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Enabling Breeding for Fruitlet Freeze Tolerance in Peach

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ENABLING BREEDING FOR FRUITLET FREEZE TOLERANCE IN PEACH

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In Partial Fulfillment
of the Requirements for the Degree
Master of Science
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Accepted by:
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ABSTRACT

Climate change is affecting the production of temperate fruit crops, with cold temperatures emerging as a critical abiotic stressor that limits plant growth and performance. Freeze damage, particularly in spring, has resulted in significant economic losses in peach production in the southeastern United States. Research efforts in peach and other *Prunus* species have primarily focused on studying dormancy-related traits associated with bloom time, such as chill and heat requirement, with fruitlet freeze tolerance not equally represented. Breeding for climate resilience in peach requires a combination of these traits to allow for late bloom via targeted chill requirement and high heat requirement, and fruitlet tolerance to late spring frosts (LSF). This study assessed fruitlet freeze tolerance in 75 peach and nectarine accessions representing modern peach breeding germplasm. Fruitlet freeze tolerance was assessed at six freezing temperatures (0 to -10ºC) using electrolyte leakage method over two seasons (2022-20223). The fruitlet freeze was described as the temperature at which 50% of tissue experiences damage, LT50 or inflection point (IP), determined from the asymmetric sigmoid curve, and as an area under the curve (AUC). The IP ranged from -3.94 °C to -10.22 °C and AUC ranged from 16-48%, with lower AUC suggesting higher tolerance. The majority of the accessions demonstrated tolerance to cold temperatures in the -4 to -6ºC LT50 and 25-35% AUC range. However, variability in tolerance was noted across different years, as well as some inconsistencies between the index of performance (IP) and AUC measurements. When categorizing accessions into tolerance groups (TGs), more stability was seen with AUC grouping across the study years. Shifts in TG assignments were also observed, particularly among extreme categories, where some accessions moved from the most tolerant (TG1 and TG3) to an intermediate tolerance group (TG2) in subsequent experimental years. A set of seven and nine nectarines were classified as most tolerant in both seasons, 2022 and 2023, using both IP and AUC, respectively. Broad-sense heritability (H^2) estimates of 0.52 and 0.80, for IP and AUC, respectively, suggested genetic control of this trait with a potential for improvement via breeding. Further research into the genetic determinants of freeze tolerance in peach fruitlets was conducted using genome-wide association studies. We identified seven significant associations between genetic markers and the trait across four chromosomes of the peach genome. Candidate gene analyses identified 144 genes in the 100kb flanking region of each significantly associated SNP marker located on Ch2 (53), Ch5 (13), Ch6 (26), and Ch7 (51). op Additional research is required to further investigate candidate genes.

The results of this study demonstrated genetic control of fruitlet freeze tolerance in peach and revealed multiple genomic regions associated with the inheritance of this trait, thus providing a foundation for breeding climate-resilient varieties. These findings support the feasibility of incorporating fruitlet freeze tolerance in climate resilience breeding for sustainable production in a changing climate.

DEDICATION

I dedicate this thesis to my wonderful family: my mom, Havva Caglar, and my lovely sister, Gamze Caglar for always encouraging me. Their love supported me in getting this work done.

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TABLE OF CONTENTS

LIST OF TABLES

Table Page

LIST OF FIGURES

CHAPTER ONE

LITERATURE REVIEW

Taxonomy and botany

Peach (*Prunus persica* [L.] Batsch) is a highly economically valuable temperate fruit tree that is widely cultivated in moderate climatic zones throughout the world and is considered the third most significant fruit crop after apples and pears (Ruiz-Sánchez et al., 2018). The peach belongs to the *Rosaceae* family, the Prunoideae subfamily, the *Prunus* genus, and the *Amygdalus* subgenus. It is native to northwest China, where it was first domesticated and cultivated over three thousand years ago (Bassi et al., 2016a). Despite this long history of cultivation, the evolutionary history of the peach remains unknown. However, a significant body of evidence suggests that the origin of the peach can be traced back to China. The *Rosaceae* family is a diverse group of plants that encompasses a wide range of fruit types, including pomes, drupes, achenes, hips, follicles, and capsules, as well as varying growth patterns that range from herbaceous to cane, bush, and tree forms. Within this family, the genus *Prunus* is particularly noteworthy, encompassing more than 430 species, including economically significant fruits such as peaches, apricots, almonds, and cherries.

A peach tree's lifespan can reach up to 30 years, with the potential to grow up to ten meters in height at full maturity. However, for commercial purposes, peach trees are usually pruned to a height and width of 3-4 meters for economic efficiency and intensive farming practices. The peak yield of a peach orchard is typically reached after 4 years of cultivation, with productivity gradually declining from year 4 through 15 (Bassi et al., 2016b). Peach trees are typically trained in an open vase form and have a wide crown structure. They have two different types of buds - central buds that are vegetative and lateral buds that serve as the center for flower production (Penso et al., 2020). The leaves of peach trees are thin-veined, averaging 7-13 cm in length and 2-3 cm in width, and are typically a dark green color with a pointed shape. The flowers of peach trees are perfect and hermaphroditic, with both male and female functional reproductive parts. They emerge from the buds before the leaves in early spring and could be single or double, approximately 3 cm in diameter. The flowers of peach trees contain five separate pink petals and sepals with one pistil and 20 to 30 stamens (Kumar & Chaudhary, 2017). There are two types of flowers which are showy and non-showy. Showy flowers have bigger, overlapping petals that open almost flat and hide the sepals. Non-showy flowers have less, thinner, narrowly cupped petals with the sepals observable between them (Chen & Okie, 2015).

The weight of a peach fruit can vary greatly, with a range of 50 to over 650 grams. However, a commercial standard requires the average weight of the fruit to be within the 150 - 230 g range and an average diameter of 7 - 10 cm (Basto et al., 2016). The peach fruit is fleshy with a single seed surrounded by the pericarp (Rodriguez et al., 2019) The pericarp is differentiated into three distinct layers: endocarp (close to the seed), mesocarp (the soft and edible part of the fruit), and exocarp (the skin) (Dardick, Chris & Callahan, 2014). Peaches are classified as a drupe due to the hardening process of the endocarp through secondary cell wall formation during its development (Rodriguez et al., 2019). Peach development is marked by three critical stages: The initial stage features rapid growth driven by a high rate of cell division, followed by the pit hardening phase, during which the endocarp begins to solidify and form the stone, and ultimately cell enlargement (Dardick et al., 2010). Sudden weather changes during these stages can cause severe problems in fruit development and reduce fruit production worldwide.

Peach industry and consumption

Peach is widely cultivated globally in temperate and subtropical regions between 30 - 45° North-South latitude (Byrne et al., 2000a). The adaptability of peach trees to various environments plays a significant role in their widespread cultivation and distribution worldwide. Global peach and nectarine production is concentrated in six countries: China, Spain, Italy, Greece, Turkey, and the United States (U.S.), which collectively accounted for more than 90-95% of total production in the 2020/2021 season. Although the annual production varies, for various reasons, the global peach and nectarine production in 2020 was estimated to be around 24,569,744 tons (on 1,491,817 ha) (*FAOSTAT, 2020.*). China is the largest producer of peaches globally, with a production of 15 million tons, followed by Spain, Italy, Turkey, Greece, and the U.S., which produced 1.306.020, 1.015.350, 892.048, 890.580, and 560.423 tons, respectively (*FAOSTAT, 2020.*).

The U.S. Department of Agriculture (USDA) National Agricultural Statistics Service (NASS) reports the main U.S. peach production in four states: California, South Carolina, Georgia, and New Jersey. California, as the largest producer, accounts for approximately two-thirds of the total national production, yielding 510,000 tons of peaches (*NASS,2021.*). However, there has been a considerable decline in the production of peaches and nectarines in the U.S. in the past 14 years, with the total yield dropping from 1,410,150 tons in 2007 to 811,270 tons in 2021 (*NASS,2021*). This decline in yield is due to several factors, such as drought, frost, competition among different plant species, and quality issues. Nevertheless, the most significant factor contributing to this decline is the impact of weather patterns resulting from climate change. Sudden weather changes and extreme weather events caused by climate change pose severe challenges to deciduous fruits production (Luedeling et al., 2013).

Dormancy and chilling requirement (CR)

The phenomenon of dormancy plays a crucial role in the phenological cycle of all deciduous species, including *Prunus*. It is comprised of three distinct phases: para-dormancy, endo-dormancy, and eco-dormancy (Lang et al., 1987a) and is crucial for the survival and adaptation of species (Allona et al., 2008). However, the increase in temperatures during winter, caused by climate change and the influence of weather oscillations such as El Nino/La Nina Southern Oscillation in the southeast U.S., has led to insufficient cold exposure and heightened vulnerability of trees to spring frosts, posing a significant threat to fruit production worldwide (Castède et al., 2014a).

Dormancy, in the context of the plant development cycle, is a multi-faceted process categorized into three distinct stages (Lang et al., 1987b). The first phase, para-dormancy, described as the absence of visible change in meristematic tissue is regulated by factors within the plant but external to the dormant form. The next phase is endo-dormancy, often recognized as the actual dormant period in a plant's life. During this phase, the growth of meristems, the plant's undifferentiated cells responsible for growth, is firmly inhibited (Romeu et al., 2014a). The final dormancy stage, eco-dormancy, is a significant period in the plant's life cycle. Here, the plant gathers warmth, like an energy reserve, during the transition from winter to spring. This energy is used to fuel the plant's flowering and leafing out, which marks the onset of a further growing season**.** The chill requirement (CR) is the cumulative cold or chilling hours a plant requires during its dormancy period to overcome the inhibitory consequences of endo-dormancy and break its dormancy. The CR is a crucial factor in determining the transitioning of deciduous stone fruit trees from endo-dormancy. To ensure successful commercial fruit production, it is imperative to accurately calculate the CR for varieties and identify the appropriate growing zones (Valentini et al., 2001). The CR exerts significant control over the peach flower bud break and subsequent reproductive development (Alburquerque et al., 2008; Kwon et al., 2020a; Romeu et al., 2014b). A strong correlation between the CR of a cultivar and the climate conditions in which it grows is essential to consider when developing and choosing cultivars to plant (Byrne & Bacon, 1992). Failure to do so can result in frost damage in regions with incidence of low temperatures in late spring, particularly for cultivars with low CR, which tend to bloom early (Gao et al., 2012; Scorza, R. & Okie, 1990). The risk of freezing damage is further heightened by the effects of global warming, which leads to a decline in winter chill accumulation (Kwon et al., 2020b) and or spikes of very cold or very warm temperatures. Insufficient chilling accumulation can pose challenges for fruit trees, particularly affecting their ability to bloom. When the required chilling hours are lacking, the blooming cycle may be delayed and prolonged. On the other hand, plenty of chilling hours followed by a swift transition to warm temperatures can expedite the blooming phase. This accelerated blooming, however, promotes the risk of spring frosts, which can potentially have adverse consequences on fruit production. Developing peach organs, especially flowers, are susceptible to frost damage during spring when air temperatures drop unexpectedly. Cultivars with medium to low chilling requirements bloom early and petal fall, or shuck split are the most vulnerable.

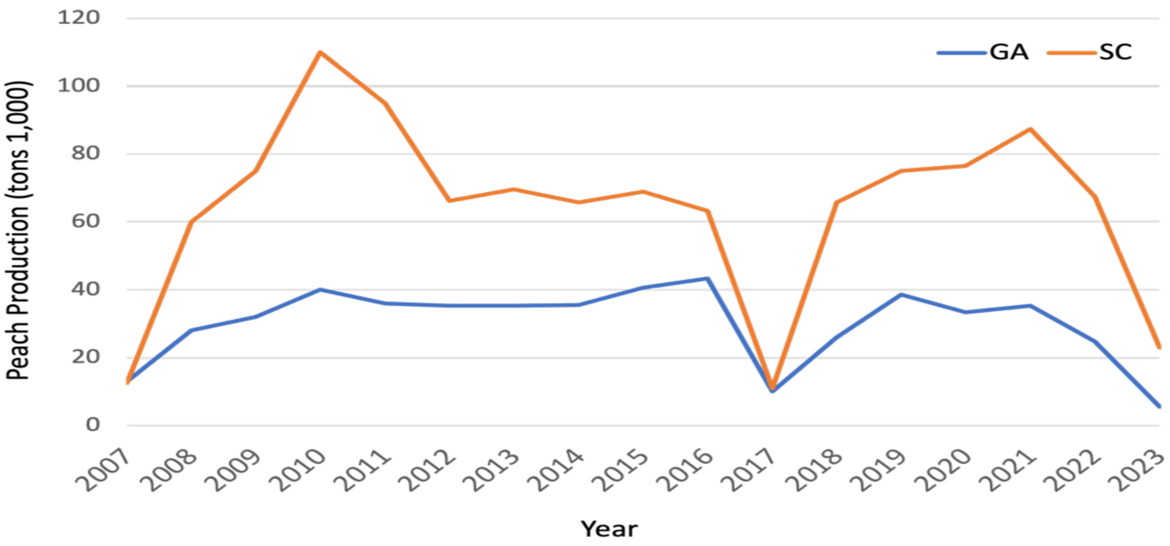

Figure 1.1. Peach production in Georgia and South Carolina in 2007-2023 period in thousand metric tons. (source NASS)

Peach production fluctuations in the southeastern region of the U.S. have been observed over the years, mainly because of late spring frost (LSF) and other weather-related factors. South Carolina and Georgia peach productions were particularly affected in 2007, 2017 and 2023 when LSF caused the production loss (Figure 1.1). In addition, the 2016/17 dormancy season was characterized by a mild winter that caused irregular flowering and delay or no leafing of some high-chill cultivars (Chen et al., 2016a). Thus, LSF caused severe damage and resulted in a 90% reduction of the peach crop (Figure 1.1). This production loss could be attributed to several factors, including the timing and intensity of the spring frost, the susceptibility of the cultivars to frost damage, and the overall climatic conditions in the region. Additionally, the impact of global warming on winter chill accumulation, the choice of lower chilling cultivars, and the changing temperature patterns could also contribute to the resilience of peach to spring frost and other weather-related stressors.

Recent studies have demonstrated the significant impact of climate change on the phenology of dormancy and blooming, increasing the risk for spring frost damage (Ramirez & Kallarackal, 2015). Early ripening cultivars are frequently favored to reach early markets and attain higher prices (Ruiz et al., 2007; Topp et al., 2008a). When early ripening is associated with early bloom and fruit set, spring frost, a numerous issue in many peach production regions, can damage flower buds, blooms, and fruitlets, resulting in a dramatic impact on peach fruit set (Rieger et al., 1991a; Smith et al., 1994).

Spring frost tolerance

Cold temperatures, including freezing, are among the critical environmental stressors for woody trees, affecting their growth, performance, and distribution (Knight & Knight, 2012; Yu et al., 2020a). Climate change has led to unexpected weather patterns and increased exposure of plants to abiotic stressors, with potentially fatal consequences (Davik et al., 2021; Kazemi-Shahandashti & Maali-Amiri, 2018a). The devastating effect of climate change is one of the most impactful phenomena on the production of temperate fruit crops (Miranda et al., 2021). While peach trees are known to be cold hardy and withstand the damaging effects of winter freezing temperatures while dormant, they are still vulnerable to spring frosts (Luedeling, 2012). The impact of spring frost and insufficient cumulative chill on peach fruit set is well-documented (Chen et al., 2016b). However, weather conditions are different every year and, even when a spring frost occurs, the temperature patterns of each frost event are different every time. Currently, there is no standardized phenotyping protocol for assessing frost tolerance in fruit development due to differences in susceptibility to freezing among cultivars and the complexity of this trait in the *Prunus* species. Studies that have assessed the freeze tolerance of developing fruitlets in peaches have focused on identifying correlations between external symptoms and frost susceptibility, including fruit size and seed damage (Assmann et al., 2008a; Rodrigo García et al., 2006a). The timing of phenological events and the maturation, reproductive success, and competitive capabilities of trees in temperate climates are heavily influenced by distinct temperature seasonality and unpredictable weather patterns throughout the year (Chuine & Beaubien, 2001; Chuine, 2010; Hasenauer et al., 1999; Myneni et al., 1997). Thus, to effectively understand the effects of early spring frosts on peach fruits, it is necessary to comprehend the types of problems this abiotic stress creates at the cellular level. While enzymes and proteins that protect cells against abiotic stressors, including cold stress, are crucial for plant survival (Yu et al., 2020b), low-temperature freezing stress can lead to irreversible injury to cell membranes and oxidative stress, resulting in physiological and metabolic modifications that impact plant growth and development (Kazemi-Shahandashti & Maali-Amiri, 2018b).

Research by Murray (2011) shows that a temperature of approximately -3°C can cause significant damage to fruitlets during petal fall with 10% damage occurring between -2°C and -3°C and up to 90% damage observed closer to -4°C. Genotypes with low CR tend to bloom early in colder regions and are more likely to be damaged by late frosts (Scorza, Ralph & Okie, 1991). However, genotypes with high CR might suffer from inadequate chilling resulting in irregular flowering with lower fruit quality (Fan et al., 2010a). Insufficient chilling can result in inconsistent bud breaks and low or no fruit set (Topp et al., 2008b). Although peach flower buds resist cold during endo-dormancy, they are susceptible to freezing temperatures during bud burst and bloom. Plants have evolved various mechanisms, such as supercooling and tolerance to intercellular ice formation, that enable them to withstand low temperatures without damaging the plant tissue. The resulting freezing-induced dehydration can permanently damage cellular components, such as cell membranes, leading to flaccidity and discoloration of the damaged tissue (Rodrigo, 2000).

Consequently, dehydration injury is accepted as the leading cause of frost damage (Wilson, 2001). The critical temperature, which determines a plant's level of cold resistance and the temperature at which tissue damage occurs, is influenced by factors such as the stage of phenological development of the bud/shoot or set of fruitlets, type of plant tissue or dew point, moisture, and pre-frost environmental conditions (Keller, 2020).

Frost tolerance is frequently used to refer to the biological capacity of a plant to survive temperatures that are only mildly below freezing (Wisniewski et al., 2014a). In recent years, various studies have demonstrated that the frequency, severity, and duration of low-temperature events, specifically SFD, are expected to increase in the 21st-century due to global warming (Augspurger, 2013; Kodra et al., 2011). This presents a significant threat to both herbaceous and woody plant species, particularly to less vital organs such as developing buds and young leaves, flowers, and fruitlets. The increased risk of spring frost damage is particularly concerning given the fact that temperate fruit trees, which account for nearly half of all global fruit production, serve as a critical source of food and income for many communities (Rodríguez et al., 2021).

An analysis of 17 peach/nectarine and 8 Japanese plum cultivars following a natural frost of 3°C (26.6°F) showed significant variation in frost resistance during bloom, as indicated by the survival of ovary in recently opened flowers. Plum cultivars Bruce and Santa Rosa and the peach cultivars June Gold and Coronet were found to be among the most tolerant. In contrast, the plum cultivar Ozark Premier, the nectarine cultivar Fantasia, and the peach cultivar Loring were among the most susceptible (Rieger et al., 1991b). Another analysis of the fruit damage caused by a 1.1°C natural frost in 28 peach genotypes of varying maturity revealed that the genotypes' tolerance to the frost varied based on measurements of percent fruit loss, suture diameter change, and other growth indices. At the time of the frost occurrence, cultivars with good leafing and a firm endocarp (pit) displayed greater tolerance to frost damage. Fruitlet survival was similarly influenced by fruit size. Fruit with suture diameters larger than 30 mm were more tolerant than those smaller than 20 mm (Assmann et al., 2008b). Szalay et al. (1999) suggested that flower buds were in the middle of the highest cold tolerance range, with the LT50 (lethal temperature for 50% mortality) between -20 and -25°C. However, the authors observed tolerance decrease and significant increase of LT50 in the following months. A good indicator of frost tolerance could also be reduction of fruit set caused by the loss (drop) of injured fruitlets (Chen et al., 2016c). There has yet to be a standardized rating or phenotyping measure created to better or more precisely evaluate frost tolerance among peach genotypes because of the trait's complexity, freezing condition variability (such as freezing temperature durability), and unpredictable occurrence.

Peach breeding and genetics

The introduction of the peach fruit to North America from China took place around 1850. Cultivar Chinese Cling, its offspring, ‘Elberta’, ’Belle of Georgia’, and ’J. H. Hale’, as well as their progeny, have been critical for peach production and breeding in the U.S. and the world (Badenes & Byrne, 2012). In 1895, Geneva, New York, became the birthplace of the first organized institutional breeding program in the Americas for peaches. Subsequently, private breeding initiatives in California were established in the early 1930s (Okie et al., 2008a). The initial aim of these programs was to develop locally adapted peaches and nectarines. However, with the advancement of technology and investment, the focus shifted towards developing high-yielding cultivars that could withstand environmental stressors and possess desirable qualities such as fruit quality, post-harvest resilience, and resistance to diseases and pests (Byrne. & Bassols Raseira, 2005; Byrne et al., 2000b). Recent shifts in climate have spurred interest in developing cultivars with lower chilling requirements, positioning adaptation to climate change as a key goal for plant breeders (Raseira et al., 2020).

Peach is a diploid species with a compact genome size of 265 Mb (Verde et al., 2017) which is approximately double that of *Arabidopsis thaliana's* genome (157 Mb) (Arumuganathan & Earle, 1991). It has a short juvenile phase and is capable of self-fertilization (Verde et al., 2017a). Among the *Rosaceae* species, peach has the most thorough genetic characterization with extensive knowledge of many genes critical to key traits. It is used as a model species for comparative and functional tree genomics and genetics studies (Arús et al., 2012; Eduardo et al., 2013; Monet et al., 1996). The availability of the reference peach genome released more than decade ago (Verde et al., 2013, Verde et al., 2017) has dramatically advanced genetic research on peaches (Aranzana et al., 2019). The quality of the genome sequence, collinearity and synteny with *Prunus* and *Rosaceae* species makes peach ideal for genetic mapping and identifying genes that determine desirable traits, including climate resilience (Bielenberg et al., 2022a; Bielenberg et al., 2015a; Cirilli et al., 2021a; Fan et al., 2010b; Shi et al., 2020).

Molecular resources

The Genome Database for Rosaceae serves as a publicly accessible repository for a wealth of molecular resources, genetic markers, linkage maps, quantitative trait loci (QTLs), and candidate genes associated with the *Rosaceae* family (http://www.rosaceae.org/; Jung et al., 2019a). This database houses the molecular resources for economically important species of *Rosaceae* family including *Prunus* genus, and peach (http://www.rosaceae.org/). More than 500,000 markers, including SNPs and SSR, 196 genetic maps were developed in *Prunus* sand utilized in QTL mapping. Approximately 1,764 QTLs associated with 163 agronomically important traits and 26,873 genes and 47,089 mRNA in the peach genome (v2.0.a1) have been reported.

The availability of a high-quality peach reference genome and 9K Infinium single nucleotide polymorphisms (SNPs) array, have enabled fast progress in mapping the traits of interest (Aranzana et al., 2019a). SNP markers have quickly emerged as the most practical and affordable markers for genetic investigations since the invention of the 9K peach Infinium array (Verde et al., 2012) and genotyping-by-sequencing (GBS) technique (Elshire et al., 2011a).

By integrating data from the peach genome with numerous SNP-based maps derived from the peach array, a consensus map was developed to assist in determining the positions and genetic sequence of markers that had not yet been mapped (da Silva Linge et al., 2018). The Genotyping-by-Sequencing (GBS) method is utilized in peach genetic mapping for its efficiency in generating a large number of SNP markers that cover the entire genome, thereby facilitating the rapid and cost-effective development of high-density linkage maps (Bielenberg et al., 2015b; Guajardo et al., 2015).

The peach genome was the first tree fruit genome sequenced in Rosaceae and has been used as a model in genetic studies to investigate the inheritance of desired traits, including climate resilience. Significant work has focused on climate-related traits, such as those influencing dormancy and bloom timing. Extensive studies on the genetic regulation of dormancy in peach have identified over 100 quantitative trait loci (QTLs) for bloom date and chill requirements. However, despite technological advances, there is still a need for more sophisticated molecular tools to help researchers and breeders address fruitlet freeze tolerance. The scientific community must prioritize and allocate substantial resources toward enhancing molecular tools to better understand and address fruitlet freeze tolerance. Despite the importance of this issue, there remains a significant gap in information regarding the mechanisms behind this phenomenon.

Quantitative trait loci (QTLs), mapping and genome-wide association analysis

QTLs in peach have been detected via bi-parental mapping (Rawandoozi et al., 2021a), pedigree-based mapping (Fresnedo-Ramírez et al., 2013) and association mapping (Cao et al., 2012; Martínez-García et al., 2013). The utilization of genome-wide association analysis (GWAS) has allowed for exploring unrelated germplasm in identification of causal genetic polymorphisms that contribute to complex traits (Riedelsheimer et al., 2012; Zhao et al., 2011). Detection of QTLs or causal genes associated with molecular markers tightly linked to trait(s) of interest has been proposed as a strategy for implementation of marker-assisted selection (MAS) in plant breeding (Xu & Crouch, 2008). However, plants' complex population structure and genetic relatedness can lead to inaccurate marker-trait associations in MAS, making it challenging to precisely identify loci that genuinely impact target traits (Chan et al., 2011; Gupta et al., 2005). To overcome these limitations in association analysis, recent studies have explored the implementation of advanced methods based on genome sequencing and SNP genotyping to facilitate QTL mapping in crops (Gupta et al., 2005; Morgante & Salamini, 2003). Hence, these complex traits require specialized methods for a deeper understanding. Approximately 1,082 QTLs have been linked to 40 agronomically relevant traits in peaches, including bloom timing, fruit quality, phytochemical content, and disease resistance (Demirel et al., 2021).

Candidate genes

In recent years, extensive research has been carried out on the expression of various genes in peach, following the sequencing of the peach genome (Verde et al., 2017). Some of this research has focused particularly on genes that are involved in the plant's response to cold (Artlip et al., 2013; Bassett et al., 2015; Jiao et al., 2017a; Wang et al., 2019). Plants, including peaches, undergo a series of molecular changes in response to freezing conditions, which are crucial for survival. These changes encompass modifications to the membrane composition, accumulation of necessary solutes, changes to the osmotic process, and regulation of plant hormone synthesis (Muthuramalingam et al., 2022). One of the significant breakthroughs in the study of freezing tolerance in plants, including peaches, has been the discovery of cold-inducible C-repeat binding factors (CBF) or dehydration responsive element binding (DREB) transcriptional activators. These activators have been found to regulate a range of genes related to the low-temperature response in plants (Wisniewski et al., 2014b). Additionally, for a plant to be able to withstand cold stress, it is essential that it has enzymes and proteins that directly protect its cells from these environmental challenges (Yu et al., 2020c). Both genetic and environmental factors influence the complexity of the freeze tolerance trait. An analysis of the peach genome has revealed the presence of two genes, PpICE1 and PpICE2, and six CBF genes believed to play a role in the plant's ability to tolerate fruitlet freezing. The five CBF genes are found together in scaffold linkage group 5, while the sixth gene is located on linkage group 2 (Wisniewski et al., 2014b). PpICE1 and PpICE2 are located on linkage groups 3 and 5, respectively. Mutations in PpICE1 and PpICE2 have been linked to increased fruitlet freeze resistance in peaches (Lee et al., 2005; Wisniewski et al., 2014c). This is due to the role of the ice-binding protein encoded by these genes in preventing ice crystal formation and protecting plant tissues. In addition, it has been observed that mutations in the ICE protein that impact freezing tolerance also affects stomatal development (Kanaoka et al., 2008). Another gene, PpDREB2A, belonging to the DREB family, has been found to be upregulated in peach fruit under freeze stress. (Jiao et al., 2017b). The CBF-COR signaling network has been widely studied as a key component in regulating freezing tolerance in plants. Genetic mapping studies have repeatedly revealed a correlation between freezing tolerance and the presence of QTLs near the CBF genes (Alonso-Blanco et al., 2005; Kang et al., 2013). These findings suggest that there may be a trade-off between freezing tolerance and overall fitness in different survival environments, with one of the QTLs being linked to the CBF2 gene as a potential candidate gene (Oakley et al., 2014). Genes that are activated and subsequently expressed during cold acclimation are vital for plant adaptation to low temperatures. For example, the upregulation of a gene responsible for a cold-regulated protein in peach fruits may enhance their freezing resistance during cold acclimation (Jin et al., 2018). Another heat shock protein gene, PpHSP70, has also shown increased expression during cold acclimation in peach fruits, suggesting a potential role in freeze tolerance (Tittarelli et al., 2009). Numerous key genes integral to cold acclimation have been successfully isolated and described. Notably, the full-length dehydrin gene PpDhn1 has been identified to exhibit varying levels of expression linked to cold resistance in evergreen and deciduous peach sibling genotypes (Artlip et al., 1997). Furthermore, the 60-kDa peach dehydrin protein (PCA60), produced in response to various environmental stressors, was purified and demonstrated antifreeze properties (Wisniewski et al., 1999).

Transcription factors play a crucial role in detecting low temperatures, initiating cold-regulated gene expression, and triggering the cold acclimatization process in plants (Wisniewski et al., 2011). For instance, the AP2/EREBP (APETALA 2/ethylene-responsive element-binding factor) family of CBF transcription factors, also known as DREB1s, play a role in the freezing tolerance of plants by binding to CBF cis-elements (5′-A/GCCGAC-3′) found in the promoters of cold-regulated genes and up-regulating their expression (Zhao, C. & Zhu, 2016; Zhao et al., 2014).

Breeding for climate resilience

Most research has been done on climate-related traits, such as dormancy-related traits that affect bloom time. Several studies have evaluated bud and flower tolerance to freezes in many cultivars of different *Prunus* species, but fruitlet freeze tolerance is relatively understudied. Melgar et al., (2022) used the electrolyte leakage method to demonstrate fruitlet freeze tolerance in 81 accessions, showing range of tolerances from -5ºC to -10ºC**,** and significant variability within peach germplasm.

Breeding efforts have accordingly focused on chilling requirement and bloom time and just recently included heat requirement in the climate-resilience traits realizing that reducing chill requirement to ensure chill satisfaction increases chances for late spring frost due to an early bloom time. We believe that the climate resilience traits should include the fruitlet freeze tolerance as well and thatthe variability reported by Melgar et al. (2022) warrants further exploration of this trait for breeding potential. Thus, my working hypothesis is that the peach germplasm diversity in fruitlet freeze tolerance can be harnessed in breeding programs to increase climate resilience in newly developed cultivars.

Project objectives

The overall aim of enabling breeding for fruitlet freeze tolerance is achieved through the following objectives:

1. Phenotype peach germplasm for fruitlet freeze tolerance and determine if this trait is genetically controlled.

2. Obtain genotyping data for the germplasm phenotyped in objective 1 using genotyping-by-sequencing.

3. Perform a genome-wide association study (GWAS) to determine regions in the peach genome associated with fruitlet freeze tolerance.

4. Investigate candidate genes associated with the fruitlet freeze tolerance.

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CHAPTER TWO

FRUITLET FREEZE TOLERANCE IN PEACH GERMPLASM

Introduction

Climate change, manifested as increase in temperature and or episodes of temperature spikes during winter, and insufficient cold exposure during dormancy increases tree vulnerability to spring frosts, and threatens fruit production worldwide (Castède et al., 2014a; Miranda et al., 2021). Freeze damage poses a significant ecological risk to stone fruit crops, and destructive spring frosts are projected to intensify due to global climate change (Gu et al., 2008). Peach (*Prunus persica* [L.] Batsch) is a highly economically valuable temperate fruit tree that is widely cultivated in moderate climatic zones throughout the world and, therefore, affected by climate change. Peach trees exhibit a remarkable adaptation to seasonal climatic variations by experiencing a period of dormancy during the winter months (Fadón et al., 2020). This evolutionary process involves shedding their leaves and entering a stage of reduced metabolic activity (endo-dormancy). Then, once trees have experienced adequate chilling temperatures, they are released from dormancy (eco-dormancy) and start developing and blooming in response to warm temperatures. Increasingly warmer winters, as well as weather patterns with more severe winter and spring temperature fluctuations, disrupt this normal pattern. Warmer winters can cause trees to bloom prematurely and expose their flowers or fruitlets to lethal freezing temperatures. This scenario has caused disastrous losses of the peach crop in the southeastern U.S. in recent years. Numerous research has focused on assessing the susceptibility of trees, buds, and flowers to freezing events (Khorshidi et al., 2014; Rieger et al., 1991c; Yu, D. J. & Lee, 2020), but the fruitlet freeze tolerance is relatively understudied (Assmann et al., 2008c; Melgar et al., 2022; Rodrigo García et al., 2006b).

Various methods are available for assessing freeze damage, such as visual evaluation of tissue discoloration, thermal analysis, measure of electrolyte leakage, and triphenyl tetrazolium chloride reduction analysis, the selection and implementation of which mostly depend on the organ or tissue that is being assessed (Yu, D. J. & Lee, 2020). Dexter et al. (1932) first noted that freezing temperatures led to the destabilization of cellular membranes and an accelerated release of symplastic solutes from cells, e.g., electrolyte leakage. The electrolyte leakage has evolved into a standard method for assessing the relative quantity of cell damage in many species in reaction to biotic and abiotic stresses, including cold stress (AniśAko & Lindstrom, 1995; Fallon & Cavender-Bares, 2018; Hatsugai & Katagiri, 2018; Kreyling et al., 2015; Lenz et al., 2013a). The electrolyte leakage, and temperature at which 50% of tissue experiences damage (LT50), used to evaluate fruitlet freeze tolerance in selected peach germplasm from the National Clonal Germplasm Repository (NCGR) in Davis, CA, and Clemson University (Melgar et al., 2022) revealed freeze tolerance to temperatures as low as -10 ºC, with majority of accessions exhibiting tolerance within -5 and -6 ºC. The highest tolerance, < -8 ºC, was observed in several cultivars from various breeding programs and released during last five decades (‘White Lady’, ‘Scarletpearl’, ‘Raritan Rose’, ‘Manon’, ‘MA Blake’, ‘Canadian Harmony’, ‘Harrow Diamond’, and ‘Sugar Giant’) suggesting that diversity for this trait in peach germplasm could be explored in breeding. However, the graphical presentation of the LT50 results revealed that the two accessions with similar LT50 values have distinctively different asymmetric sigmoid curve patterns. The authors suggested that further investigation is needed to determine how best to describe the level of freeze damage in the fruitlets. The research highlighted the possibility of harnessing the genetic potential for freeze tolerance in peach breeding to address the effect of changing climate and predicted more frequent late spring frosts on stone fruit production (Chamberlain et al., 2019a; Inouye, 2008; Martin et al., 2010; Ru et al., 2023). These findings suggested that breeding for improved fruitlet freeze tolerance in peaches might be possible.

The breeding goals in peach breeding programs have evolved significantly over the decades (Byrne, David, 2005; Okie et al., 2008b). Initially, the focus was primarily on improving traits related to yield, disease resistance, and adaptability to specific growing regions. However, as consumer preferences and environmental concerns evolved, breeding goals shifted towards quality attributes, such as fruit flavor, appearance, and nutritional content (Gasic et al., 2022; Worthington & Clark, 2021). Importance of breeding for climate resilience or plasticity became more emphasized with production disruptions caused by climate change. However, focus was on the dormancy related traits, such as lowering chilling requirement and delaying bloom time (Campoy et al., 2019; Fan et al., 2010c), and not fruitlet freeze tolerance.

Therefore, we expanded the Melgar et al. (2022) study by evaluating fruitlet freeze tolerance and heritability in modern peach breeding germplasm. Our hypothesis was that observed diversity in peach fruitlets' ability to tolerate low temperatures is genetically controlled and can be used in breeding to incorporate fruitlet freeze tolerance in newly developed cultivars and provide recommendations to growers. The results of this study provide the foundation for further understanding the genetics behind this trait and support development of molecular tools to enable breeding of new peach cultivars with enhanced freeze tolerance.

Materials and methods

Peach and nectarine germplasm

Fifty-one peach and nine nectarine cultivars from the Clemson University *Prunus* germplasm collection and 15 advanced selections from Clemson University peach breeding program (CUPBP) were used for evaluation of their fruitlet tolerance to freezing temperatures following the protocol described in Melgar et al. (2022). The germplasm consisted of heirlooms, cultivars released or patented in the U.S. in the last five decades, and advanced selections from the CUPBP (Table 2.1). The material is maintained at the Clemson University Musser Fruit Research Center (Latitude: 34.639038, Longitude: −82.935244) in Seneca, SC, under warm, humid, moderate climate and standard commercial techniques for irrigation, fertilization, and pest-disease control. The trees used in the study were 7 - 10 years old, grafted on Guardian® rootstock and trained as perpendicular V or open-center vase. Chilling requirement (CR) in chill hours (CH), bloom dates in Julian date (JD), and heat requirement (HR) in growing degree hours (GDH) for this material were previously described in Demirel et al. (2021) and Atagul et al. (2022).

