with the ELISA kit (CEA759Ge, Enzyme-linked Immunosorbent Assay Kit for Gibberellic Acid (GA), Lifeome, Cloud-Clone Corp. USA). All regents were diluted according to the instruction in the kit using diluents provided with kit. All samples, standards, and blank (50 µl of each) were added to plate. Detection Reagent A (50 µl) was added immediately to each well and plate was incubated at 37 ºC for 1 hour. The solution was aspirated and washed 3 times with 1X wash solution. Then 100 µl Detection Reagent B was added to plate and incubated at 37 ºC for 30 minutes after covering plate with sealer. The solution was aspirated and washed 5 times with 1X wash solution. The plate was covered with new sealer after adding 90 µl of Substrate solution and incubated at 37 ºC for 20 minutes. Stop solution (50 µl) was added to each well after incubation. Microplate reader (Epoch, Bio-TEK, Instruments Inc. Winooski, VT) was used to read the plate at 450 nm.

Statistical Analysis

The absorption reading was compared to a standard curve based on different concentrations of standards (gibberellin) (provided with essay kit). A standard curve was constructed with the log of GA concentration on y-axis and absorbance on x-axis.

The data was analyzed using PROC GLIM in Statistical Analysis System (Version 9.4; SAS Institute Inc., Cary, NC). A one-way analysis of variance (ANOVA) was performed to determine difference in GA concentration in temperature treatments and also cultivar/rootstock combinations, and the differences were analyzed using the LSMEANS with LINES statement at $\alpha = 0.05$.

RESULTS

Visual Observations

To visually observe and compare the damage after the temperature treatments branches were divided into four categories; number of branches have live flowers (male catkins), number of branches with green healthy leaves, number of branches with only one terminal green bud, and number of branches dead/damaged. The visual observations of eight temperature treatments i.e., 4 ºC, 2 ºC, 0 ºC, -2 ºC for 4 and 8 hours at different stages are discussed in the following paragraphs. While the observations of -6 ºC for 4 and 8 hours' treatment will be discussed separately in secondary and tertiary bud growth section.

Stage 1

The branches collected from three pecan cultivars at stage 1 (outer bud scale shed stage) were observed after three weeks of low temperature treatments (Figure 1 and Figure 2.). The majority of the Maramec/Colby (MC) branches were able to produce large leaves and catkins after all the treatments (Figure 2. B-E). In case of Pawnee/Peruque (PP), branches also produced leaves and catkins, but most of the branches were damaged or died after low-temperature exposure (Figure 2. G-J). PP branches treated with -2 ºC for 4 and 8 hours had more leaves and catkins, compared to all other temperature treatments. Kanza/Giles (KG) branches showed bud break and catkins growth after all the temperature treatments and controls but catkins were very small (Figure 2. L-N). Almost all the KG branches that were treated for 4 hours of duration had better leaf growth or catkin growth than branches that were treated for 8 hours. Overall, when branches were treated at stage 1, the majority of the branches in KG were in categories of only terminal bud live or small catkins, MC were branches with large green leaves, and PP were dead/damaged branches.

Stage 2

The branches treated with different low-temperature at stage 2 (a week after outer bud scale shed stage) were visually observed after three weeks of low temperature treatments (Figure 3). In KG, most of the branches had only terminal green buds or were damaged after all low-temperature treatments. Only a few of the branches produced leaves. The majority of the MC branches were able to produce leaves after temperature treatments. PP branches showed more damage compared to production of live catkins and leaves. Overall, most of the branches treated at stage 2 in KG were damaged/dead or with terminal green buds only whereas MC had green healthy leaves. In PP, the larger number of branches in the category of damaged/dead, followed by live male catkins and leaves.

Stage 3

The branches treated with different low-temperature at stage 3 (early bloom stage) were visually observed after two weeks of treatments (Figure 4). In KG and PP, the most of branches had damaged/dead leaves and catkins after low-temperature treatments. Whereas, majority of the MC branches had green leaves or terminal green buds after temperature treatments, but they were falling down when touched.

The branches collected at stage 3 showed the most damage, followed by stage 2, while the stage 1 branches had the least damage.

Secondary and Tertiary bud break/growth

The growth of secondary and tertiary buds was observed in the branches treated with -6 ºC temperature for 4 and 8 hours as well as in controls after manual removal of primary buds (Figure 5 and 6). At stage 1, the number of branches with secondary and tertiary buds (in percentage) varied among the three cultivars, treatments, and controls (Figure 5.). In case of -6 ºC for 4 hours, KG and PP had more number of branches with secondary bud break and growth (33%), while MC there was no secondary buds' break. For 8 hours (-6 ºC), KG and PP showed

similar higher number of branches with secondary bud growth (26% and 24%, respectively) compared to MC which had 8% branches with secondary bud growth. On the other hand, the controls with removal of primary buds had comparatively higher secondary bud growth in all cultivars; PP (85% branches), KG (62%), and MC (22%). There was 0% tertiary bud growth in all 3 cultivars for 4 hours' duration controls. However, PP and MC showed little tertiary bud growth when treated with 8 hours (4% in both).

At stage 2, the trend of secondary bud growth among the cultivars was opposite to stage 1 (Figure 6.). At this stage, MC had the highest number of branches with secondary bud growth for 4 hours (55%) and controls (100%). While PP and KG branches showed lesser secondary bud break which was 22% (for each cultivar) in 4 hours' duration. In controls, KG had second highest number of branches with secondary bud growth (44%), while 0% in PP. However, with 8 hours of -6 ºC, there were lesser secondary bud growth in PP (33%), MC (17%), KG (0%) branches. The tertiary bud growth was seen in MC (11% branches) when treated with -6 ºC for 4 hours, and in PP (17%) when treated with -6 ºC for 8 hours.

Pollen viability

The fresh pollens from Kanza/Colby (KC) was observed by FDA to compare with other temperature treated pollens later. The observation of preliminary tests showed that there were no differences in florescence of pollen grains observed after short low temperature exposure i.e., 2 °C and -2 °C for 4 and 8 hours as well heat exposure of 30 °C for 2 hours. Also there were no differences in low (-20 °C for 12 hours) and high (35 °C for 12 hours) temperature exposed pollen structure and florescence as most of the pollens were green fluorescent (Figure 7A, B, C). However, some variation in florescence and distorted pollen structures were seen after heat exposure of 35 °C for 2 days and 10 days (Figure 7 D, E).

Stigma viability

The stigmas from different cultivar/rootstock combinations were observed under dissecting microscope without using any test solution (Figure 8.). In the preliminary Hydrogen peroxide test, when fresh pecan stigmas (without any temperature treatments) were observed, clear differences in bubble intensity/amount was seen in green and dark color (appeared dead) stigmas from KC, KG, MC and PP (Figure 9.). There were many big bubbles on stigma surface and peripheral areas of some stigmas while in others stigma surface accumulated many small and compact bubbles (Chen et al., 2013). The bigger and more number of air bubbles indicate presence and more activity of peroxidase enzyme in stigma surface cells and viability of stigma surface cells. Out of the ten PP stigmas observed, only 2 had small and compact bubbles on their stigma surfaces (20 May, 2021). Further PP and KM stigmas were again tested after 4 days (24 Ma, 2021) showed no bubble formation on their surface even though stigma surfaces looked green (live) (Figure 10.). In the case of low temperature (-2 °C for 8 hours) and control KG stigmas, there was clear difference in bubbles formation from different stigma samples among the treatment stigmas as well controls (26 May, 2021).

In Baker's procedure, no staining was observed after 20 minutes, 1 and 2 hours of incubation of pistil flower samples in the test solution (Figure 11). There was some dark on green stigmas kept for 15 hours in the test solution. Stained areas indicate receptive or viable part of stigma surface. Some pistillate flowers with colored stigma such as PP stigma was red colored and dark colored in other cultivars were also incubated with green colored stigmas in the test solution. It was difficult to see the staining in those colored stigma or test those stigmas with this procedure.

Carbohydrate analysis

Sugars and starch content from the bark and wood tissue from the upper part of branch samples of 3 pecan cultivar/rootstock combinations; Pawnee/Peruque (PP), Maramec/Colby

(MC), and Kanza/Giles (KG) treated with 4 different temperature treatments i.e., 4 °C, 2 °C, 0 °C, and -2 °C (each for 8-hour duration) and controls were analyzed at stage 1 and stage 2. The three-way cultivar × treatments × stage interaction was significant in bark sugar, bark starch, wood sugars, and wood starch content (Table 2.).

Sugar content in pecan bark

Stage 1 (Outer bud scale shed stage): In KG, the bark sugar content in branches treated with -2 °C was significantly lower than branches treated with 2 °C, 4 °C, and controls (Figure 12.). In MC, -2 °C treated branch samples had significantly lower bark sugar concentration compared to those treated with 2 °C and control samples. Similarly, in PP and the control, 4 °C, and 0 °C branch samples had significantly higher sugar in comparison to -2 °C treated samples. Further, there was no significant difference in sugar content in control samples (without low-temperature treatment) among the three pecan cultivars. However, there were significant differences among the cultivars treated with low-temperature treatments. For instance, for 4 °C treated MC (37.51 mg/g DW) branch samples had significantly lower sugars in the bark compared to KG (71.49 mg/g DW) and PP (44.49 mg/g DW).

Stage 2 (a week after outer bud scale shed stage): In KG and MC, controls, 2 °C, and 0 °C treated branches had significantly higher sugar level in the bark than those treated with -2 °C temperatures. There were no significant differences in the bark sugar concentration in PP branch samples from all temperature treatments and controls. Further, controls and 4 °C treated branches showed significant differences among the cultivars. For example, in temperature treatment of 4 °C, KG branch samples (41.21 mg/g DW) exhibited significantly higher sugar than MC branch samples (21.95 mg/g DW). However, there were no significant variations in the bark sugar among the cultivars from other temperature treatment samples.

Furthermore, there were significant differences between stage 1 and stage 2 bark sugar concentration in PP and KG. Such as stage 1 samples (71.49 mg/g DW) from KG treated with 4 °C had significantly more sugars compared to stage 2 samples (41.22 mg/g DW). While MC branch samples from stage 1 and stage 2 did not show significant variation in bark sugar from all temperature treatments and controls as well.

Starch content in pecan bark

Stage 1 (Outer bud scale shed stage): In KG, the bark starch concentration in branches treated with 4 °C, 0 °C, and -2 °C was significantly higher than branches treated with 2 °C and controls (Figure 13.). For instance, starch content in the bark of 4 °C treated branch samples was 6.81mg/g DW, while in 2 °C treated samples it was 3.59 mg/g DW. A similar trend was seen in MC and PP, as the 4 °C, 0 °C, and -2 °C treated samples had significantly higher starch levels compared to branches treated with 2 °C and controls. Further, there was no significant difference in starch content in control samples (without low-temperature treatment) among the three pecan cultivars. However, there were significant differences among the cultivars treated with low temperatures of 0 °C and 2 °C. For instance, for -2 °C treated MC (5.22 mg/g DW) branch samples had significantly lower bark starch content compared to KG (6.81 mg/g DW) samples.

Stage 2 (a week after outer bud scale shed stage): In KG, control branches had significantly higher starch amount (4.45 mg/g DW) in the bark than those treated with 4 °C temperature (2.70 mg/g DW). There was significant variation in MC branch samples treated with different low temperatures, such as 2 °C treated set had 4.43 mg/g DW starch content in the bark while 0 °C and 4°C sets had 2.89 mg/g DW and 2.24 mg/g DW. In case of PP, only 2 °C treated and control branch samples significantly differed from each other in the starch levels. Additionally, control branches showed significant differences among the cultivars. For example, KG branches (4.45 mg/g DW) exhibited significantly higher bark starch levels compared to PP

branch samples (1.98 mg/g DW). However, there were no significant variations observed in starch content in the bark among all cultivars from low-temperature treatments.

Moreover, there were significant differences between stage 1 and stage 2 bark starch concentration in all the cultivars branch samples treated with -2 °C, 0 °C, and 4 °C. For example, -2 °C treated samples from stage 1 in KG had 6.81 mg/g DW starch content and stage 2 samples had 3.72 mg/g DW starch content which was significantly lower. Whereas in control sets, stage 2 KG samples had significantly higher starch content (4.45 mg/g DW) than stage 1 KG controls (2.29 mg/g DW). However, 2 °C treated branch samples from stage 1 and stage 2 did not show significant variation in the starch levels.

Sugar content in pecan wood

Stage 1 (Outer bud scale shed stage): In KG, the wood sugar content in branches treated with 4 °C and 2 °C was significantly higher than branches treated with 0 °C (Figure 14.). While in MC and PP, there were no significant differences in sugar content in the control and low-temperature treatment samples. Further, there was no significant difference in sugar content in control samples (without low-temperature treatment) among the three pecan cultivars. However, there were significant differences among the cultivars treated with low-temperature treatments. For instance, for 2 °C treated KG (37.62 mg/g DW) branch samples had significantly higher sugar in the wood compared to PP (17.17 mg/g DW) and MC (17.30 mg/g DW).

Stage 2 (a week after outer bud scale shed stage): In KG, control branches had a significantly higher sugar level in the wood than those treated with all low temperatures. Whereas, no significant differences were measured in the wood sugar concentration in PP and MC branch samples from all temperature treatments and controls. Further, there were no significant variations in the wood sugar among the cultivars from low-temperature treatment and control samples except for 4°C treatment. In temperature treatment of 4°C, KG branches had a

sugar concentration of 20.39 mg/g DW which was significantly higher than PP samples (10.04 mg/g DW).

Furthermore, there were significant differences between stage 1 and stage 2 wood sugar concentration in KG and PP. Such as stage 1 samples from KG treated with 4 °C had significantly more wood sugar (41.83 mg/g DW) compared to stage 2 samples (20.39 mg/g DW). While MC branch samples from stage 1 and stage 2 did not show significant variation in wood sugar content from all temperature treatments and controls as well.

Starch content in pecan wood

Stage 1 (Outer bud scale shed stage): In all three cultivars, the wood starch concentration in branches treated with 4 °C, 2 °C, 0 °C, -2 °C temperatures and controls was not significantly different (Figure 15.). For instance, starch content in the wood of 4 °C treated branch samples of KG, MC, and PP was 2.48, 1.75, 1.97 (mg/g DW), respectively, which was not significantly different. Further, there was no significant difference in starch content in control samples as well as low-temperature treated samples among the three pecan cultivars.

Stage 2 (a week after outer bud scale shed stage): In KG, controls branches had significantly higher starch amounts (6.97 mg/g DW) in the wood than those treated with 4 °C and 0°C temperatures (1.49 mg/g DW and 2.07 mg/g DW). There was significant variation in MC branch samples treated with different low temperatures, such as controls and 4 °C treated set had 1.48 mg/g DW and 1.87 mg/g DW starch content in the wood which was significantly higher than 2 °C, 0 °C, and -2 °C treated sets. In case of PP, only -2 °C treated samples significantly differed from others in the starch levels. Additionally, controls and 2 °C treated branches showed significant differences among the cultivars in wood starch concentration. For example, in control KG branches (6.97 mg/g DW) exhibited significantly higher wood starch levels compared to PP (2.22 mg/g DW) and MC (1.48 mg/g DW) branch samples. However, there were no significant

variations observed in starch content in the bark among all cultivars from other low-temperature treatments.

Moreover, there were significant differences between stage 1 and stage 2 wood starch concentration in the branch samples treated with -2 °C, 2 °C, and controls. For example, -2 °C treated samples from stage 1 in PP and MC had 1.93 mg/g DW (in both) starch content and stage 2 samples had 5.15 mg/g DW and 5.54 mg/g DW starch content which was significantly higher than stage 1. Similarly, at stage 2, KG control samples had significantly higher starch content (6.97 mg/g DW) than stage 1 KG controls (1.76 mg/g DW). However, 4 °C and 0 °C treated branch samples from stage 1 and stage 2 did not show significant variation in the wood starch levels in all three cultivars.

Gibberellin Analysis

The GA concentration observed from leaf samples of different pecan cultivars/rootstock combinations after temperature treatments as well as controls is shown in Figure 16. No significant differences in the GA concentration was observed among the cultivar/rootstock combinations and low temperature treatments, except for Pawnee/Peruque (PP). In PP, GA levels was significantly higher in leaves samples collected from branches treated with 2 °C temperature (179.89 ug/g) than -2 °C (17.19 ug/g), controls (42.27 ug/g), and fresh collected samples (18.16 ug/g). In KG, MC, and KM, there was higher level of GA in -2 °C treatments leaves but it was not significantly different from 2 °C treatment and controls.

DISCUSSION

Visual Observations

Spring low temperature damage mainly targets developing buds, new leaves, reproductive organ, which are comprised of soft and fresh tissues (Hosseinpour et al., 2018).

From our chamber study we observed that the branches at stage 3 (early bloom) were severely and the most damaged after all low temperature treatments compared to stage 2 and stage 1 samples. The branches treated with various low temperature at stage 1 (outer bud scale shed stage), were able to produce leaves and catkins with some extent of damage. The branch samples from stage 2 (one week after outer scale shed) also showed leaves and small catkin formation, but they were comparatively more damaged than stage 1 branch samples. The freeze/frost injury is highly dependent on the phenological growth stage of the bud/flower (Hosseinpour et al., 2018; Aygun, and San, 2005). In pecans, freeze induced minimal harm to dormant or enlarged buds, but caused significant damage to buds during the stage of leaf expansion (Spark,1992; Farokhzad et al., 2018; Grauke and Pratt, 1992). Similarly, in almond, higher damage at full bloom stage than at popcorn stage (which is earlier stage) has been observed by spring frost (Hosseinpour et al., 2018).

Differences in the damage/injury among the cultivars after low temperature exposures was observed. For stage 1, MC branches were the least damaged and were able to produce big green leaves. In case of PP, branches were also able to produce leaves and catkins, but a larger number of branches were damaged/dead. Similarly, in KG, branches showed small catkins and leaves, but most of the branches were damaged or with only terminal live bud. At stage 2, MC branches were the least damaged, followed by PP, and KC (the most damaged). The low temperature tolerance varies among buds/flowers of different cultivars and, in some cases, is higher among different species (Hosseinpour et al., 2018). During the spring, severity of low temperature damage varying among genotypes/cultivars in almonds, apples, and sour cherry (Aygun, and San, 2005; Imani et al., 2012; Szpadzik et al., 2009).

Furthermore, there was only a slight difference in damage between 4-hour and 8-hour duration. Most of the branches under 8-hours showed more damage than 4-hours. The damage extent was directly related to low temperature intensity and duration (Larsen, 2010).

The selection of superior cultivars generally based on climatic adaption, high production, and good quality of kernel and nut. The spring freeze damage can be one of the important limiting factors for tree nut production for pecans, walnut, pistachio, almond, etc. Evaluation and understanding of spring freeze damage among the cultivars is important for the selection and development of trees with a broader climatic adaption. The spring freeze tolerant cultivars with high values of kernel quality and quantity could be selected for cultivation from the breeding programs (Khadivi et al., 2019). For instance, spring freeze damage has been considered as a factor during examining cultivars for selection in walnut (Khadivi et al., 2019) and almonds (Imani and Mahamadkani, 2011).

Secondary and Tertiary bud break/growth

Overall, the control branches with no temperature treatment but primary bud removed manually, had the highest number of secondary bud break/growth in both stage 1 and stage 2, which might be due the complete absence of primary buds on branch as in treated branches the primary buds were still present even they were damaged/killed by low temperature treatment. Another reason of more secondary bud growth in controls (without primary) might be less stress conditions compared to low temperature treated branches. At stage 1, KG and PP showed more secondary bud growth compared to MC. However, at stage 2, MC branches had more secondary bud break than KG and PP. In pecans, after the death of primary bud, the secondary bud break and growth in the field conditions has been observed after naturally occurring spring freeze (Madden, 1980; Reid, 2020). The variation among the pecan cultivars to produce growth from secondary buds has also been reported (Wells, 2008). Furthermore, secondary buds have lesser potential than primary to break and produce leaves and pistillate flowers (Malstrom, et al., 1982).

In case of tertiary bud break, only PP had tertiary bud growth at both stages only when treated with -6 ºC for 8 hours. MC also showed some tertiary bud growth when treated with 4

hours (stage1) and 8 hours (stage 2). Whereas, KG and all the control branches had 0% tertiary bud growth. The tertiary buds have lesser potential to break and produce leaves and flowers compared to secondary buds as it will only grow if both primary and secondary buds are damaged (Caspari et al., 2003). The tertiary buds have lesser potential to produce leaves and pistillate flowers compared to primary and secondary buds (Malstrom, et al., 1982).

Pistillate flower development may be prevented or impeded simply because of the terminal position of the inflorescence when assimilate reserves are low (Lockwood and Sparks, 1978; Yates and Sparks, 1994).

If the primary shoot is damaged or destroyed, for example by spring frost, the secondary or tertiary bud may grow. Secondary or tertiary buds may also develop shoots in grapes when vines are pruned hard or when the parent vine exhibits high vigour. However, both the secondary and tertiary buds are considerably less fruitful (Bennett, 2002).

Pollen viability

This was preliminary experiment in which we observed the pollen viability by FDA test. The FDA test has been successfully used for testing pollen viability after various low and high temperature exposure in nut trees such as walnut and hazelnut (Novara et al., 2017; Ozcan et al., 2019). After a short exposure of low and high temperature (4, 8, and 12 hours), there was no differences in pollen florescent. This indicates that both low and high temperature (-20 °C and 30 °C for 12 hours) exposures failed to reduce pollen viability at mature pollen stage. Recently, it has been observed that pecan pollen can maintain nearly 50% viability even when stored at -80 °C for a year (Wang et al., 2021). Similarly, hazelnut pollen can maintain its high viability at -30 C for long time (Novara et al., 2017). However, longer heat exposure (35 °C for 2-10 days) can lead to distort pollen structure and caused change in florescence in very few pollen grains. In field pea, pollen viability was observed using FDA test after 4-7 days of heat exposure (35 °C). They

found the heat stress can effect pollen viability and vigor as it increased the time taken by pollen tube to geminate and reach the ovary (Conner, 2011; Jiang et al., 2019; Sparks and Yates, 2002).

This suggested that pecan mature pollen grains are more resilient to low as well as high temperature stresses. However, instead of influencing mature pollen viability, freezing temperature might affect the pollen development. Therefore, in future, we will be observing paraffin sections of pecan male catkins to observe pollen development.

To investigate the impact of environmental factors on pecan pollen viability in detail, we need a highly reliable, reproducible, and less time consuming approaches to observe pollens. FDA test might be good method in combination with other dyes methods or in vitro method to ensure high efficiency of pollen viability testing.

Stigma viability

Variation in shape of stigma surface among pecan cultivars was observed such as round and big stigma surface in PP and MC, while elongated boat shape in KG, KC, and KM. Hydrogen peroxide determined the stigma viability and receptivity on the basis of amount and intensity of bubbles from stigma surface. We observed clear differences in bubble formation (in number and size) among cultivars on different days. For instance, in PP there were more bubbles formation on May 14 (approximately 5 days after pollen shed in PP) compared to no bubbles formation on May 20. Further, in green and dark colored stigmas from KG (collected May 26) we observed almost no bubble formation and these samples were collected from 2° bud growth after the freeze event. This method is an inexpensive and easy to perform, however, presence of tissue damage can lead to misleading results (Souza et al., 2016). This method could be a good approach for stigma viability when used in combination with other methods to increase efficiency of results. Papillae degeneration and the production of exudate have been associated with the beginning of stigma viability in tree crops (Yi et al., 2006).

The Baker's procedure based on alcohol hydrogenase activity in stigma surface cells, in which stigma stained dark brown-purple. However, this test doesn't produce stain for every species (Dafni and Maués, 1998). The incubation time of samples in test solution also varied from 20-40 minutes to 12-18 hours among species (Dafni and Maués, 1998; Gupta et al., 2015). In our observation we did not observe any stain on stigma after 20 minutes, 1 and 2 hours' incubation. However, we observed staining on green stigmas after 15 hours. Some pistillate flowers with colored stigma such as PP stigma was red colored and dark colored in other cultivars were also incubated with green colored stigmas in the test solution. It was difficult to see the staining in those colored stigma or test those stigmas with this procedure.

Carbohydrates analysis

The sugars from three pecan cultivar/rootstock combinations showed difference in content after various low temperature treatments, while the pattern of change was similar among the cultivars. In our study, we observed a significant decrease in the bark sugar levels for both stages, in low temperature (especially at -2 ºC in all pecan cultivars) treated branches compared to controls. Further, MC bark sugars significantly reduced in 4 ºC temperature treated branches at both stages and it has better growth than other two cultivars at 4 ºC treatment. This might indicate that MC was less affected by 4 ºC and it utilized its bark and wood sugar for buds, new leaves, catkins growth and development. Similar kind of results were reported in peach after controlled freezing treatments, where sugar content was lower in freezing temperature treatments (Yun et al., 2014). Although, sugar content is generally expected to increase in low temperature conditions, as seen in samples from natural freeze experiment (discussed in chapter 3). This could be explained by the fact that branches are no longer attached to trees, which limited the translocation of sugars to shoot apex from nearby and lower parts to support higher demand in apical portion. In case of field conditions, higher sugars content in apical portions after freeze might be due to mobilization of sugar from lower and nearby parts to apical portions in order to

support higher sugar demand during low temperature. Carbohydrates can be used to enhance cold hardiness or to support metabolic processes (Morin et al., 2007). The bud growth is supported by the carbohydrates content near the shoot apex as well as those transported by the vascular system (Tixier et al., 2017).

Overall there was higher sugar content in both bark and wood in KG, which was the most damaged compared to MC and PP. This could be due to differences in growth stages of different cultivars (during sampling time, we collected branch samples from KG, MC, and PP on same day); in field during sampling time KG had a little behind in term of bud break, and new leaves compared to more growth on MC and PP, which indicate that these cultivars were using their sugars rapidly for new growth. Similarly, in chamber conditions after low temperature, 'Kanza' has the least growth in term of bud breaks and growth, leaves and catkins formation compared to other cultivars. However, higher total soluble sugar content was observed in the most resistant Eucalyptus genotype after controlled experiment (Leborgne et al., 1995). Similarly, in walnut under freezing conditions, significant increase in sugar in tolerant genotypes compared to sensitive genotypes (Panahi et al., 2021).

At stage 1, the starch content in bark samples treated with low temperatures was significantly higher than controls (except 2 ºC). Starch is the major carbohydrate reserve in trees. Starch may be utilized as a secondary source when demand exceeds concurrent photoassimilate supply (Bustan et al., 2011). On the other hand, at stage 2, there were no significant variations in bark starch levels after low temperatures. This could be related to more damage and lesser growth after low temperature at stage 2 compared to stage 1. At bud break stage which is before the leafing period, the carbohydrates taken up by buds are derived from the mobilization of storage organ reserves (Alves et al., 2007). Such as glucose is generated from starch degradation or by the cleavage of sucrose (Cho et al., 2018). Rapid and efficient mobilization of stored carbohydrates is necessary to sustain the bud growth after bud break (Simões et al., 2014). In

apple during initial growth stages, during ON year, lower concentration of soluble sugars and starch was observed compared to OFF year leaves. This might be because of sugars being used by developing flowers (Fan et al., 2016).

Further, no significant variation was seen in wood starch among all temperature treatments and cultivars at stage 1. No significant changes in starch content were reported in hazelnut cultivars after low temperature treatments (Wanjiku and Bohne, 2016). Similarly, in Eucalyptus no significant variation in starch content was observed in chamber and field conditions (Leborgne et al., 1995). However, starch content in wood and bark from stage 2, showed significant variation among cultivars in control samples, which indicate toward difference in growth rate and utilization of starch among different cultivars.

Some interesting observation was seen in this experiment such as at both growth stages, MC bark sugar levels showed significant rapid decline even after 4 °C temperature exposure, whereas wood sugar also had decline but not significantly.

Gibberellin Analysis

This experiment was aimed to observe changes in GAs in terminal new leaves after low temperature treatments in comparison to controls (with no temperature treatment) during the bloom stage in pecans. Cold and GA both accelerated plant bolting and flowering through inducing the GA content in the shoot apices (Song et al., 2019). In our experiment, we observed significant higher GA level in 2 °C treated samples only in Pawnee/Peruque (PP). In KG, MC, and KM, there was higher level of GA in -2 °C treatments leaves but it was not significantly different from 2 °C treatment and controls. Similarly, higher GA and other hormones concentration after low temperature (2 °C) has been observed in Chinese cabbage (Song et al., 2019). In cotton, GhDREB1 improves plant tolerance to low temperature and is negatively regulated by GA_3 (Shan et al., 2007). A subset of GA biosynthesis genes is activated in response

to low temperature (4 °C), resulting in increased levels of bioactive GAs and transcript abundance of GA-inducible genes in imbibed Arabidopsis thaliana seeds (Yamauchi et al., 2004). On the other hand, in fruit tree, it has also been demonstrated that low temperature can reduce endogenous GAs in citrus and mango (Potchanasin et al., 2009). In our experiment, no significant differences in the other temperature treated and controls were observed in the endogenous concentration of GAs in leaves from all cultivars/rootstock combinations.