Phenotyping fruitlet freeze tolerance

One hundred fruitlets per accession were collected at the shuck-off stage with a diameter not exceeding 13 mm and transported to the laboratory during two seasons, 2022 and 2023. Fruitlet freeze tolerance was evaluated using the electrolyte leakage method (Melgar et al., 2022). Five fruitlets were placed into individual test tubes, with three replicates per accession and temperature, and immersed in ethylene glycol–water refrigerated bath (AP 20R-30, VWR, Radnor, PA, USA). Six treatments using different freezing temperatures: 0 ºC, -2 ºC, -4 ºC, -6 ºC, -8 ºC, and -10 ºC were applied, starting at 0ºC and decreasing 2 degrees every hour. After one hour of exposure to each temperature, the three replicates corresponding to each specific temperature were withdrawn from the water bath, covered with Parafilm® (Bemis, Neenah, WI, USA) and placed in a refrigerator (4°C) to thaw gradually. After one hour in the refrigerator, 10 ml of deionized water was added to each tube and the tubes were shaken at 200 rpm overnight at room temperature, for at least 12 hours. The next day each tube was vortexed for a few seconds and electrical conductivity (EC1) was measured using a conductivity meter (Fisher Scientific Accumet AP85, Thermo Fisher Scientific, Waltham, MA, USA) to determine the amount of solutes from the fruitlets released into the solution. In the final step, the test tubes were autoclaved for 20 min at 121°C to completely disrupt the cell membranes and release all solutes contained within the cell into the surrounding solution. Following autoclaving, samples were allowed to cool down at room temperature and electrical conductivity was again measured (EC2). Electrolyte leakage was calculated as the ratio of ion leakage from freeze injury: EL = EC1 × 100/EC2 (Barranco et al., 2005). The ratio of ion leakage at each temperature was used to develop a graphical interpretation of freeze tolerance throughout decreasing temperatures using SigmaPlot 13.0 (Systat, San Jose, CA, USA) and the following equation (Sigmoidal, Sigmoid, 4 Parameter):

$$f = y0 + a/(1 + exp(-(x - x0)/b)),$$

where *x0* = the temperature at the inflection point (LT50); *b* = Hill's slope of the curve (i.e., steepness of the curve at *x0*); *y0* = the lowest electrolyte leakage value (at 0°C); and *a* = difference in electrolyte leakage between the minimum and maximum temperature.

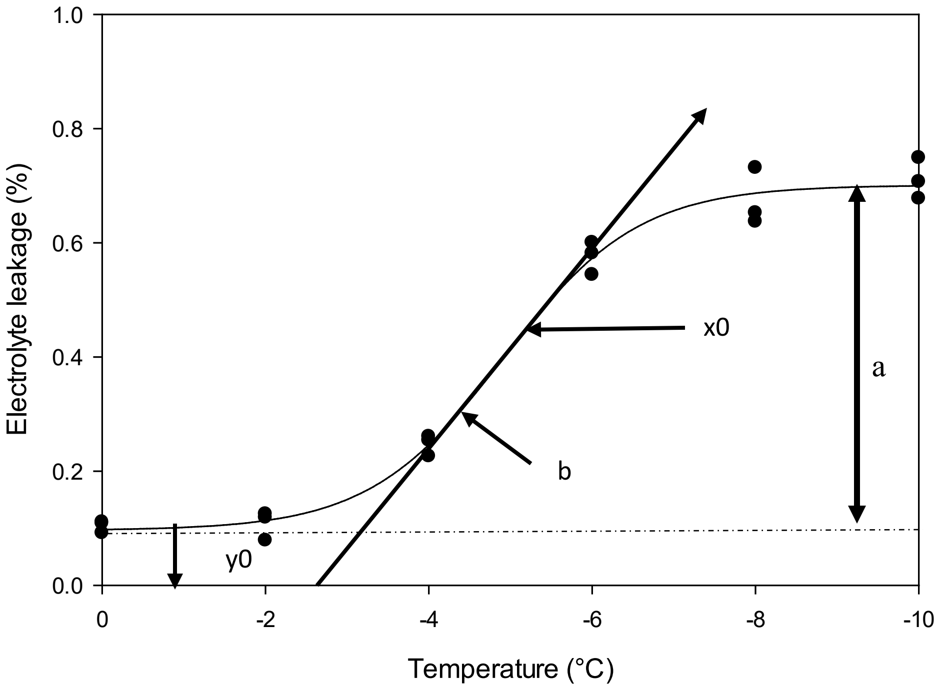

Figure 2.1. Electrolyte leakage (Y axis) vs Temperature (X axis), x0, temperature at the inflection point (LT50); b, slope of the curve (steepness of the curve at x0); y0, lowest electrolyte leakage value (at 0 °C); a, difference in electrolyte leakage between minimum and maximum temperature; and b, Hill's slope of the curve (steepness of the curve at x0).

The temperature at which 50% of the ion leakage (LT50) occurred, termed the inflection point (IP), and the area under the sigmoidal curve (AUC) were recorded. In addition, the material was grouped into three tolerance groups (TG) based on the IP and the AUC. The TG1 (high tolerance), contained material exhibiting no freeze damage at temperatures < -6ºC and with AUC

< 25%. The TG2 (intermediate tolerance), contained material exhibiting damage at temperatures from -5 to -6ºC and having AUC between 25-35%; and the TG3 (low tolerance), contained material exhibiting damage to temperature >-5ºC and having AUC > 35%.

Statistical data analyses

Statistical analyses were performed in R Studio version 2023.03.1 and SPSS v. 27 (IBM SPSS Statistics for Windows, Version 27.0. Armonk, NY: IBM Corp)

Data normality and equal variance assumption was determined using the Shapiro – Wilk test. Spearman's correlation coefficient was used to estimate the relationship between two variables ranked on an ordinal scale.

Broad-sense heritability (H^2) was calculated as:

$$H^2 = \sigma g^2 / (\sigma g^2 + \sigma e^2/n),$$

where $\sigma g2$ is the genetic variance, σe is the environmental variance and n is the number of years.

Data was organized in six datasets using the trait (IP, AUC) values obtained in each experimental season (2022-2023) and average value: IP2022, IP2023, IP_Ave, AUC2022, AUV2023 and AUC_Ave.

Results

Diverse peach (66) and nectarine (9) germplasm, consisting of cultivars important for the southeast U.S. peach industry (heirlooms and cultivars patented within the last five decades) was evaluated for fruitlet freeze tolerance (Table 2.1; Figure 2.1). The majority of cultivars were released within the last three decades of the 20th century (1971-1999). Some of them are still grown in the southeast U.S. and used as standards in regional trials or represent important breeding parents used in peach breeding programs. This germplasm is adapted to or evaluated for suitability in the southeast U.S. with chilling requirement ranging from 500 to over 1,000 CH, majority being in the 600-900 CH range, bloom time best linear unbiased prediction (BLUP) value from 62 – 80 JD and estimated minimum heat requirement from 1,362 – 7,039 GDH.

Fruitlet freeze tolerance was estimated for 75 and 71 accessions in 2022 and 2023 season, respectively. Due to a damage from late spring frosts in 2023, fruitlets of peach cultivars FlavrBurst™, Messina®, Rich May and Carored, were not included in the electrolyte leakage study. Inflection point (IP) and area under the curve (AUC) were used to determine the level of tolerance and group cultivars in tolerance groups (Table 2.1).

IP ranged from -3.94°C (IP2022) to -10.22°C (IP2023), with similar overall average IP (IP_Ave = -5.72ºC) observed in both years (Table 2.2). Significant differences between IP ranges in the two experimental years were observed, with a wider range (-4.02 to -10.22 ºC) observed in IP2022 and a narrower range (-3.94 to -7.79 ºC) in IP2023 dataset. IP distribution was skewed towards lower tolerance in 2022 and did not exhibit normal distribution (Shapiro Wilk test; W = 0.91135, p-value = 6.484e-05) (Figure 2.2). However, bimodal normal distribution (Shapiro Wilk test; W = 0.97354, p-value = 0.1385) was observed in IP2023 dataset (Figure 2.2).

Table 2.1. Characteristics of the U.S. modern peach breeding germplasm used in this study, including fruitlet freeze tolerance and classification in freeze tolerance groups based on IP (inflection point, °C) and AUC (area under the curve, %). Fruit type – FT; origin – country (state); CR - chilling requirement in chill hours (CH); HR - heat requirement in growing degree hours (GDH); BD - bloom date (BLUP) in Julian Days (JD).

Accession[1]	FT	Origin	Released	CR (CH)	HR (GDH)	BD (JD)	Fruitlet Freeze						Grouping			
							2022		2023		Average		2022		2023	
							IP	AUC	IP	AUC	IP	AUC	IP	AUC	IP	AUC
Elberta	P	US (GA)	1889	800	5112	75	-5.29	32.59	-5.93	35.35	-5.61	33.97	2	2	2	3
Redrose	P	US (NJ)	1940	850	1654	75	-6.4	26.35	-5.72	33.58	-6.06	29.97	1	2	2	2
Loring	P	US (MO)	1946	800	3667	73	-5.14	42.43	-5.41	27.32	-5.28	34.88	2	3	2	2
Coronet	P	US (GA)	1953	588	5934	69	-6	38.67	-5.82	39.08	-5.91	38.88	2	3	2	3
Redglobe	P	US (MD)	1954	850	1654	75	-5.96	24.69	-5.43	17.69	-5.70	21.19	2	1	2	1
June Gold	P	US (CA)	1959	650	4879	68	-4.04	45.67	-5.49	33.32	-4.77	39.50	3	3	2	2
Parade	P	US (CA)	1960	800	3762	72	-7.63	22.48	-5.32	18.52	-6.48	20.50	1	1	2	1
Jefferson	P	US (VA)	1960	850	3578	77	-4.22	34.46	-5.34	28.66	-4.78	31.56	3	2	2	2
Cresthaven	P	US (MI)	1963	950	5506	74	-4.62	35.72	-5.51	27.05	-5.07	31.39	3	3	2	2
Empress	P	US (CA)	1964	650	5400	69	-5.84	31.56	-5.36	32.37	-5.60	31.97	2	2	2	2
Big Red	P	US (FL)	1969	750	5193	72	-5.32	29.86	-5.01	25.36	-5.17	27.61	2	2	2	2
O'Henry	P	US (CA)	1970	800	4876	74	-5.53	28.03	-5.3	28.86	-5.42	28.45	2	2	2	2
Brightstar	P	US (CA)	1976	708	6242	74	-7.62	23.98	-7.54	23.45	-7.58	23.72	1	1	1	1
Topaz	P	US (MO)	1976	700	-	-	-6.01	23.28	-5.6	24.75	-5.81	24.02	1	1	2	1
Biscoe	P	US (NC)	1977	900	5380	73	-5.65	34.44	-5.92	28.97	-5.79	31.71	2	2	2	2
Majestic	P	US (LA)	1979	750	5869	73	-4.66	37.06	-5.47	35.18	-5.07	36.12	3	3	2	3
Sunprince	P	US (GA)	1982	800	3402	71	-7.77	21.95	-5.95	21.95	-6.86	21.95	1	1	2	1
Silver Gem	N	US (CA)	1982	-	-	-	-6.55	20.91	-6.75	22.13	-6.65	21.52	1	1	1	1

Accession	FT	Origin	Released	CR (CH)	HR (GDH)	BD (JD)	Fruitlet Freeze						Grouping			
							2022		2023		Average		2022		2023	
							IP	AUC	IP	AUC	IP	AUC	IP	AUC	IP	AUC
Ruston Red	P	US (LA)	1982	800	-	-	-4.88	33.05	-4.64	32.66	-4.76	32.86	2	2	3	2
Goldcrest	P	US (CA)	1984	650	4598	66	-5.75	35.75	-4.65	36.63	-5.20	36.19	2	3	3	3
Juneprince	P	US (GA)	1985	650	4598	67	-5.57	32.63	-6.2	29.85	-5.89	31.24	2	2	1	2
Fireprince	P	US (GA)	1985	850	5796	73	-4.13	34.27	-5.75	28.72	-4.94	31.50	3	2	2	2
Caroking	P	US (SC)	1987	636	5824	71	-4.46	40.11	-4.04	44.16	-4.25	42.14	3	3	3	3
Tra-Zee	P	US (CA)	1988	800	5915	80	-5.29	28.47	-5.39	29.05	-5.34	28.76	2	2	2	2
Carogem	P	US (SC)	1989	850	2178	75	-6.35	28.15	-5.9	25.3	-6.13	26.73	1	2	2	2
Scarlet Pearl	P	US (GA)	1989	750	5531	72	-4.68	38.57	-6	35.06	-5.34	36.82	3	3	2	3
Summer Fire	N	US (CA)	1990	-	-	-	-8.62	20.93	-6.16	23.62	-7.39	22.28	1	1	1	1
Rich May	P	US (CA)	1991	800	3402	70	-5.39	35.31	-	-	-5.39	35.31	2	3	-	-
Summerprince	P	US (GA)	1992	850	5242	71	-6.75	22.59	-7.24	24.76	-7.00	23.68	1	1	1	1
Glory	P	US (GA)	1992	800	5280	74	-6.25	21.85	-5.4	15.75	-5.83	18.80	1	1	2	1
Rich Joy	P	US (CA)	1992	850	-	-	-4.25	44.13	-4.37	39.06	-4.31	41.60	3	3	3	3
Arctic Pride	N	US (CA)	1993	800	2846	69	-10.22	19.36	-6.04	23.8	-8.13	21.58	1	1	1	1
Flameprince	P	US (GA)	1993	850	5661	72	-5.11	32.13	-6.21	29.56	-5.66	30.85	2	2	1	2
Guardian®	P	US (SC)	1993	850	-	-	-4.99	25.98	-5.79	25.57	-5.39	25.78	3	2	2	2
P.F. 23	P	US (MI)	1993	700	5287	69	-4.84	32.03	-5.8	34.82	-5.32	33.43	3	2	2	2
Arctic Gold	N	CFZ	1995	-	-	-	-5.98	23.11	-5.65	22.38	-5.82	22.75	2	1	2	1
Autumn Red	P	US (CA)	1996	800	5284	78	-4.48	31.97	-5.42	33.35	-4.95	32.66	3	2	2	2
Sweet September	P	US (CA)	1997	850	2350	80	-8.28	25.35	-5.97	25.44	-7.13	25.40	1	2	2	2
Autumnprince	P	US (GA)	1997	800	3901	73	-7.26	26.71	-6.29	26	-6.78	26.36	1	2	1	2
Snow Prince	P	US (CA)	1997	-	-	-	-6.21	23.92	-6.59	20.01	-6.40	21.97	1	1	1	1
Rubyprince	P	US (GA)	1997	850	-	-	-5.42	31.87	-7.79	27.14	-6.61	29.51	2	2	1	2
Juneprincess	N	US (GA)	1997	850	6117	73	-4.02	18.7	-4.08	24.49	-4.05	21.60	3	1	3	1
Arctic Belle	N	US (CA)	1998	900	6076	75	-6.9	21.02	-6.98	23.94	-6.94	22.48	1	1	1	1

Accession	FT	Origin	Released	CR (CH)	HR (GDH)	BD (JD)	Fruitlet Freeze						Grouping			
							2022		2023		Average		2022		2023	
							IP	AUC	IP	AUC	IP	AUC	IP	AUC	IP	AUC
Arctic Blaze	N	US (CA)	1998	750	4476	69	-6.88	19.82	-5.39	23.07	-6.14	21.45	1	1	2	1
Sureprince	P	US (GA)	1998	950	5260	73	-5.89	36.89	-6.24	37.52	-6.07	37.21	2	3	1	3
Valley Sweet	P	US (CA)	1998	700	4485	64	-5.49	32.21	-5.98	29.21	-5.74	30.71	2	2	2	2
Joanna Sweet	P	US (CA)	1999	800	5112	75	-4.96	31.86	-5.63	34.7	-5.30	33.28	3	2	2	2
John Boy	P	US (PA)	2000	800	4365	74	-5.37	31.84	-6.35	28.24	-5.86	30.04	2	2	1	2
China Pearl	P	US (NC)	2001	1100	-	-	-4.65	36.53	-3.94	35.74	-4.30	36.14	3	3	3	3
Arrington	N	US (AR)	2002	750	5193	72	-6.5	22.53	-4.97	32.69	-5.74	27.61	1	1	3	2
Westbrook	N	US (AR)	2002	750	4791	73	-6.26	23.54	-7.32	23.23	-6.79	23.39	1	1	1	1
White River	P	US (AR)	2002	700	-	-	-5.27	34.31	-5.7	29.89	-5.49	32.10	2	2	2	2
Julyprince	P	US (GA)	2004	850	1654	75	-4.05	47.76	-4.91	40.38	-4.48	44.07	3	3	3	3
Carored	P	US (SC)	2005	612	6197	69	-5.49	35.52	-	-	-5.49	35.52	2	3	-	-
Augustprince	P	US (GA)	2006	850	1547	75	-6.25	25.16	-5.38	32.18	-5.82	28.67	1	2	2	2
Early Augustprince	P	US (GA)	2006	800	5112	74	-5.95	23.42	-6.61	16.14	-6.28	19.78	2	1	1	1
Messina®	P	US (NJ)	2006	-	-	-	-5.95	33.57	-	-	-5.95	33.57	2	2	-	-
FlavrBurst™	P	US (WV)	2008	-	-	-	-4.9	33.31	-	-	-4.90	33.31	3	2	-	-
SummerFest™	P	US (WV)	2010	-	-	-	-5.6	23.27	-6.21	23.93	-5.91	23.60	2	1	1	1
Sweetstar	P	France	2010	-	-	-	-4.75	30.75	-7.06	26.27	-5.91	28.51	3	2	1	2
SC-01	P	US (SC)	-	800	2706	66	-4.75	37.28	-5.05	37.95	-4.90	37.62	3	3	2	3
SC-02	P	US (SC)	-	564	5947	62	-5.62	32.7	-5.32	30.02	-5.47	31.36	2	2	2	2
SC-03	P	US (SC)	-	972	3877	77	-5.36	28.96	-5.34	27.77	-5.35	28.37	2	2	2	2
SC-04	P	US (SC)	-	996	3052	76	-4.58	31.16	-5.6	33.68	-5.09	32.42	3	2	2	2
SC-05	P	US (SC)	-	1068	4147	78	-5.18	32.46	-4.74	34.69	-4.96	33.58	2	2	3	2
SC-06	P	US (SC)	-	1116	4856	72	-6	34.87	-5.27	27.35	-5.64	31.11	2	2	2	2
SC-07	P	US (SC)	-	972	1362	70	-5.58	23.27	-7.24	24.48	-6.41	23.88	2	1	1	1
SC-08	P	US (SC)	-	800	5095	69	-5.43	24.31	-6.85	27.66	-6.14	25.99	2	1	1	2

Accession	FT	Origin	Released	CR (CH)	HR (GDH)	BD (JD)	Fruitlet Freeze						Grouping			
							2022		2023		Average		2022		2023	
							IP	AUC	IP	AUC	IP	AUC	IP	AUC	IP	AUC
SC-09	P	US (SC)	-	708	5473	71	-4.93	35.46	-5.72	35.57	-5.33	35.52	3	3	2	3
SC-10	P	US (SC)	-	516	5997	62	-5.72	26.79	-5.97	26.32	-5.85	26.56	2	2	2	2
SC-11	P	US (SC)	-	1068	3779	73	-5.85	30.04	-5.64	31.74	-5.75	30.89	2	2	2	2
SC-12	P	US (SC)	-	540	7039	67	-6.43	27.13	-6.05	25.89	-6.24	26.51	1	2	1	2
SC-13	P	US (SC)	-	516	6863	65	-5.7	28.32	-6.58	28.21	-6.14	28.27	2	2	1	2
SC-14	P	US (SC)	-	-	-	-	-5.44	30.24	-5.29	29.8	-5.37	30.02	2	2	2	2
SC-15	P	US (SC)	-	-	-	-	-5.27	31.5	-5.78	28.17	-5.53	29.84	2	2	2	2

[1] Accessions are ordered based on the year of release. Peach – P and nectarine – N. FlavrBurst™ (KV00398); Messina® (NJ352); SummerFest™ (KV981175)

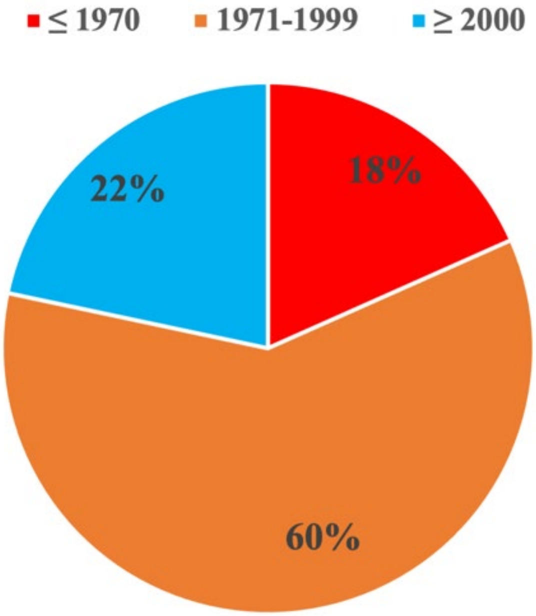

Figure 2.2. Distribution of peach and nectarine germplasm by release year

The highest negative temperature at which fruitlet damage was observed was -3.94ºC, in peach 'Sweetstar', in IP2023, and the lowest temperature at which the highest fruitlet tolerance was observed was -10.22ºC in nectarine 'Arctic Pride' in IP2022. The remaining accessions fell within the range of freezing temperatures between these two extremes with the majority showing fruitlet tolerance in -4 to -6ºC range in both datasets (Table 2.1; Figure 2.2). When IP values were used in grouping the accessions in tolerance groups (TG) 26.67% of material grouped as least (TG3) and most tolerant (TG1) and 46.67% were in the intermediate group (TG2) in 2022 (Table 2.1). A similar grouping was observed with IP2023 data with 28% characterized as most tolerant (TG1), 54.6% as intermediate (TG2), 12% as least tolerant (TG3) and 5.4% not applicable (NA). While most cultivars and selections maintained the group in both datasets, some discrepancies were observed. The most apparent change was from extreme groups to intermediate, with the most extreme change observed for peach cultivar 'Sweetstar' that moved from TG1 in 2022 to TG3 in

2023. The most tolerant group based on the IP2022 data consisted of cultivars released in the last century with few heirlooms such as ‘Redrose’ and ‘Parade’ released in 1940 and 1960, respectively, and three newer releases, two nectarines from University of Arkansas, ‘Westbrook’ and ‘Arrington’ released in 2002, and one peach from USDA, Byron, GA, ‘Augustprince’ released in 2006. Interestingly, seven nectarine cultivars, Arctic Belle, Arctic Blaze, Arctic Pride, Arrington, Silver Gem, Summer Fire, and Westbrook, were grouped in the most tolerant group based on IP2022, with five of them (Arctic Belle, Arctic Pride, Silver Gem, Summer Fire, and Westbrook) being classified in the same most tolerant group in 2023. Selections from the CUPBP were mostly grouped in TG2 (75 and 73% in 2022 and 2023, respectively) with SC-12 being the most tolerant in both 2022 and 2023.

Broad sense heritability of 0.52, was obtained with IP data from both seasons (Table 2.2).

Table 2.2. Descriptive statistics of fruitlet freeze tolerance observed in peach and nectarine germplasm in 2022 and 2023 season for inflection point (IP) and area under curve (AUC).

Trait	N	Min	Max	Mean	SD	H^2
IP-2022 (ºC)	75	**-10.22**	-4.02	-5.68	1.1	0.52
IP-2023 (ºC)	71	-7.79	**-3.94**	-5.76	0.79	
AUC-2022 (%)	75	18.7	**47.76**	30.12	6.49	0.85
AUC-2023 (%)	71	**15.75**	44.16	28.83	5.86	
IP_Ave (ºC)		-9	-3.98	-5.72		
AUC_Ave (%)		17.23	45.96	29.48%		

N, number of analyzed samples; SD, standard deviation; H^2, broad sense heritability; Ave; average. Extreme values are bolded.

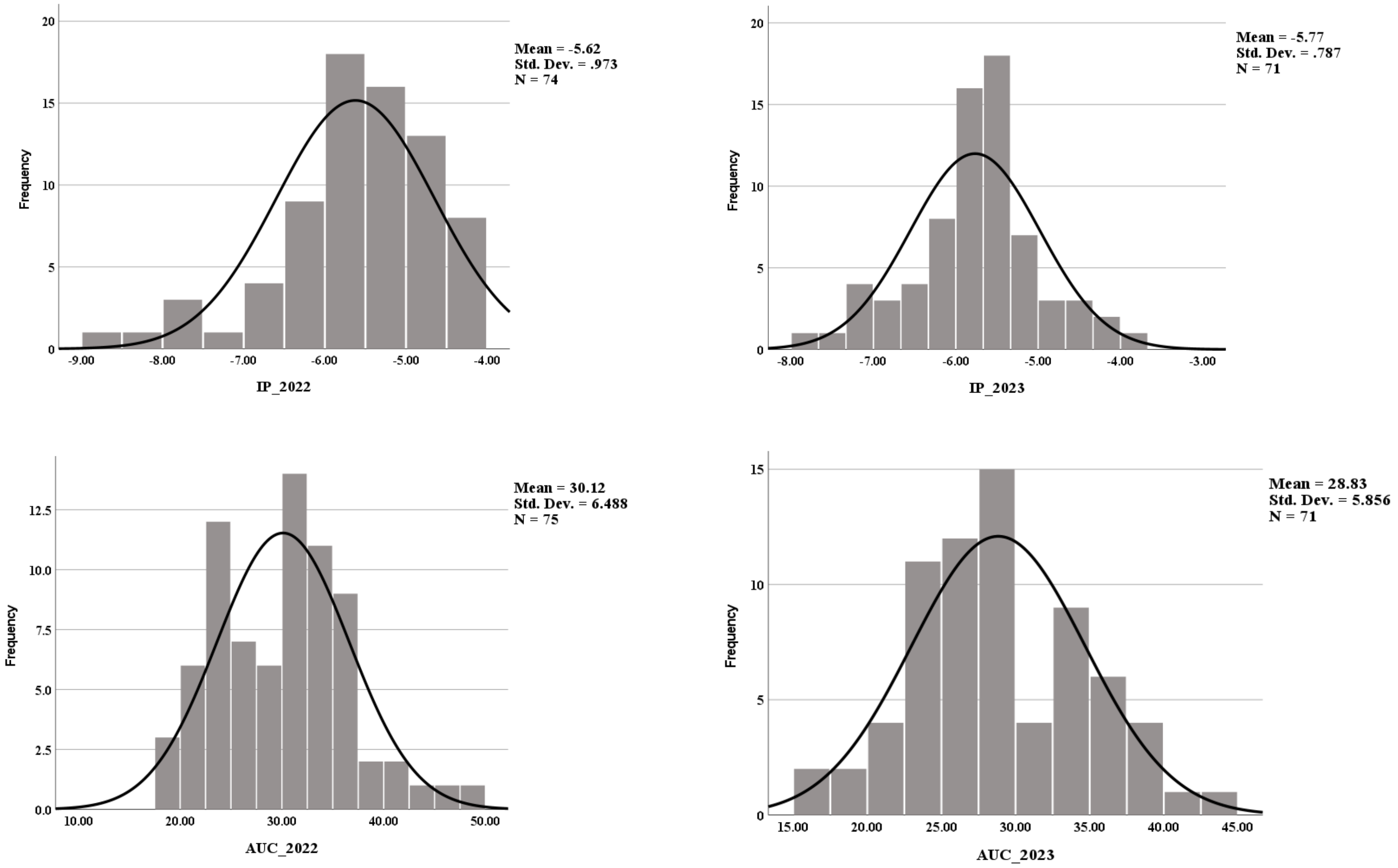

Figure 2.3. Frequency distribution of Inflection Point (IP) and area under the curve (AUC) of fruitlet freeze tolerance within the peach and nectarine germplasm in 2022 and 2023 season.

Fruitlet freeze tolerance was obtained by using the parameters i inflection point (IP) and area under the curve (AUC). IP determined the temperature at which 50% of the material (fruitlets) show sign of freeze damage (Figure 2.3; Appendix A and B) but could not accurately represent the performance of the fruitlets under the freezing temperatures as divergence and steepness of the sigmoid curve are not well represented by its value. For example, the same IP of approximately -5.9ºC was observed in 'Glory', 'Fireprince', and 'Julyprince' (Figure 2.4, red arrow), but the actual temperature when fruitlets were starting to experience damage differed. Fruitlets of 'Julyprince' began to show damage at -2ºC, 'Glory' at -4ºC and fruitlets of 'Fireprince' did not show any freeze damage until below -5 ºC (Figure 2.4). This difference is also reflected in not normal distribution of the IP data and in grouping based on the IP value, as cultivars with distinct divergence in curve slopes are grouped together (Appendix A and B).

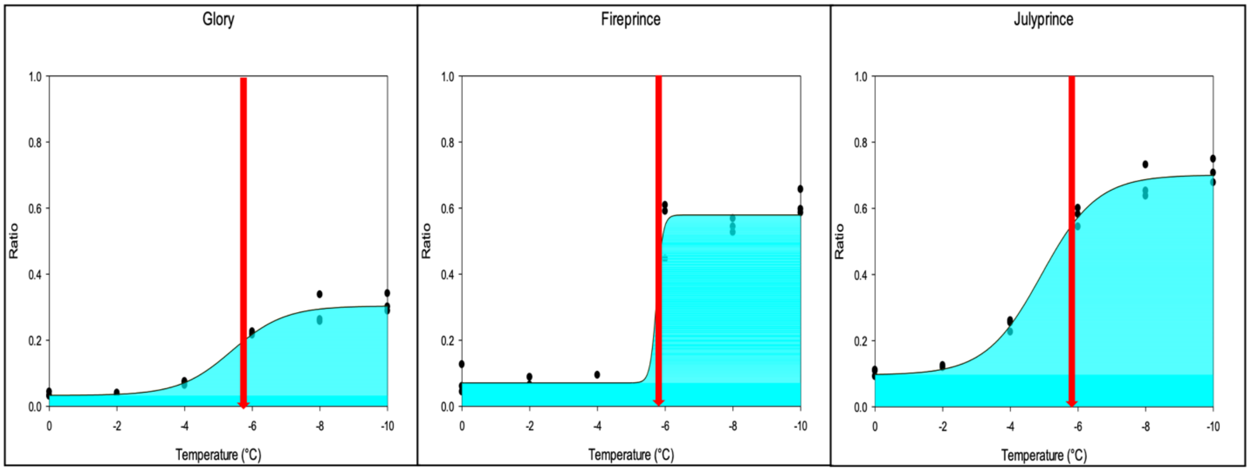

Figure 2.4. Patterns of fruitlet freeze response to freezing temperatures observed in three peach cultivars in 2023. Sigmoid curve and inflection point (red arrow) showing 50% of ion leakage. Area under the curve is shaded blue. Ratio is electrolyte leakage.

Therefore, to obtain a more accurate description of the fruitlet freeze tolerance, we calculated the AUC for each accession as the curve's steepness shows the initiation and rate of leakage. Even though the three cultivars, in the example above, showed similar IP the curve slopes and therefore AUC were quite different. The AUC observed in the three cultivars shown in Figure 3 ranged from 22% in ‘Glory’, 29% in ‘Fireprince’ to 47% in 'Julyprince', suggesting the highest fruitlet freeze tolerance in ‘Glory’ and lowest in ‘Julyprince’ (Figure 2.4; Table 2.1). In this example, it is worth noting that although AUC values for ‘Glory’ make it appear more tolerant than ‘Fireprince’, 'Fireprince's fruitlets showed almost two degrees higher tolerance as they did not begin to leak electrolytes until lower temperature (-6°C) and therefore should be considered having higher fruitlet freeze tolerance (Figure 2.3). That means a higher percentage is less tolerant.

Overall AUC in all peach and nectarine accessions ranged from 15.75% in 2023 to 47.76% in 2022 (Table 2.2). AUC was normally distributed (Shapiro Wilk test; W = 0.96973, p-value = 0.06932 in 2022; 0.98839, p-value = 0.7603 in 2023) in both years and showed bimodal distribution (Figure 2). The AUC interval was similar in the two experimental years, 29.06% and 28.41% in 2022 and 2023, respectively (Table 2.2). The lowest AUC was calculated in 2023, with 13.08% less fruitlet damage than the average, while the highest damage occurred in 2022, 17.64% freezing damage more than the mean (Table 2.2). Overall, the lowest AUC, 15.75%, was observed in ‘Glory’ in 2023, while the highest damage was detected in fruitlets of ‘Julyprince’ in 2022. Commercial peach cultivars Julyprince, June Gold, Rich Joy, and Caroking exhibited high degree of susceptibility to freeze tolerance, with AUC values of 47.7, 45.6, 44.1, and 40.1%, respectively (Table 2.1). Interestingly, the same cultivars had the lowest IP range, - 4.03 and - 4.45 °C. Based on IP, the highest fruitlet resilience to freezing temperatures was observed in nectarines ‘Arctic Pride’ and ‘Summerfire’ and peaches ‘Rubyprince’ and ‘Sweet September’.

Grouping of peach and nectarine accessions based on the observed AUC values revealed majority of material in intermediate group TG2 (50 and 53% in 2022 and 2023, respectively), and 28 and 26% of accessions in most tolerant (TG1), and 22 and 17% in the least tolerant (TG3) group in AUC2022 and AUC2023 datasets, respectively (Table 2.1). Most accessions maintained their group regardless of season with few changing from most (‘Arrington’ and SC-8) or least (‘June Gold’, ‘Loring’ and ‘Cresthaven’) tolerant to intermediate. Similar to IP grouping, AUC grouping classified all nine nectarines as most tolerant (TG1) in both years, except ‘Arrington’ which moved to TG2 in 2023 (AUC=27%), with 5% difference in AUC between the two seasons and only 2% over the group threshold. Two CUPBP selections SC-8 and SC-7 were in the most tolerant group in 2022 with SC-7 in 2023 having AUC of 27%, 2% above the threshold for the TG1. The CUPBP selection SC-12 was grouped as intermediate in both AUC2022 and AUC2023 datasets, with 2 and 0.86%, respectively, above the threshold of the TG1.

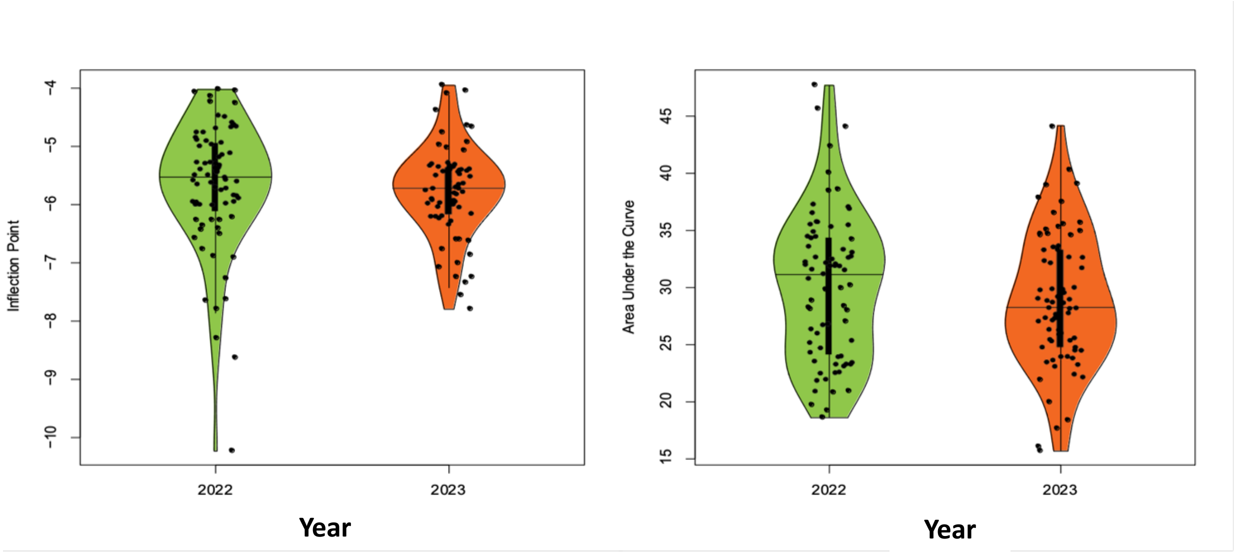

Figure 2.5. Inflection Point and Area Under the Curve distribution in modern peach breeding germplasm evaluated over two years.

Similar to IP, high broad sense heritability ($H^2=0.85$) was obtained for AUC, revealing high ratio of total genetic variance for fruitlet freeze tolerance in total phenotypic variance (Table 2.2).

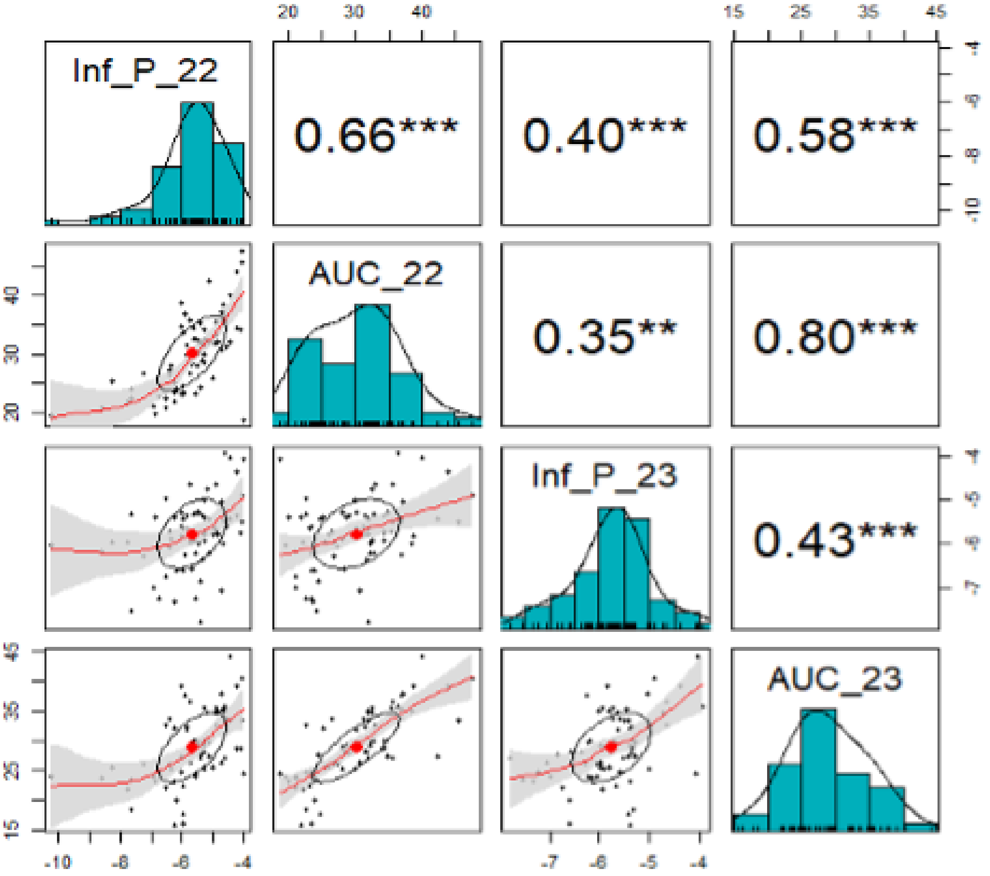

Figure 2.6. Correlation between inflection point (IP) and Area under the curve (AUC) among years (2022 to 2023). Asterisks indicate the Spearman correlation coefficient at a significance level of $p < 0.01$ (**) and $p < 0.001$ (***).

Comparison of the median positions within the IP violins revealed similarities in the distribution of IP values across the two experimental years (Figure 2.5). The median line within the AUC violins indicated a higher median for 2022 compared to 2023 season. Furthermore, the width of the violin plots reveals that the AUC distribution was broader in comparison to the IP distribution, suggesting a higher degree of variability reflected by the AUC data.

Spearman correlation analysis revealed highly significant positive correlation among all datasets (Figure 2.6). The lowest correlation was observed for IP between years (0.40), while the highest correlation was observed between AUC datasets, 0.80 and IP2022 and AUC2022 (0.66) (Figure 2.6).

Discussion

We have evaluated fruitlet freeze tolerance in the peach germplasm important to the southeast U.S. peach industry. This research addressed the pressing demand for a standardized phenotyping protocol to assess the freeze tolerance of developing fruitlets, a trait of critical importance to temperate fruit production. Although electrolyte leakage is commonly used to measure tolerance of other tissues (typically leaves), this method is frequently utilized in plant physiology to assess plant response to stress (Dionisio-Sese & Tobita, 1998). The connection between freezing-induced damage is associated with the rupture of biological membranes (Ziegler & Kandler, 1980) that is measured via the electrolyte leakage technique. During the initial phase of spring, after transition from endo- to eco- dormancy and prior to bud burst, there is a gradual reduction in freezing resistance as temperatures ascend, culminating with the emergence of fruitlets (Yu et al., 2020a). Upon the initiation of growth, freeze tolerance becomes permanently diminished, and plants are unable to undergo a process of re-acclimation to lower temperatures (Rapacz, 2002; Repo, 1991; Sakai & Larcher, 2012a). Dormancy and de-acclimation stages are well researched in temperate fruit trees and peach (Griffith & Einhorn, 2023; Liu, J. & Sherif, 2019). However, little is known about fruitlet freeze tolerance. Breeding for climate resilience in peach trees in addition to dormancy related traits (chilling and heat requirement) to delay the bloom time must include fruitlet freeze tolerance to ensure peach production sustainability. Therefore, to support breeding for fruitlet freeze tolerance in peach we evaluated modern peach breeding germplasm for fruitlet freeze tolerance using the electrolyte leakage method and expressed phenotypic differences as IP and AUC.

Fruitlet freeze tolerance observed in the peach germplasm evaluated in this study was variable across seasons (2022 and 2023) and traits (IP and AUC), with AUC exhibiting better stability. Commercial peach cultivars Julyprince, June Gold, Rich Joy, and Caroking exhibited high degrees of susceptibility to freeze tolerance, with AUC values of above 40% (Table 2.1). Furthermore, these cultivars had the lowest IP range, - 4.03 and - 4.45°C, close to the critical temperature (~ -3ºC) for causing damage to fruitlets (Murray, 2020). Cultivars Arctic Pride, Rubyprince, Summerfire, and Sweet September exhibited remarkable tolerance to freezing conditions, as evidenced by their significantly reduced ion leakage in comparison to other cultivars (19-27%) and the temperature at the IP close to -10°C (Table 2.1). This suggested that fruitlets of these cultivars have a high tolerance to low temperatures which is in agreement with previous reports (Melgar et al., 2022).

Lower correlation between IP datasets posed difficulty in comparing data from different studies. However, the values were similar when the data from the Melgar et al. (2021) were used to calculate AUC.

The outcomes of this study indicate that fruitlet freeze tolerance in peach germplasm shows a diverse range of responses, with LT50 values ranging from -3.94°C to -10.22°C. Furthermore, the accessions exhibited varying degrees of vulnerability to freeze tolerance, with AUC values ranging from 16% to 48%. While all three approaches were effective in explaining fruitlet freeze tolerance, it is worth noting that the IP value was most variable and vulnerable to methodological error. For example, the observed difference in IP values could be attributed to variation in fruitlet sampling due to slight differences in fruitlet size and or timing between sampling and analysis that could affect healing of the scar after fruitlet detachment from the branch, thus, increasing electrolyte leakage and skewing the results. Another important point is the steepness of the curve. When the slope is highly inclined, most fruits are damaged simultaneously. In opposition, on a gentler slope, some fruits may be damaged sooner, while others may be affected later. The timing of damage to specific fruits on a less steep slope can also be crucial in determining overall crop success. The difference of just one degree in tolerance in peach production could mean having the peach crop or not, so it is important to further describe the fruitlet freeze tolerance. Even though the AUC was obtained from sigmoid curve developed using LT50 data, this study showed that AUC approach reduces data variability and, therefore, might be more precise in predicting cultivar's response to freezing temperatures. This is also supported by the assignment of cultivars to tolerance groups using IP and AUC, as lower group assignment mismatch was observed for AUC than IP values. Furthermore, the mismatches observed in the AUC group assignment were on the borderline of the TG threshold.

Interestingly, nectarine fruitlets showed high tolerance in both seasons suggesting that the pubescence might influence fruitlets susceptibility/tolerance to freezing temperatures. Single gene mutation from pubescent (*G*-) to glabrous (*gg*) skin on chromosome 5 is the difference between the peach and nectarine phenotype (Dirlewanger et al., 2006; Vendramin et al., 2014a). Other subtle differences in the flesh density and texture between peach and nectarine are suggested and speculated to be attributed to the pleiotropic effect of the single gene mutation, but the research documenting them is lacking. One could speculate that nectarine fruitlet increased tolerance to freezing temperatures observed in this study is due to the origin of the material or with morphological characteristics of the fruit tissues e.g., lower water content in the nectarine fruit tissue than in peach (Wen et al., 1995). The formation of intercellular or intracellular ice crystals can lead to cell death. Several studies on grape plants suggested that freeze damage during spring is caused by the formation of intercellular ice rather than intra-cellular ice formation (Wilson, 2001). The generation of a water vapor gradient between the interior and exterior of cells directly contributes to intercellular ice crystal formation. Furthermore, cells become dehydrated as water moves from the interior to the exterior of the cell and accumulates on intercellular ice crystals, causing a loss of turgor (Poling, 2008). That might be the potential reason why we observed higher tolerance in nectarines than peaches.

Studies mostly focused on the trichome morphology and differences between peach and nectarine but never on other aspects of fruit morphology (Vendramin et al., 2014b). It would be interesting to explore this line of thought and determine the cause of higher tolerance to freeze in nectarine fruitlets in laboratory experiments, especially since we do not have field observation to support this observation nor it was reported in the literature (Blake, 1932). The lab data suggests significant differences between peach and nectarines, thus, expanding the fruitlet freeze evaluation to include more nectarine cultivars might help explain observations made here.

Tolerance group assignment based on the IP and AUC could be valuable information for advising growers about cultivars whose young fruit tolerate low temperatures so they can incorporate this information when choosing the cultivars to plant in areas more prone to freezing. They can also use this information to arrange special protection (e.g., wind machines, irrigation) when they have limited resources to reduce the impact of late spring (radiation) frosts, as a change in just a few degrees could mean a difference between full production or a total crop loss. Thus, this study may be a steppingstone for growers and county agents to incorporate information on the freezing tolerance of fruits according to their area and minimize possible economic damage. This information can also be useful to breeders that consider climate resilience as an important trait.

The observed variation of IP among cultivars suggests that both IP and AUC would be beneficial for estimating fruitlet freeze tolerance through multiple conditions or time and could be used for informing growers, county agents and breeders about potential of cultivars to tolerate low spring temperatures.

Increased frequency in occurrence of spring frosts all over the world is putting emphasis on adding this trait in breeding efforts for climate resilience. It is crucial to conduct more in-depth investigations into the influence of climate characteristics of plants. As evidenced with the peach crop losses in the southeast U.S. in the last two decades, ensuring satisfaction of chill requirement and increasing heat requirement to delay bloom are no longer enough to ensure sustainability of peach production in the changing climate. Thus, fruitlet freeze tolerance should be included in the suite of traits when breeding for climate resilience. Furthermore, broad sense heritability estimated in this study supports genetic control of the fruitlet freeze tolerance in peach and nectarine germplasm and potential for improvement of this trait via breeding. This is the first extensive study into the peach fruitlet freeze tolerance that lays foundation for further investigation into the genetic control of this trait.

Conclusion

Climate change and increased occurrence of freezing temperature events during spring in the southeastern region of the United States, require better understanding of the genetic control of all climate resilience traits (chilling and heat requirement, bloom time and fruitlet freeze tolerance) to ensure sustainability of peach production. This study showed that peach germplasm is variable in fruit freeze tolerance. The wide diversity of fruitlet freeze tolerance, and broad-sense heritability (H^2) estimates of 0.52 and 0.85 for LT50 and the area under the curve, respectively, observed in peaches suggests that this trait is genetically controlled and has the potential to be used in breeding. Consequently, the results of this study lay the groundwork for future investigation into the regions of the peach genome responsible for controlling this trait.

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CHAPTER THREE

GENOME WIDE ASSOCIATION STUDY OF FRUITLET FREEZE TOLERANCE IN PEACH GERMPLASM

Introduction

In temperate climates, the most significant risk of frost injury to woody plant species occurs during blooming and fruitlets onset in spring (Inouye, 2000; Vitasse et al., 2014). Even plant varieties that can survive very low temperatures during the winter season are significantly impacted by relatively minor temperature variations that can arise during the processes of flowering and fruit set in the spring. Incidents of spring frost have caused severe financial losses for the agriculture industry. For example, in 2017, severe frost occurrences caused losses across Europe of 3.3 billion euros (Lamichhane, 2021). France witnessed considerable damage to its vineyards in 2021, with frost involving at least half of the 680 vineyards (Dai et al., 2023). Similar to Europe, the U.S. agriculture has also reported crop loss due to spring freezes, with the latest reported in news outlets for grape, apple, and stone fruit industries in 2023 (Lamichhane, 2021). Notably, blooming begins earlier due to climate change, while the likelihood of isolated frost nights during spring has remained strong (Liu et al., 2018). The increasing trend in late spring frost events, particularly in the southeastern U.S. states of Georgia and South Carolina, has resulted in more than 50% crop losses over the past five years. In response, breeding programs are now prioritizing climate resilience and adaptability in the development of new cultivars to mitigate the production challenges posed by climate change.

In early spring, before bud break, buds in temperate climatic zones are in a phase of ecodormancy, and their resistance to frost decreases progressively as temperature rises, reaching a minimum as fruitlets emerge (Lenz et al., 2013b; Sakai & Larcher, 2012b). After the post-bloom stage, even relatively short freezing exposures lasting just a few hours can lead to substantial yield losses, ranging from partial to complete (Bassi & Monet, 2008). Hence, the period extending from the initiation of floral bud break to the occurrence of spring frost stands as a key and vulnerable period for producers. A significant limitation to peach production is the potential occurrence of spring frosts during the flowering and fruitlet period. Floral bud break and the avoidance of potentially lethal spring freezing temperatures are contingent upon two temperature requirements. For peach floral buds to develop, a quantitative exposure to chilling temperatures, often called the chilling requirement (CR), followed by the quantitative accumulation of warm temperatures, known as the heat requirement (HR) is needed (Bielenberg et al., 2022b). For proper flowering and fruit development, peaches require a certain amount of chill during the winter, known as "winter chill." However, the ongoing global warming tendency is making winters milder, resulting in declined winter chill and earlier blooming of peach trees. This difference in climate conditions poses a severe threat to peach production. The most effective way to control spring frost and facilitate sustainable and eco-friendly peach cultivation is to grow cultivars adapted to the environmental conditions. Understanding adaptive approaches to minimize the effects of climate change on phenology steps like CR, HR, and bloom time and the consequences of spring frost to flowers and fruitlets is essential to address the impact of spring frost due to fluctuations in global temperatures on significant phenological features (Hatfield & Prueger, 2015; Yu et al., 2020b). Cultivars with low chilling requirements guarantee that the trees will bloom, as these requirements are efficiently met. However, earlier bloom makes the crop vulnerable to the risks of brief warm spells, often called "false spring." Such conditions can significantly increase the likelihood of crop damage resulting from following freezing temperatures (Bielenberg et al., 2022b; Chamberlain et al., 2019b; Chen et al., 2016d). In the recent climate context, cultivars that bloom early are at risk of frost damage, especially in spring. On the other hand, late-blooming cultivars may encounter issues such as insufficient fruit set (Sønsteby et al., 2019; Vanalli et al., 2021) due to limited pollination. Late-blooming cultivars may also be exposed to higher temperatures, which can further impact fruit development. Some reasons why late-blooming cultivars may face challenges in achieving optimal fruit sets are pollination timing, unfavorable weather conditions, limited pollinator activity, competition for resources and genetic factors.

The genetics of dormancy related traits, such as CR and bloom date (BD) have been subject to extensive research within the *Prunus* (Abbott et al., 2015; Castède et al., 2014b; Fadón, E. & Rodrigo, 2018; Fan et al., 2010d; Olukolu et al., 2009; Rawandoozi et al., 2021b; Zhebentyayeva et al., 2014). However, limited research has been directed towards genetic mechanisms controlling HR (Cirilli et al., 2021b; Fan et al., 2010d; Romeu et al., 2014c) in peach and fruitlet freeze tolerance has not been the subject of genetic studies (Melgar et al., 2022).

Peach is an economically significant crop with abundant genetic resources (Aranzana et al., 2019b; Jung et al., 2019b). The genetic variability of cultivated peach has been affected by three main forces: inbreeding, random drift, and heterosis (Micheletti et al., 2015). The selfing and the targeted selection have resulted in a significant reduction in genetic variability compared to other cultivated species of the genus (Byrne, D. H., 1990; Mnejja et al., 2010). In addition, the the small number of parents used in the breeding programs have further contributed to the issue (Aranzana, et al., 2003a; Cao et al., 2014; Verde et al., 2013). Peach is one of the most genetically characterized species with vast resources publicly available. (Ahmad et al., 2011; Aranzana, et al., 2012; Cao et al., 2014; Jung et al., 2019c; Verde et al., 2013). Trait genetics in peach has been investigated via bi-parental mapping, pedigree-based mapping and association mapping and many important marker-trait associations have been converted into DNA tools (Aranzana et al., 2019c).

Despite extensive efforts in comprehending the genetic mechanisms governing CR, HR, and BD in peach, only few resulted in the DNA test for MAS (Demirel et al., 2021). However, the fruitlet freeze tolerance research is lacking. Thus, the primary goal of this study is to understand the genetic factors responsible for fruitlet freeze tolerance by determining the region(s) in peach genome that is associated with this trait.

Materials and methods

Plant material

A diverse set of 107 peach and nectarine accessions representing heirloom cultivars, landraces and modern U.S. peach breeding germplasm evaluated in Chapter II and Melgar et al. (2022) study was assembled based on already available genotyping data obtained previously in the CUPBP (Table 3.1). Out of 107 accessions, 53 are maintained in the National Clonal Germplasm Repository (NCGR) in Davis, and 54 were maintained in Clemson University (CU) *Prunus* collection. Fruitlet freeze tolerance was estimated in 107 accessions in four experimental years 2018 (80 accessions), 2021 (47 accessions), 2022 (36 accessions) and 2023 (33 accessions). A subset of this material was evaluated in previous study by Melgar et al. (2022) during 2018 and 2021, and a subset was described in Chapter II of this work.

Phenotyping

Phenotyping protocols for fruitlet freeze tolerance are described in Chapter II. Phenotypic data were organized in trait by year of study datasets. Eight datasets were assembled: IP2018, IP2021, IP2022, IP2023, AUC2018, AUC2021, AUC2022, AUC2023. In addition, the best linear unbiased prediction (BLUP) values of IP and AUC per accession were obtained using a mixed effect model. The BLUP was chosen instead of a standard arithmetic mean because of unbalanced annual IP and AUC data available for the study. Most accessions were phenotyped for at least two years and only 4 were phenotyped for all 4 years (Table 3.1), respectively.

Descriptive statistical analyses were conducted as described in Chapter II.

DNA extraction and genotyping

DNA was extracted from young leaf tissue following the protocol described by (Edge-Garza et al., 2014). DNA quantity and quality were assessed by a spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and Bioteck (Biotek HT Synergy Multidetection microplate reader, Winooski, VT, USA), and submitted to the University of Minnesota Genomic Center for genotyping-by-sequencing (GBS) (Elshire et al., 2011b). ApeKI restriction enzyme was used, and libraries were sequenced on the NovaSeq6000 Illumina (Illumina, San Diego, CA, USA).

Processing of sequence reads

Sequence reads were processed using TASSEL 4.0 and 5.0 GBS pipelines (Bradbury et al., 2007) with default parameters. Eight scaffolds of the peach genome v2.0 (Verde et al., 2017b) were obtained from the Genome Database for Rosaceae (www.rosaceae.org) (Jung et al., 2018; Verde et al., 2017c) and used as the reference for alignment of sequenced reads with Bowtie v2.1 (Langmead & Salzberg, 2012). SNP calls for all genotypes were exported as vcf files. Filtering for genotype quality and missing data were performed using PLINK (Purcell et al., 2007). SNPs and accessions with more than 20% missing data were omitted from further analysis. Furthermore, SNPs with MAF lower than 0.05 and those that deviated from Hardy–Weinberg equilibrium (HWE) at the significance threshold of 0.001 were discarded from further analysis. SNPs were named based on the scaffold number, and base pair position within the peach genome v2.0 build, as explained in Bielenberg et al. (2015c).

Population structure analysis and genome wide association study (GWAS)

The population structure was investigated using a Bayesian clustering in fastSTRUCTURE (Raj et al., 2014). Number of clusters (K) ranging from 1 to 10 were tested using the default priors. The chooseK.py script provided in fastSTRUCTURE was used to estimate the reasonable range of K for the appropriate model complexity. The admixture proportions of each genotype were visualized by DISTRUCT plots (Rosenberg, 2004).

The GWAS was performed using the compressed general linear model (GLM) implemented in the TASSEL 5.0 (Bradbury et al., 2007). The Q matrices representing the degree of membership in underlying populations, were obtained in TASSEL. Significant marker-traits associations were visualized via Manhattan plot and quality of data was validated via Q-Q plots. Bonferroni threshold of 5.6×10^{-6} was applied to determine significant marker-trait associations.

Candidate gene analysis

A systematic search within 200 and 100kb flanking windows surrounding significant SNPs was conducted to compile the predicted candidate genes associated with fruitlet freeze tolerance traits. A BED file was created with chromosome and position and extracted from the *Prunus persica* v2.1 genome annotation file (.gff3) using bedtools (https://bedtools.readthedocs.io). Functional data was incorporated from the annotation file from Genome Database for Rosaceae (GDR) (GDR): https://www.rosaceae.org) (Jung et al., 2019b).

Results

Phenotypic datasets

Inflection point (IP), ranged from -3.94°C observed in (IP2023) dataset to -20.98°C observed in (IP2018), with similar overall average IP (-5ºC to -6ºC) observed in 3 years except in 2018 (-7.01ºC) (Table 3.1).

Area under the curve (AUC) in all material ranged from 4.48% fruitlet damage in 2018 to 47.76% in 2022 (Table 3.2). AUC was normally distributed in all datasets (Shapiro Wilk test; W = 0.97186, p-value = 0.3112 in 2021, W = 0.97852, p-value = 0.6953 in 2022 and W = 0.97639, p-value = 0.6733 in 2023) except in AUC 2018 (Shapiro Wilk test; W = 0.95431, p-value = 0.006132). The AUC interval in the four experimental years, ranged from 23.36% to 32.32%. The lowest AUC was calculated in 2018, with 4.48%, while the highest damage occurred in 2022, 47.76% (Table 3.2).

Table 3.1. A diverse set of 107 peach and nectarine accessions representing heirloom cultivars, landraces and modern U.S. peach breeding germplasm. FT – Fruit type; P – Peach; N – Nectarine; IP - inflection point; AUC - area under the curve. [1]CU -Clemson University peach breeding; NCGR - National Clonal Germplasm Repository in Davis, CA.

Accessions	**Origin**	**FT**	**2018**		**2021**		**2022**		**2023**		**BLUPs**		**Location**[1]
			IP	**AUC**	**IP**	**AUC**	**IP**	**AUC**	**IP**	**AUC**	**IP**	**AUC**	
745	Poland	P	-5.85	33.06	-5.75	24.78	-	-	-	-	-5.86	31.01	DPRU.736
880329	Pakistan	P	-20.98	16.58	-5.89	22.62	-	-	-	-	-7.47	24.92	DPRU.1472
880330	Pakistan	P	-6.13	19.47	-5.39	22.35	-	-	-	-	-5.85	25.78	DPRU.1473
880332	Pakistan	P	-6.02	18.39	-6.03	15.88	-	-	-	-	-5.91	23.31	DPRU.1774
6820040	Italy	P	-5.4	38.1	-	-	-	-	-	-	-5.82	34.76	DPRU.1971
Admiral Dewey	US (GA)	P	-5.27	27.98	-6.22	28.63	-	-	-	-	-5.85	30.61	DPRU.1119
Allstar®	US (MI)	P	-7.63	9.08	-	-	-	-	-	-	-6.08	20.7	CU
AR-233	Uzbekistan	P	-5.96	39.35	-	-	-	-	-	-	-5.88	35.37	DPRU.721
Baby Crawford	US (CA)	P	-5.2	39.79	-	-	-	-	-	-	-5.79	35.58	DPRU.2139
Bai Mang Pen Tao	China	P	-5.89	21.01	-	-	-	-	-	-	-5.87	26.48	DPRU.2019
Big Red	US (FL)	P	-8.02	7.7	-6.01	26.9	-5.32	29.86	-5.01	25.36	-6.03	23.54	CU
Biscoe	US (NC)	P	-7.26	18.47	-	-	-5.65	34.44	-5.92	28.97	-6.07	26.51	CU
Bolinha	Brazil	P	-6.06	26.6	-5.92	23.27	-	-	-	-	-5.9	28.41	DPRU.3259

Accessions	**Origin**	**FT**	**2018**		**2021**		**2022**		**2023**		**BLUPs**		**Location**
			IP	**AUC**	**IP**	**AUC**	**IP**	**AUC**	**IP**	**AUC**	**IP**	**AUC**	
BR-2	Brazil	P	-7.1	15.67	-6.16	24.69	-	-	-	-	-6.04	25.3	DPRU.1985
Brightstar	UC (CA)	P	-	-	-	-	-7.62	23.98	-7.54	23.45	-6.43	22.52	CU
Calmar	US (CA)	P	-6	31.76	-	-	-	-	-	-	-5.89	31.69	DPRU.980
Candor	US (NC)	P	-5.67	39.69	-	-	-	-	-	-	-5.85	35.53	CU
Carmen	US (CA)	P	-6.02	31.68	-	-	-	-	-	-	-5.89	31.65	DPRU.2142
Carogem	US (SC)	P	-	-	-	-	-6.35	28.15	-5.9	25.3	-6.12	24.48	CU
Caroking	US (SC)	P	-	-	-4.62	35.03	-4.46	40.11	-4.04	44.16	-5.62	35.53	CU
Carored	US (SC)	P	-6.24	16.76	-6.1	35.22	-5.49	35.52	-	-	-5.95	29.48	CU
Cascata332	Brazil	P	-6.82	23.22	-7.09	12.3	-	-	-	-	-6.1	23.72	DPRU.3203
Cascata519	Brazil	P	-6.66	14.34	-	-	-	-	-	-	-5.96	23.25	DPRU.1761
Cerrito	Brazil	P	-5.85	26.18	-	-	-	-	-	-	-5.87	28.98	DPRU.1661
China Pearl	US (NC)	P	-	-	-	-	-4.65	36.53	-3.94	35.74	-5.74	30.63	CU
Chugdar	Pakistan	P	-5.61	34.33	-7.27	24.71	-	-	-	-	-6	31.4	DPRU.1477
Churko	Pakistan	P	-6.38	30.94	-4.41	25.61	-	-	-	-	-5.78	30.59	DPRU.1479
Coronet	US (GA)	P	-	-	-	-	-6	38.67	-5.82	39.08	-6.08	32.42	CU
Cresthaven	US (CA)	P	-	-	-	-	-4.62	35.72	-5.51	27.05	-5.9	27.53	CU
Criollo	Guatemala	P	-7.01	15	-6.48	14.57	-	-	-	-	-6.06	21.78	DPRU.1790
Cumberland	US (NJ)	P	-9.82	13.54	-5.57	23.87	-	-	-	-	-6.26	24.34	CU

Accessions	**Origin**	**FT**	**2018**		**2021**		**2022**		**2023**		**BLUPs**		**Location**
			IP	**AUC**	**IP**	**AUC**	**IP**	**AUC**	**IP**	**AUC**	**IP**	**AUC**	
De Wet	S. Africa	P	-6.07	21.81	-6.79	23.27	-	-	-	-	-5.99	26.84	DPRU.1610
Demeur	US (CA)	P	-6.02	31.59	-	-	-	-	-	-	-5.89	31.61	DPRU.2144
DPRU.2228.20B	China	P	-6.91	14.47	-	-	-	-	-	-	-5.99	23.31	DPRU.2228
DPRU.2495	China	P	-5.64	29.68	-	-	-	-	-	-	-5.84	30.68	DPRU.2495
Early Augustprince	US (GA)	P	-	-	-	-	-5.95	23.42	-6.61	16.14	-6.15	19.95	CU
EarlyStar®	US (MI)	P	-5.58	39.61	-	-	-	-	-	-	-5.84	35.49	CU
Elberta	US (GA)	P	-	-	-6.24	32.25	-5.29	32.59	-5.93	35.35	-6.04	30.83	CU
Empress	US (CA)	P	-	-	-	-	-5.84	31.56	-5.36	32.37	-6.01	27.9	CU
Federica	Italy	P	-5.98	33.95	-6.17	19.77	-	-	-	-	-5.92	29.66	DPRU.2270
FlavBurst®	US (WV)	P	-5.75	40.48	-	-	-4.9	33.31	-	-	-5.82	33.48	CU
Fortyniner	US (CA)	P	-5.23	41.57	-	-	-	-	-	-	-5.8	36.44	DPRU.1355
Garnet Beauty	Canada	P	-5.26	42.08	-	-	-	-	-	-	-5.8	36.69	CU
Gilgalo	Pakistan	P	-5.85	36.62	-	-	-	-	-	-	-5.87	34.04	DPRU.1496
Glenglo	US (WV)	P	-6.11	8.61	-	-	-	-	-	-	-5.9	20.47	CU
Glory	US (GA)	P	-	-	-7.31	9.51	-6.25	21.85	-5.4	15.75	-6.18	17.76	CU
Glowingstar®	US (MI)	P	-6.98	11.88	-	-	-	-	-	-	-6	22.05	CU
Golden Queen Improved	New Zealand	P	-5.2	32.06	-5.66	31.51	-	-	-	-	-5.78	32.88	DPRU.1576
Harrow Beauty	Canada	P	-15.84	25.18	-	-	-	-	-	-	-7.05	28.5	CU

Accessions	**Origin**	**FT**	**2018**		**2021**		**2022**		**2023**		**BLUPs**		**Location**
			IP	**AUC**	**IP**	**AUC**	**IP**	**AUC**	**IP**	**AUC**	**IP**	**AUC**	
Harrow Diamond	Canada	P	-8.05	9.91	-7.19	10.29	-	-	-	-	-6.24	18.72	CU
Hiley	US (GA)	P	-6.84	15.99	-	-	-	-	-	-	-5.99	24.05	DPRU.537
Hunshu	Pakistan	P	-6.24	29.64	-	-	-	-	-	-	-5.92	30.66	DPRU.1480
Jade™	France	N	-4	20.14	-	-	-	-	-	-	-5.65	26.06	CU
Jefferson	US (VA)	P	-	-	-6.14	24.82	-4.22	34.46	-5.34	28.66	-5.87	27.81	CU
Joanna Sweet	US (CA)	P	-	-	-6.16	14.92	-4.96	31.86	-5.63	34.7	-5.97	26.22	CU
John Boy	US (PA)	P	-8.87	7.65	-	-	-5.37	31.84	-6.35	28.24	-6.24	23.02	CU
Julyprince	US (GA)	P	-	-	-	-	-4.05	47.76	-4.91	40.38	-5.77	35.81	CU
Juneprince	US (GA)	P	-7.24	22.46	-4.05	38.96	-5.57	32.63	-6.2	29.85	-5.92	30.27	CU
Juneprincess	US (GA)	N	-	-	-	-	-4.02	18.7	-4.08	24.49	-5.68	21.13	CU
Kawanakajima Hakutou	Japan	P	-	-	-7.69	20.37	-	-	-	-	-6.2	25.8	DPRU.2466
Khanda	Pakistan	P	-6.55	29.58	-	-	-	-	-	-	-5.95	30.63	DPRU.1481
Kijewska Zapala	Poland	P	-6.08	17.12	-	-	-	-	-	-	-5.9	24.59	DPRU.1606
Lola	Russia	P	-7.31	21.06	-5.13	25.22	-	-	-	-	-5.95	27.23	DPRU.1586
Loring	US (MO)	P	-	-	-6.59	14.31	-5.14	42.43	-5.41	27.32	-6.01	26.86	CU
Lutkoo	Pakistan	P	-6.26	30.1	-7.81	20.66	-	-	-	-	-6.12	28.7	DPRU.1483
Majestic	US (LA)	P	-	-	-6.03	26.1	-4.66	37.06	-5.47	35.18	-5.91	30.37	CU
Miller'sLate	US (CA)	P	-5.54	34.11	-6.56	23.92	-	-	-	-	-5.91	31.07	DPRU.1276

Accessions	**Origin**	**FT**	**2018**		**2021**		**2022**		**2023**		**BLUPs**		**Location**
			IP	**AUC**	**IP**	**AUC**	**IP**	**AUC**	**IP**	**AUC**	**IP**	**AUC**	
Muir Peach	US (CA)	P	-5.01	30.18	-4.77	28.03	-	-	-	-	-5.67	31.13	DPRU.1177
Nata	n/a	P	-5.7	31.77	-6.03	21.52	-	-	-	-	-5.87	29.52	DPRU.663
O'Henry	US (CA)	P	-	-	-6.35	23.58	-5.53	28.03	-5.3	28.86	-6.01	25.97	CU
PF17	*US (MI)*	P	-13.92	6.67	-	-	-	-	-	-	-6.82	19.53	CU
PF23	US (MI)	P	-	-	-	-	-4.84	32.03	-5.8	34.82	-5.95	28.86	CU
Pallas	US (GA)	P	-5.65	35.3	-5.96	29.82	-	-	-	-	-5.86	33.38	DPRU.539
Peregrine	UK	P	-6.11	26.72	-4.11	25.46	-	-	-	-	-5.72	29.16	DPRU.654
PF15A	US (MI)	P	-8.02	7.37	-	-	-	-	-	-	-6.13	19.87	CU
Prarie Schooner	US (IL)	P	-6.14	27.05	-	-	-	-	-	-	-5.9	29.41	DPRU.1569
Premier	Brazil	P	-5.92	27.12	-6	28.6	-	-	-	-	-5.89	30.32	DPRU.2268
Ranniaya	Russia	P	-5.81	31.33	-6.54	25.87	-	-	-	-	-5.94	30.8	DPRU.669
Raritan Rose	US (CA)	P	-9.47	7.46	-	-	-	-	-	-	-6.3	19.91	DPRU.2171
Redglobe	US (MD)	P	-7.96	6.93	-	-	-5.96	24.69	-5.43	17.69	-6.12	18.49	CU
Red Gold	US (MO)	N	-7.51	15.77	-	-	-	-	-	-	-6.07	23.94	CU
Redhaven	US (MI)	P	-5.14	21.98	-	-	-	-	-	-	-5.79	26.95	CU
Rich May	US (CA)	P	-6.03	30.49	-	-	-5.39	35.31	-	-	-5.9	30.87	CU
Rogany Goy	Russia	P	-	-	-	-	-4.25	44.13	-4.37	39.06	-5.74	34.19	DPRU.1566

Accessions	**Origin**	**FT**	**2018**		**2021**		**2022**		**2023**		**BLUPs**		**Location**
			IP	**AUC**	**IP**	**AUC**	**IP**	**AUC**	**IP**	**AUC**	**IP**	**AUC**	
Ruston Red	US (LA)	P	-	-	-	-	-4.88	33.05	-4.64	32.66	-5.83	28.49	CU
Sanguine Tardeva	US (CA)	P	-6.07	33.64	-5.96	18.73	-	-	-	-	-5.91	29.22	DPRU.2175
SC5	US (SC)	P	-	-	-4.86	34.83	-5.18	32.46	-4.74	34.69	-5.78	31.27	CU
Scarlet Pearl	US (GA)	P	-9.44	7.3	-6.17	15.29	-4.68	38.57	-6	35.06	-6.2	24.8	CU
Scarletprince	US (GA)	P	-11.26	10.97	-5.27	27.18	-	-	-	-	-6.38	24.58	CU
Sulina	Brazil	P	-6.07	23.59	-6.19	25.5	-	-	-	-	-5.93	28.15	DPRU.1954
Summerprince	US (GA)	P	-	-	-	-	-6.75	22.59	-7.24	24.76	-6.3	22.49	CU
Sunprince	US (GA)	P	-	-	-	-	-7.77	21.95	-5.95	21.95	-6.28	21.37	CU
Sureprince	US (GA)	P	-8.19	13.67	-4.8	39.7	-5.89	36.89	-6.24	37.52	-6.1	31.03	CU
Sweet Breeze	US (CA)	P	-7.6	12.19	-	-	-	-	-	-	-6.08	22.2	CU
Sweet Dream	US (CA)	P	-8.33	9.16	-	-	-	-	-	-	-6.16	20.73	CU
Sweet Scarlet	US (CA)	P	-8.51	4.48	-	-	-	-	-	-	-6.18	18.47	CU
Sweetstar	France	P	-	-	-	-	-4.75	30.75	-7.06	26.27	-6.07	25.65	CU
Takashur I	Pakistan	P	-6.22	29.89	-5.42	23.72	-	-	-	-	-5.87	29.63	DPRU.1486
Tebana	Italy	P	-5.9	38.08	-5.46	29.86	-	-	-	-	-5.84	34.3	DPRU.2008
Tho Muang	Thailand	P	-7.43	17.81	-	-	-	-	-	-	-6.06	24.93	DPRU.1484
Tra-Zee	US (CA)	P	-	-	-	-	-5.29	28.47	-5.39	29.05	-5.96	25.81	CU
Turquesa	Brazil	P	-5.97	27.71	-5.99	27.54	-	-	-	-	-5.9	30.16	DPRU.1667

Accessions	Origin	FT	2018		2021		2022		2023		BLUPs		Location
			IP	AUC	IP	AUC	IP	AUC	IP	AUC	IP	AUC	
Tuscan Cling	US (MS)	P	-5.89	29.91	-	-	-	-	-	-	-5.87	30.79	DPRU.655
Valley Sweet	US (CA)	P	-	-	-	-	-5.49	32.21	-5.98	29.21	-6.04	27.08	CU
Villa Ada	Italy	P	-7.25	25.18	-	-	-	-	-	-	-6.03	28.5	DPRU.1977
Winblo Sport	US (NC)	P	-6.1	8.68	-	-	-	-	-	-	-5.9	20.5	CU
Yumyeong	South Korea	P	-12.23	14.21	-5.73	26.07	-	-	-	-	-6.53	25.28	DPRU.1612

Table 3.2. Descriptive statistics of fruitlet freeze tolerance observed in peach and nectarine germplasm evaluated for fruitlet freeze tolerance. IP, inflection point; AUC, area under curve; BLUPs (best linear unbiased prediction) value.