GA_3 application significantly increased the number of male flowers, total flowers, and male: female flower ratio per branch (Hassankhah et al., 2018). Higher levels of GAs facilitate differentiation of male flowers (Fan et al., 2017). GA improves anther cold tolerance by regulating stress-responsive genes in almond (Li et al., 2021).

The relationship between GA signaling and cold stress tolerance is still not fully understood (Colebrook et al., 2014). However, GA is clearly involved in a wide range of responses to both mild and severe abiotic stress, and a better understanding of the role of GA signaling in these responses would be a significant step towards better understanding and improving growth and stress responses of plants under adverse environments (Colebrook et al., 2014).

Conclusion

The differences in damage to buds, leaves, and flowers after various low temperature treatments was observed among three pecan cultivars and growth stages. Maramec/Colby showed the least damage at all three growth stages i.e., outer bud scale shed (stage I), one week after outer bud scale shed (stage II), and early bloom stage (stage III), while Kanza/Giles showed the maximum damage at all three growth stages. Further, at stage 1, Maramec/Colby did not showed damage after low temperature treatments (even at -2 ºC), at stage II, it showed damage after 0 ºC and -2 ºC treatment and at stage III, it showed damage after 4 ºC treatment. In case of

Kanza/Giles, it showed damage at stage I after all low temperature treatment (including 4 ºC). Similarly, at stage II and stage III, Kanza/Giles showed sever damage after all low temperature exposure. In case of Pawnee/Peruque, stage III was more sensitive to low temperature (4 ºC for 4 hours) compared to stage I and stage II. Further, in growth chamber conditions after low temperature treatments (-2 ºC for 8 hours) significantly lower bark soluble sugar content was observed at stage 1 and stage II. The decline might be due to utilization of sugars to protect from low temperature. From this chamber study, we can get an idea about damage extent in the field conditions to pecan cultivars by low temperature during spring at different stage growth.

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Tables and Figures

Table 1. Controlled temperature, humidity, and light conditions in the growth chambers

Time	Temperature (F)	Temperature (C)	Relative Humidity(%)	Light duration/intensity
0:00	58.1	14.5	75.7	0
1:00	57.0	13.9	77.9	0
2:00	56.1	13.4	79.5	0
3:00	55.3	12.9	80.7	0
4:00	54.7	12.6	81.5	0
5:00	54.2	12.3	82.7	0
6:00	53.7	12.1	83.7	0
7:00	54.3	12.4	83.0	11
8:00	56.9	13.8	78.3	22
9:00	59.7	15.4	72.1	33
10:00	62.5	16.9	66.2	33
11:00	65.0	18.3	60.9	33
12:00	67.0	19.4	56.9	33
13:00	68.6	20.3	54.0	33
14:00	69.8	21.0	52.0	33
15:00	70.7	21.5	50.2	33
16:00	70.9	21.6	49.4	33
17:00	70.5	21.4	49.8	33
18:00	69.4	20.8	51.4	11
19:00	67.1	19.5	55.6	0
20:00	63.8	17.7	55.6	0
21:00	61.7	16.5	66.0	0
22:00	60.2	15.7	69.5	0
23:00	59.1	15.1	72.4	0
	Light intensity: 0 - No light/lights off, 11 and 22- low light, 33-full light			

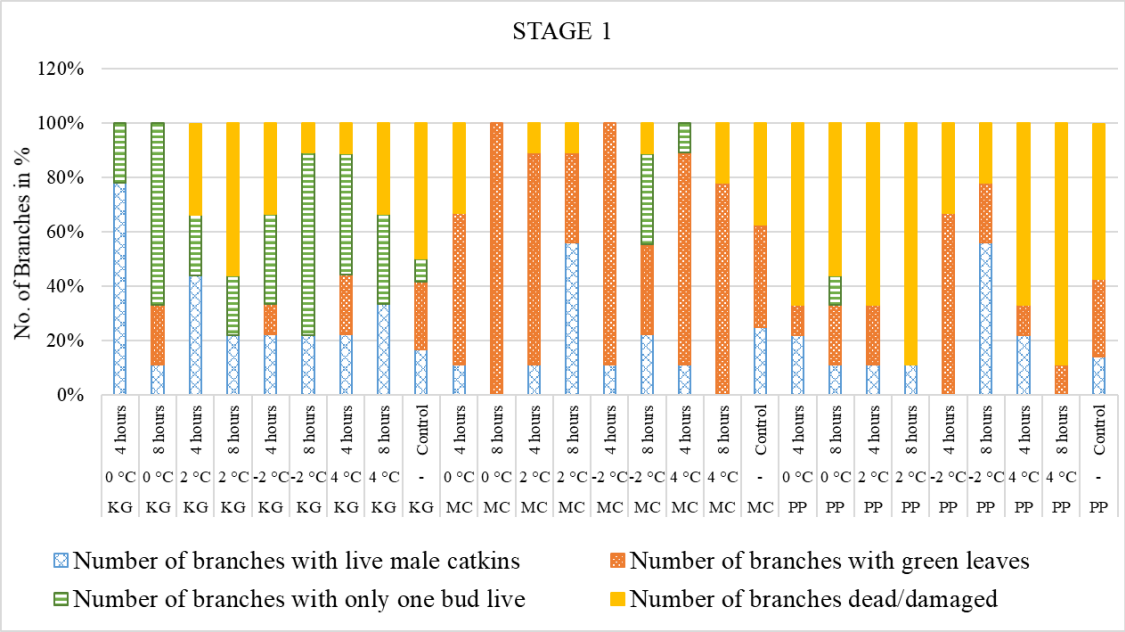

Figure 1. Visual observation of stage 1: branches after treating with 8 different temperatures and controls of 3 pecan cultivars KG (Kanza/Giles), MC (Maramec/Colby), and PP (Pawnee/Peruque).

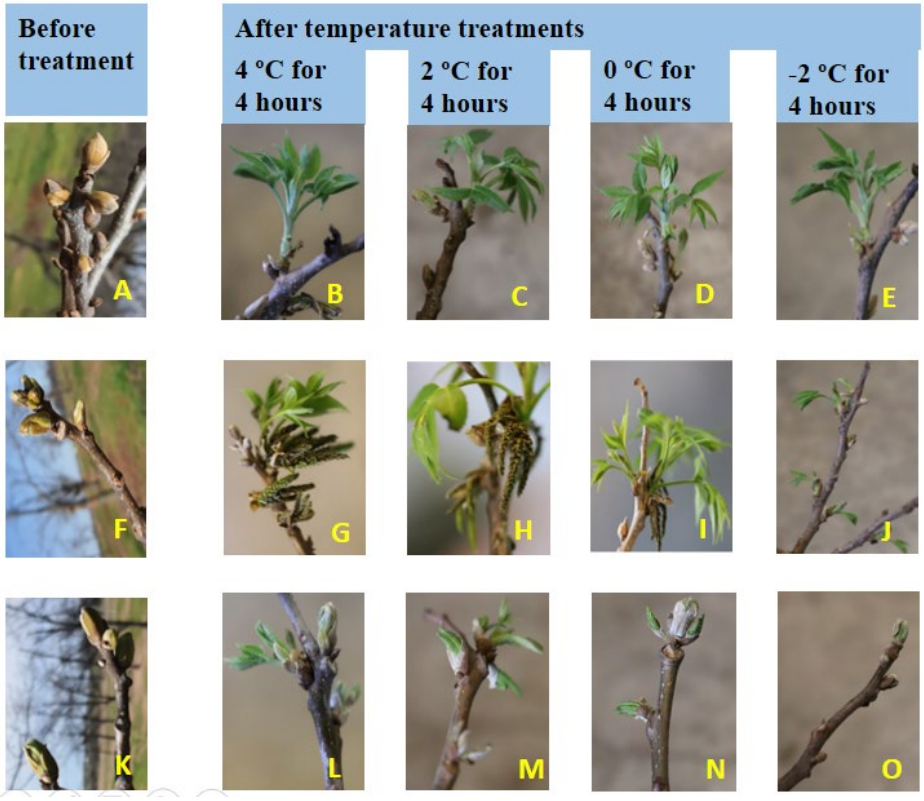

Figure 2. Visual observation of stage 1: branches before treatment and after treated with low temperatures from 3 pecan cultivars Maramec/Colby (A-E), Pawnee/Peruque (F-J), and Kanza/Giles (K-O).

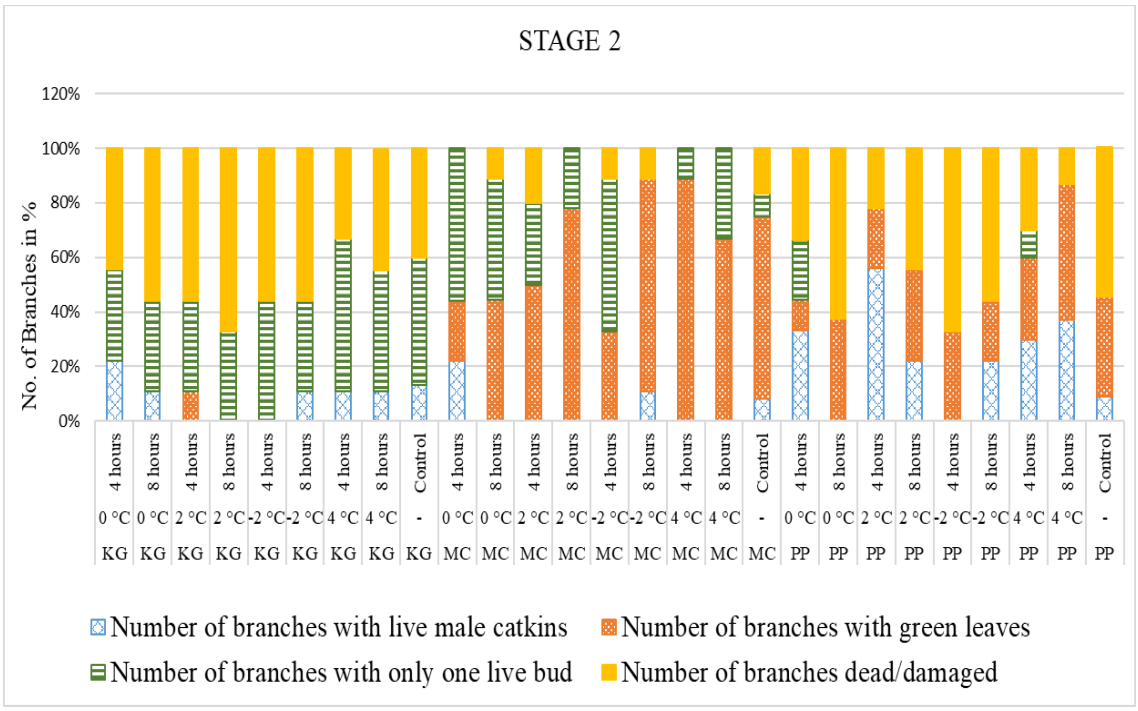

Figure 3. Visual observation of stage 2: branches after treating with 8 different temperatures and controls of 3 pecan cultivars KG (Kanza/Giles), MC (Maramec/Colby), and PP (Pawnee/Peruque).

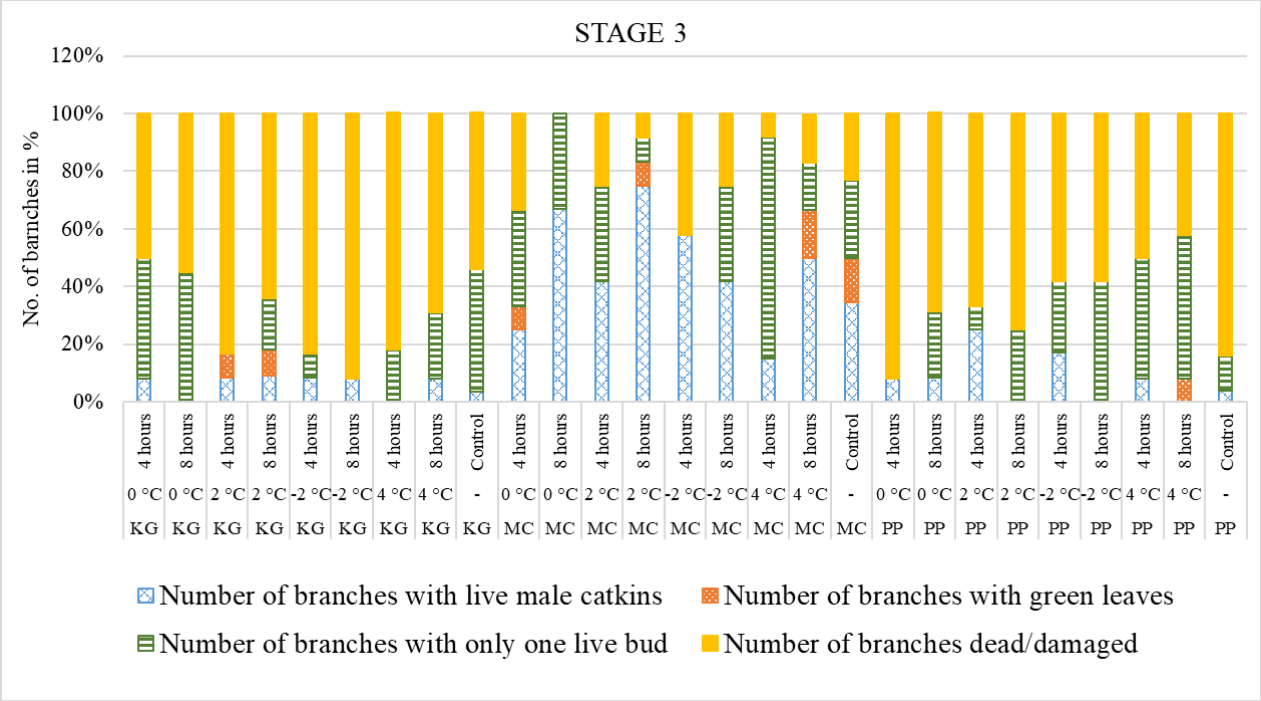

Figure 4. Visual observation of stage 3: branches after treating with 8 different temperatures and controls of 3 pecan cultivars KG (Kanza/Giles), MC (Maramec/Colby), and PP (Pawnee/Peruque).