Trait	**N**	**Minimum**	**Maximum**	**Mean**
IP2018	80	-20.98	-4	-7.01
AUC2018	80	4.48	42.08	23.36
IP2021	47	-7.81	-4.05	-5.96
AUC2021	47	9.51	39.7	24.42
IP2022	36	-7.77	-4.02	-5.36
AUC2022	36	18.7	47.76	32.32
IP2023	33	-7.54	-3.94	-5.59
AUC2023	33	15.75	44.16	29.97
BLUP_IP	107	-7.47	-5.62	-6.00
BLUP_AUC	107	17.76	36.69	27.60

N, number of analyzed samples.

The width of the violin plots reveals that the AUC distribution was broader than the IP distribution except for IP 2018, suggesting a higher degree of variability reflected by the AUC data (Figure 3.1 and 3.2).

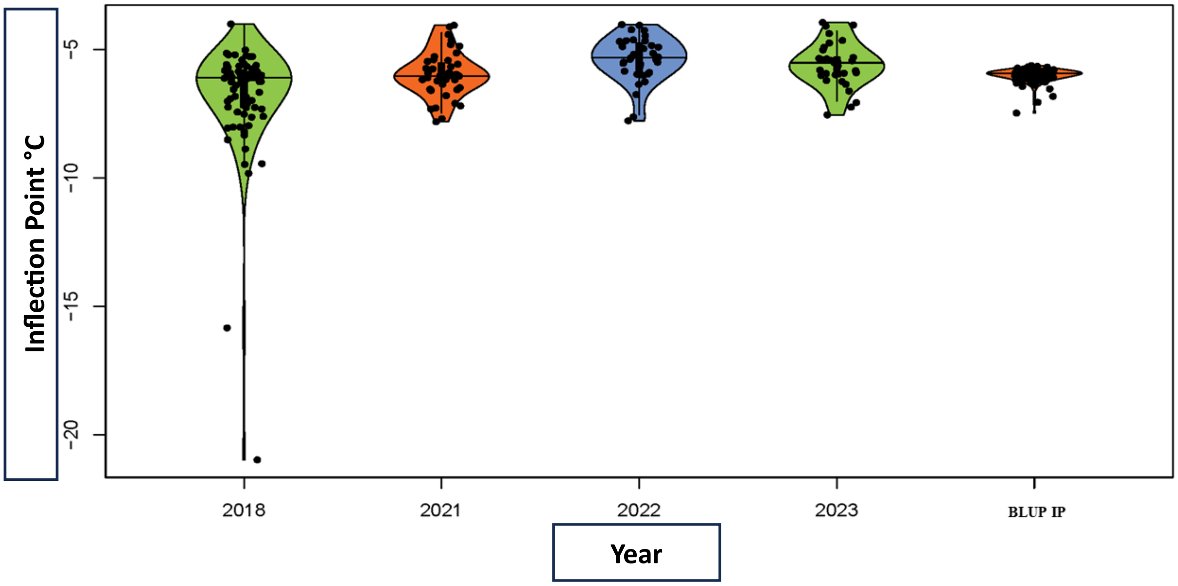

Figure 3.1. Inflection Point distribution in modern peach breeding germplasm evaluated over four years and BLUP value.

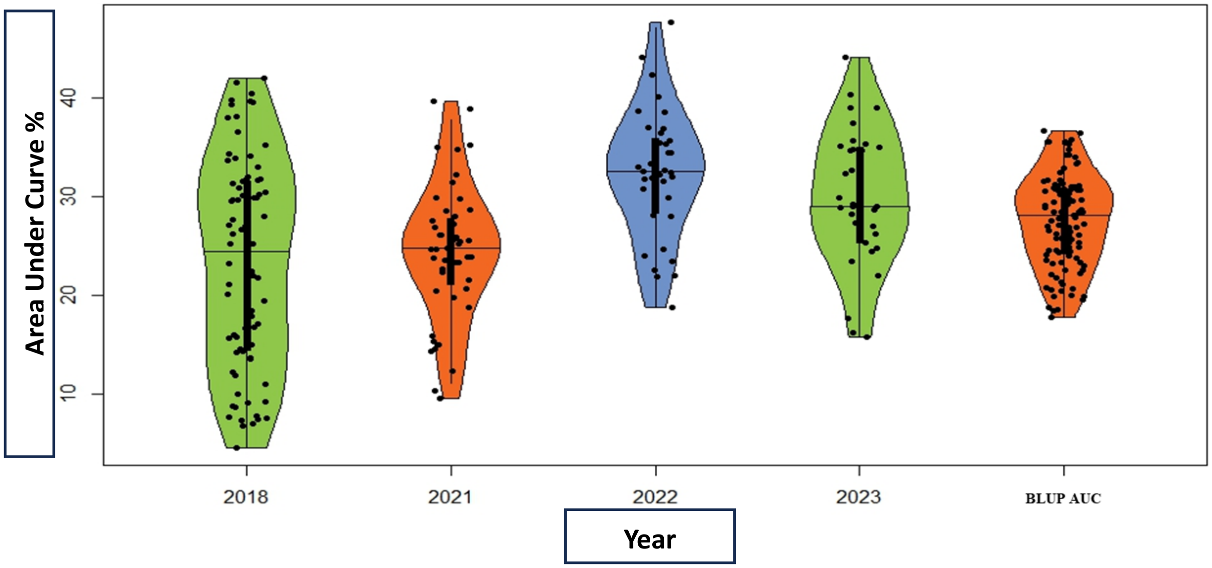

Figure 3.2. Distribution of the area under the curve (AUC) phenotypes in modern peach breeding germplasm evaluated over four years and BLUP value.

Spearman correlation analysis revealed some significant positive correlations among all datasets (Figure 3.3). A strong correlation was observed between IP_BLUP and IP2018 with 0.88 and between AUC BLUP and AUC 2018 (0.93) and AUC 2023 (0.91).

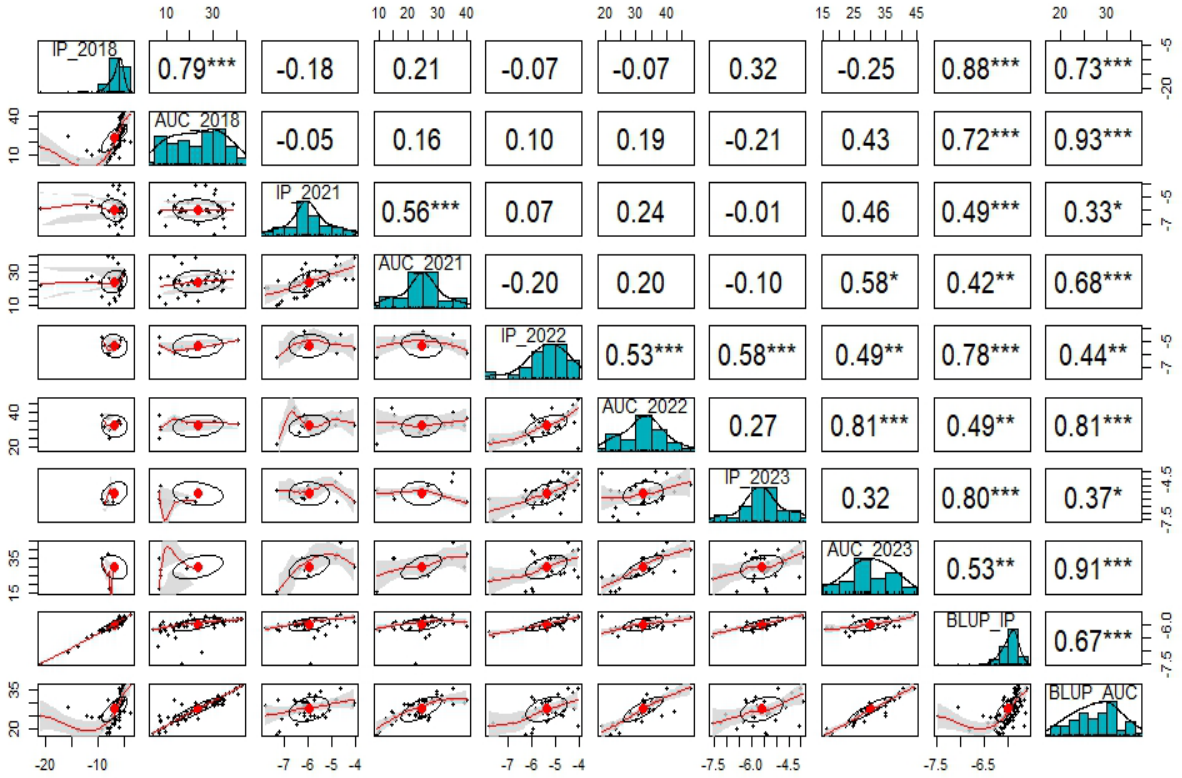

Figure 3.3. Correlation between inflection point (IP) and Area under the curve (AUC) among years (2018 to 2023). Asterisks indicate the Spearman correlation coefficient at a significance level of $p < 0.05$ (*), 0.01 (**) and $p < 0.001$ (***)

Detection of SNPs

Genotyping by sequencing resulted in 182,958 SNPs. After filtering for low genotype calls, MAF and HWE a total of 13,470 SNPs were retained for further analyses. The genotyping rate in the 107 accessions was 94%. SNP distribution over the eight chromosomes is shown in Figure

3.4. Chromosome 1 had the highest (2,457), while Chromosome 5 had the lowest number of SNPs (816).

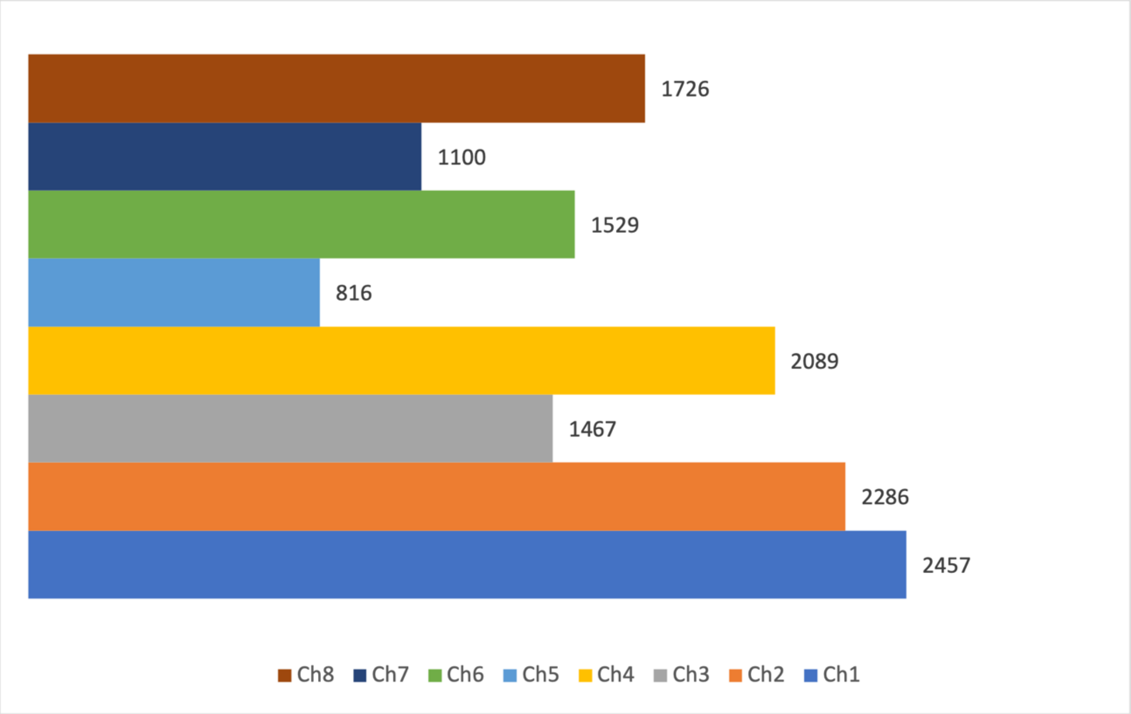

Figure 3.4. SNP distribution across eight chromosomes of the peach genome.

Population structure and genome wide association study

Population structure analysis with fastSTRUCTURE suggested that K=4 best explained model complexity that maximizes marginal likelihood, and K=5 best explained structure in the data. Analysis of origin and pedigree of the material suggested that K=4 best explained the population structure in this material (Figure 3.5). Majority of accessions (63%) were classified as admixed which was expected due to the most of material belonging to the modern peach breeding germplasm (Appendix C.). Remaining accessions were grouped in four populations. Population one (colored red) included 10 accessions, majority being landraces from China (DPRU.2495, Churko, Hunshu, Khanda, Lutkoo, Takashuri, 880332, Chugdar, Rogany Goy and AR-233).

Population two (colored green) included 11 cultivars of North American origin (O'Henry, Sweet Scarlet, Tra-Zee, Valley Sweet, FlavrBurst, John Boy, Loring, Carored, Sweet Breeze, Coronet and Joanna Sweet). Population three (colored light blue) included four cultivars, three of Asian origin (Kawanakajima Hakutou, Yumyeong and BaiMangPenTao) and cultivar Cumberland. Population four (colored purple) included 15 accessions, cultivars and landraces with mixed origin from Brazil, Italy, France and U.S. Majority of admixed accessions were a mixture of population 2 and 4.

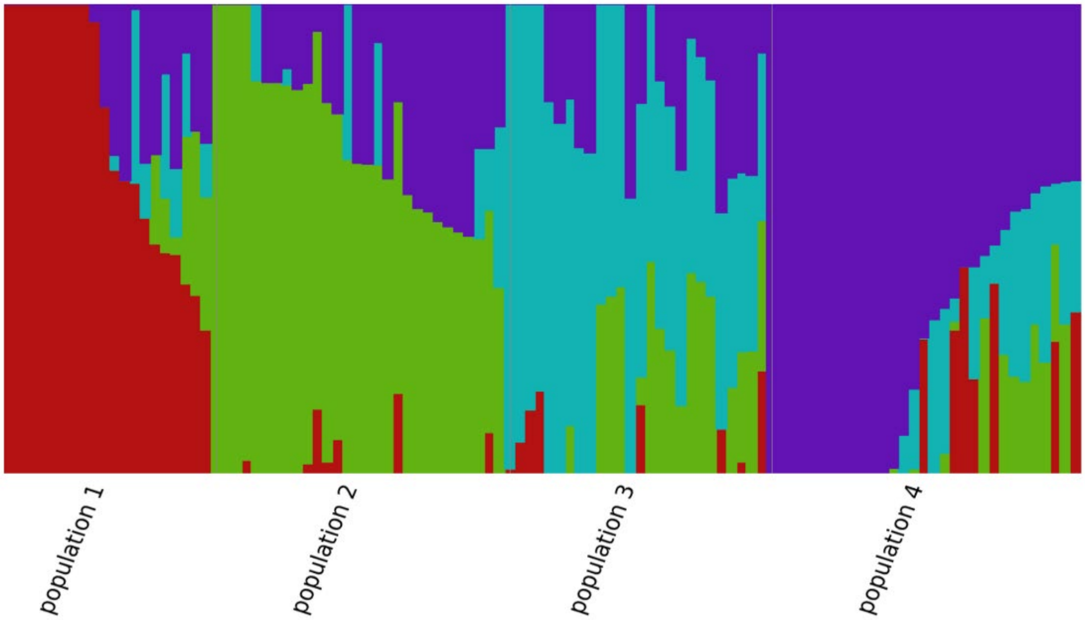

Figure 3.5. The population structure of 107 accessions revealed by fastSTRUCTURE.

Association mapping via general linear model (GLM) using all available dataset only revealed significant associations with the IP2018 and IP_BLUP datasets (Appendix D). Seven different marker-trait associations were detected after Bonferroni correction (p-value ≤ 0.05) (Figure 3.5).

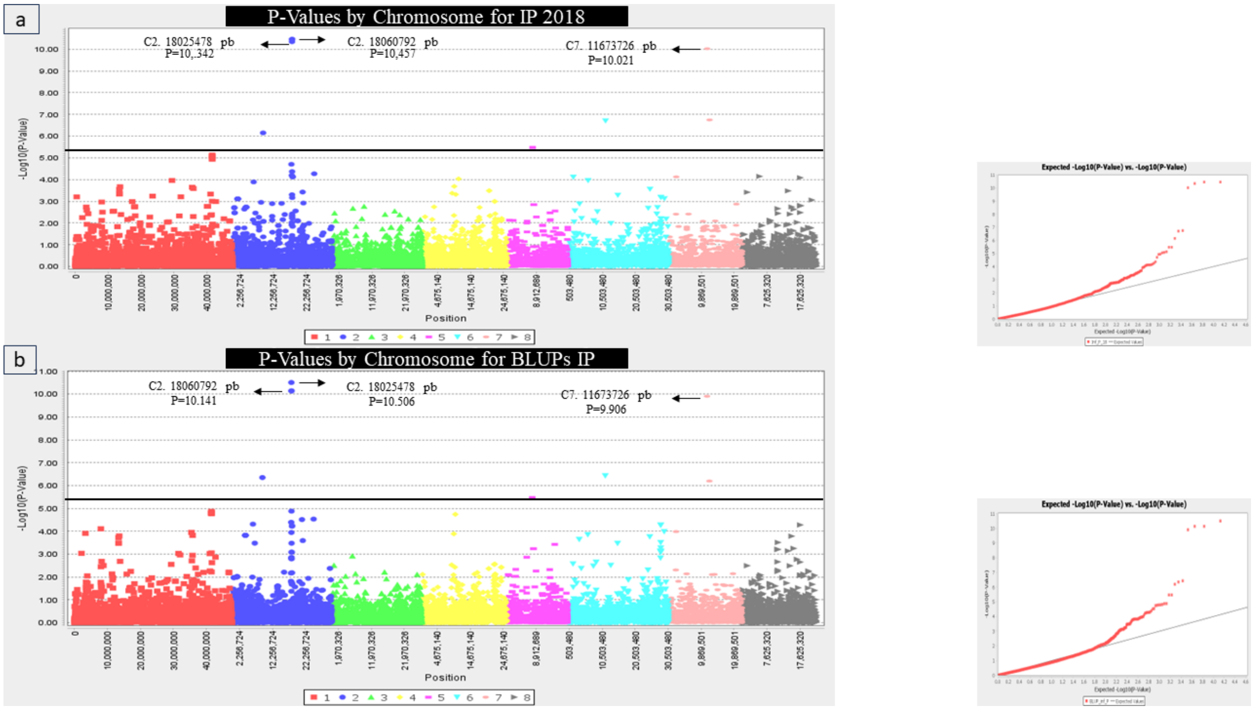

Figure 3.6. Manhattan plot showing genome-wide association (GWAS) using general linear model (GLM) for Inflection Point (IP) using IP2018 (a) and BLUP2018 (b) datasets. Each color represents a different chromosome. The horizontal solid line represents the Bonferroni significance threshold 5.6 x 10^{-6} at a significance level of $P < 0.05$. Quantile–quantile (Q-Q) plots show that the quantile points lie on the theoretical normal line.

Significantly associated markers were detected on four chromosomes (Ch), Ch2 (3), Ch5 (1), Ch6 (1) and Ch7 (2) (Figure 3.6). Two markers on Ch2, S2_18060792 and S2_18025478, and a single marker on chromosome 7, S7_11673726, were the highest associated with both IP2018 and IP_BLUP ($P = 10^{-10}$) (Figure 3.6. a, b). The other significantly associated markers were S2_9245044 on Ch2, S5_772006 on Ch5, S6_11447678 on Ch6, and S7_12386756 on Ch7 (Table 3.3).

Table 3.3. GLM analysis for the most significantly associated markers with IP 2018 - BLUPs IP based on Bonferroni multiple tests using GLM.

Trait	SNP name	P-value	Major Allele	Minor Allele
IP-2018	S2_18060792	10.457	A	G
	S2_18025478	10.342	T	C
	S2_9245044	6.144	C	T
	S5_7720068	5.477	T	C
	S6_11447678	6.706	T	C
	S7_11673726	10.021	A	C
	S7_12386756	6.746	G	T
BLUPs IP	S2_18060792	10.141	A	G
	S2_18025478	10.506	T	C
	S2_9245044	6.351	C	T
	S5_7720068	5.468	T	C
	S6_11447678	6.429	T	C
	S7_11673726	9.906	A	C
	S7_12386756	6.198	G	T

Candidate gene analysis

A total of 144 genes were identified in the 100kb flanking region of each significantly associated SNP (Appendix E). Out of 144 genes, 53 were located on Ch2, 13 on Ch5, 26 on Ch6, and 51 on Ch7. Most genes found in the vicinity of the two markers with the strongest association with the fruitlet freeze tolerance on Ch2 do not have known function or their functional annotation is not clearly associated with the freeze tolerance. Out of all candidate genes with available functional annotation Prupe.5G064100, on chromosome 5, and Prupe.6G142400 and Prupe.6G142600 on chromosome 6, all identified as part of the hydroxyproline-rich glycoprotein

(HRGP) family proteins, and have been indicated in the environmental adaptation and cold tolerance. In addition, Prupe.6G142400 and Prupe.6G142600 were annotated as late Embryogenesis Abundant (LEA) proteins, a class of proteins synthesized in plants during the late phases of seed development or under environmental stress conditions, such as dehydration, cold, or high salinity.

Discussion

Peach material included in this research represents modern peach breeding germplasm from Clemson University and the National Clonal Germplasm Repository (NCGR) in Davis. Fruitlet freeze tolerance was evaluated in 107 accessions in four experimental years. The BLUPs value was preferred due to the unbalanced annual IP and AUC data available for the research. A subset of this material was evaluated in a previous study by Melgar et al. (2022) in 2018 and 2021. This dataset helped to increase the sample size for the genome-wide association study. As noted previously, slight variation in the temperatures in the fruitlet period followed by spring can result in significant economic losses. The differences between IP means in the experimental years were observed, with a wide average (-7.01C) observed in 2018 and a narrower IP average (-5.36 to -5.96 ºC) between 2021 and 2023. The lowest IP value was observed in 2018 in fruitlets of landrace accession 880329 from Pakistan (-20.98ºC), whereas fruitlets or peach cultivar China Pearl in 2023 had -3.94ºC (Table 3.1). The possible reasons might be the differences in leakage in the cultivars shipped from California due to scars and the differences in the number of samples between years.

The population structure showed distinct origin-based grouping. Analysis of origin of the material suggested K=4 best explained the population structure and most accessions (63%) were classified as admixed, which was assumed due to the vast majority of the material belonging to the modern peach breeding germplasm.

The phenotypic variation observed in the peach germplasm utilized in this study allowed for detection of 4 regions in peach genome associated with the fruitlet freeze tolerance. Seven significantly associated SNPs were associated with the fruitlet freeze on 4 chromosomes (2, 5, 6 and 7). Investigating the 100 kb upstream and downstream from each SNP revealed 144 candidate genes. Many candidate genes had not been annotated. Among all genes the Hydroxyproline-rich glycoprotein (HRGP) associated with SNP on chromosome 5 was described to play a vital role in support and strength to the cell wall and is also is detected in response to various environmental stressors, such as drought, salinity, and cold, suggesting that HRGPs could be involved in the plant's adaptation to adverse conditions (Showalter et al., 2010). Plants often face various abiotic stressors, such as environmental temperature changes, and in response, they have evolved different adaptive mechanisms, including modifications to their cell wall structure (Pinski et al., 2019). Cold stress leads to downregulating numerous photosynthesis-related proteins while upregulating proteins involved in cell wall remodeling (Janmohammadi et al., 2015). Thus, HRGB supports and strengthens the cell wall, helping plants withstand cold stress. Furthermore, Late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein associated with SNPs on chromosome 6 are a set of proteins that are produced in plants during the late stages of seed development or under conditions of environmental stress, such as dehydration, cold, or high salinity. These proteins are assumed to play a critical role in protecting cells and cellular structures from damage triggered by these stress conditions. The hydroxyproline-rich glycoprotein (HRGP) family is a subgroup of LEA proteins. LEA proteins may have antifreeze properties, helping control ice crystal formation and cell damage during cold stress (Arora & Wisniewski, 1994; Yu et al., 2020d). Furthermore, research of the peach genome indicates that peaches have two ICE genes and six CBF genes (Wisniewski et al., 2014d). Regarding peach, five of the CBF genes are located on chromosome 5 (10,054,488 to 10,086,243), while the other is on chromosome 2 (24,801,153 - 24,802,102). On the other hand, ICE1 and ICE2 genes are located on chromosomes 3 (21739624 - 21746437) and 5 (13261248 - 13258470), respectively. Even though the association mapping studies revealed seven significant markers, none of them were close to our findings. However, including more phenotypic with available GBS data in the future can help us find more significant markers that might be close to that region.

Fruitlet freeze tolerance in peach is a complex process that involves a range of physiological, biochemical, and molecular adaptations to survive in freezing conditions. Further research is needed to investigate some of the candidate genes and their potential involvement in the freeze tolerance.

Conclusion

This study provides preliminary findings on genetic control of the fruitlet freeze tolerance in peach genome. Association mapping revealed 7 significant marker-trait associations. Preliminary candidate gene investigations determined 144 genes in the 100kb flanking region of each significantly associated SNP located on Ch2 (53), Ch5 (13), Ch6 (26), and Ch7 (51). Candidate genes associated with fruitlet freeze tolerance on chromosomes 5 and 6 were indicated in the abiotic stress. These regions of the peach genome may provide new information for further studies on fruitlet freeze tolerance in peach. Additional research is required to further investigate candidate genes and to understand their role in the fruitlet freeze tolerance.

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CHAPTER FOUR

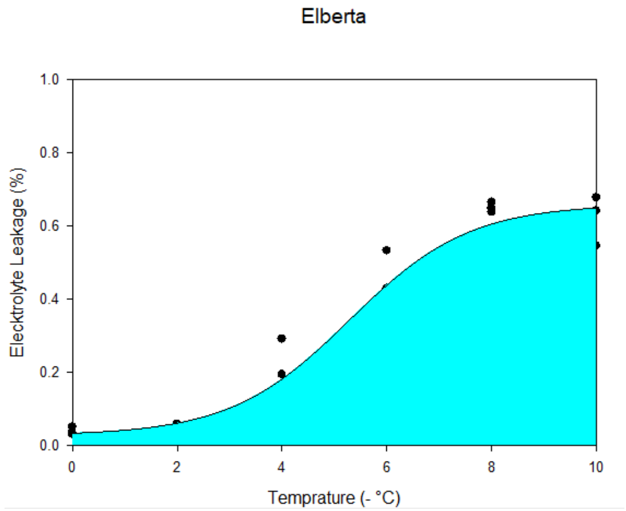
The increasing occurrence of freezing temperatures during spring in the southeastern United States poses a significant challenge to peach and nectarine production in the region. The outstanding losses experienced in the peach industry due to recurrent spring frosts have prompted a more thorough investigation into these traits. The breeding efforts now include development of climate resilient cultivars. The primary aim of this study was to understand the genetic factors responsible for fruitlet freeze tolerance by determining the region in the peach genome associated with this trait. Therefore, this study assessed fruitlet freeze tolerance in peach and nectarine accessions representing modern peach breeding germplasm. Fruitlet freeze tolerance was evaluated at six freezing temperatures (0 to -10ºC) using the electrolyte leakage method over two seasons (2022-20223). Most accessions showed tolerance in the -4 to -6ºC LT50 and 25-35% AUC range. Furthermore, seven and nine nectarines were classified as most tolerant in both seasons, 2022 and 2023, using IP and AUC, respectively. Tolerance groups proposed in this study are of direct benefit for peach growers as they now have additional information to consult when choosing what to plant and also how to manage already planted cultivars.

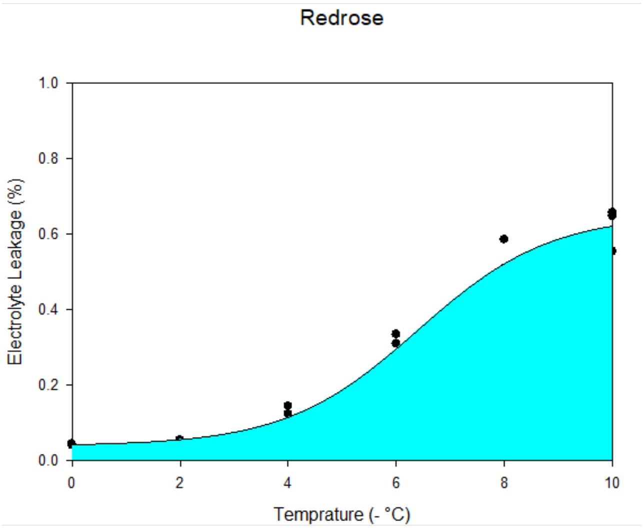
Broad-sense heritability (H^2) estimates of 0.52 and 0.80 for IP and AUC presented genetic control of this trait with a potential for improvement via breeding. The genetic control of the fruitlet freeze tolerance in peaches was further investigated via genome-wide associations, revealing seven significant marker-trait associations across the whole genome. Preliminary candidate gene analyses determined 144 genes in the 100kb flanking region of each significantly associated SNP located on Ch2 (53), Ch5 (13), Ch6 (26), and Ch7 (51). These findings highlighted the first step in understanding genetic control of fruitlet freeze tolerance in peach, and highlights some significant markers that are playing a role in it. Out of all candidate genes with available functional annotation, Prupe.5G064100 on chromosome 5 annotated as hydroxyproline-rich glycoprotein (HRGP) family proteins and late Embryogenesis Abundant (LEA) proteins (Prupe.6G142400 and Prupe.6G142600) on chromosome 6, have been indicated in the environmental adaptation and cold tolerance. Additional research is required to investigate candidate genes further. Phenotypic information could serve in parent selection and support efforts to maintain and expand the peach germplasm diversity to mitigate the effects of climate change. These findings emphasize the importance of combining genetic information into breeding efforts and germplasm management to ensure the long-term sustainability of peach production under changing climatic conditions.

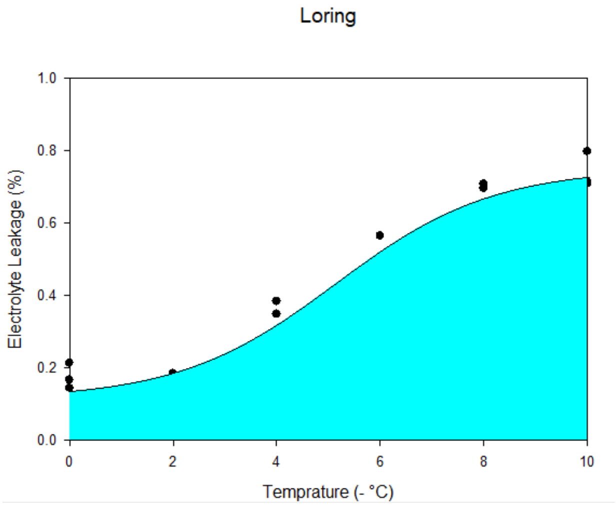
APPENDICES

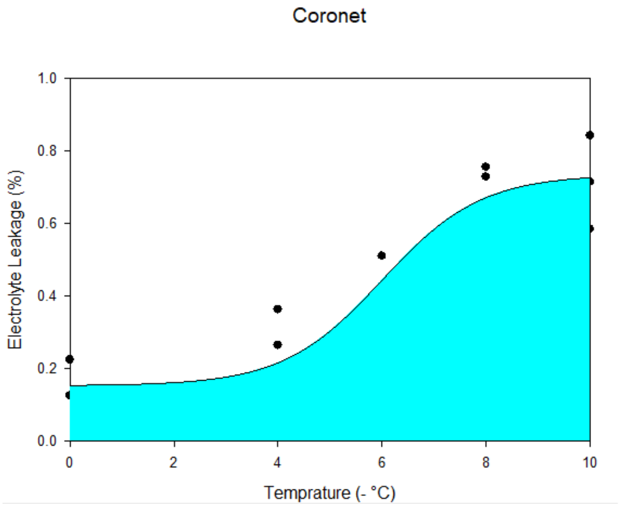
Appendix A

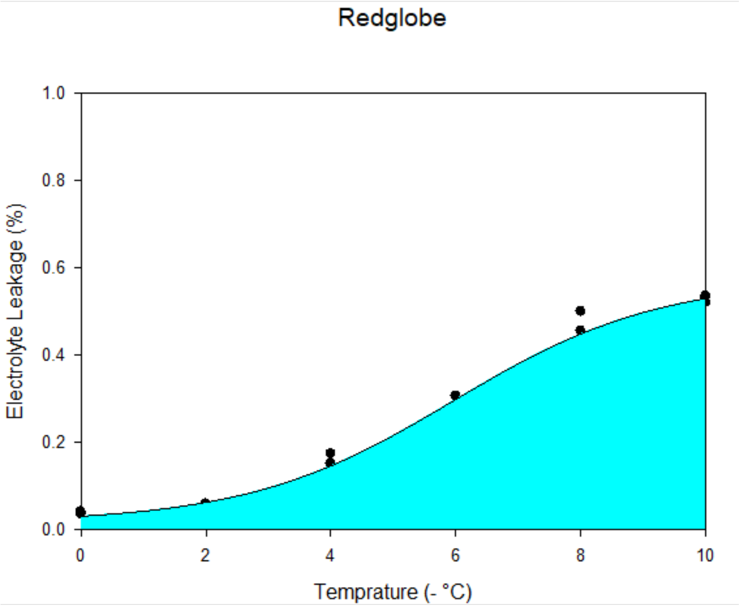
Fruitlet freeze response to freezing temperatures observed in peach and nectarine accessions listed by release year in 2022.







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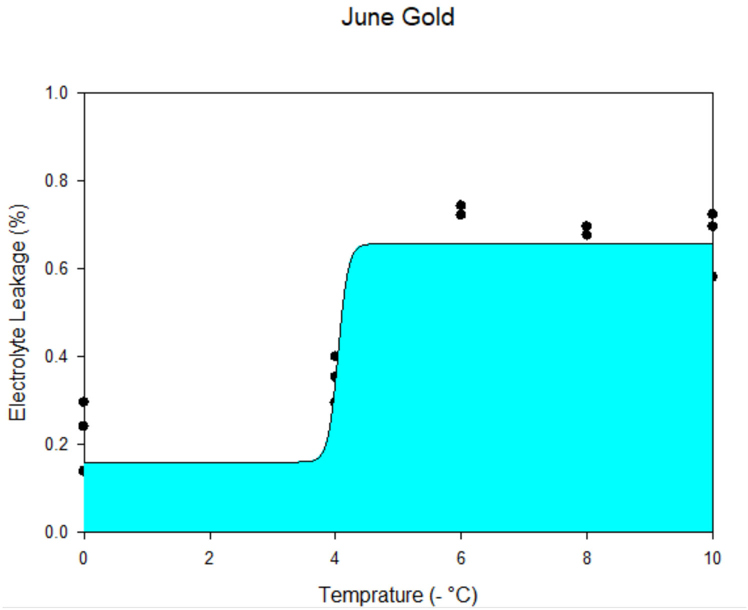
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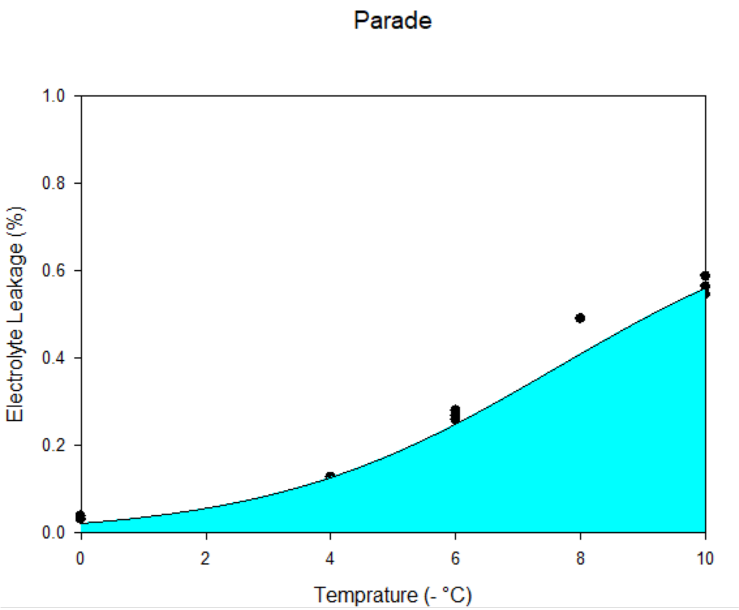
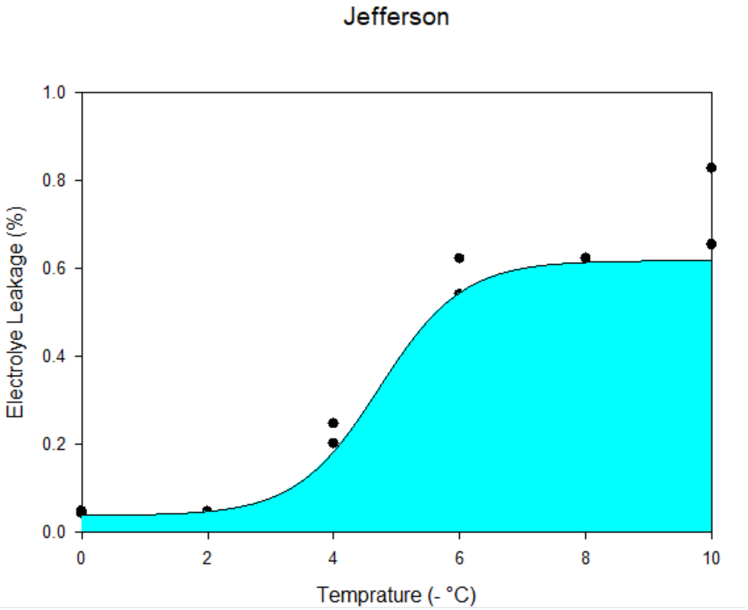
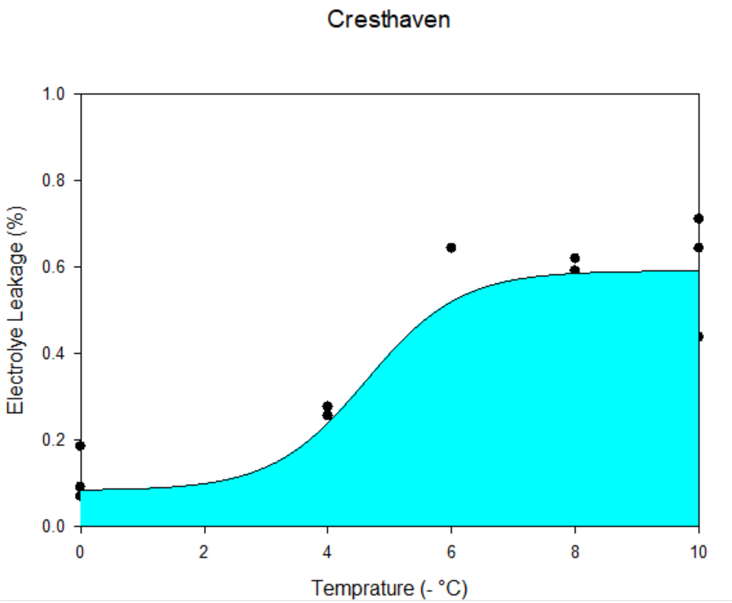
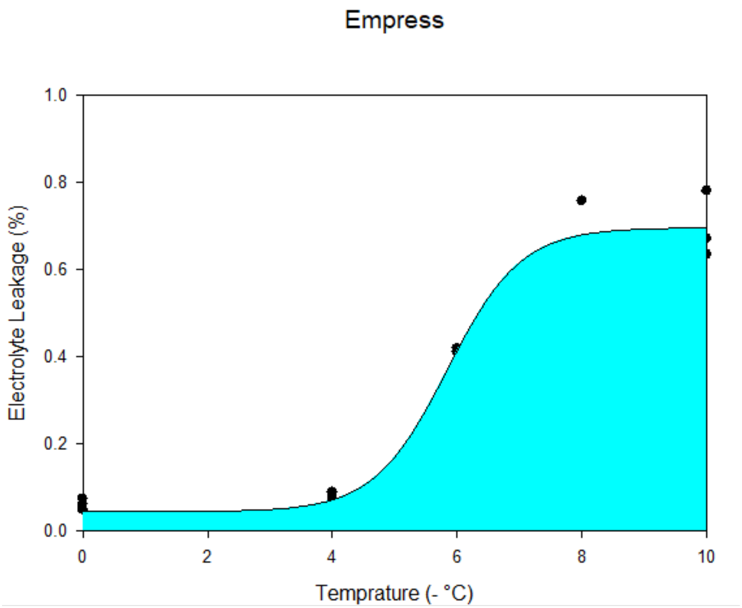
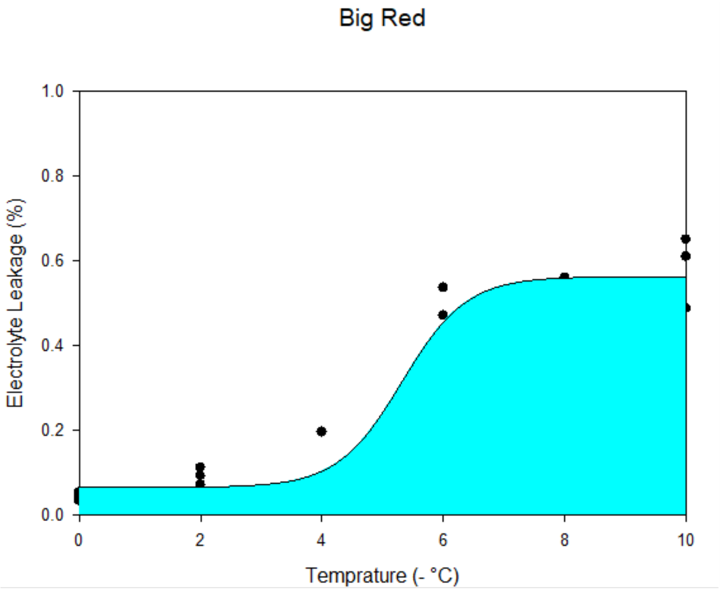
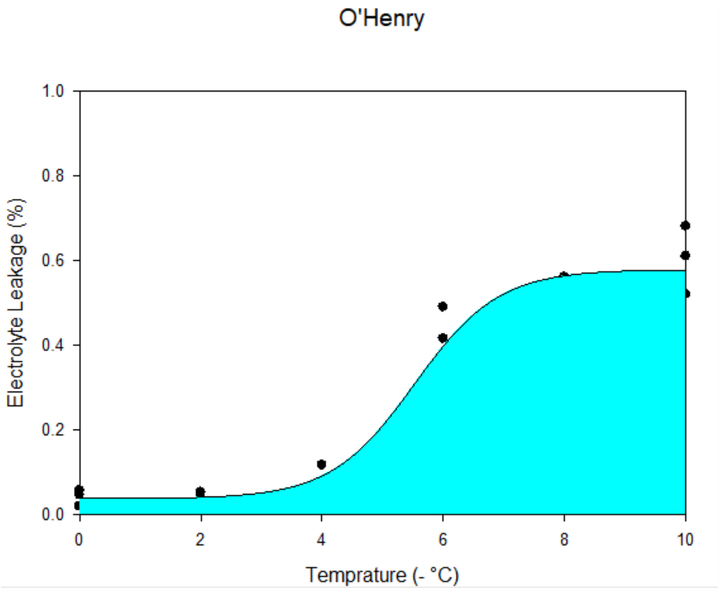

Parade

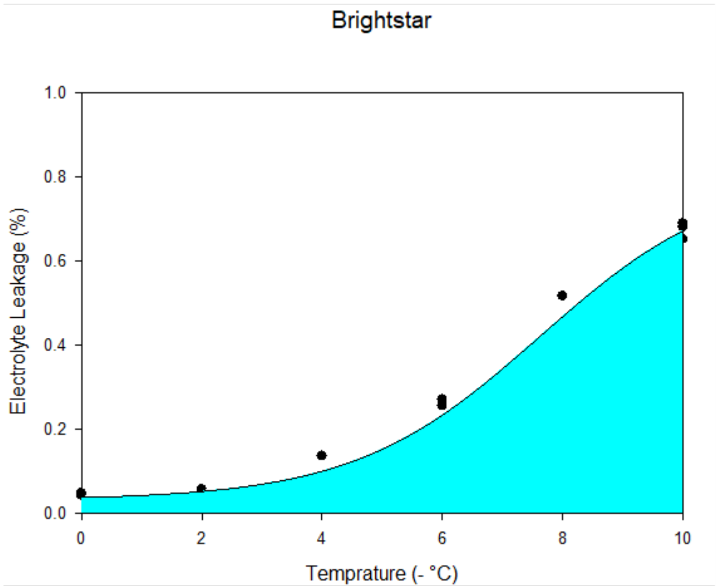

Jefferson

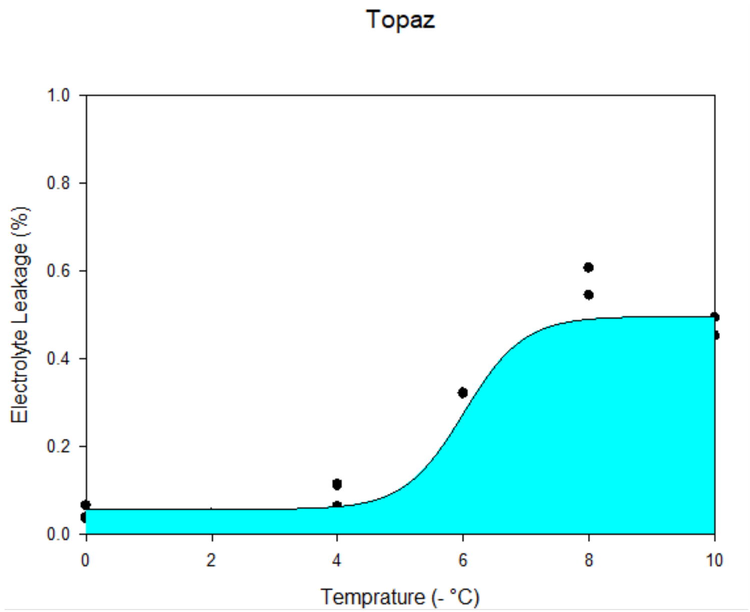

Cresthaven
Electrolye Leakage (%)
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
Temprature (- °C)


Empress
Electrolyte Leakage (%)
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
Temprature (- °C)


Big Red
Electrolyte Leakage (%)
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
Temprature (- °C)


O'Henry
Electrolyte Leakage (%)
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
Temprature (- °C)


Brightstar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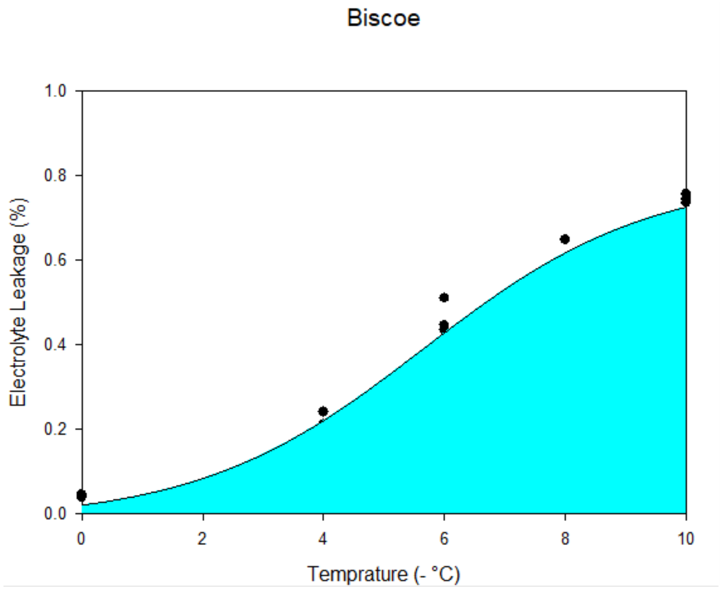
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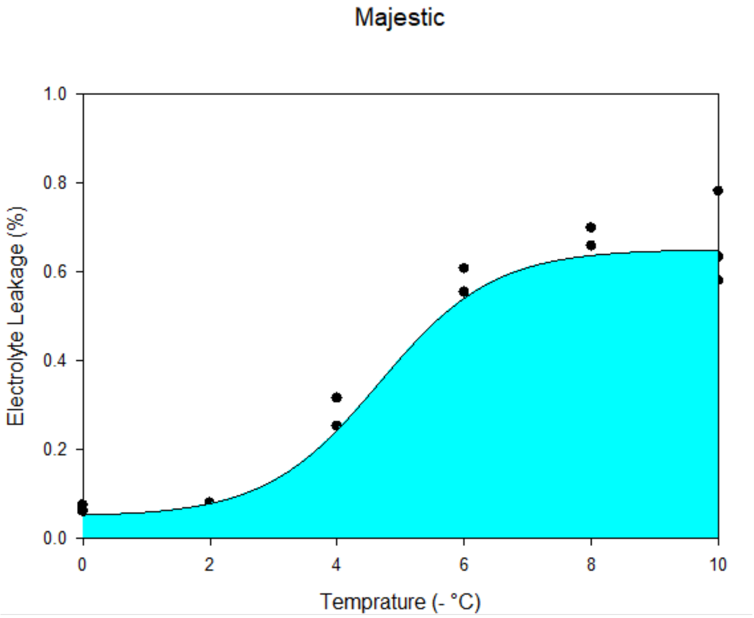
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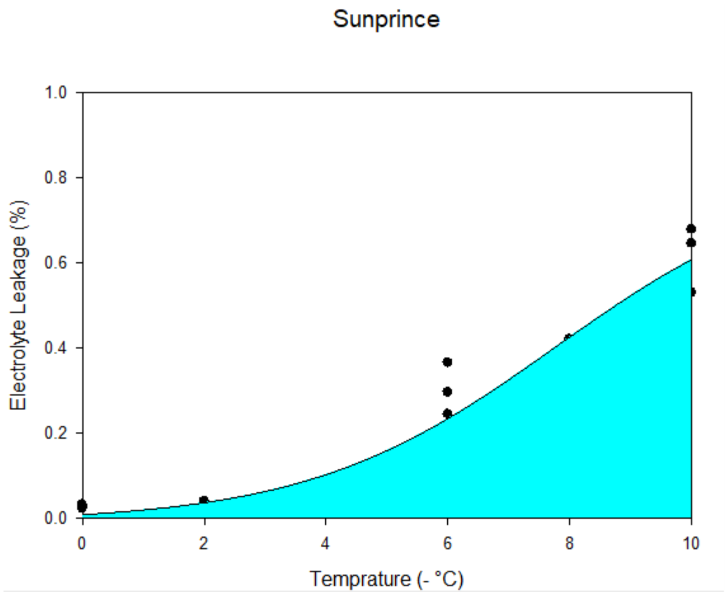
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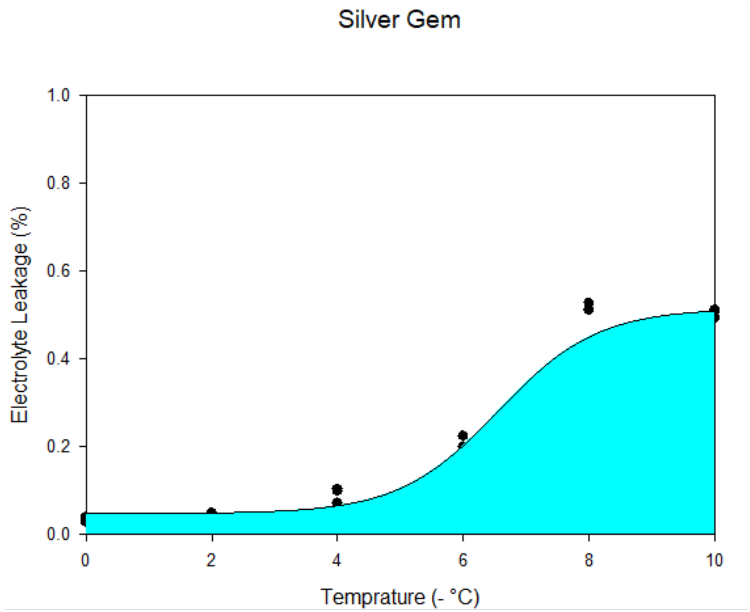
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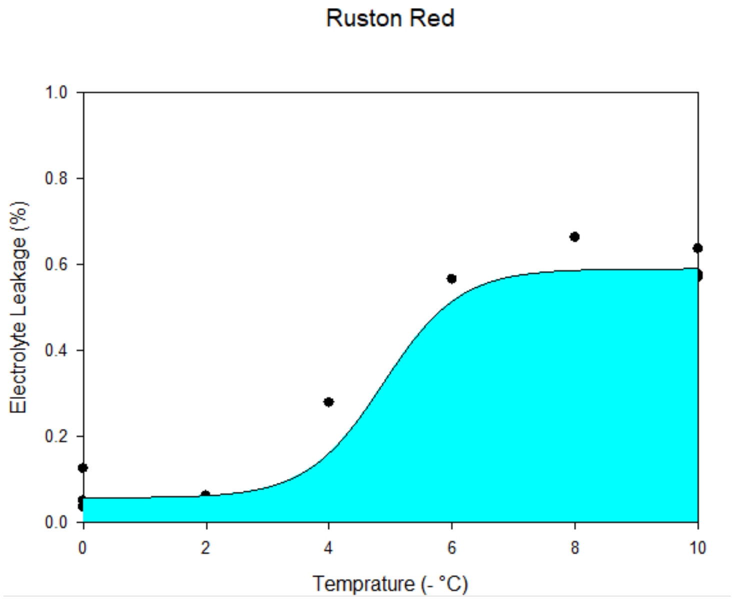
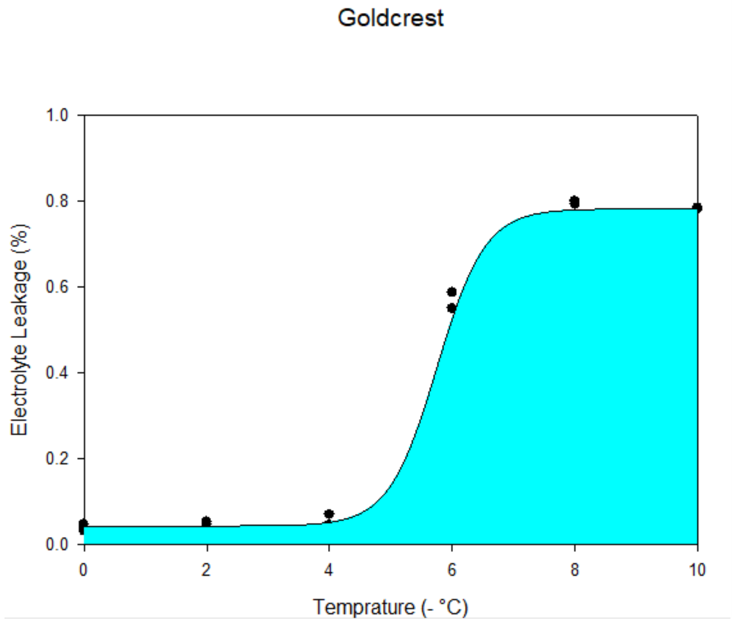
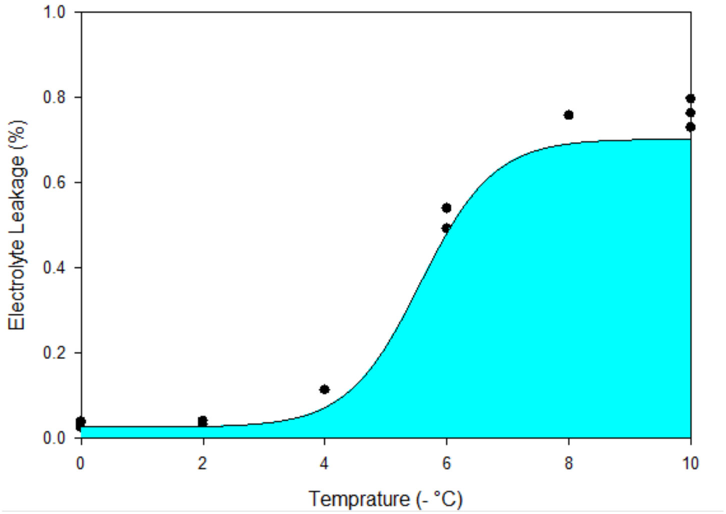
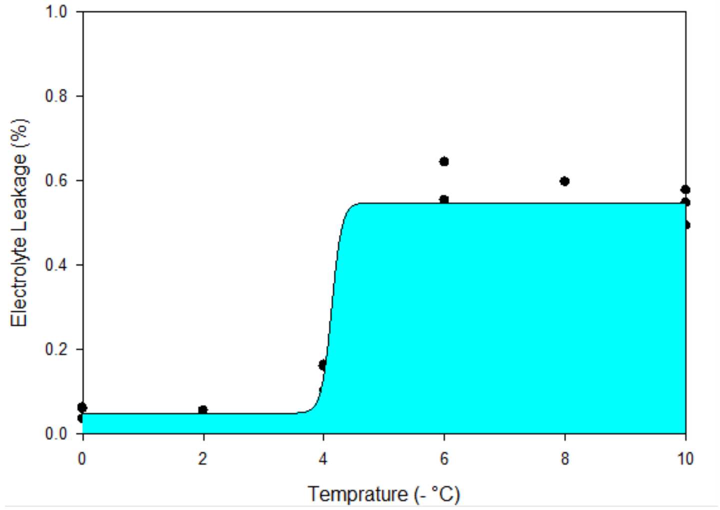
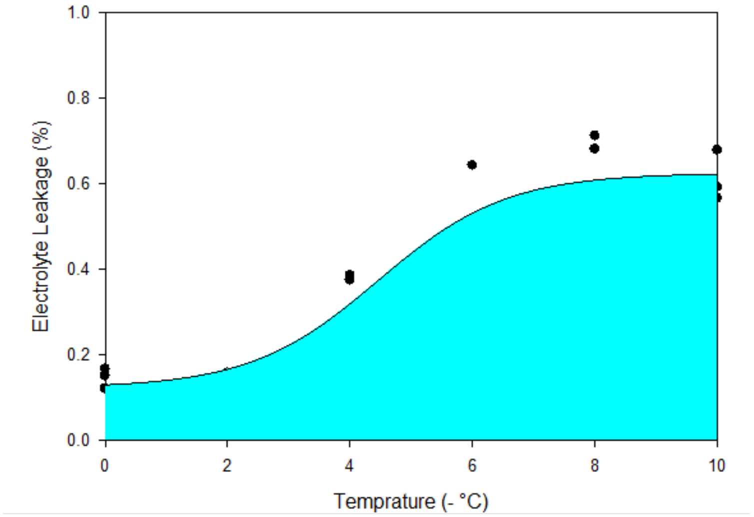
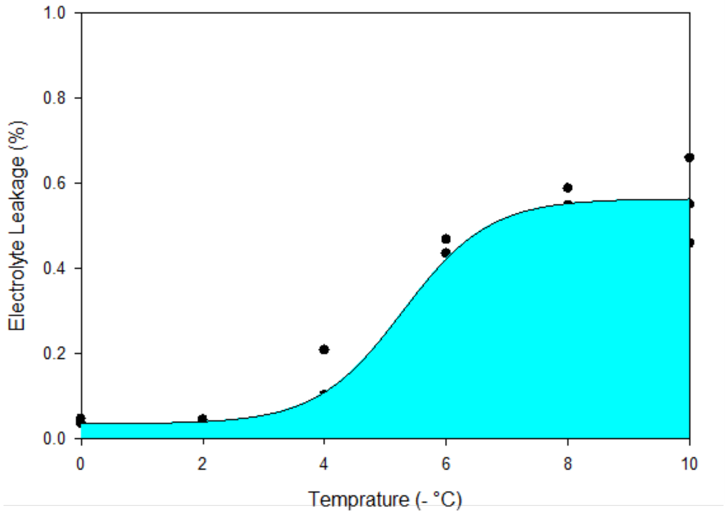

Ruston Red


Goldcrest


Juneprince


Fireprince


Caroking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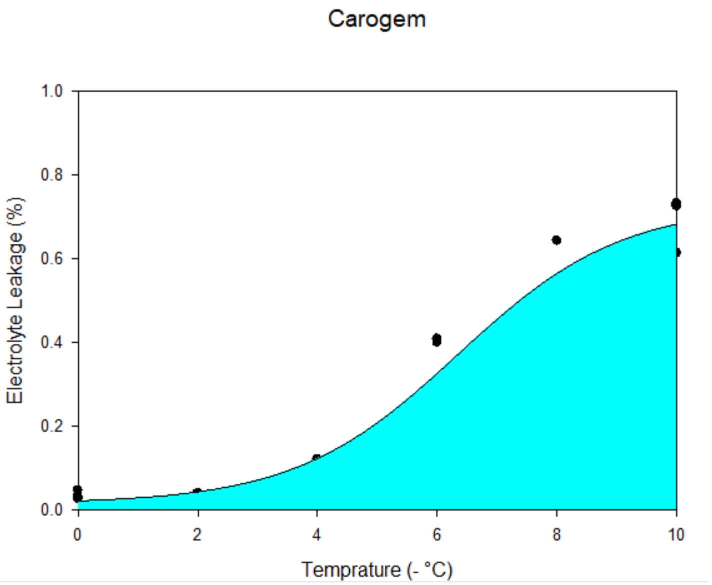
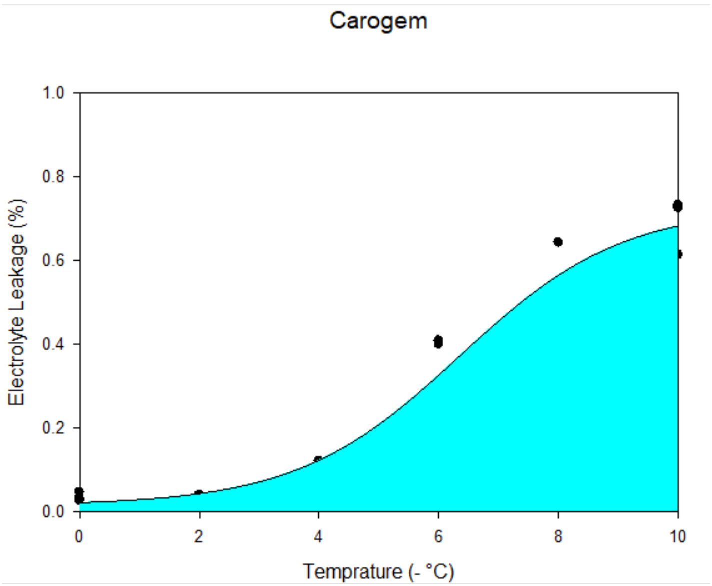
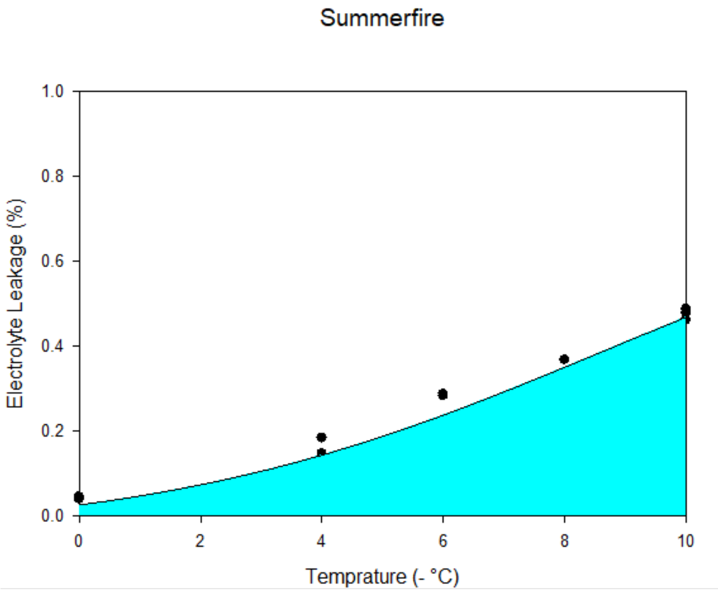

Carogem


Carogem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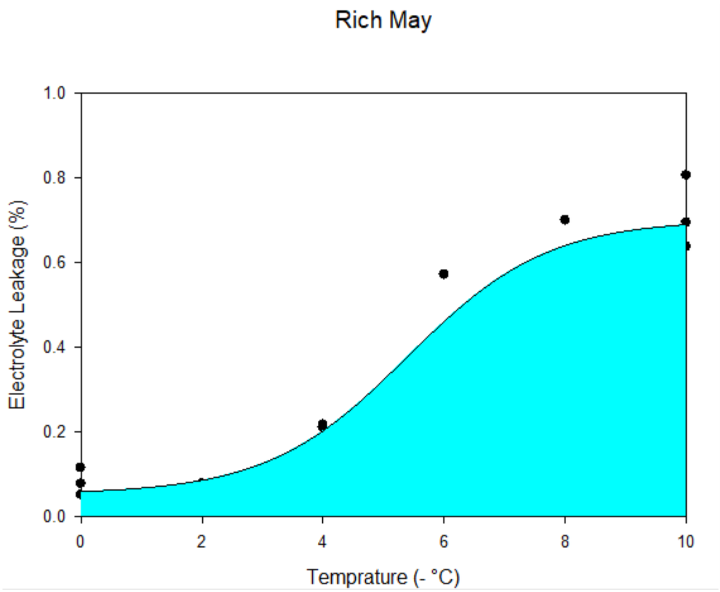
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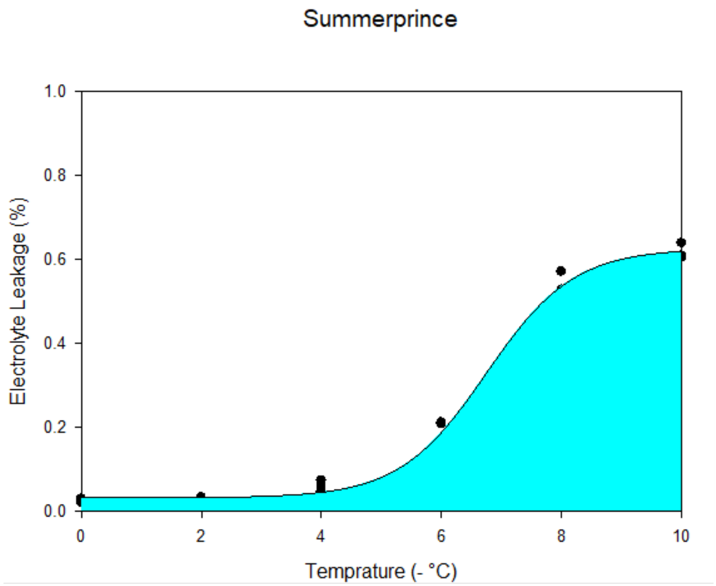
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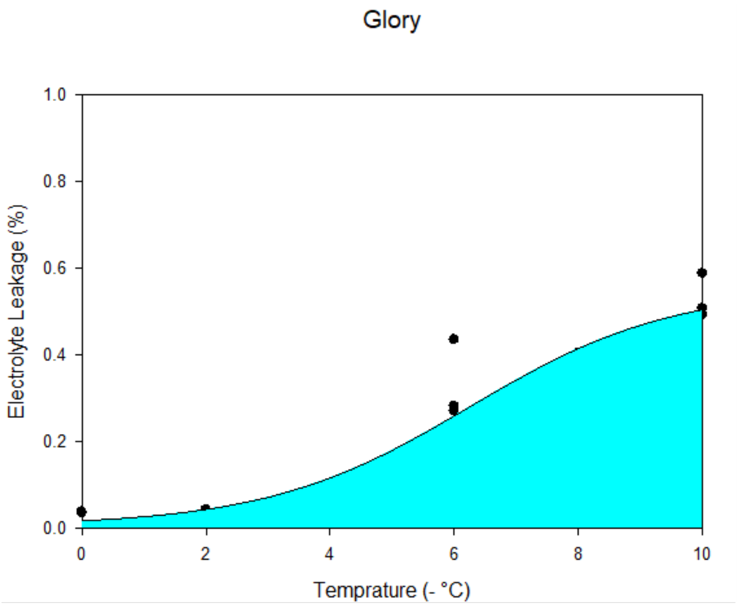
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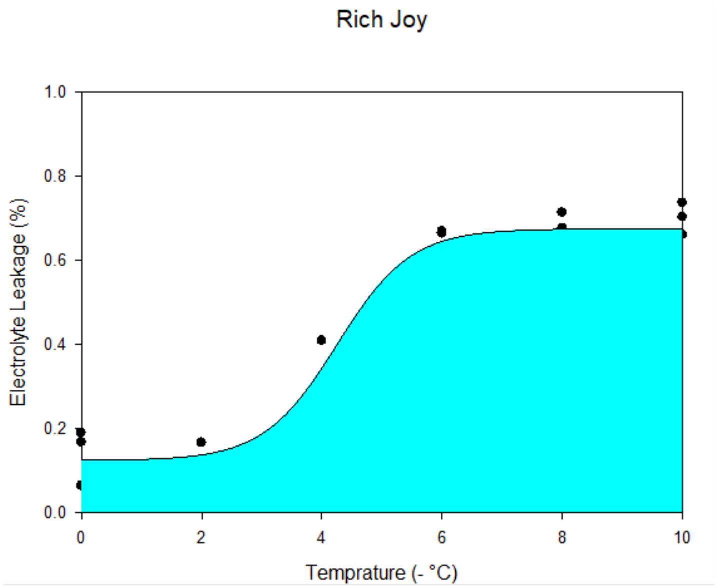
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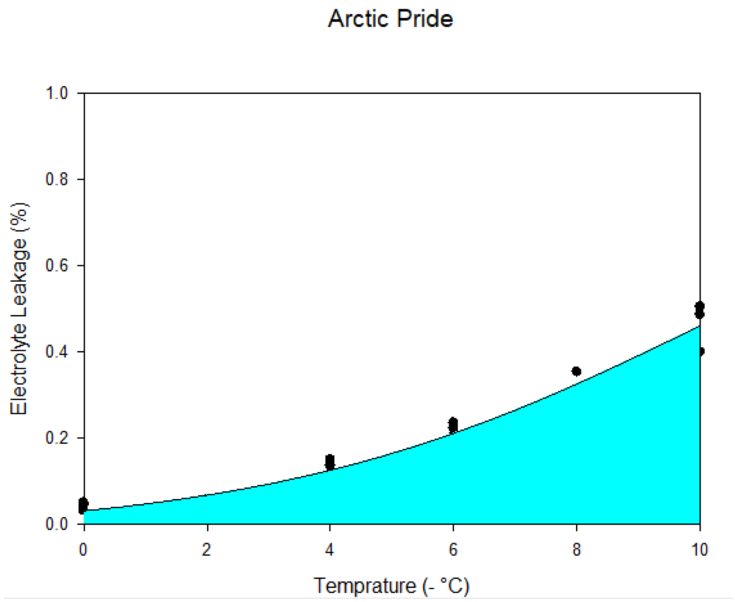
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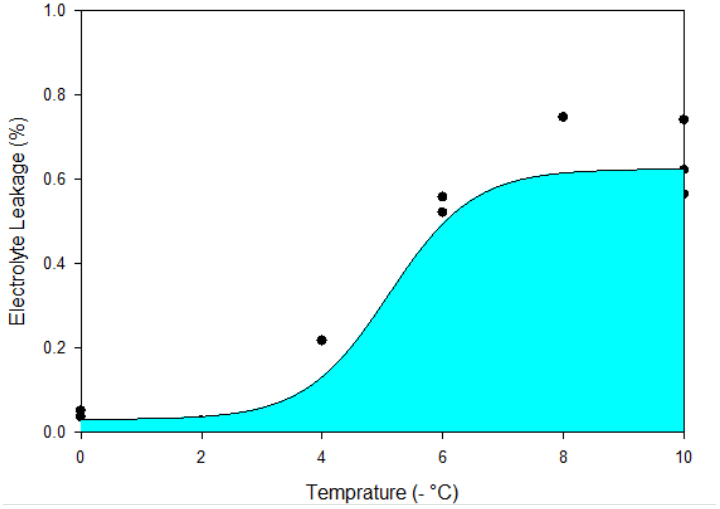
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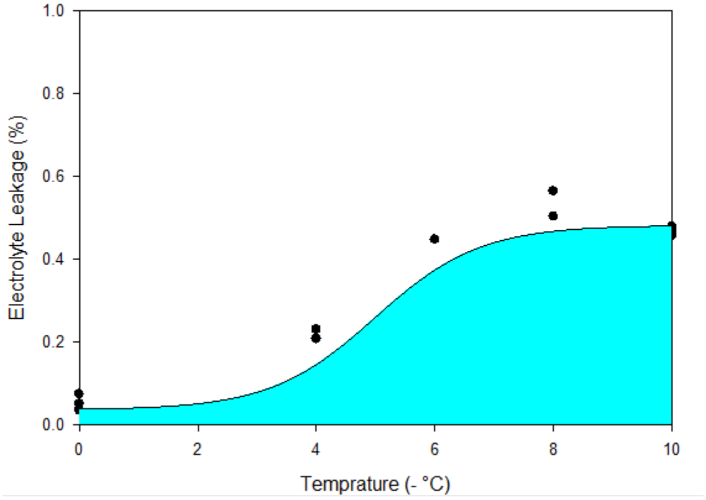
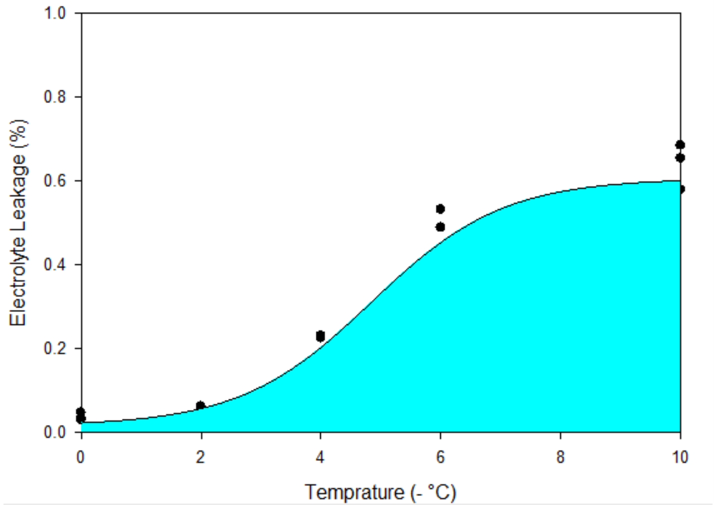