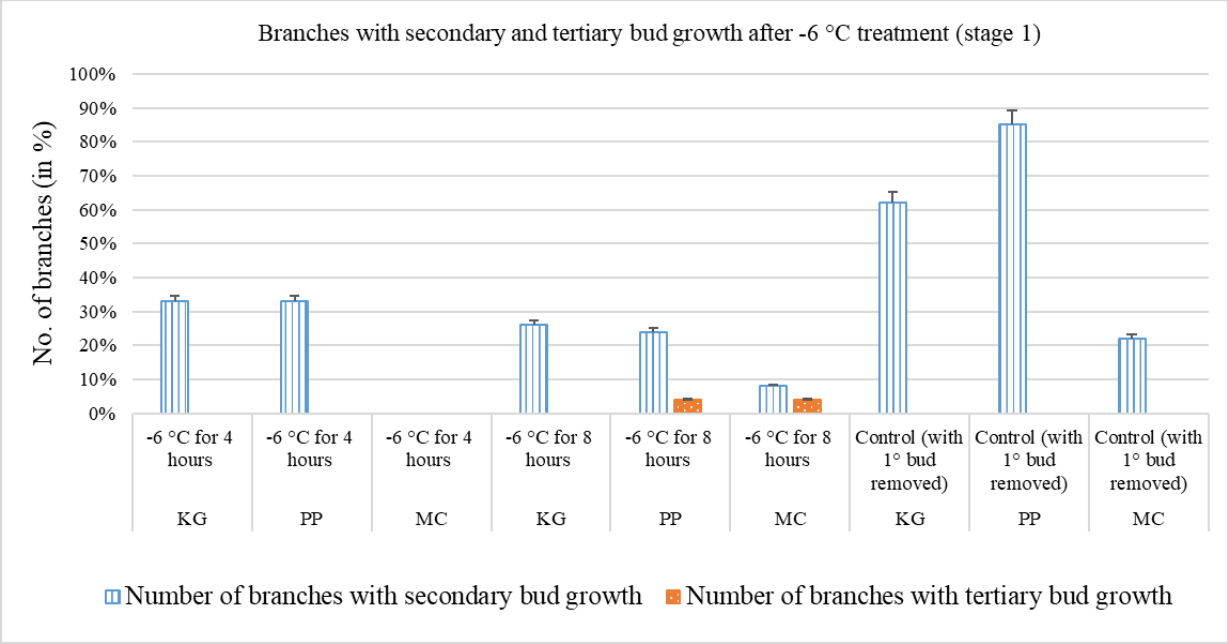

Figure 5. The secondary and tertiary bud growth observation of stage 1 branches after treating with -6 °C temperature and controls of 3 pecan cultivars KG (Kanza/Giles), MC (Maramec/Colby), and PP (Pawnee/Peruque).

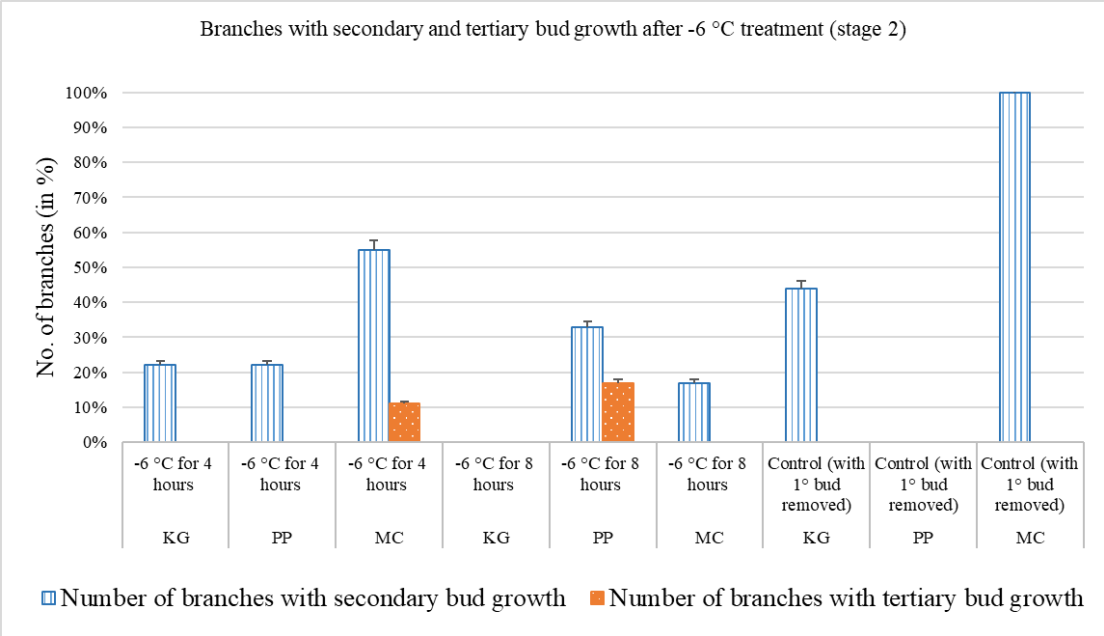

Figure 6. The secondary and tertiary bud growth observation of stage 2 branches after treating with -6 °C temperature and controls of 3 pecan cultivars KG (Kanza/Giles), MC (Maramec/Colby), and PP (Pawnee/Peruque).

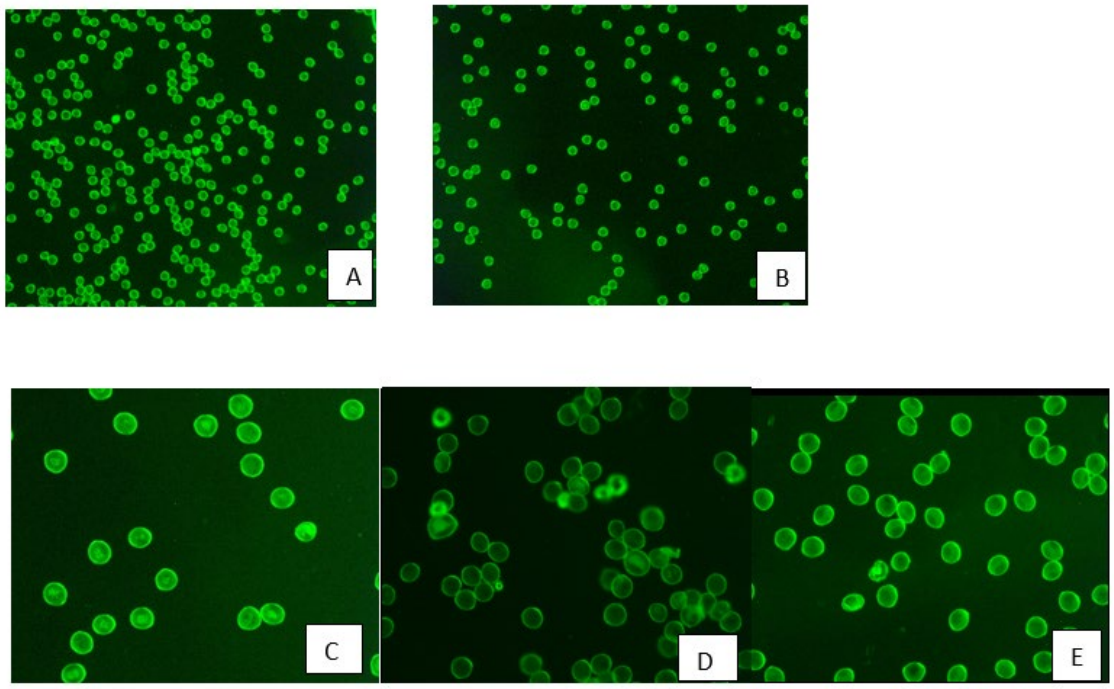

Figure 7. Florescein diacetate test FDA: (A) Floresecnt pollen grains after heat exposure (30 °C for 12 hours, KC) under 4X, (B) After low temperature exposure (-20 °C for 12 hours, MC) under 4X, (C) After low temperature exposure (-20 °C for 12 hours, MC) under 10X, (D) After heat exposure (40 °C for 10 days, MC) under 10X, and (E) After heat exposure (40 °C for 2 days, KM) under 10X.

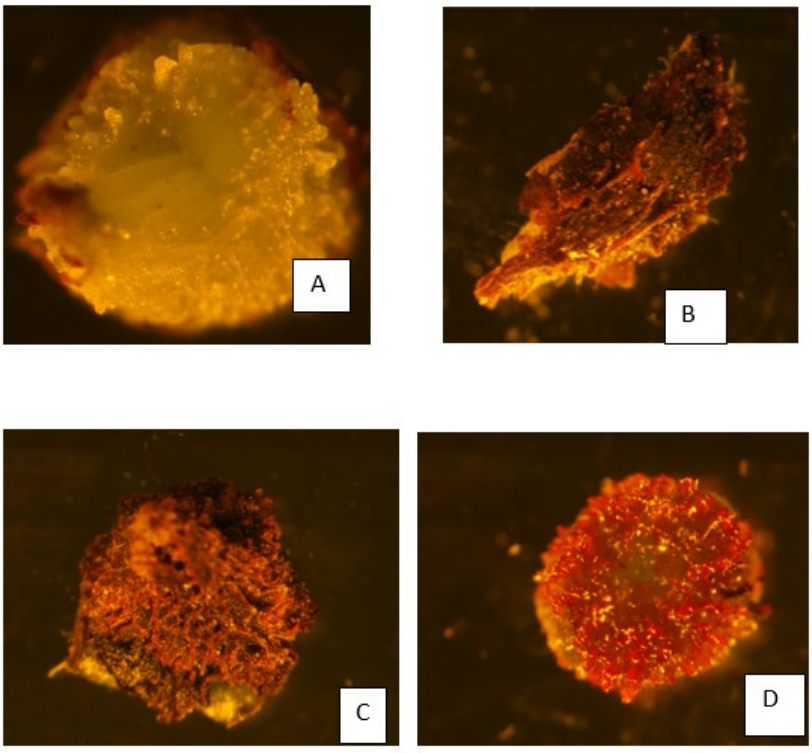

Figure 8. Pecan stigmas: (A) KC stigmas green, (B) KC stigmas dark colored, (C) MC stigmas, and (D) PP stigmas red.

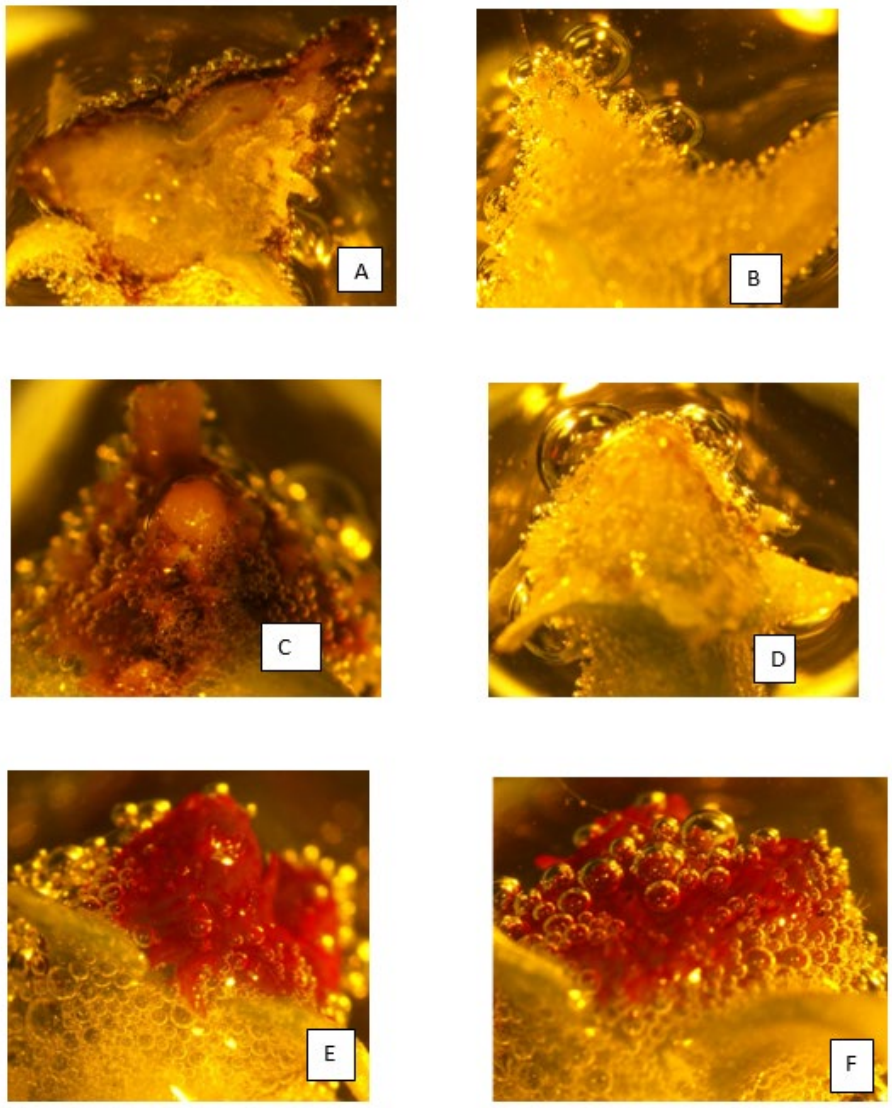

Figure 9. Stigma viability using hydrogen peroxidase (3%) test: (A) KG stigmas with almost no bubbles from stigma surface, (B) KG stigmas with larger bubbles from surface, (C) MC no bubbles from stigma surface, (D) MC stigmas with larger bubbles from surface, (E) PP stigmas with fewer small bubbles from stigma surface, and (F) PP stigmas with larger bubbles from surface.

Figure 10. Stigma viability using hydrogen peroxidase (3%) test: (A) PP stigma with almost no bubbles from stigma surface, (B) KM stigma with almost no bubbles from stigma surface, (C) KG almost no bubbles from stigma surface.