Guardian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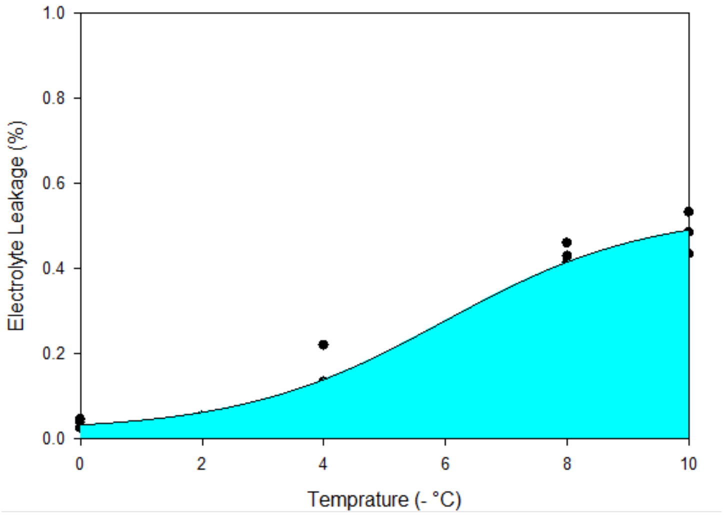
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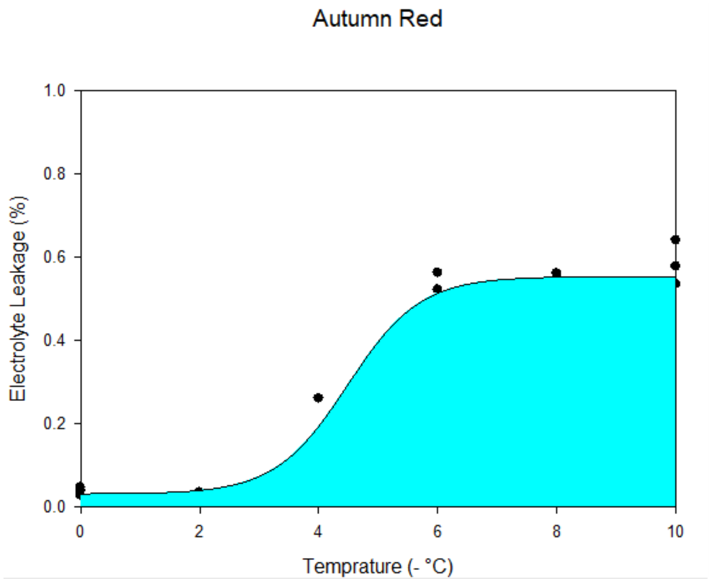
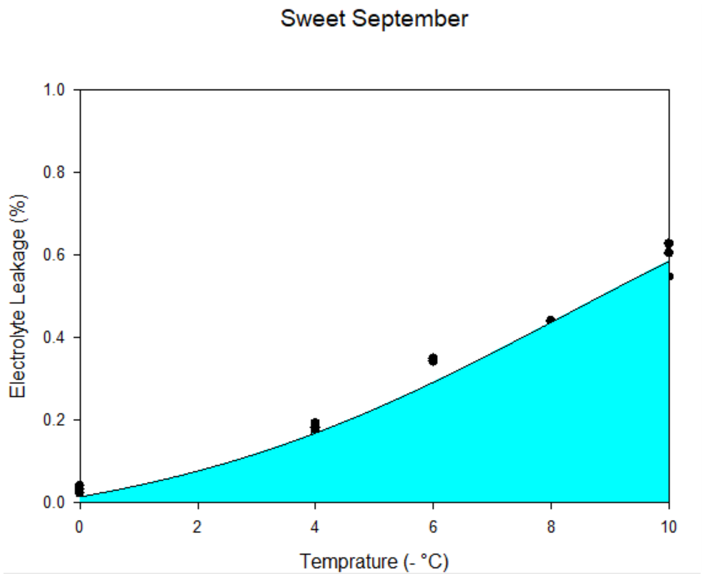
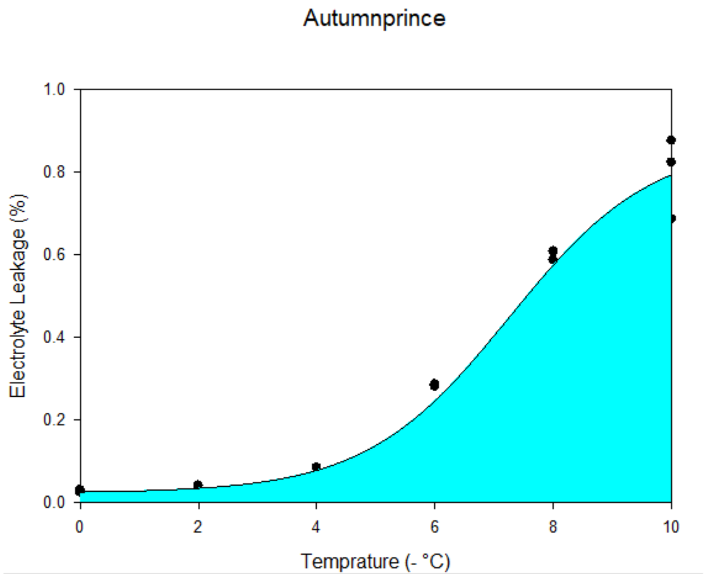
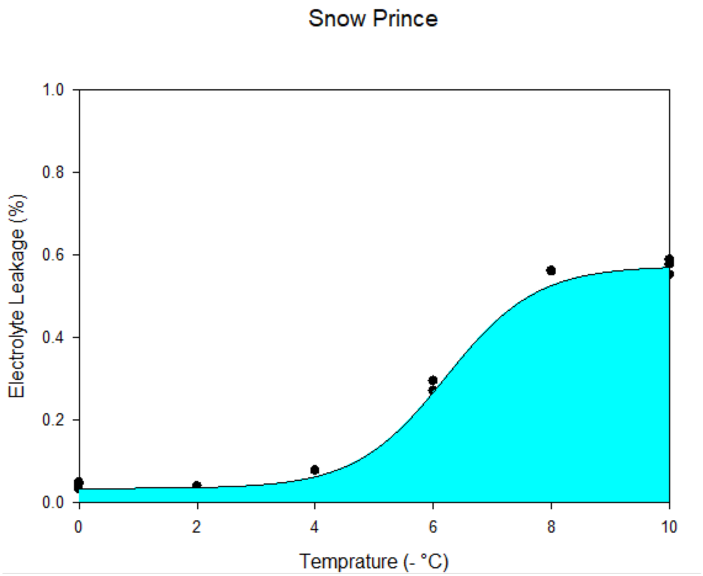
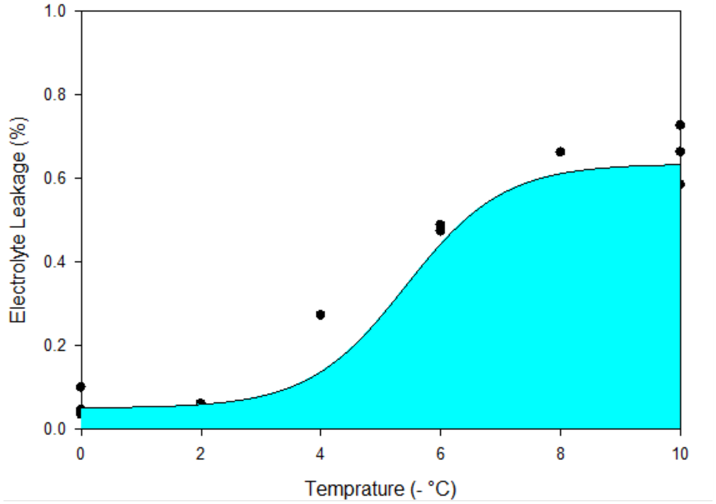

Autumn Red
Electrolyte Leakage (%)
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
Temprature (- °C)

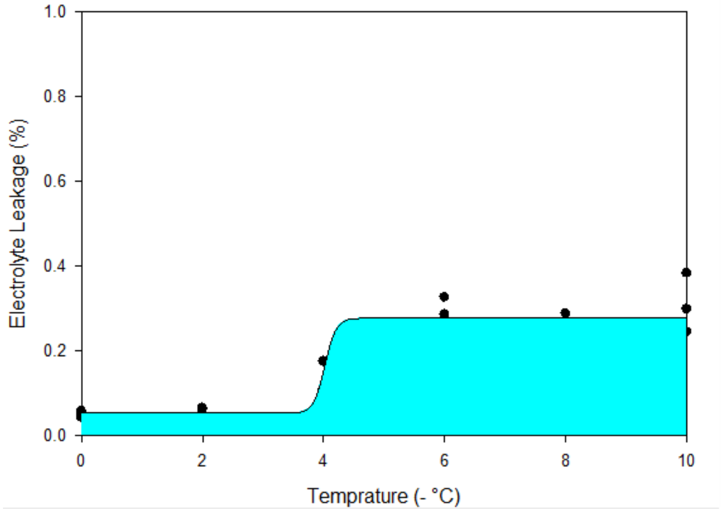

Sweet September
Electrolyte Leakage (%)
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
Temprature (- °C)


Autumnprince
Electrolyte Leakage (%)
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
Temprature (- °C)


Snow Prince
Electrolyte Leakage (%)
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
Temprature (- °C)



Rubyprince



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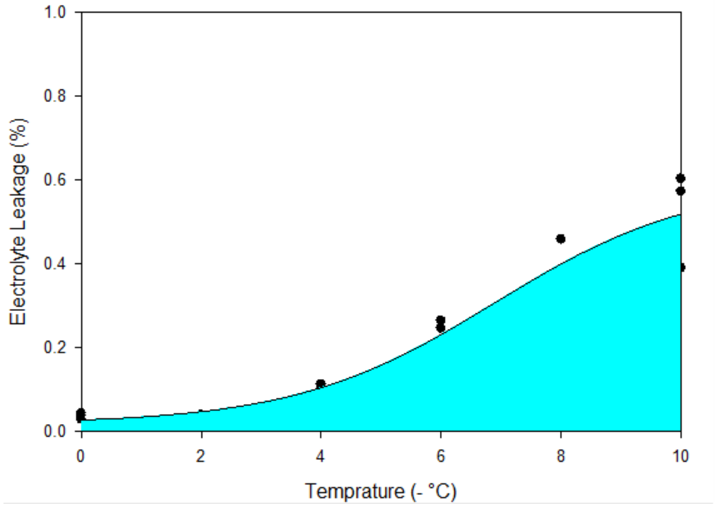
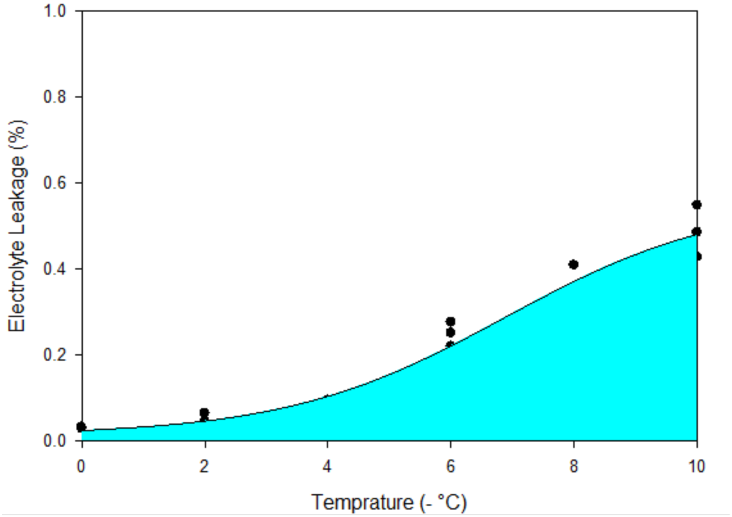
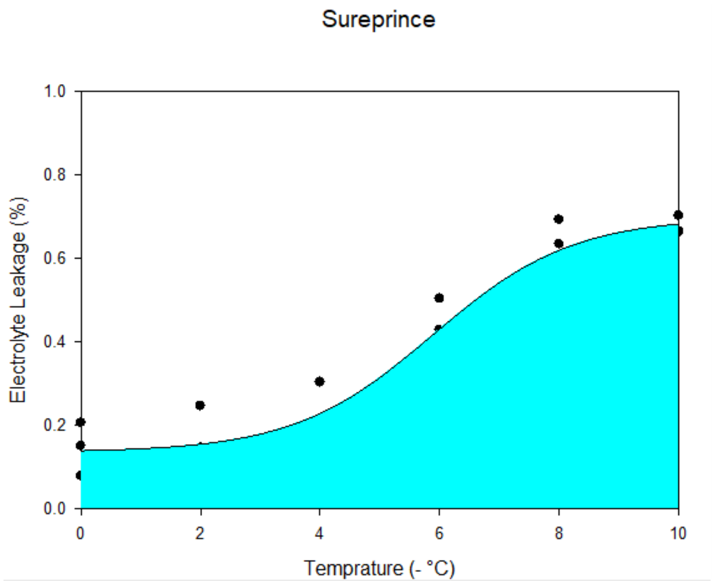
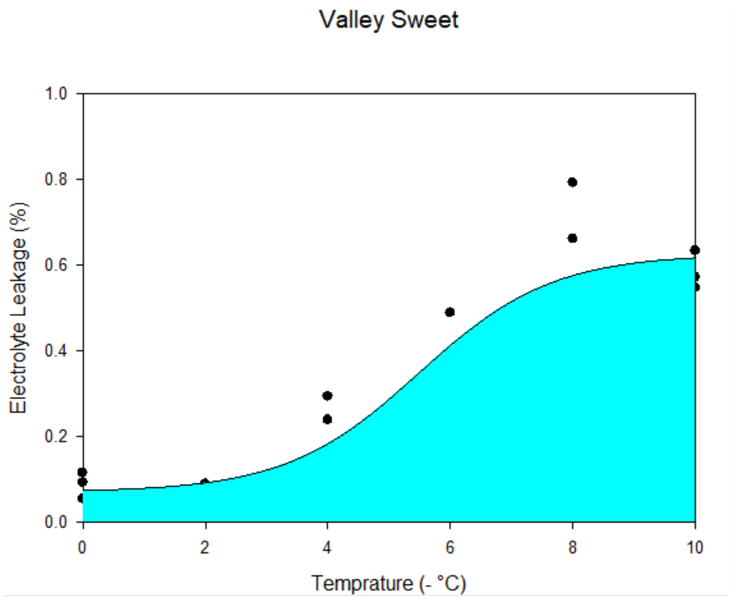


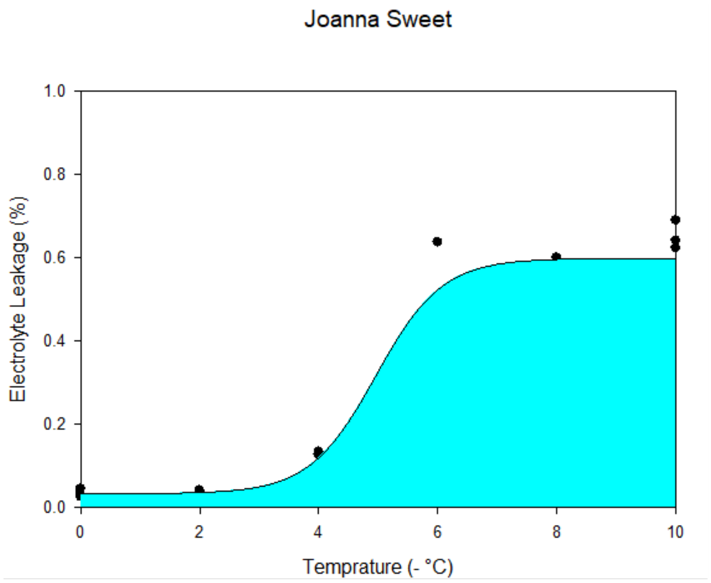
Arctic Belle

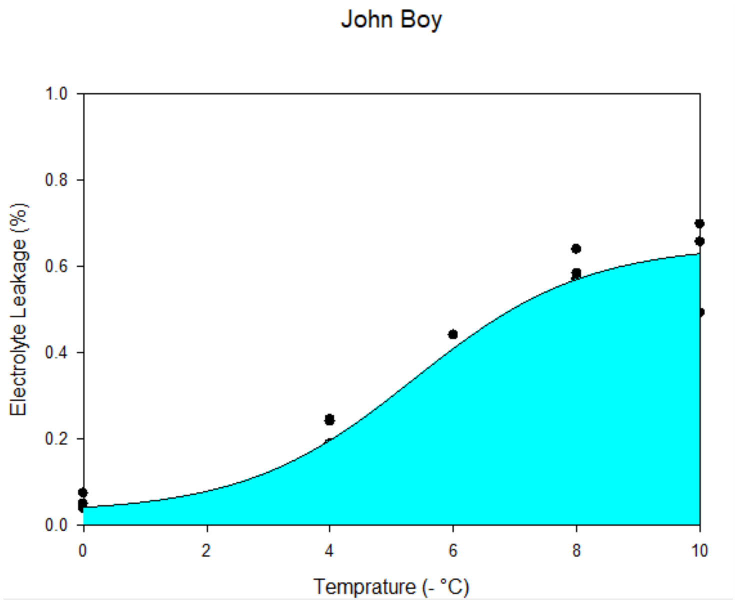


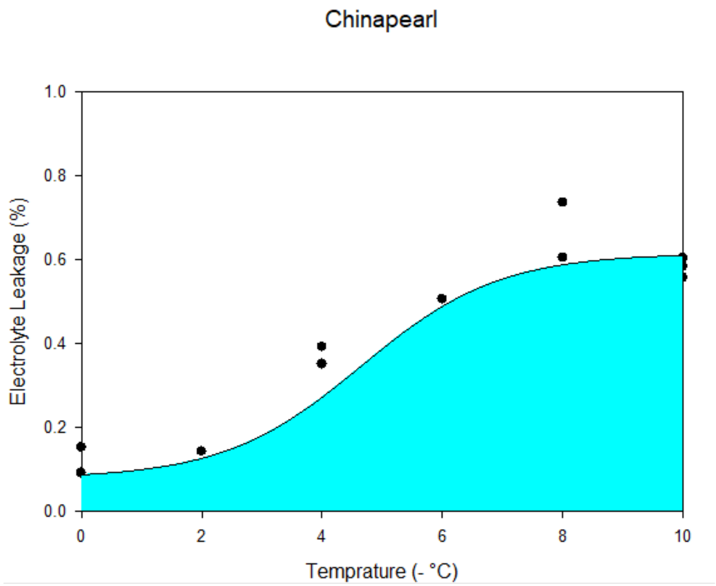
Arctic Blaze

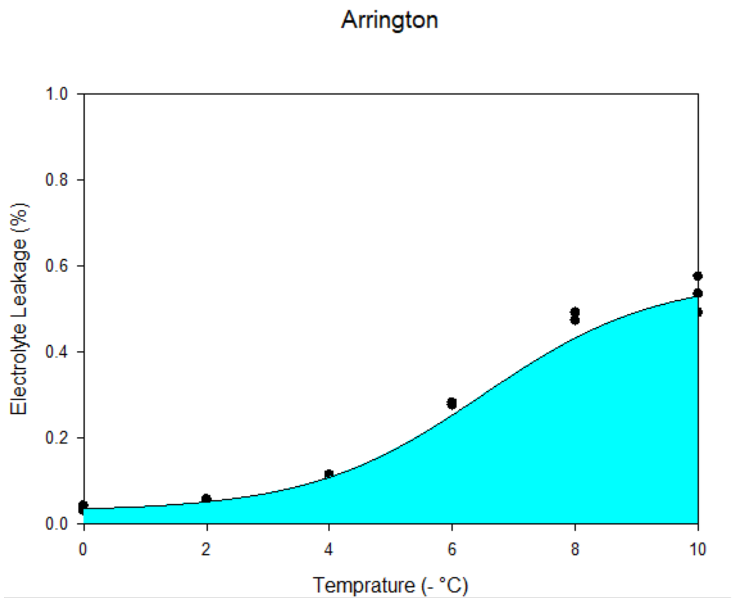

Sureprince
Electrolyte Leakage (%)
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
Temprature (- °C)

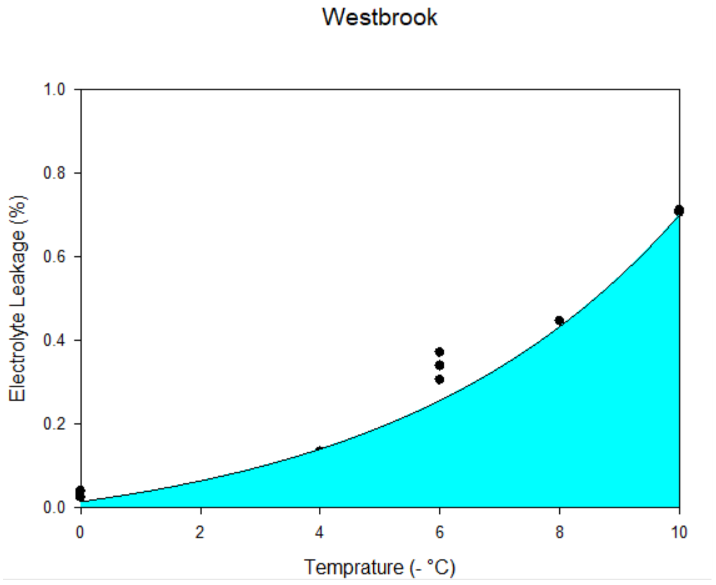

Valley Sweet
Electrolyte Leakage (%)
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
Temprature (- °C)

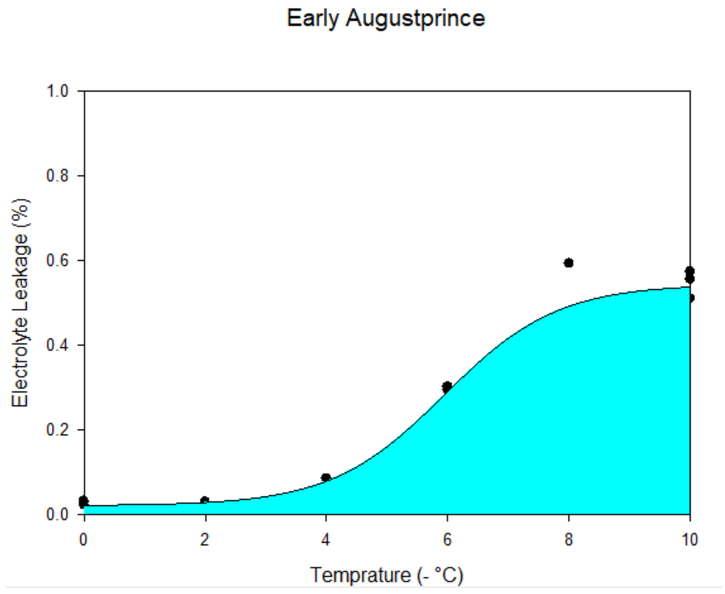

Joanna Sweet
Electrolyte Leakage (%)
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
Temprature (- °C)


John Boy
Electrolyte Leakage (%)
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
Temprature (- °C)

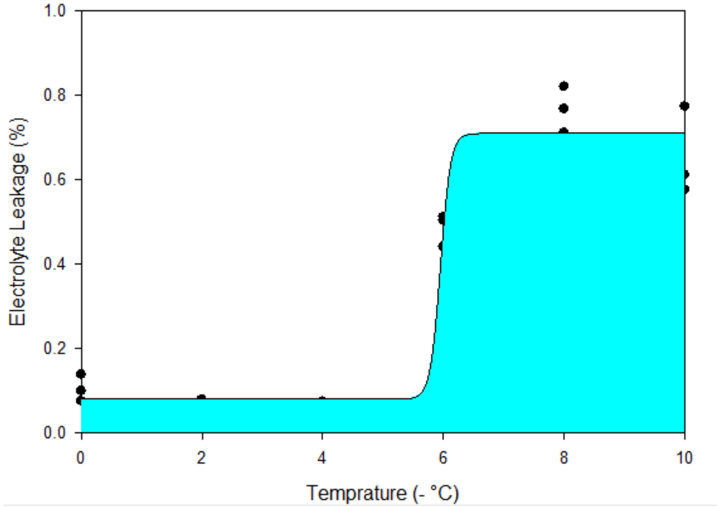

Chinapearl


Arrington

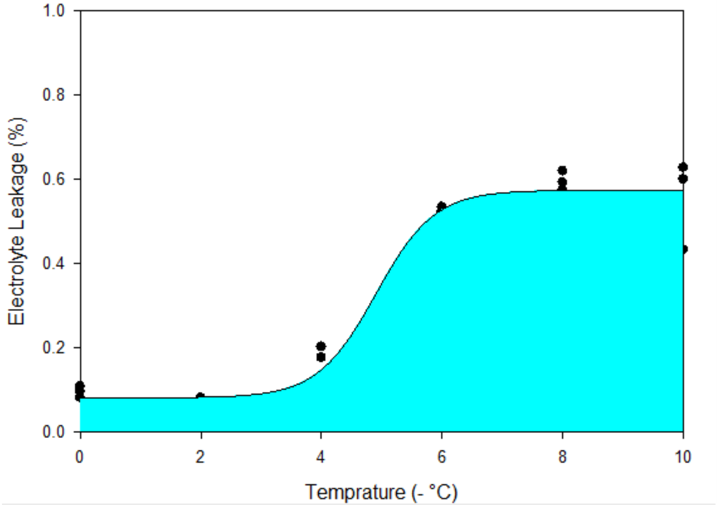

Westbrook


Early Augustprince

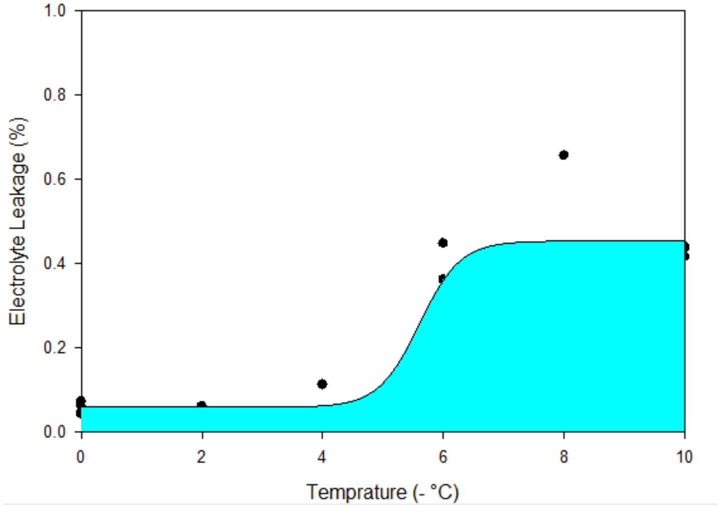
Messina



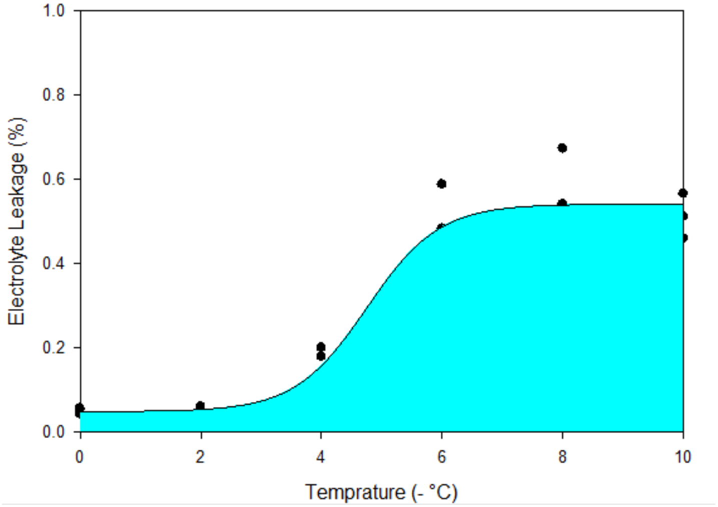
Flavrburst

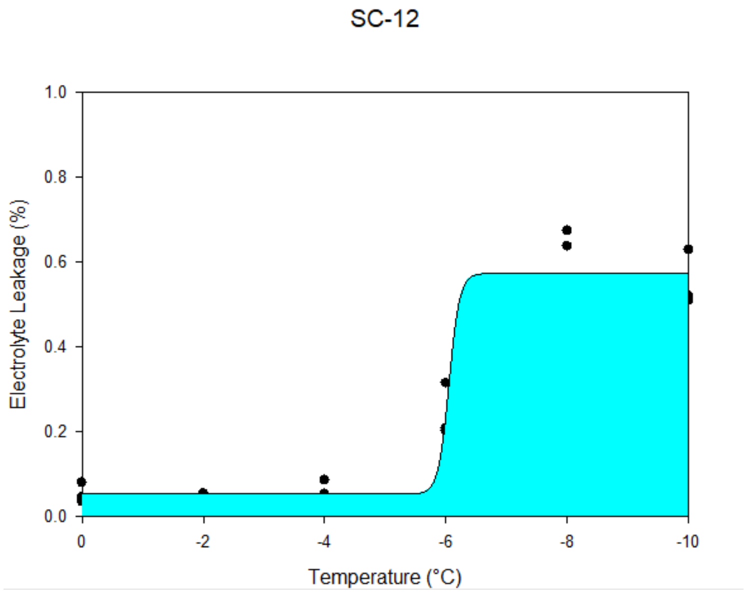


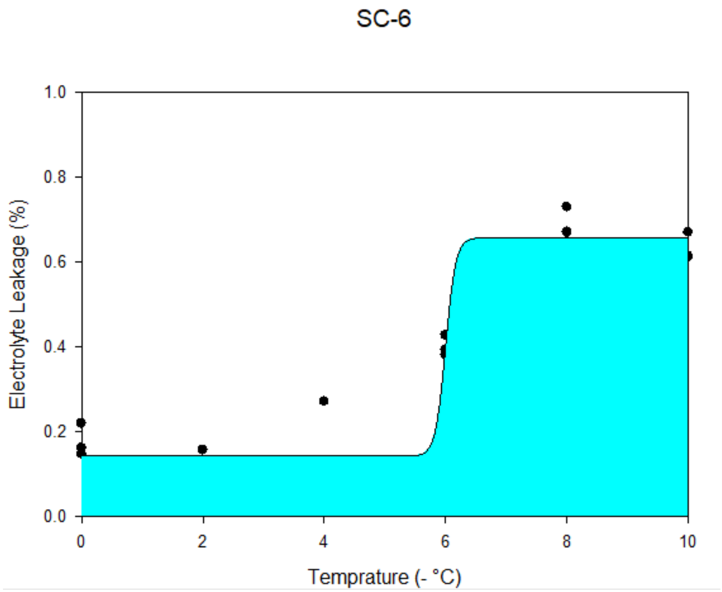
Summerfest

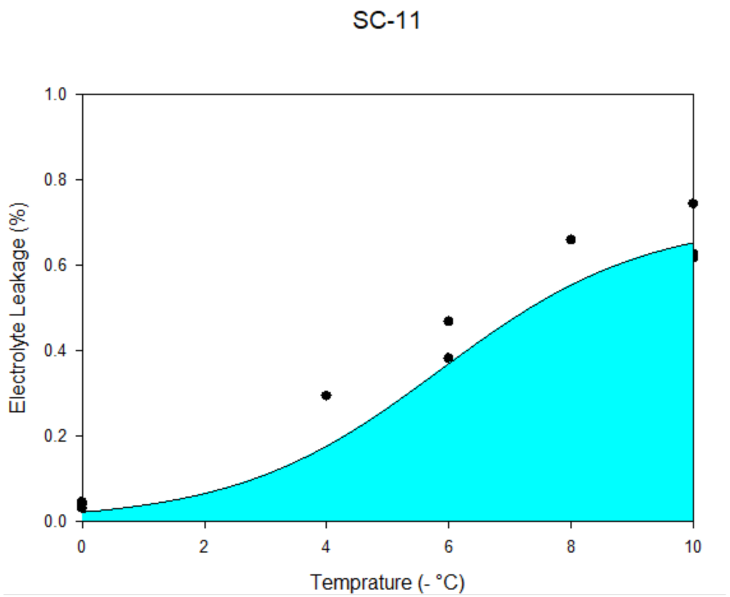


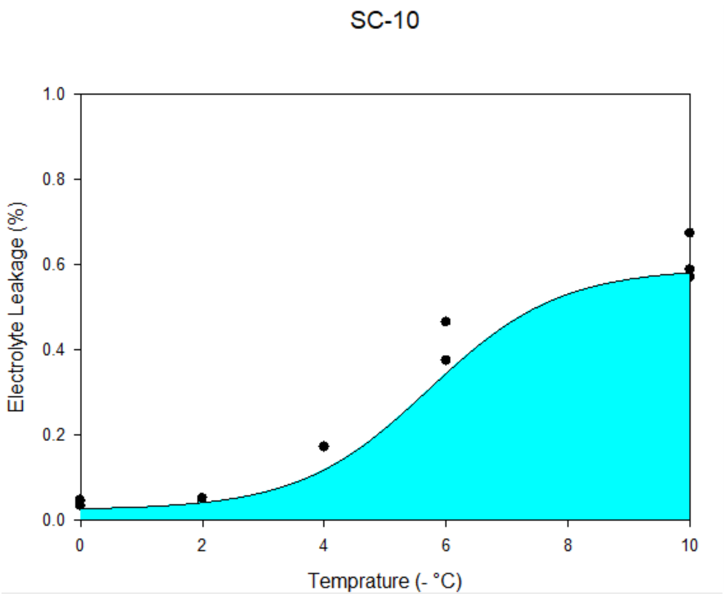
Sweetstar

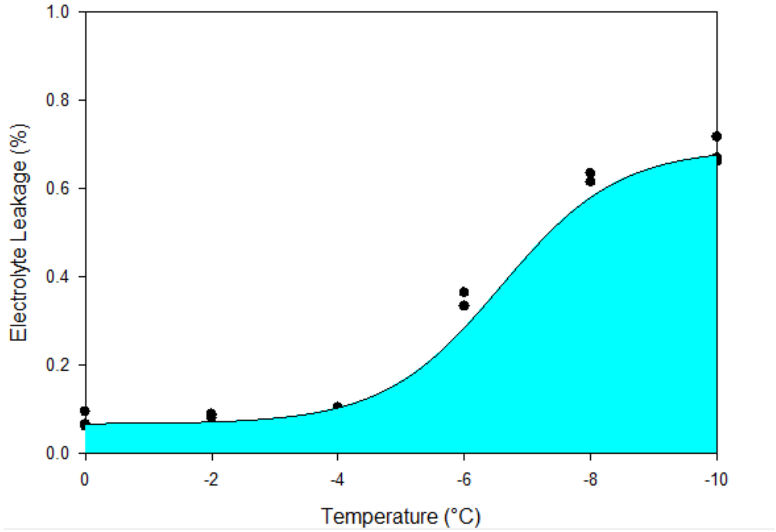



SC-12
Electrolyte Leakage (%)
1.0
0.8
0.6
0.4
0.2
0.0
0
-2
-4
-6
-8
-10
Temperature (°C)