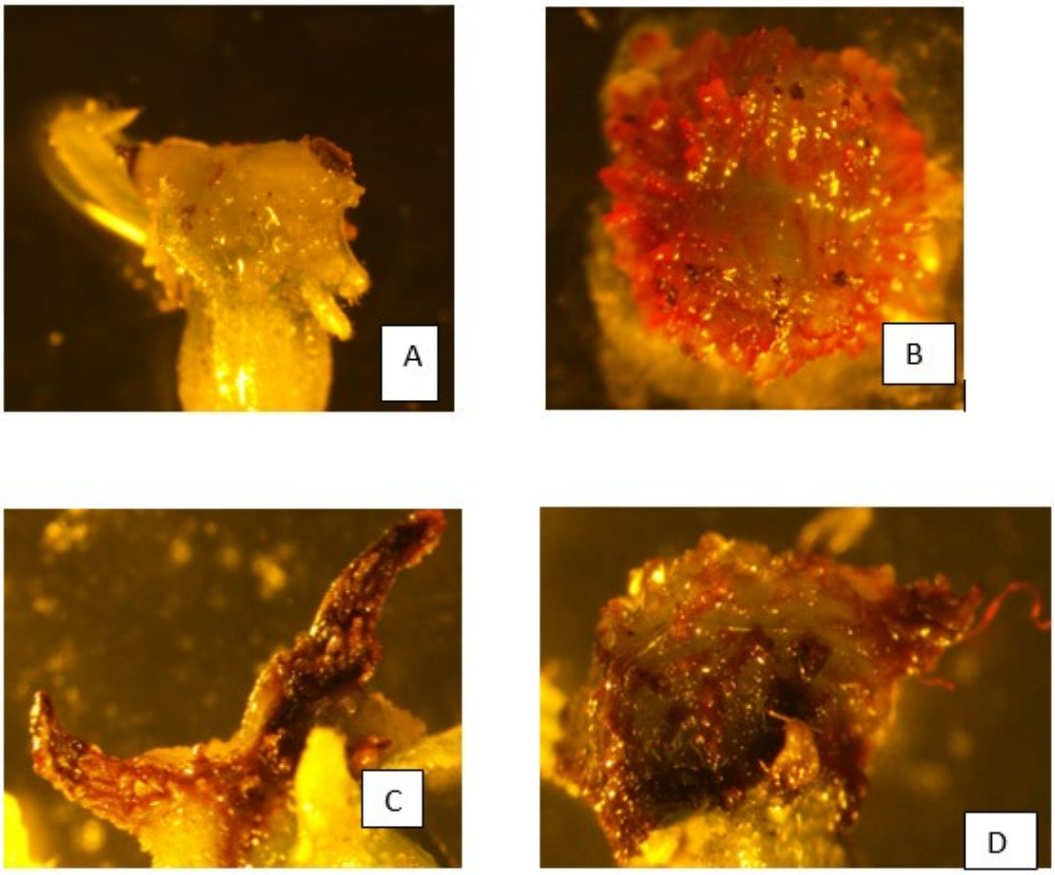

Figure 11. Stigma viability using Baker's procedure: (A) KG stigma with almost no any stain on stigma surface (after 2 hours in test solution), (B) PP stigma with almost no any stain on stigma surface (after 2 hours in test solution), (C) and (D) KG stigma with dark color surface (after 15 hours in test solution).

Table 2. Summary ANOVA table for bark sugars (in mg/g DW), bark starch (in mg/g DW), wood sugars (in mg/g DW), and wood starch (in mg/g DW) content in pecans.

Source	Bark Sugars	Bark Starch	Wood Sugars	Wood Starch
Cultivar (CV)	***	***	***	*
Treatment (T)	***	***	***	***
CV×T	***	***	***	***
Stage (S)	***	***	***	***
CV×S	***	NS	***	**
T×S	***	***	***	***
CV×T×S	***	***	***	***

* Significant at the 0.05 probability level; **significant at the 0.01 probability level; ***significant at the 0.001 probability level; NS not significant

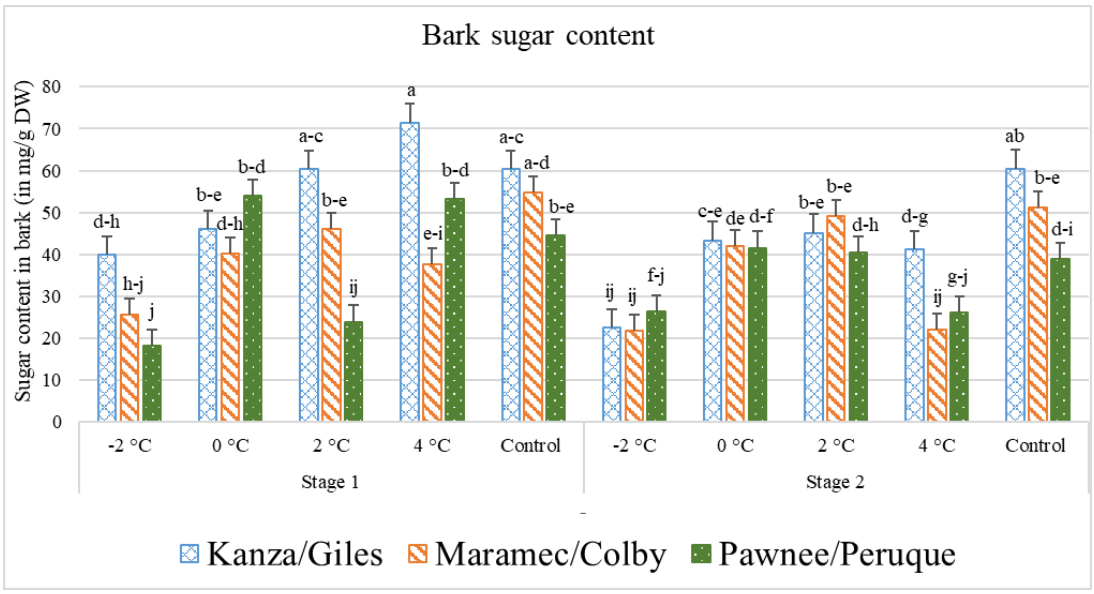

Figure 12. Soluble sugar content in the bark (in mg/g DW) for four different temperature treatments (-2 °C, 0 °C, 2 °C, and 4 °C for 8 hours) and controls of 3 pecan cultivars Kanza/Giles, Maramec/Colby, and Pawnee/Peruque at stgae 1 and stage 2.

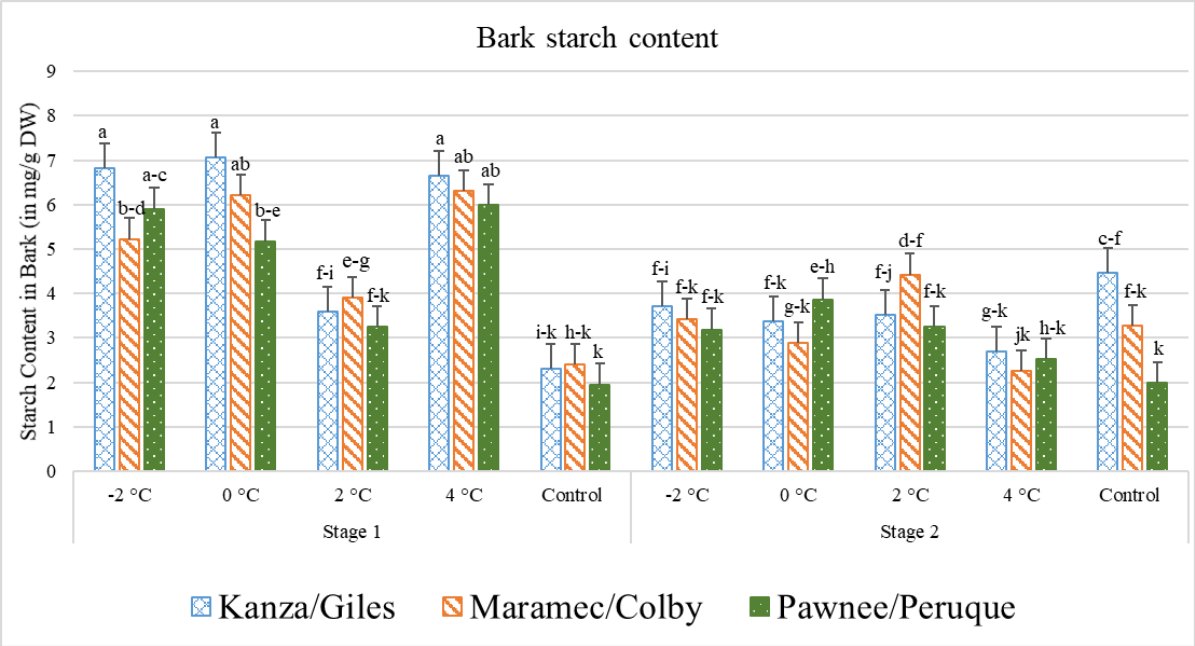

Figure 13. Starch content in the bark (in mg/g DW) for four different temperature treatments (-2 °C, 0 °C, 2 °C, and 4 °C for 8 hours) and controls of 3 pecan cultivars Kanza/Giles, Maramec/Colby, and Pawnee/Peruque at stage 1 and stage 2.

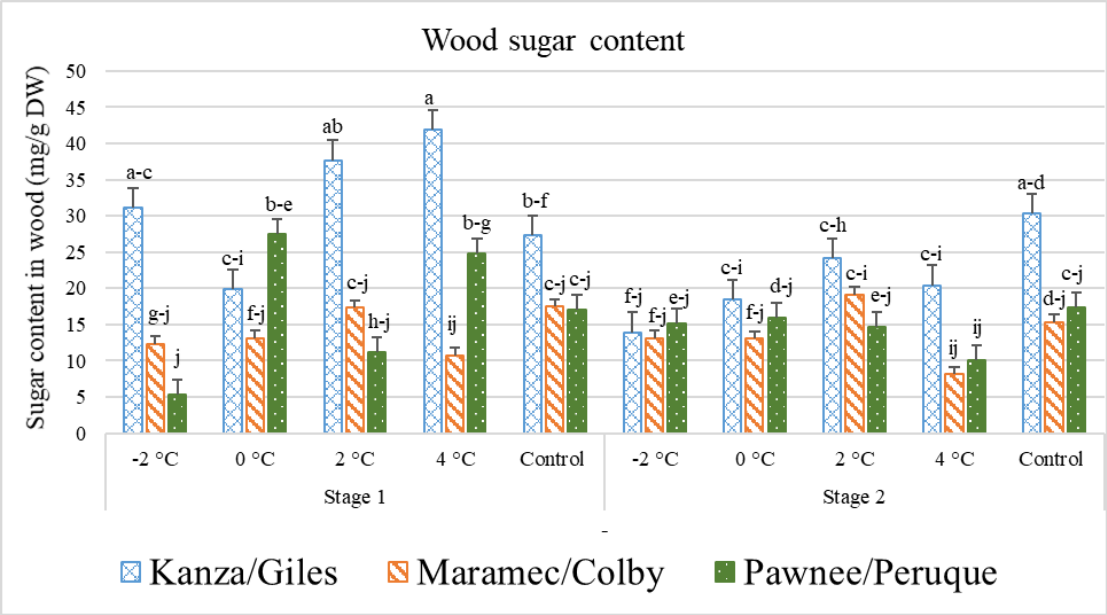

Figure 14. Soluble sugar content in the wood (in mg/g DW) for four different temperature treatments (-2 °C, 0 °C, 2 °C, and 4 °C for 8 hours) and controls of 3 pecan cultivars Kanza/Giles, Maramec/Colby, and Pawnee/Peruque at stage 1 and stage 2.

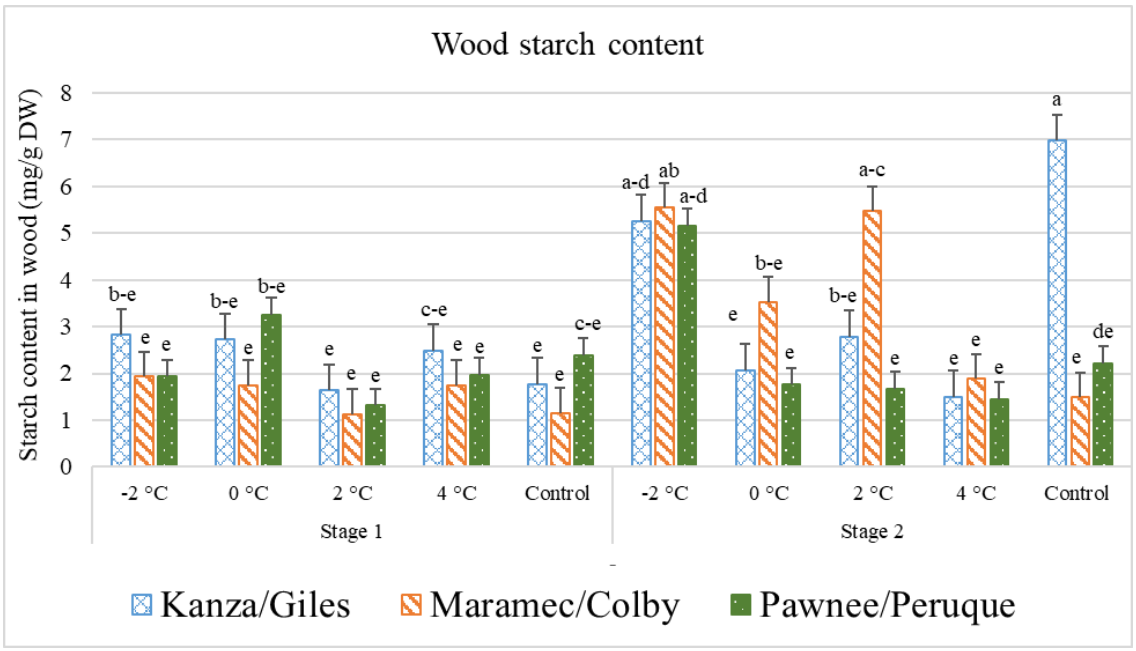

Figure 15. Starch content in the wood (in mg/g DW) for four different temperature treatments (-2 °C, 0 °C, 2 °C, and 4 °C for 8 hours) and controls of 3 pecan cultivars Kanza/Giles, Maramec/Colby, and Pawnee/Peruque at stage1 and stage 2.