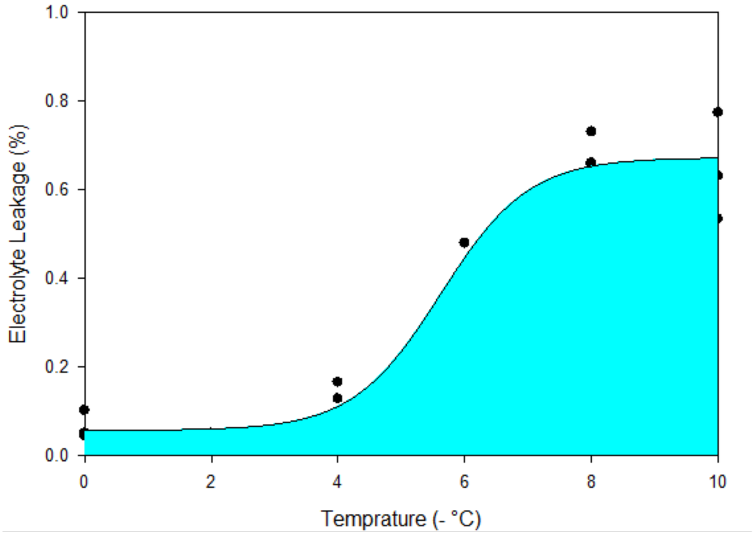

SC-6
Electrolyte Leakage (%)
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
Temprature (- °C)


SC-11
Electrolyte Leakage (%)
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
Temprature (- °C)

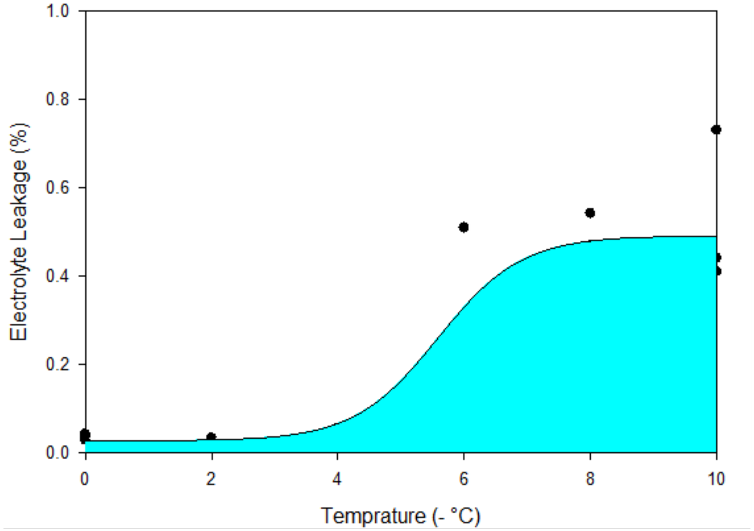

SC-10
Electrolyte Leakage (%)
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
Temprature (- °C)



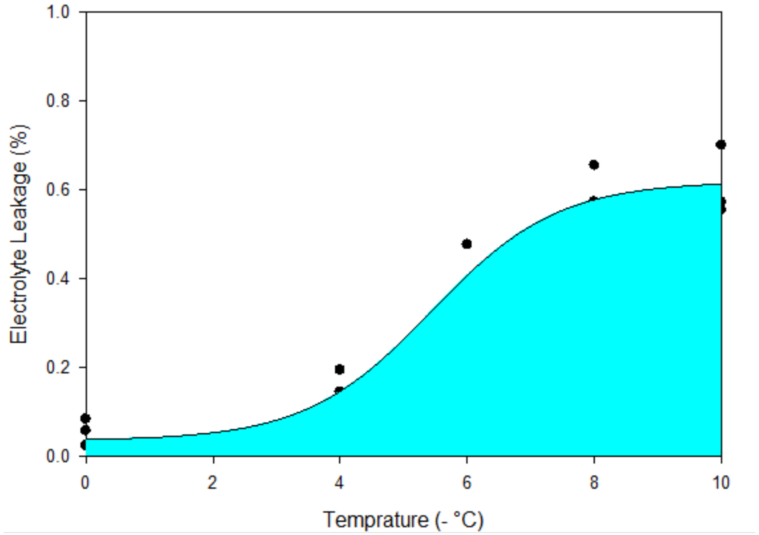
SC-13



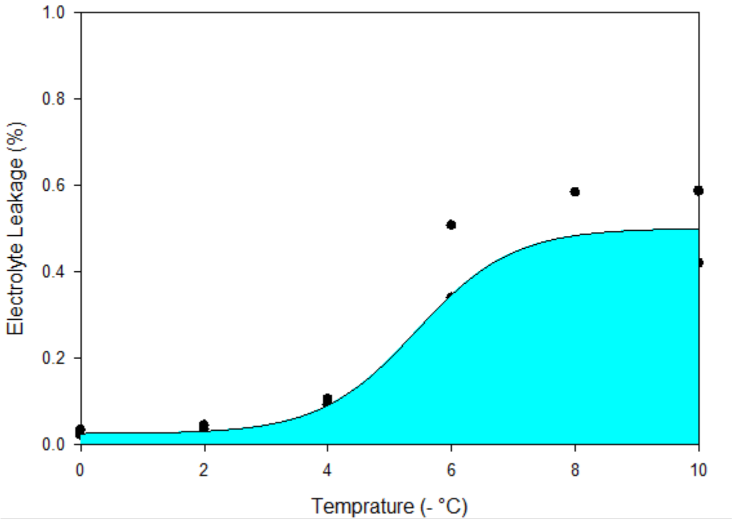
SC-2



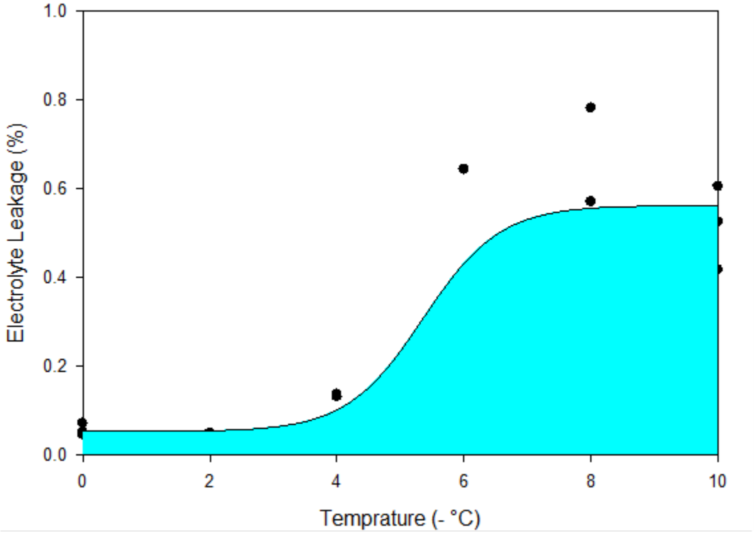
SC-7



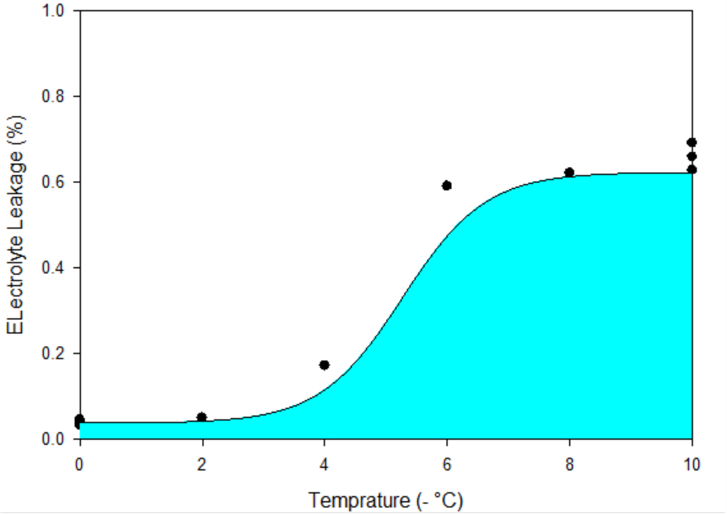
SC-14



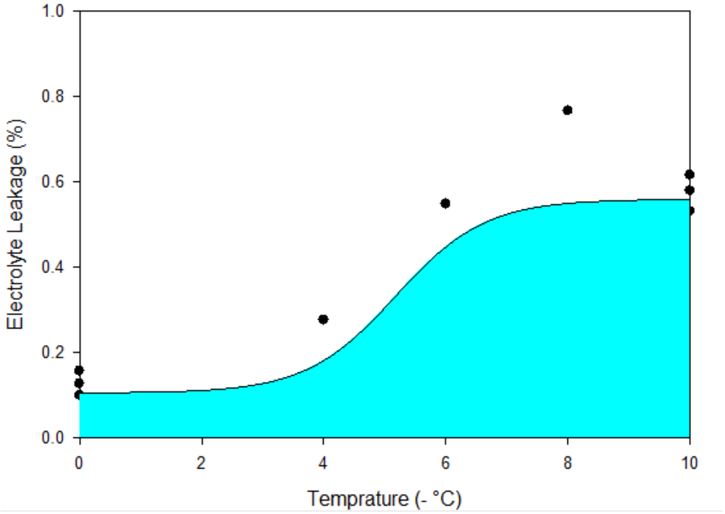
SC-8



SC-3



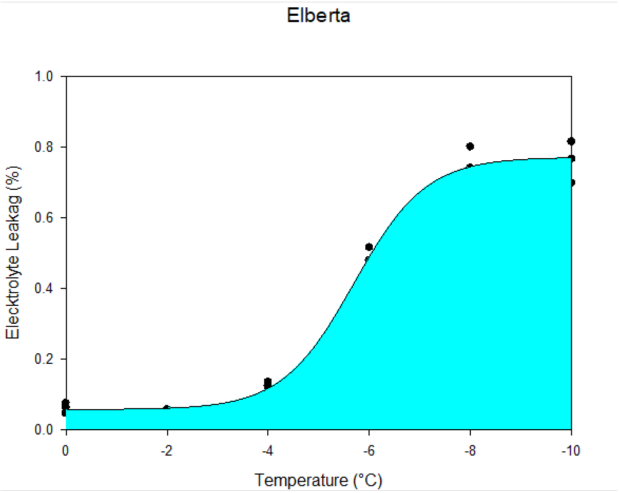
SC-15

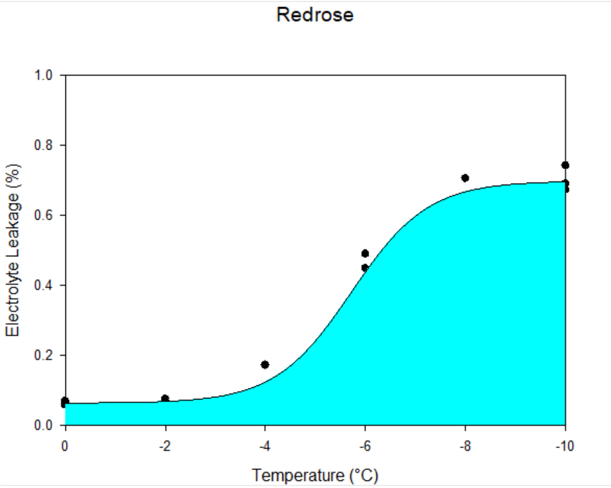


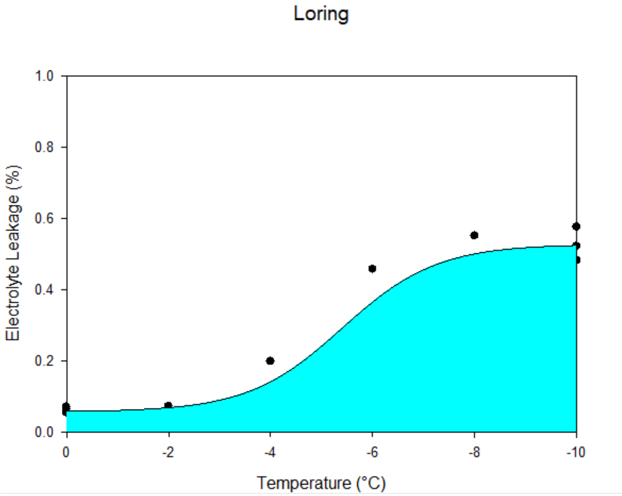
SC-5

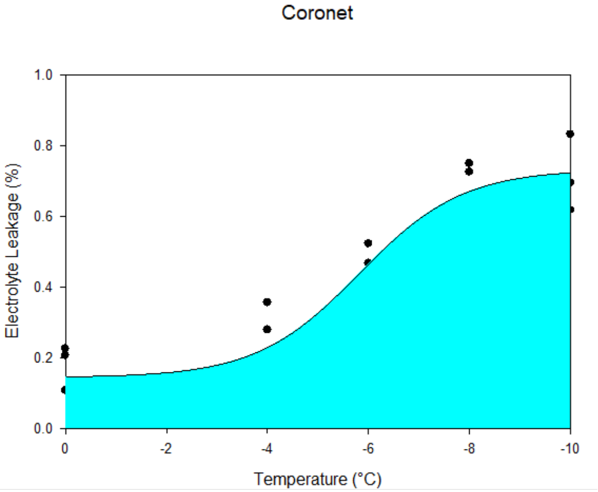
Appendix B

Fruitlet freeze response to freezing temperatures observed in peach and nectarine accessions listed by release year in 2023.