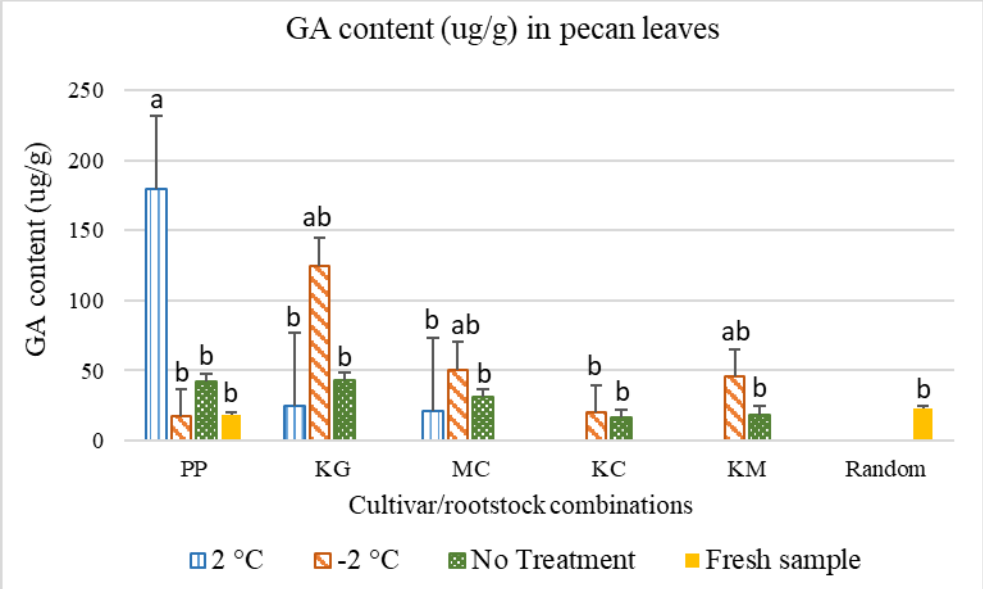

Figure 16. Gibberellins (GA) concentration (in ug/g) in leaves samples from different pecan cultivar/rootstock combinations; Pawnee/Peruque (PP), Kanza/Giles (KG), Maramec/Colby (MC), Kanza/Colby (KC), Kanza/Mount (KM) after different temperature treatments and controls.

CHAPTER III

EVALUATION OF PECAN CULTIVARS AGAINST SPRING FREEZE

ABSTRACT

Pecan [*Carya illinoinensis* (Wangenh.) K. Koch] is a member of the *Juglandaceae* family. The US is the world's largest pecan producer with an average yearly production of 250 to 300 million pounds. During the spring season, pecan trees break their bud dormancy and produce new leaves and flowers. Storage carbohydrates are thought to support the bloom and early vegetative growth during this time until new leaves reach full photosynthetic activity. Spring freeze is known for its damaging effects on pecan bud and flower growth and development. A recently experienced spring freeze in Oklahoma was on April 21, 2021 (with less than 0 ºC for 6 hours). Damage in the leaf, bud, and catkins damage was observed among the cultivar/rootstock combinations in this experiment. Kanza/Mount showed the maximum degree of damage to terminal leaves, buds, and catkins, while Maramec/Colby had the minimum damage confined to leaves only. Further, a significant difference in the sugar and starch content in the pecan bark and wood tissue was observed. The most prominent change in carbohydrate was detected in the bark sugar levels as it rises in all the cultivars/rootstock combinations after the freeze.

INTRODUCTION

Pecan [*Carya illinoinensis* (Wangenh.) K. Koch] is a member of the *Juglandaceae f*amily. Pecan is native to the United States/ North America (Thompson, 2017). The US is the world's largest pecan producer with an average yearly production of 250 to 300 million pounds. The US produced approximately 302 million pounds of pecan nuts in 2020 which is 18 % higher than 2019 pecan production (USDA, 2021). Among all the total nuts (in shell) produced, 95.30% (288 million pounds) were of "improved" varieties and about 4.70% (14.2 million pounds) were produced from "native" and "seedling" varieties (USDA, 2021). The "natives" and "seedlings" are pecan varieties that have not been propagated (non-grafted) and are produced from open-pollinated seeds (naturally or by humans). Whereas "improved" pecan varieties are those that a scion has been grafted or budded onto a rootstock (Grauke and Starr, 2014). In "native" or "seedling", every seed is genetically different from each other and they have diverse nut quality (size, shape, and kernel percentage) (Wells, 2014; Sanderlin, 2015). The grafted or improved varieties/cultivars consist of two different genetically combined parts to form a graft union and they form a morphologically uniform orchard. The rootstock forms the root structure and the scion forms the above-ground part and canopy (Wells, 2014). There are different budding and grafting techniques for pecan propagation to combine rootstock and scion (Fabrizio et al., 2018). The rootstock-scion interactions led to one tree with two genetically different parts. The name of cultivars comes from the scion name which is selected for desired traits. The selection of compatible scion and rootstock type is important for healthy grafted pecan orchards.

Temperature, especially low and freezing, is an important abiotic factor that affects the growth of pecan trees at various growth stages. Freeze injury/damage to pecan trees can occur in the autumn before they have acclimated to the cold (Cade, 2001; Smith et al., 1993), during winter dormancy (Wood, 1986), or in the spring (Malstrom, 1982) during and after bud differentiation processes have initiated. Spring low temperature injury is usually confined to the

newly developing shoots, buds, and leaves followed by growth resumption by secondary and sometimes tertiary buds (Smith et al., 1993). Damage by frost or low temperature at this time is measured in terms of the extent of injury and death of buds, leaves, and inflorescences. Cold damage to young and old trees is a frequent problem in Oklahoma and it can be influenced by rootstock (Carroll, and Smith, 2017). Oklahoma recently experienced a spring freeze on April 21, 2021 (with temperature less than 0 ºC for 6 hours).

Carbohydrates such as starch, sucrose, and glucose play an important role in the floral induction signaling and energy across various flowering processes (Chen et al., 2018). In the spring season, when floral induction begins, stored carbohydrates particularly those from roots, serve as a substrate for bloom development (Lockwood and Sparks, 1978). Carbohydrate (starch, sucrose, etc.,) contents in leaves, wood, branches, shoots, and roots of various trees including pistachio, pear, orange, chestnut, peach, apple, olive, pecan, and walnut have all been investigated with variable degrees to relation to blooming (Smith and Waugh, 1938; Spann et al., 2008; Zwieniecki and Lampinen, 2015). In the tissues of healthy trees, patterns of starch and soluble sugars reflect normal physiological function and tree vitality. Variations in these patterns may represent changes in physiological performance and could be helpful in determining the physiology of trees under environmental stress (Wong et al., 2003). The variation in carbohydrate contents in pecan leaves and shoots from bud break to natural leaf fall has been studied (Kim and Wetzstein, 2005). The two major parts of the vascular system in plants that play a crucial role in carbohydrate transport and storage are the xylem and phloem. Their names are derived from Greek *xylon* (means wood) and *phloos* (means tree bark). Xylem mainly transports water and minerals from the root to top part of trees, while phloem transports the food (sugars) to different parts of the tress (Jensen et al., 2016). The wood and bark tissue sample's carbohydrate content will be analyzed in this study.

This chapter will discuss the different pecan cultivar/rootstock combinations affected by a natural spring freeze (April 21, 2021) and changes in carbohydrate (sugars and starch) content before and after the freeze event.

OBJECTIVE

To evaluate different pecan cultivars for their extent of damage to new leaves and male flowers (catkins) by spring freeze occurring on April 21, 2021.

HYPOTHESIS

- Pecan cultivar and rootstock impacts the freeze susceptibility or resistance degrees of pecans
- Carbohydrate content of pecan tissues is correlated to spring freeze damage in pecan

MATERIAL AND METHODS

This experiment was conducted to study the effect of a late spring freeze on pecan leaf and flower structures in the field and labs at Oklahoma State University, Stillwater. Five pecan cultivar/rootstock combinations (approximately 27 year-old) used in the experiment were Pawnee/Peruque (PP), Kanza/Giles (KG), Kanza/Mount (KM), Kanza/Colby (KC), and Maramec/Colby (MC), grown in Cimarron Valley Research Station (97° 02'13" W 35°58'55" N), in Perkins, Oklahoma. This experiment was conducted from April 19, 2021 to September 12, 2021. Twenty branch samples of each cultivar/rootstock combination were collected from the middle canopy part of trees on April 19, 2021; one day before the expected freezing temperature in Perkins. The size of collected branch samples was approximately 30 cm from the terminal bud and they were immediately placed into water bucket to prevent wilting. To check temperature conditions in the orchard, we used Mesonet website and HOBO data loggers which were installed on random trees in the orchard. The freeze occurred in the night April 21, 2021 during which the

temperature was below 32F (0 ºC) for 6 hours. A day after freezing temperature (April 21, 2021), twenty branch samples per cultivar/rootstock combinations were collected from the middle canopy of trees. We checked all trees of each cultivar/rootstock combinations. The branches were collected in such a way that represent the overall damage of cultivar/rootstock combination in the orchard. For example, if most trees had damaged terminal buds, we collected more number of branches with terminal buds' damage in the set of twenty for that cultivar/rootstock combination. From the twenty branch samples collected a day before and day after the freeze event, three branches per each cultivar/rootstock combination were immediately sampled for carbohydrate analysis.

Carbohydrate- sample preparation

The branch samples size of 30 cm from terminal bud was collected and placed immediately into water bucket to prevent wilting. Each branch was divide in three parts; apical, middle, and basal (approximate size 4-5 cm) from the terminal bud. Wood and bark was separated manually on the same day they were collected from the field from all branches. Then wood and bark samples were chopped using pruning shears and scissors and placed in the labelled envelopes (2.25×3.5") and dried in oven (Isotemp oven Model 655F, Fisher Scientific) for 2-3 days at 75 ºC. After drying, the samples were kept at room temperature until further use. The bark and wood tissues were ground first into small pieces using Willey mill grinder (in Perkins) and placed into 2 ml Eppendorf with 4 mm bearing balls (Precision Chrome Steel G25, UXCELL, China) in it. Then Mini-Beadbeater 96 (Biospec Products, Bartlesville, OK) in NRC lab was used to make fine powder of samples collected in 2 ml Eppendorf. The fine powder (25-27 mg) was used to quantify the sugar and starch content using a colorimetric method (anthrone reagent method).

The anthrone reagent (0.1%) was prepared fresh for every use by dissolving 0.1g of anthrone in 100 mL of concentrated chilled H_2SO_4.

Extraction and Assay procedure for sugars and starch

The extraction and assay procedure is same as described in experiment 1(chapter 2).

Statistical analysis

The absorption reading was compared to a standard curve based on different standard concentrations of glucose and total carbohydrate content was expressed as mg/g DW. The data was analyzed using PROC GLM in Statistical Analysis System (Version 9.4; SAS Institute Inc., Cary, NC). A one-way analysis of variance (ANOVA) was performed to determine the effects of freeze on sugar and starch concentration in different cultivar/rootstock combinations, and the treatment differences were analyzed using the LSMEANS with LINES statement at $\alpha = 0.05$. During the preliminary analysis, significant two way interactions were observed for sugars in bark samples and sugars and starch in wood samples.

RESULTS

Visual observations

The Figure1. A-E represent the growth stage of all five cultivar/rootstock combinations in Cimarron Valley Research Station before the freeze event i.e. April 19, 2021. At that time Pawnee/Peruque (PP) had large catkins and lesser terminal leaves because it is type-I or protandry. Maramec/Colby (MC), Kanza/Giles (KG), Kanza/Mount (MC), and Kanza/Colby (KC) had smaller catkins compared to PP and more leaves because they are type-II or protogyny. Further, all Kanza cultivars (on three different rootstocks) had lesser and smaller catkins compared to MC and more terminal leaves growth. After the low temperature on April 21, 2021, visual differences in the terminal bud, leaf, and catkin damage were observed amongst the five

pecan cultivar/rootstock combinations (Table 1. and Figure 1. F-J). KM depicted the maximum damage followed by KC and KG, while PP and MC were the least damaged by the spring freeze. 'Kanza' cultivars on three different rootstocks showed different degrees of damage in term of damage to damage to catkins, terminal leaves and buds. For example, in KM a large number of the branches had severely damaged catkins, leaves, and terminal buds, while in KG the most of the damage was seen on tips of terminal leaves and some terminal buds. Similarly, the cultivar 'Maramec' and 'Kanza' grafted on rootstock 'Colby' showed differences in their susceptibility to freezing temperatures. These observations may indicate that both the pecan cultivar and rootstock type affect the freeze damage extent or susceptibility to spring freeze. Additionally, the lower canopy branches showed a higher level of damage than the upper canopy branches. Furthermore, we observed the secondary bud break and development of leaves and pistillate flowers in KG after the terminal new leaves and terminal buds were damaged by low temperature. PP has almost no or very few secondary bud growth and pistillate flowers development from those secondary buds.

Carbohydrate analysis

The two-way cultivar × freeze interaction was significant in bark sugar, wood sugars, and wood starch content (Table 2.). Even though the two-way interaction was non-significant (at 0.05 probability level) for bark starch, but it is very close to 0.05 as its p-value was 0.052.

Sugar content in pecan bark

The bark sugar level increased significantly after the freeze event in all 5 cultivar/rootstock combinations compared to before the freeze event bark sugar (Figure 2.). Before the freeze event, among all 5 cultivar/rootstock combinations, there was no significant difference in the bark sugar content. While they all had significant increases in sugars after the freeze. For instance, KM bark sugar concentration increased from 60.33 mg/g DW to 83.31 mg/g

DW. Further, there was significant lower bark sugar content in PP (71.94 mg/g DW) after the freeze event compared to all 3 Kanza cultivars on three different rootstocks i.e., Mount, Colby, and Giles (83.31, 82.40, and 82.74 mg/g DW, respectively).

Starch content in pecan bark

There was significant difference in the bark starch content of the before freeze samples among cultivar/rootstock combinations (Figure 3.). Before freeze event, MP branch samples (12.95 mg/g DW) had significant higher bark starch compared to PP (9.88 mg/g DW) and KG (9.51 mg/g DW). There was a change in the starch levels after the freeze in all cultivar/rootstock combinations but it was significant only for PP. In PP, starch content was significantly lower in the after freeze samples (6.96 mg/g DW) than the before freeze samples (9.88 mg/g DW). After the freeze event, MC still had significant higher starch in bark than PP and KG. Overall, the after freeze bark samples exhibited significantly lower starch levels than the before freeze samples.

Sugar content in pecan wood

There was a significant difference in the wood sugar level among the five cultivar/rootstock combinations the before freeze event (Figure 4.). KM (60.1 mg/g DW) and KC (58.23 mg/g DW) exhibited significantly higher wood sugar levels than KG (43.51 mg/g DW) and MC (40.59 mg/g DW). There were significant variations in wood sugar content after the freeze in all 3 Kanza cultivars on 3 different rootstocks. In case of MC and PP, the difference between before the freeze wood sugar levels was non-significant. Furthermore, the concentration of wood sugar in the after freeze samples varied among the cultivar/rootstock combinations. For instance, wood sugar content was significantly lower in MC (40.74 mg/g DW) than KG (53.11 mg/g DW).

Starch content in pecan wood

There was a significant difference in the wood starch level among the cultivar/rootstock combinations before the freeze event (Figure 5.). In comparison to PP, KG, and MC (24.85, 18.54, and 9.25 mg/g DW, respectively), KM and KC (42.86 and 38.92 mg/g DW, respectively) had significantly higher starch content in the wood. There was a significant lower wood starch content after the freeze in KM and KC branches compared to the before freeze samples. However, the difference in wood starch was not significant in the other cultivar/rootstock combinations before and after the freeze. Additionally, the concentration of wood starch in the after freeze samples varied among the cultivar/rootstock combinations. For instance, wood starch was significantly higher in all Kanza cultivars (such as KC 29.71 mg/g DW) than MC (11.61 mg/g DW). Overall, there was significantly lower starch content in the after freeze wood samples than the before freeze samples.

Discussion

Visual observations

The spring bud and flower formation is an important physiological process that is influenced by external factors like temperature, photoperiod, and precipitation. The damage to pecans by spring freeze has been observed in the past. For instance, severe damage to pecan leaves and catkins by late spring freeze (28 F) was reported by Wells, (2007). Some cultivars showed more injuries by severe freeze events (April 7, 2009) while other cultivars showed lesser damage (Smith and Cheary, 2010). Rootstock seedlings affect a cultivar's resistance or susceptibility to low-temperature damage during any of the freeze events (Sanderlin, 2000). The combination of scion and rootstock significantly alters the freeze impact indicating a close relationship between the seedling rootstock's effect on scion phenology and freeze tolerance (Grauke and Pratt, 1992). In the present experiment, similar observations were seen; differences in the degree of damage in all cultivar/rootstock combinations. 'Kanza' cultivar on three different

rootstocks (Mount, Colby, and Giles) had different extents of damage to terminal leaves, buds, and catkins. Moreover, cultivar 'Maramec' and 'Kanza' grafted on the same rootstock (Colby) had differences in damage by the spring freeze. MC showed almost no damage to leaves and catkins while KC had severe damage to leaves, terminal buds and catkins. Cultivar and rootstock affect the bud break date, a heritable trait that is associated with their provenance. The rootstock which break their bud dormancy later compared to other rootstock can results in late bud break on scion/cultivar which is grafted on them. 'Kanza', 'Mount', and 'Colby' cultivars break their bud dormancy later than other cultivars. At the time of freeze event (April 21, 2021), all the Kanza with rootstocks Mount, Colby, and Giles was late in bud and leaves growth compared to PP and MC. We observed lesser damage on MC and PP and greater damage on KG, KM, and KG. However, the opposite trend was observed by Smith and Cheary (2010) after freeze on April 9, 2009, in which 'Kanza', 'Mount', and 'Colby' had least bud damage than 'Pawnee' (all grafted on Giles). The difference in damage by these two freeze events (2009 and 2021) could be due to difference in time of freeze event and growth stage. During 2009 freeze they compared different bud growth stages and 'Kanza' was at the least advanced bud growth stage (outer bud scale intact) and 'Pawnee' was at the more advanced bud stage (outer bud scale shed). The outer bud scale intact stage is considered as more resistant to low temperature than outer bud scale shed stage.

One important factor that might be a reason for differences in the damage in cultivars is their location in the orchard. In our research station, KC and KM trees are planted at some distance from the MC, PP, and KG. Spring frost injury has been shown to vary within an orchard (Miranda et al., 2005). 'OK642' an advanced selection from Oklahoma showed little damage to buds in the east field (had higher elevation) and the greatest bud damage in west field after freeze on April, 2009 (Smith and Cheary, 2010).

A pecan tree can have a wide canopy (nearly 12 m) and height (21 m). Because of this wide structure of pecan trees, temperature and growth vary within the tree canopy. A radiation freeze, in which the temperature near the soil surface and lower branches is lower than the temperature in the upper branches, leads to an increasing temperature gradient from bottom to top of the canopy. This results in more damage to the lower branches compared to the upper branches (Graham, 2020; Sparks, 2005; Reid, 2020a; Reid, 2018a, b; Charrier et al., 2015). In the present experiment, we mainly evaluated and collected the samples from middle tree canopy (approximately from same height) from all cultivar/rootstock combinations. But we also observed the difference in the damage in upper and lower canopy after the spring freeze. The branches on lower canopy showed more damage compared to upper branches. A similar damage trend has been observed in the past (Reid, 2020; Wells, 2007).

After the damage of primary buds, secondary buds may break and produce pistillate flowers in some cultivars (Wells, 2007). The epicormic branch growth from lateral buds after apical dominance loss because of severe freeze (-5 °C) was also seen in Oak Ridge, Tennessee (Gu et al., 2008). Similarly, in pecan secondary bud growth was reported followed by a freeze damaged or killed terminal primary buds (Madden, 1980; Reid, 2020b). We also observed the secondary bud break and pistillate flower formation in KG after the terminal buds and leaves were damaged. In KG, the secondary bud growth was observed on 2 or 3 trees out of approximately 25 trees (in one row in the orchard). However, in MC and PP almost no or very few secondary bud growth was observed after the freeze.

Carbohydrate

Soluble sugars and starch content in pecan tissues has been analyzed and reported in the past (Taylor, 1937; Su et al., 2021; Melendez et al., 2021, Kim and Wetzstein, 2005; Wood, 2014). Taylor (1937) reported the soluble sugars content ranged from approximately 46-97 mg/g

DW and starch content ranged from 4-23 mg/g DW from pecan stem sections. Recently, Melendez et al., (2021) reported soluble sugars (approximately total 120 mg/g DW) and starch content of leaves (ranged from 30-40 mg/g FW) from 9-year old pecan trees. In our experiment we observed approximately similar higher soluble sugars and lower starch content from pecan branch samples. Soluble sugar content in pecan bark ranged from 60-83 mg/g DW and wood 40-60 mg/g DW. While starch content in bark tissue ranged from 9-13 mg/g DW and wood tissue 9-45 mg/g DW. However, at flowering stage, lower soluble sugar content (approximately 26 mg/g DW) and starch content (25 mg/g DW) was observed in stem samples from 15-year-old 'Stuart' pecans (Kim and Wetzstein, 2005). But they observed higher starch content (60 mg/g DW) in leaf samples. In case of grafted pecan stem samples from one-year-old seedling, lower sugar content (20-39 mg/g FW) compared to higher starch content (30-80 mg/g DW) was observed by Su et al. (2021). From the above mentioned studies, we can observe when there were higher soluble sugars in tissues, starch was lower, indicate relationship between soluble sugars and starch in tissues.

The frost damage resistant and susceptible pistachio rootstocks showed an almost similar level of soluble sugars in their leaves when measured during normal environmental conditions (no freeze event) (Sorkhan et al., 2011). These findings are similar to our results; there was no significant difference in bark sugar level among different cultivars/rootstock combinations a day before freeze event. However, we observed a significant increase in bark sugar content in all the cultivars one day after the freeze event. While there was a significant reduction in the bark starch content immediately after the freeze day (especially in the PP). A decrease in starch along with a simultaneous increase in sugars has been reported in grapefruit' trees under the low-temperature conditions (Rodrigues and Ryan, 1960). The depletion of starch in all tree organs was associated with an increase in soluble carbohydrate levels. The interconversion of starch/soluble carbohydrates is very classical and has been reported in many investigations (Charrier et al., 2013); The relationship between frost hardiness and glucose + fructose + sucrose (GFS) in the

walnut branches has been reported (Charrier et al., 2013). The higher carbohydrate content (as cryoprotectants) along with less intracellular water are the key to survive very low temperatures (Charrier et al., 2015). The inverse levels of sugars in stem and leaves during the pecan bloom stage have been observed. The transport of sugars from leaves to support stem growth can cause a reduction in sugars in leaves and increases in stem sugars (Kim and Wetzstein, 2005).

The total carbohydrate content represents the pool of carbon that can be used either to improve cold hardiness via hydrolysis to soluble carbohydrate forms or to support metabolic processes of the tree (Morin et al., 2007). There was difference among the cultivar/rootstock combinations in bark sugar after the freeze event such as all the 'Kanza' cultivars on 3 different rootstocks (Mount, Colby, and Giles) had significantly higher sugars than PP. That might be due to the lesser utilization of sugars in 'Kanza' cultivars to support the leaves and flower growth. On the other hand, PP consumed their sugars quickly as they had catkins growth than 'Kanza' cultivar. 'Kanza' cultivar was little behind compared to PP, MC in bud and shoot growth. 'Kanza' break their bud dormancy later in the spring compared to other cultivars. The difference in the levels of starch and soluble sugars among the cultivars has been reported in oak trees (Morin et al., 2007). The late growth genotype had the highest content of soluble sugars in comparison with early and mid-season growth walnut genotypes, while the opposite trend for starch content (Farokhzad et al., 2018).

For wood sugar, there was a significant change in sugars after the low temperature, especially in 'Kanza' cultivar on 3 different rootstocks. However, difference in wood sugar before and after freeze was non-significant in PP and MC. Their lower wood sugar content may indicate the utilization of sugars to support their branch growth as well as to protect them from the low temperature.

There was a significant higher wood starch in KM and KC compared to PP and MC in samples collected a day before freeze. The one reason for this might be the presence of higher stored carbohydrates from the previous season and comparatively less demand in the current season to support buds and leaves as most of the canopy part in KM and KC was lost due to the freeze last year (October, 2020). Almost all the trees of KC and KM in our orchard experienced canopy/limbs damage by the freeze (October, 2020). As in pecan, branch and root tissues are the sites for carbohydrate reserves produced during the current season in summer and early autumn (Lockwood and Sparks, 1978). These stored carbohydrates serve as a substrate for flower growth in the spring season (Lockwood and Sparks, 1978). There was significant decrease in wood starch after the freeze event in KM and KC. The rapid decline in starch level at the bloom time in pecan indicates its significance in flowering and fruiting (Smith and Waugh, 1938). The stored starch in xylem parenchyma is converted to soluble carbohydrates and released into the xylem sap during bud growth in the spring (Tixier et al., 2017). In our experiment, after freeze event, wood starch decreased and bark sugars increased. In avocado, decrease in starch content coincidence with an increase in glucose and sucrose under low temperature was observed by Rodrigues and Ryan (1960).

The soluble carbohydrate concentrations are associated with higher resistance to cold stress (Levitt, 1980; Charrier et al., 2015). Accumulation of carbohydrates reduced the freezing point of the cell sap and delay the ice crystal formation (Mutlu et al., 2013). In *Arabidopsis*, it was observed that accessions with very low sugar levels showed low freezing tolerance (Zuther et al., 2012). However, we observed the opposite pattern; the most damaged cultivar/rootstock combinations had the maximum sugar content while the least damaged had lower sugar content. This might be due to differences in the physiology of the model plant and nut tree. Further, difference in sugar content may be due to differences in growth stage (bud break time) of these cultivar/rootstock combination.

In the present experiment, the sugar concentration was higher in the pecan bark compared to wood tissue. A similar trend has been reported in other trees such as *J. regia* and oak trees for bark and wood sugars during the spring season (Bonhomme et al., 2010; Tixier et al., 2017; Bazot et al., 2013). On the other hand, wood exhibited higher starch content than the bark. The xylem parenchyma plays an important part in starch storage and also accumulates soluble sugars during the spring (Tixier et al., 2017).

Conclusion

The difference in damage extent in terms of damage to terminal buds, leaves, and catkins was observed among 5 cultivar/rootstock combinations by the spring freeze occurred on April 21, 2021. 'Kanza' cultivar grafted on three different rootstocks (Mount, Colby, and Giles) showed variations in damage extent for example Kanza/Mount showed maximum damage to terminal buds, leaves, and catkins, while Kanza/Giles had comparatively lesser damage to leaves. 'Maramec' and 'Kanza' on same rootstock Colby had different extent of damage as Maramec/Colby had almost no damage to leaves, buds, and catkins, while Kanza/Colby had leaves, catkins damage after the freeze event. This indicate cultivar and rootstock affect the damage extent by freeze in pecans. A significant increase in the bark sugar in all cultivars/rootstock combinations one day after the freeze event was observed. The increased sugars might be needed for energy as well as to protect tissues under low temperature conditions. The evaluation of the degree of damage to buds and bloom in different pecan cultivar/rootstock combinations by spring freeze is important to better understand its effects on pecans as well as for future studies.

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Tables and Figures

Table 1. Damage observed in pecan cultivar/rootstock combinations after the freeze (based on randomly collected 20 branches from each).

Cultivar/ Rootstock	**Number of branches with no damage**	**Number of branches with leaves damage only**	**Number of branches with terminal bud, catkins and leaves damage**
Kanza/Mount	40%	15%	45%
Kanza/Colby	45%	15%	40%
Kanza/Giles	50%	35%	15%
Pawnee/Peruque	90%	10%	0
Maramec/Colby	95%	5%	0

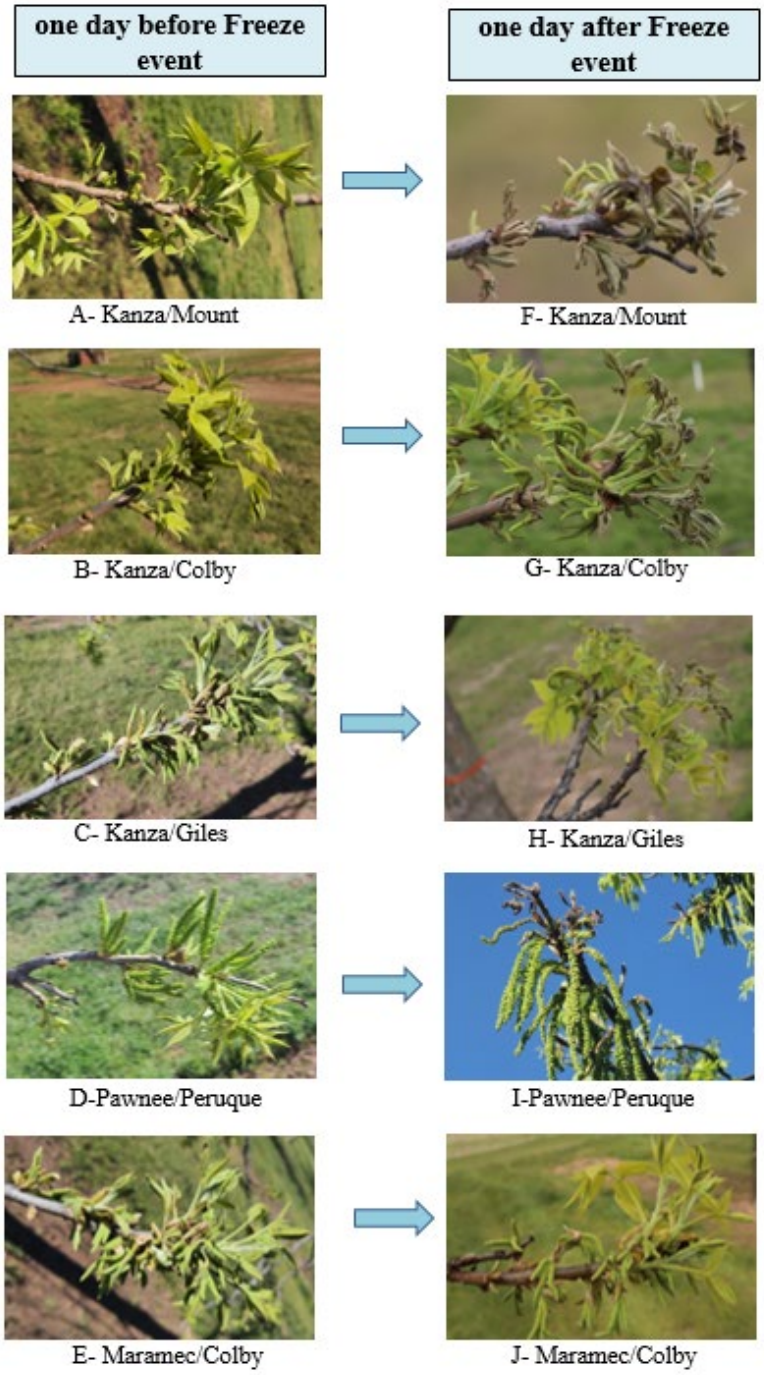

Figure 1: A-Kanza/Mount, B-Kanza/Colby, C-Kanza\Giles, D-Pawnee-Peruque, E-Maramec/Colby: Showing green terminal tips, catkins, and leaves. F- Kanza/Mount and G-Kanza/Colby: showing the terminal bud, catkins, and leaves damage. H- Kanza/Giles and I-Pawnee/Peruque: Showing the terminal leaves damage. J- Maramec/Colby: Showing almost no damage to leaves and catkins.

Table 2. Summary ANOVA table for bark sugars (in mg/g DW), bark starch (in mg/g DW), wood sugars (in mg/g DW), and wood starch (in mg/g DW) content.

Source	Bark Sugars	Bark Starch	Wood Sugars	Wood Starch
Cultivar (CV)	*	***	***	***
Freeze (F)	***	**	*	***
CV×F	*	NS	***	***

* Significant at the 0.05 probability level; **significant at the 0.01 probability level; ***significant at the 0.001 probability level; NS non-significant

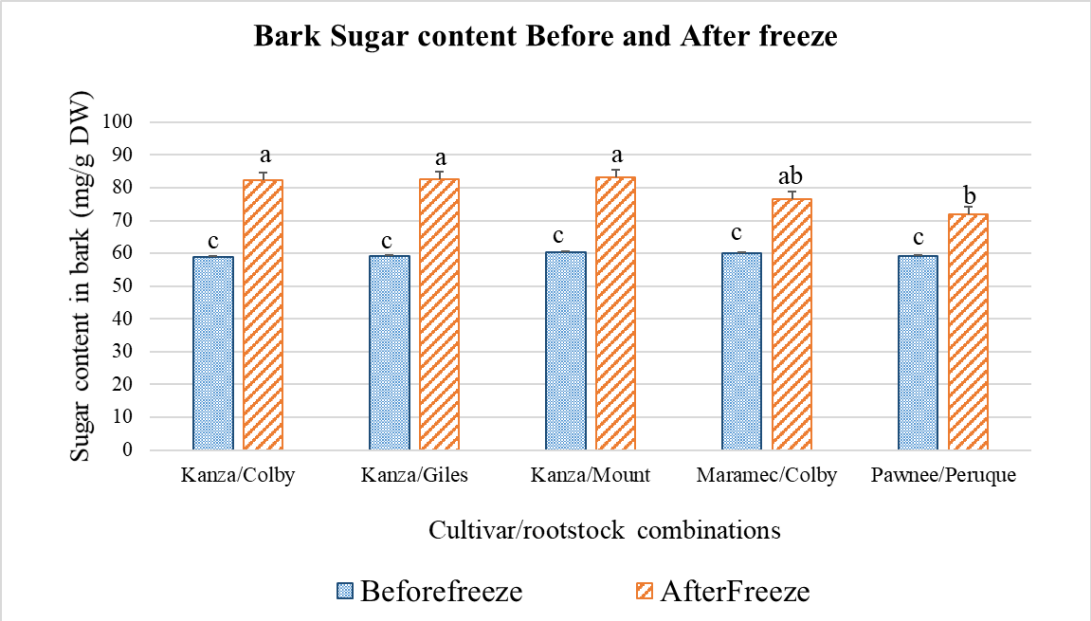

Figure 2. Soluble sugar content in the bark (in mg/g DW) for five pecan cultivar/rootstock combinations before and after freeze samples.

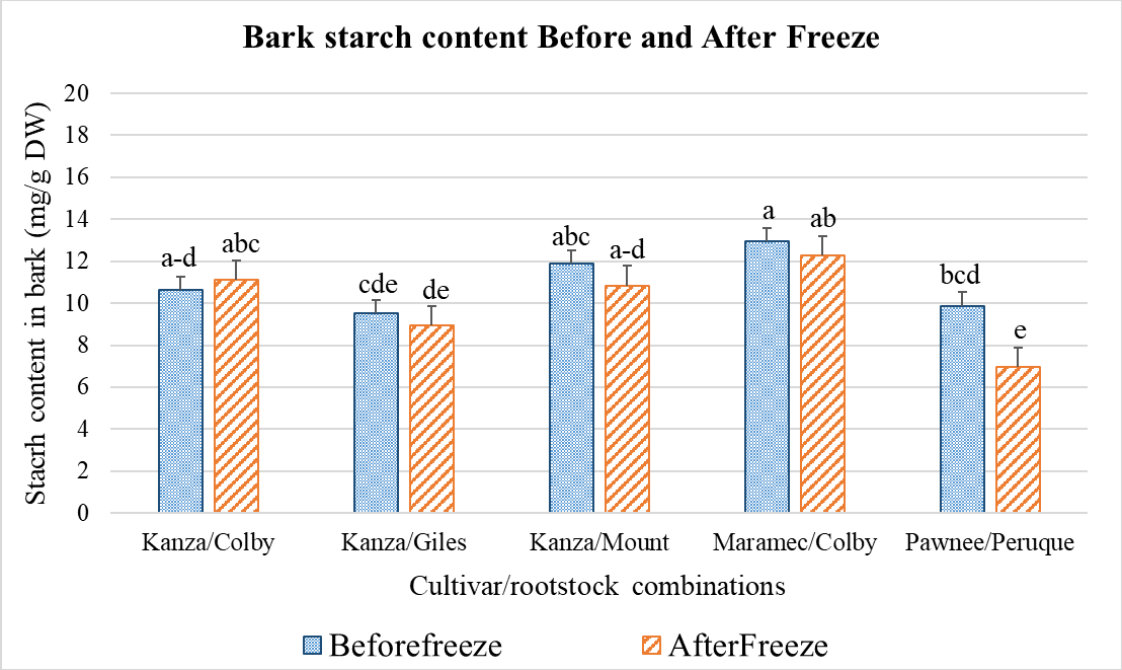

Figure 3. Starch content in the bark (in mg/g DW) for five pecan cultivar/rootstock combinations before and after freeze samples.

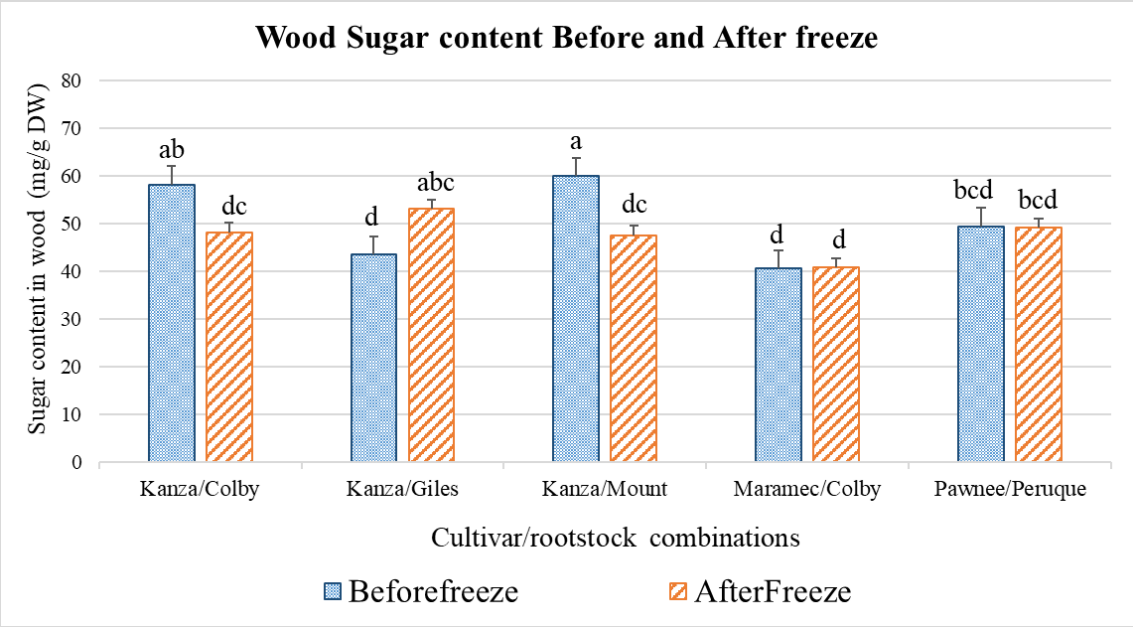

Figure 4. Soluble sugar content in the wood (in mg/g DW) for five pecan cultivar/rootstock combinations before and after freeze samples.

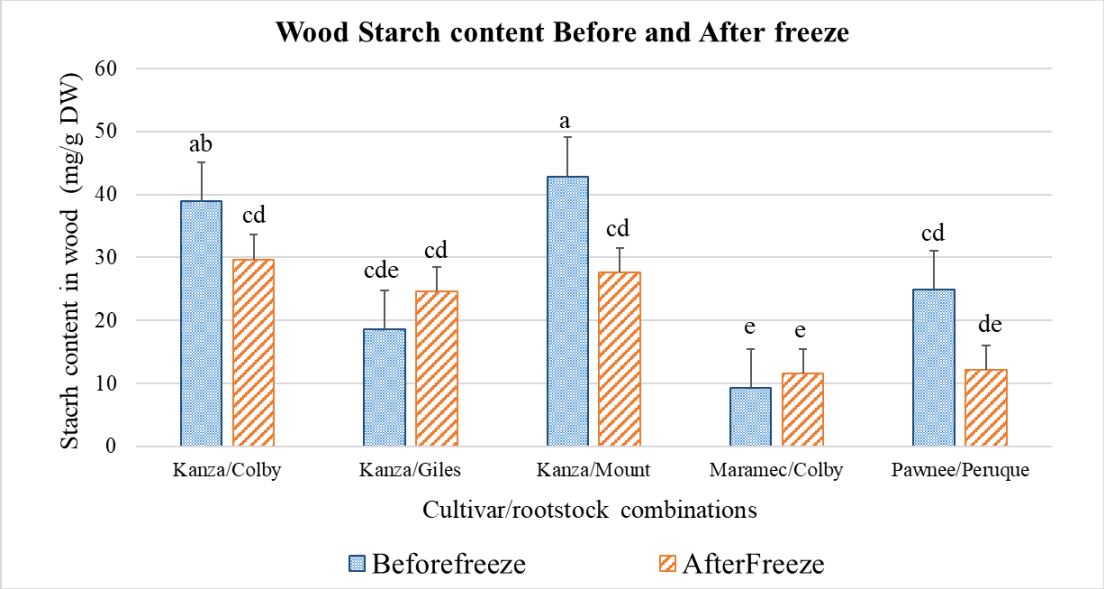

Figure 5. Starch content in the wood (mg/g DW) for five pecan cultivar/rootstock combinations before and after freeze samples.

CHAPTER IV

CONCLUSION

The spring low temperature is one of the important limiting factors for horticultural crops production including nut trees such as pecans. During the spring season, a single incident with a temperature of a few degrees below zero for few hours can cause significant injury or death of flower buds. The huge crop losses have been experienced in the past due to sudden spring freeze event. This research was aimed to study the impact of spring freeze on pecan buds/flowers during the spring season.

There are numerous factors that contribute to pecan susceptibility or resistance to low temperature. The freeze chamber experiment and the natural spring freeze event (on April 21, 2021) observations suggested that the degree of damage/injury extent varied among cultivars, bud/flowers growth stages, intensity and duration of low temperature, and in the field within tree canopy, orchard location. Further, the significant variations in carbohydrates (sugars and starch) content observed in both the filed spring freeze event and low-temperature treatments indicate their role and translocation during the low-temperature conditions.

The evaluation of the degree of damage to buds and bloom in different pecan cultivar/rootstock combinations by spring freeze is important to better understand its effects on pecans as well as for future studies. Overall, this research provides information about different aspects related to spring freeze conditions in pecans.

VITA

AMANDEEP KAUR

Candidate for the Degree of

Master of Science

Thesis: THE EFFECT OF SPRING FREEZE ON BLOOM QUALITIES IN PECANS

Major Field: Horticulture

Biographical:

Education:

Completed the requirements for the Master of Science in Horticulture at Oklahoma State University, Stillwater, Oklahoma in December, 2021.

Completed the requirements for the Master of Science in Botany at Punjab Agricultural University, Ludhiana, India in 2018.

Experience: Graduate Research Assistant in Pecan Research Project in the Department of Horticulture and Landscape Architecture, Oklahoma State University, Stillwater, OK.

Field/Technical Assistant in "Genetic Enhancement of Minor Pulses" project in the Department of Plant Breeding and Genetics, Punjab Agricultural University, Ludhiana, India in 2019.

Professional Memberships:
American Society for Horticulture Science
Crop Science Society of America
American Society of Agronomy
Soil Science Society of America