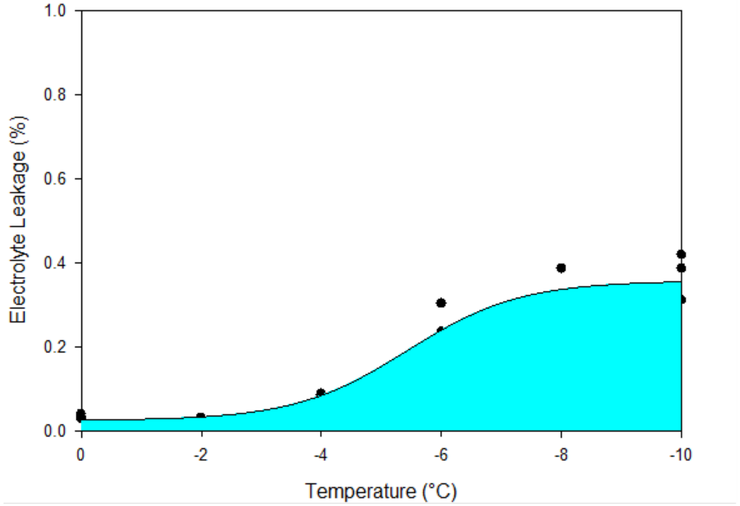




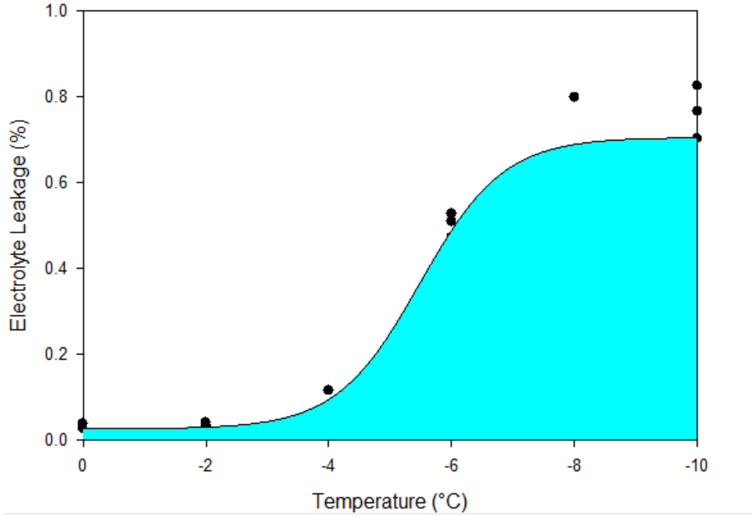




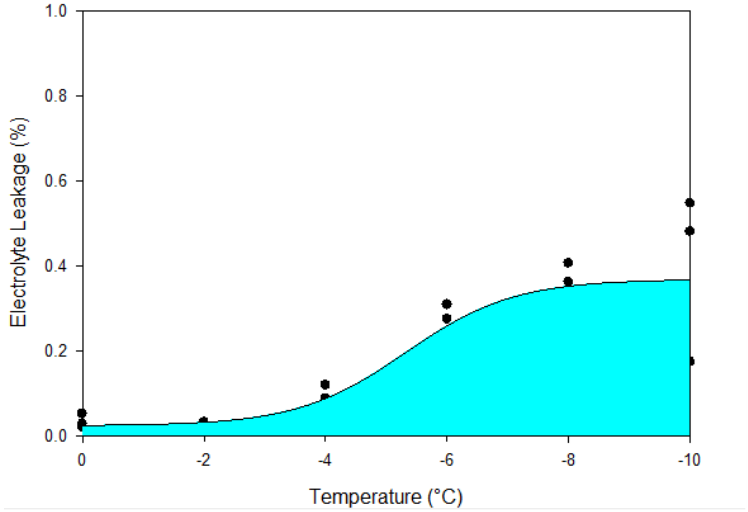
Redglobe



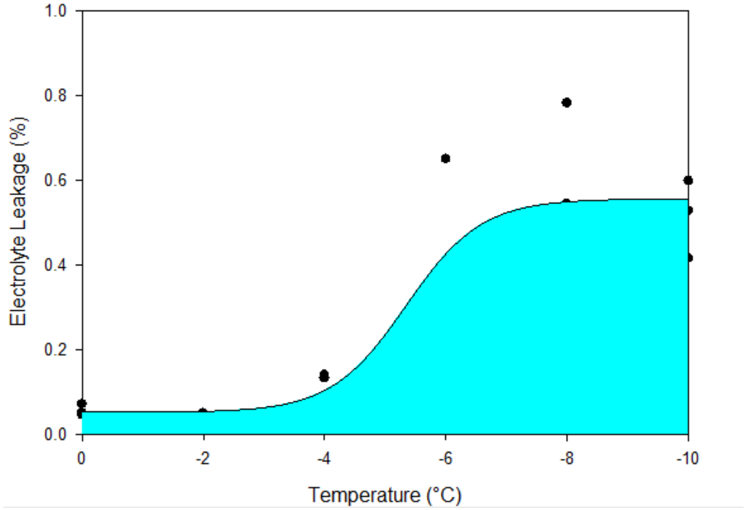
June Gold

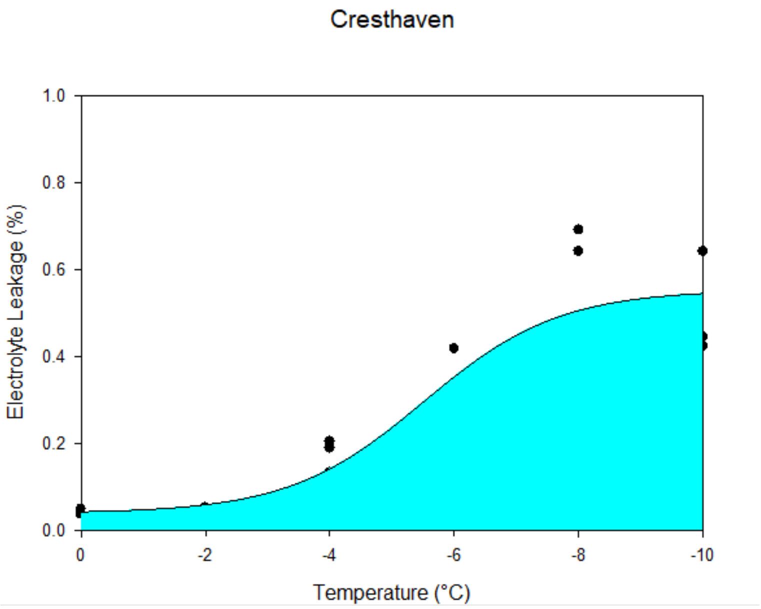


Parade

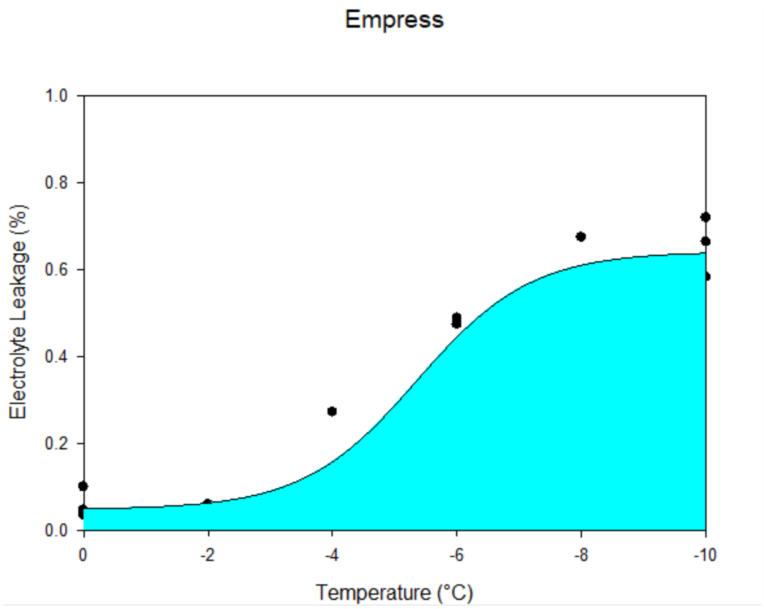


Jefferson

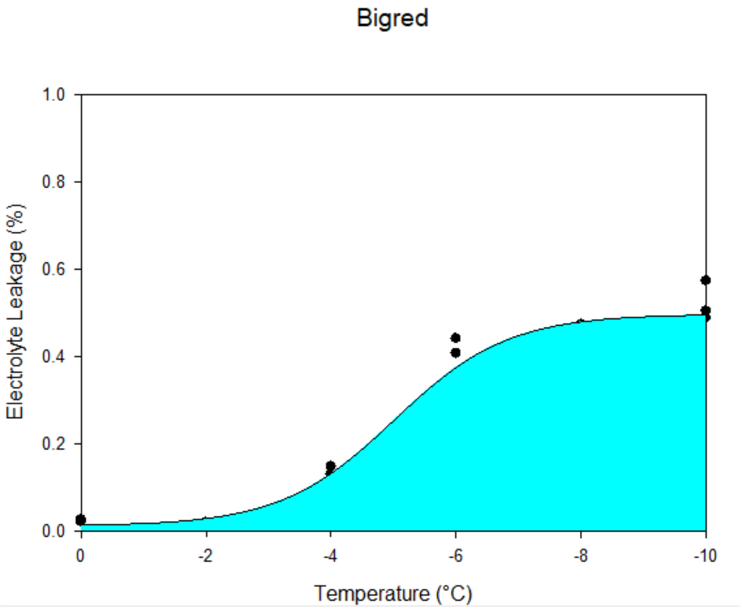




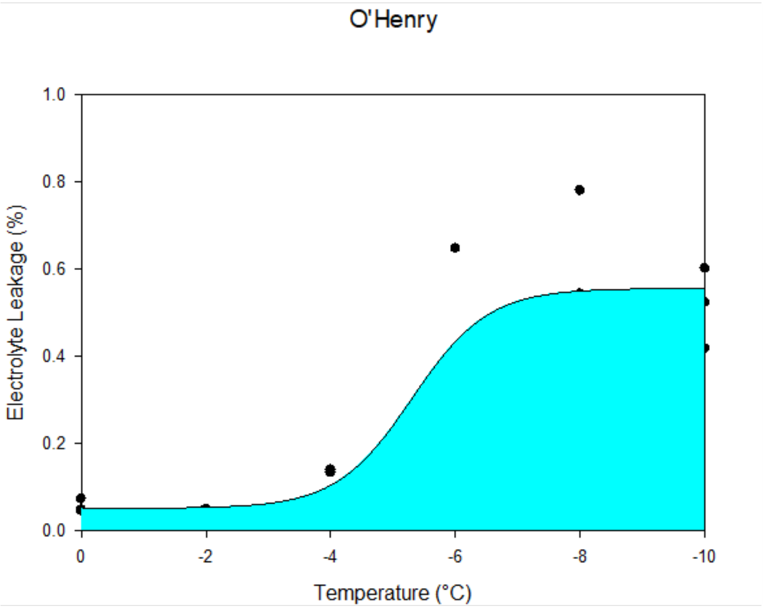
Cresthaven



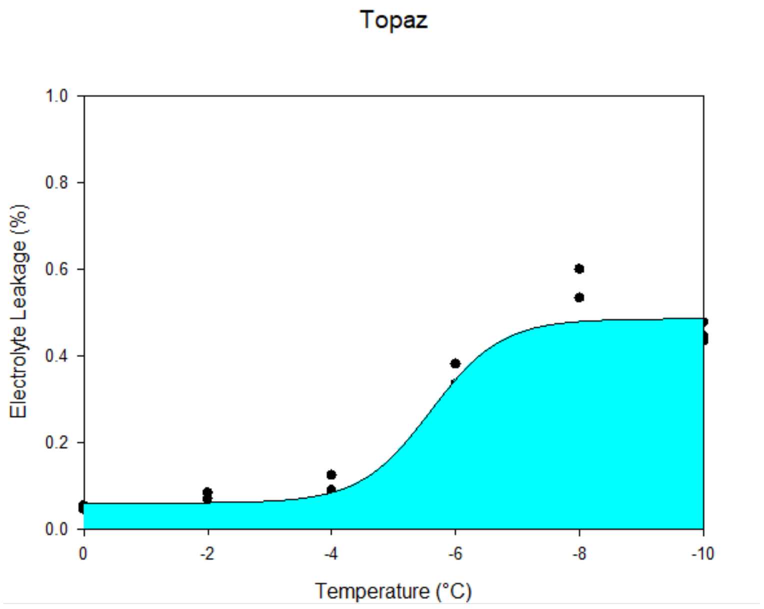
Empress

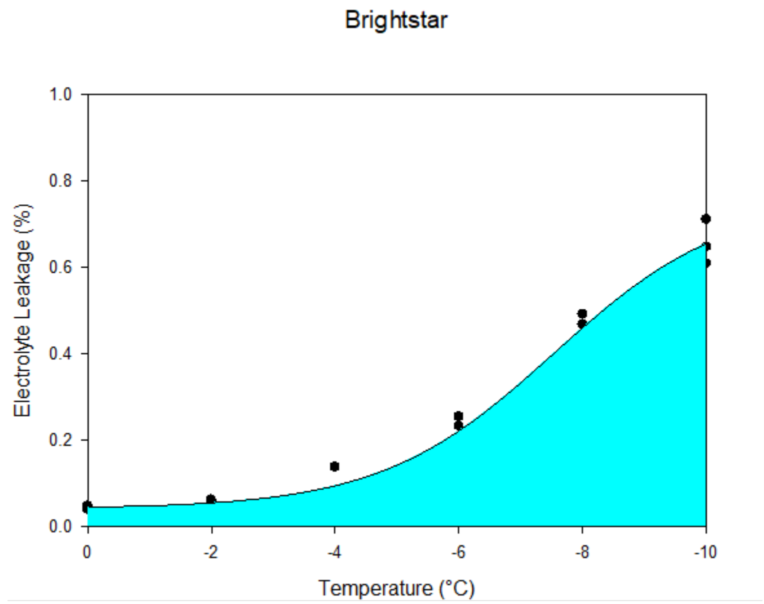


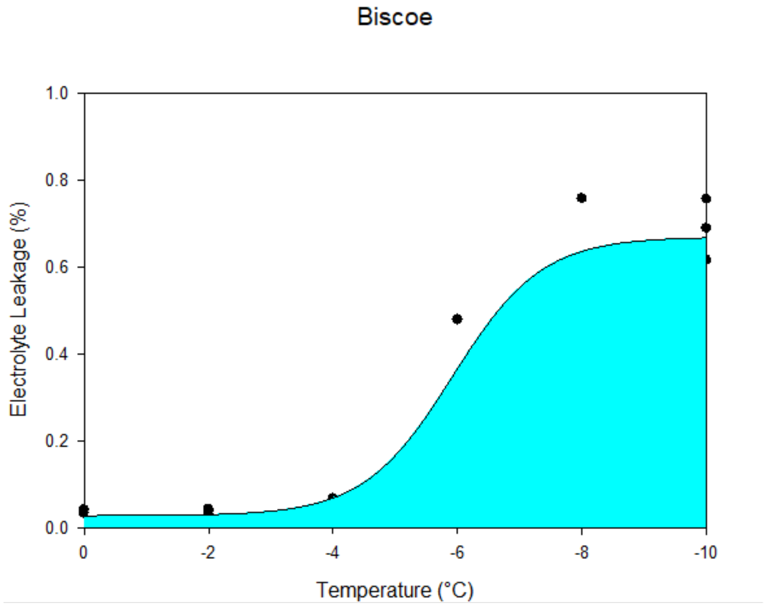
Bigred

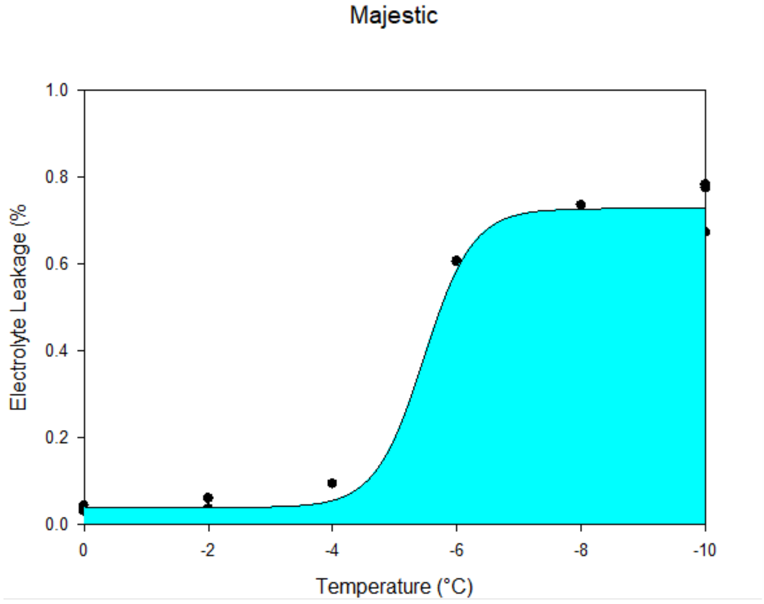


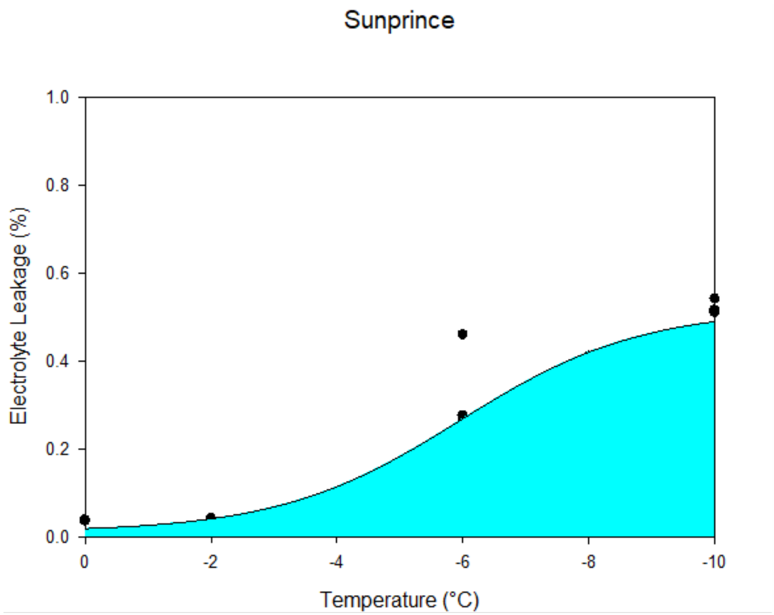
O'Henry

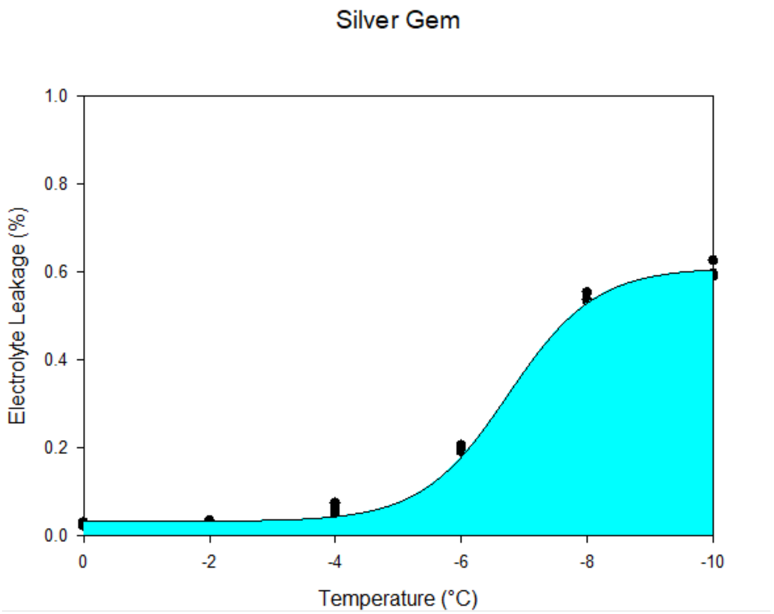

Topaz

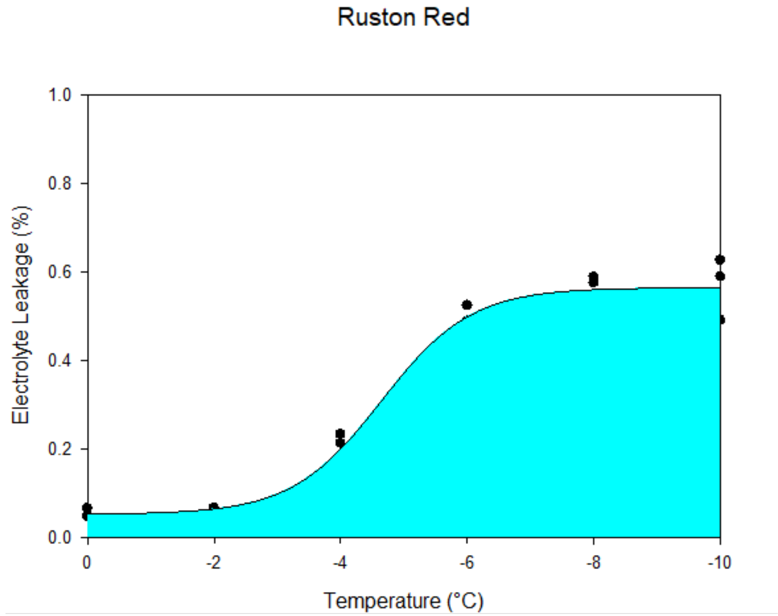

Brightstar

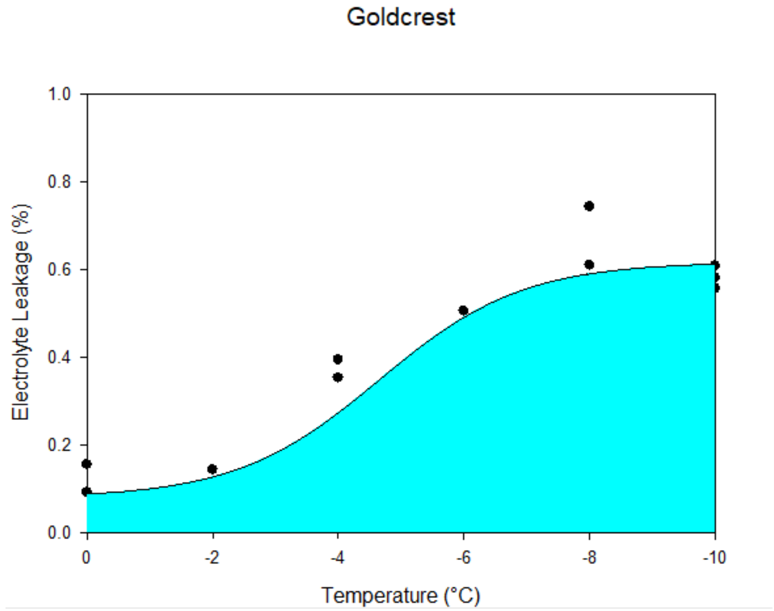

Biscoe

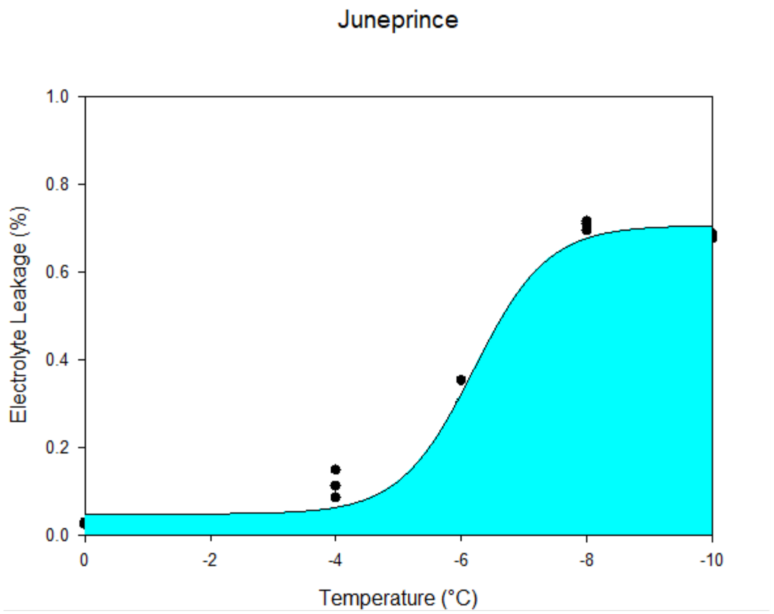

Majestic

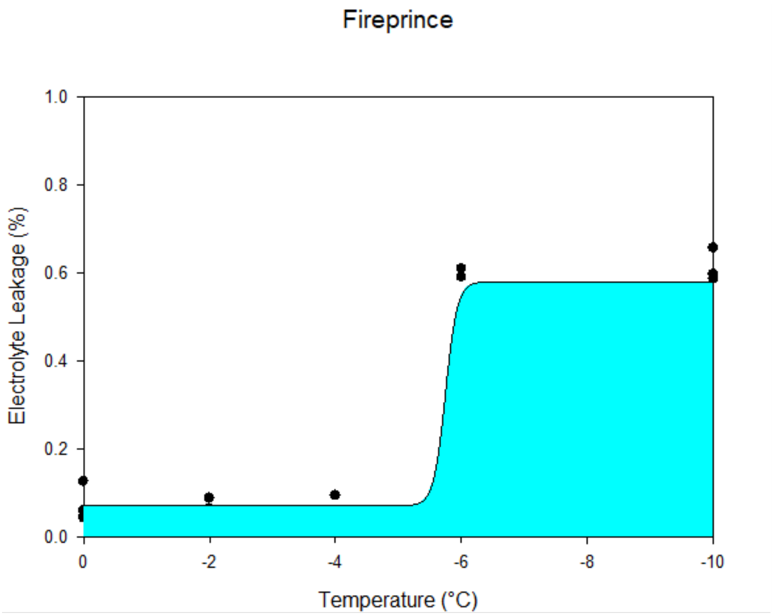

Sunprince

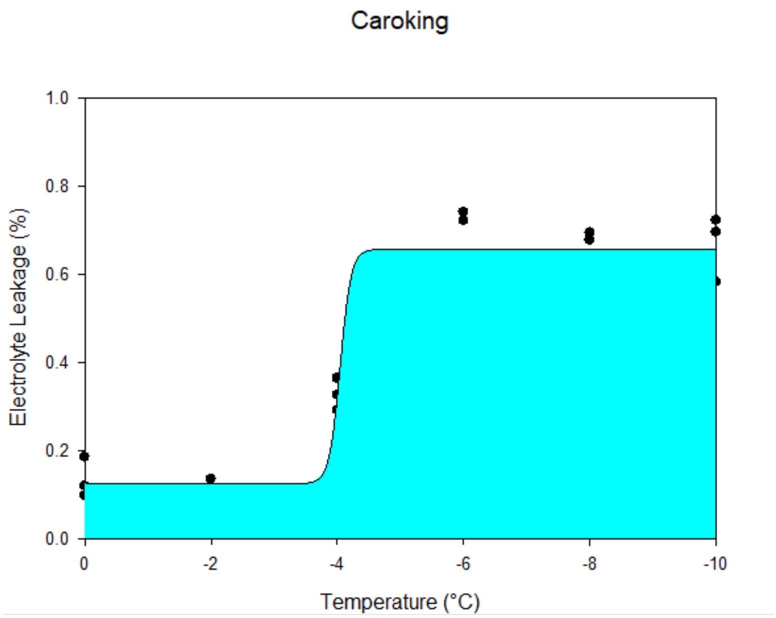

Silver Gem

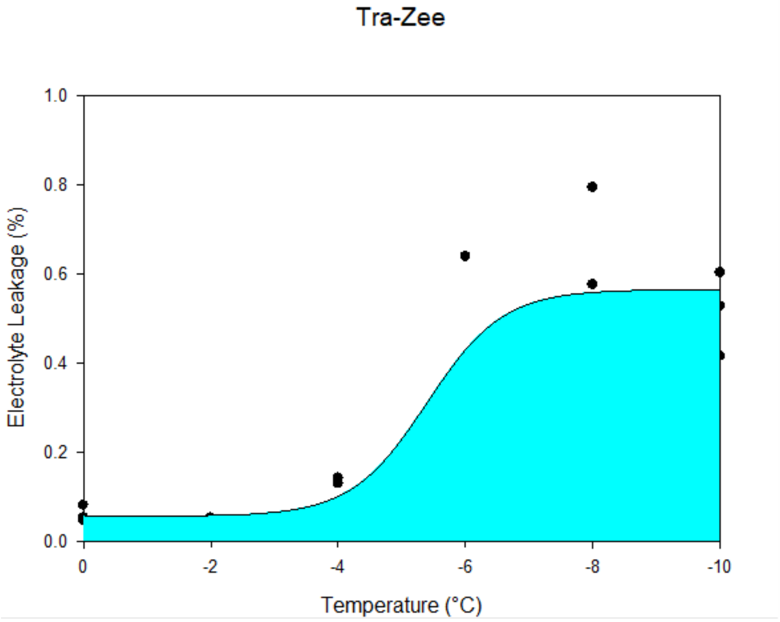

Ruston Red

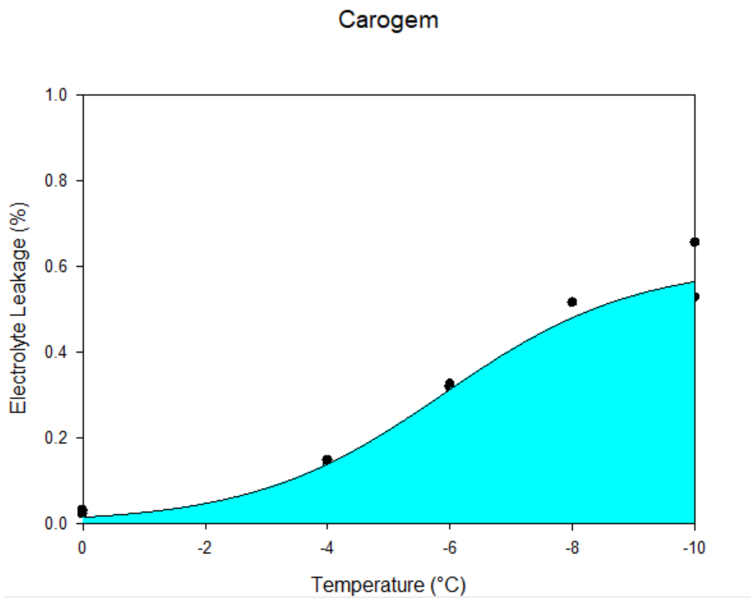

Goldcrest

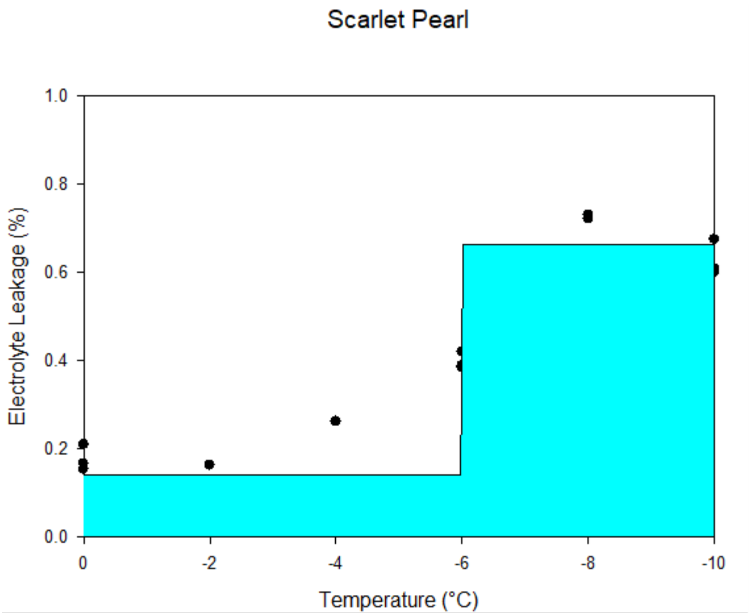

Juneprince
Electrolyte Leakage (%)
1.0
0.8
0.6
0.4
0.2
0.0
0
-2
-4
-6
-8
-10
Temperature (°C)

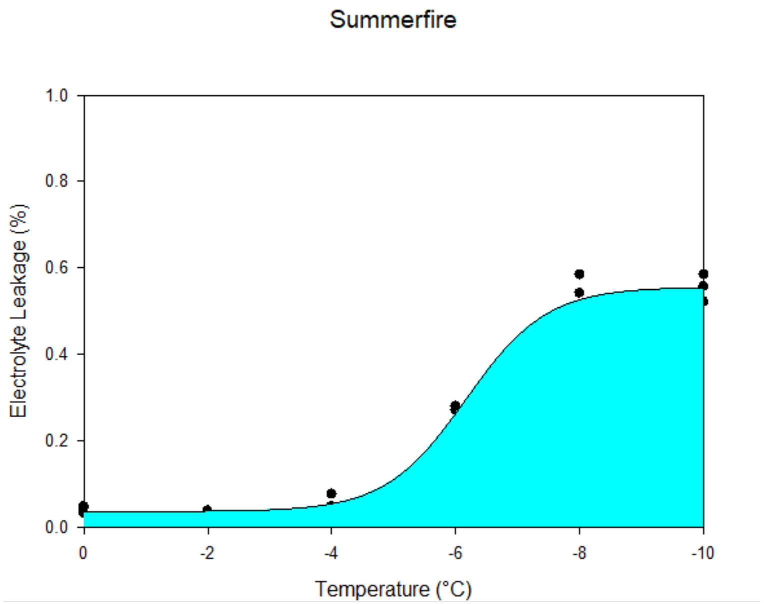

Fireprince
Electrolyte Leakage (%)
1.0
0.8
0.6
0.4
0.2
0.0
0
-2
-4
-6
-8
-10
Temperature (°C)

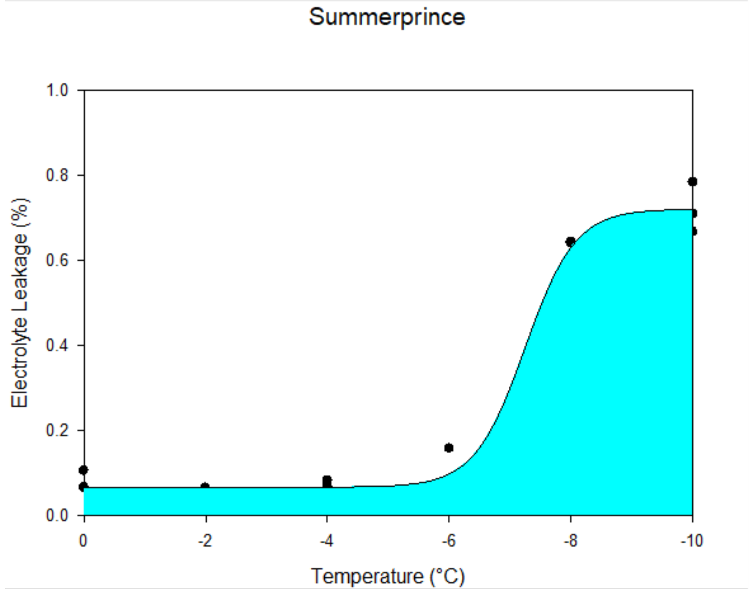

Caroking
Electrolyte Leakage (%)
1.0
0.8
0.6
0.4
0.2
0.0
0
-2
-4
-6
-8
-10
Temperature (°C)

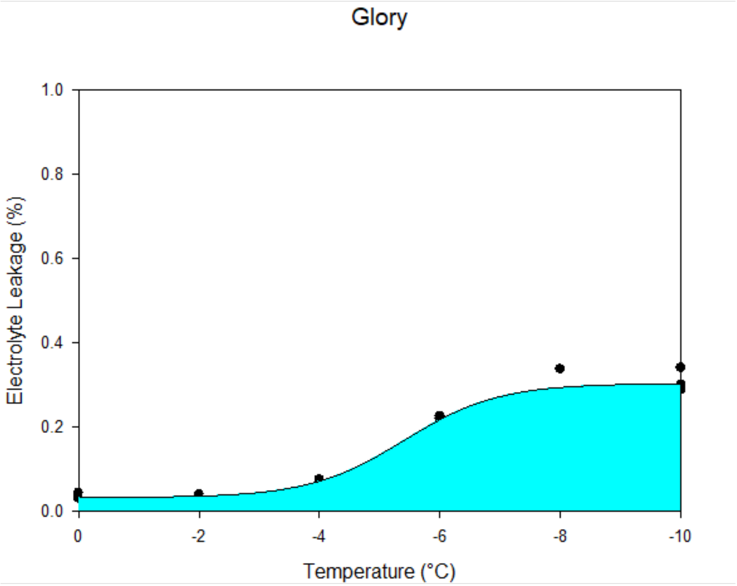

Tra-Zee
Electrolyte Leakage (%)
1.0
0.8
0.6
0.4
0.2
0.0
0
-2
-4
-6
-8
-10
Temperature (°C)

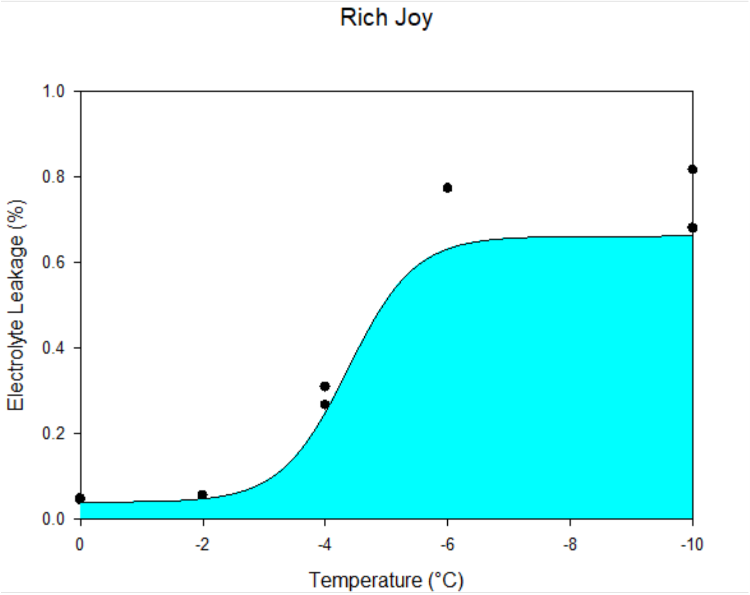

Carogem

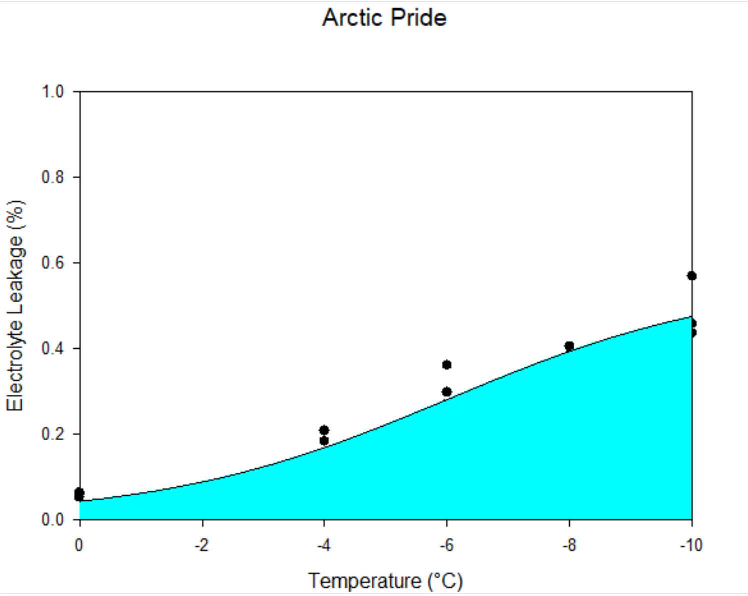

Scarlet Pearl

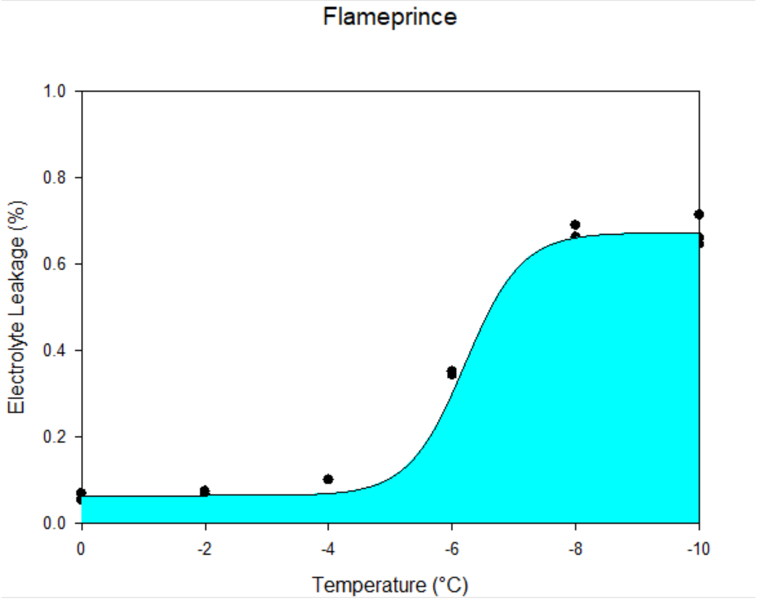

Summerfire

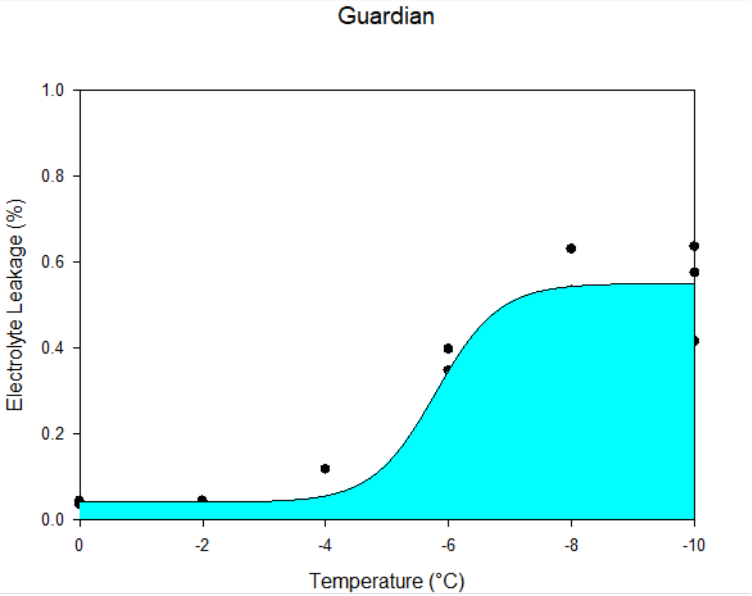

Summerprince


Glory

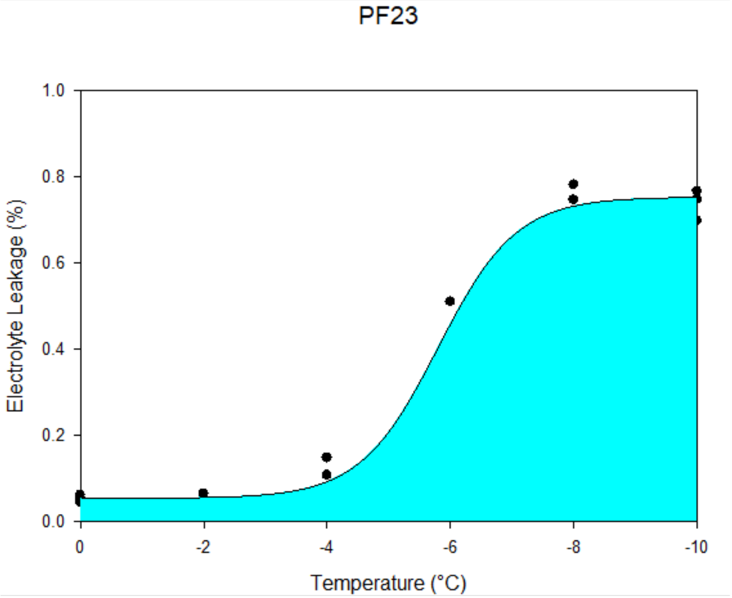

Rich Joy


Arctic Pride

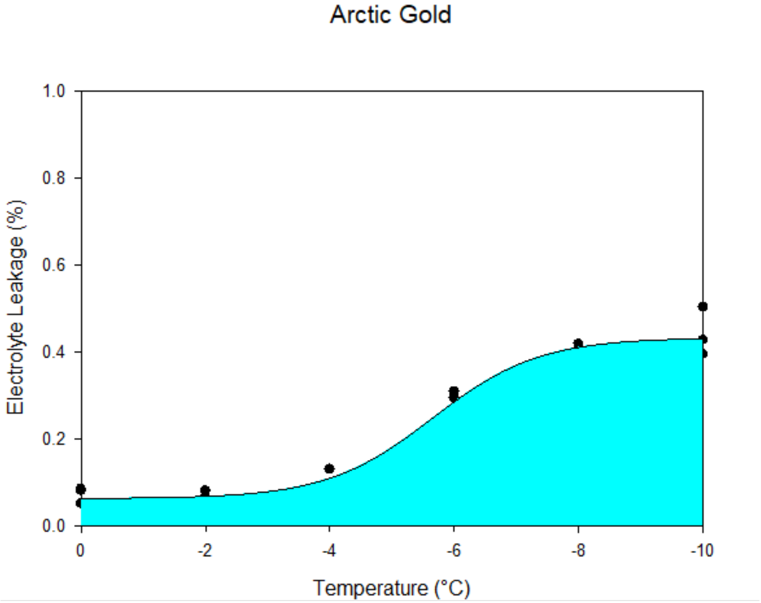

Flameprince



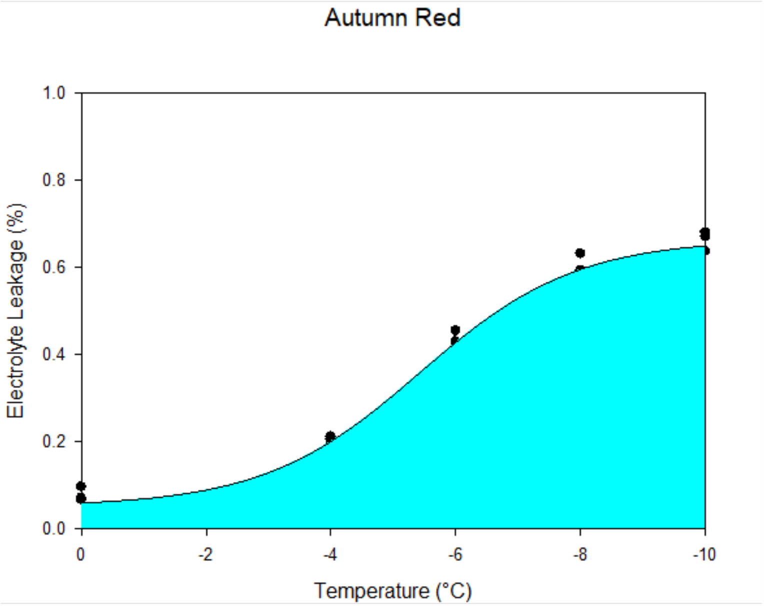
Guardian



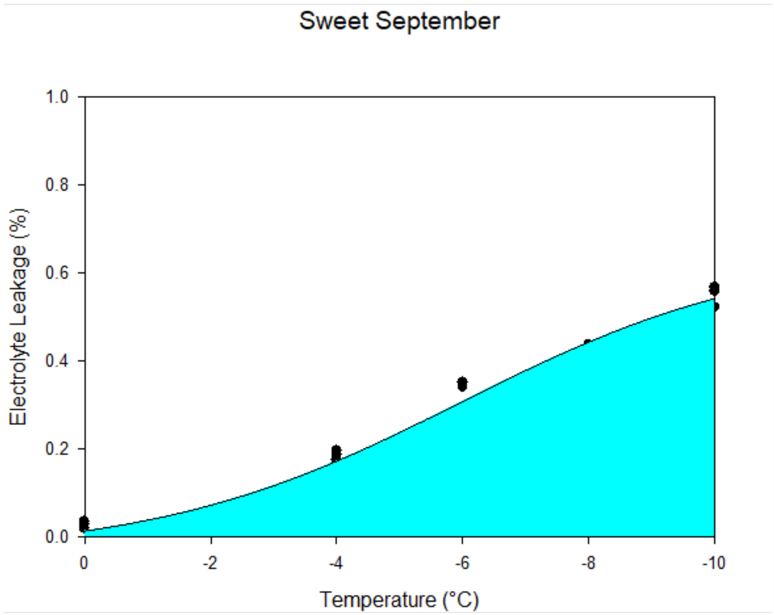
PF23

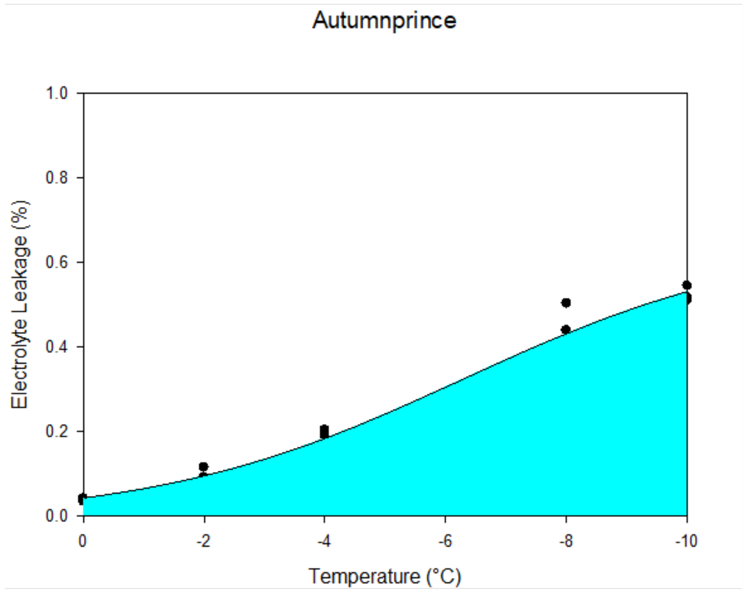


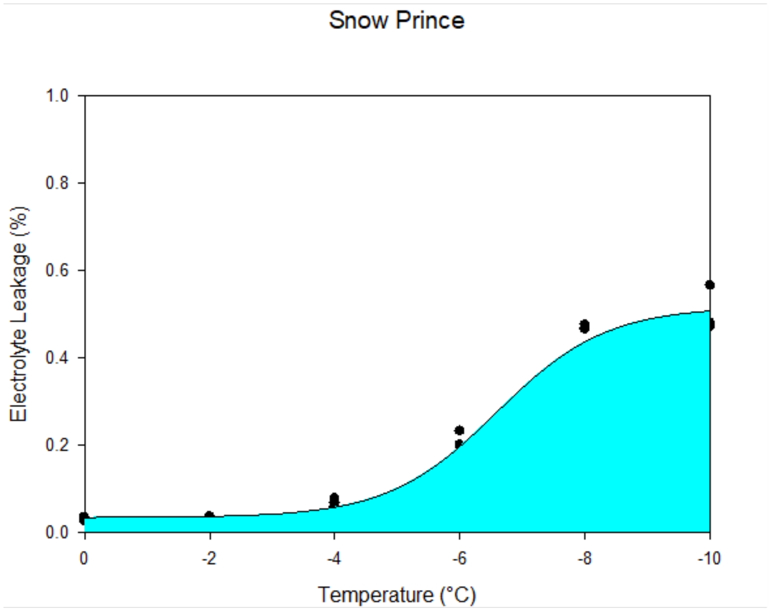
Arctic Gold

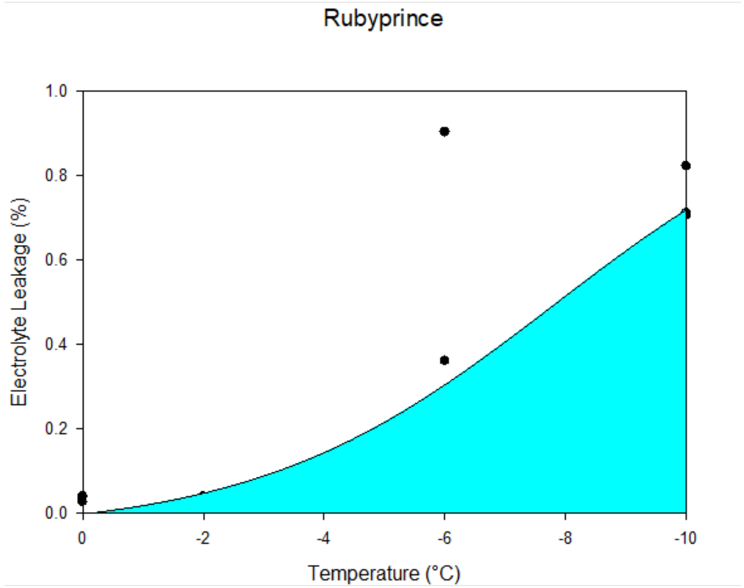


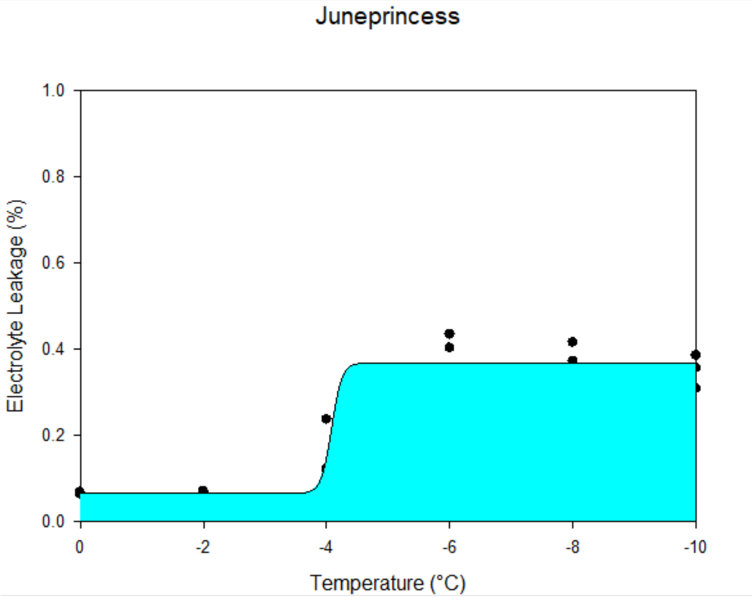
Autumn Red

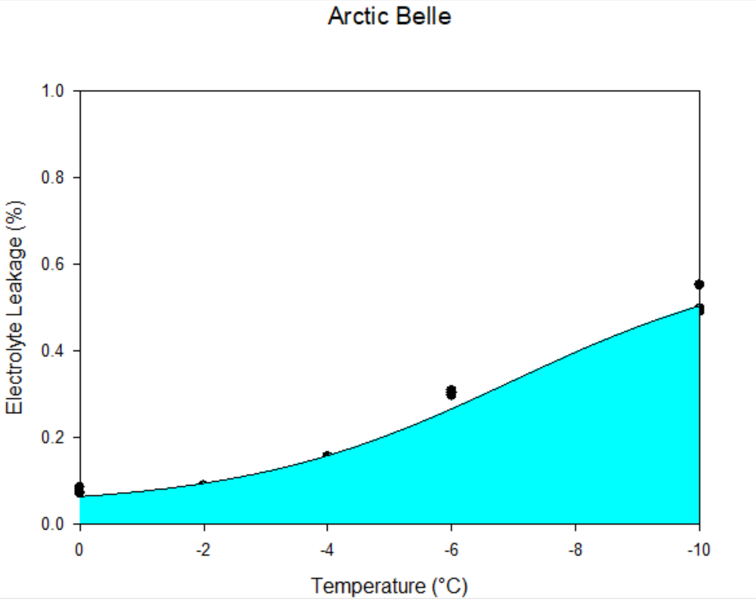

Sweet September

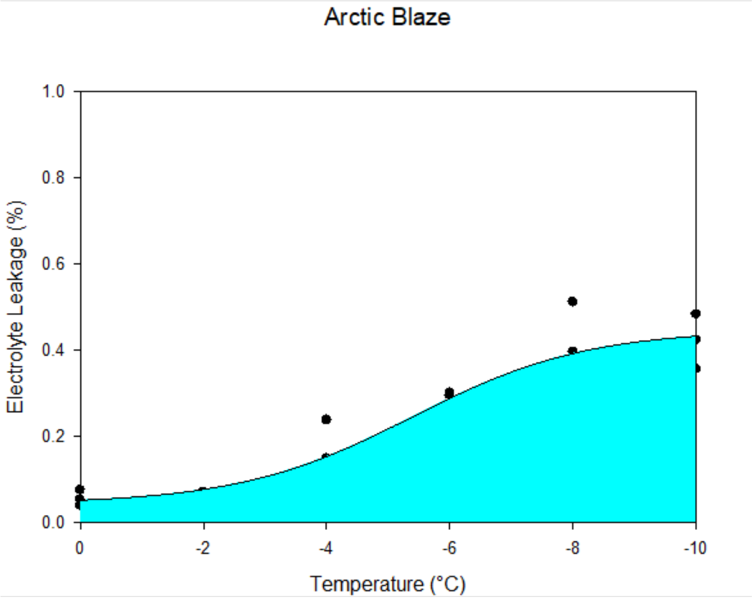

Autumnprince

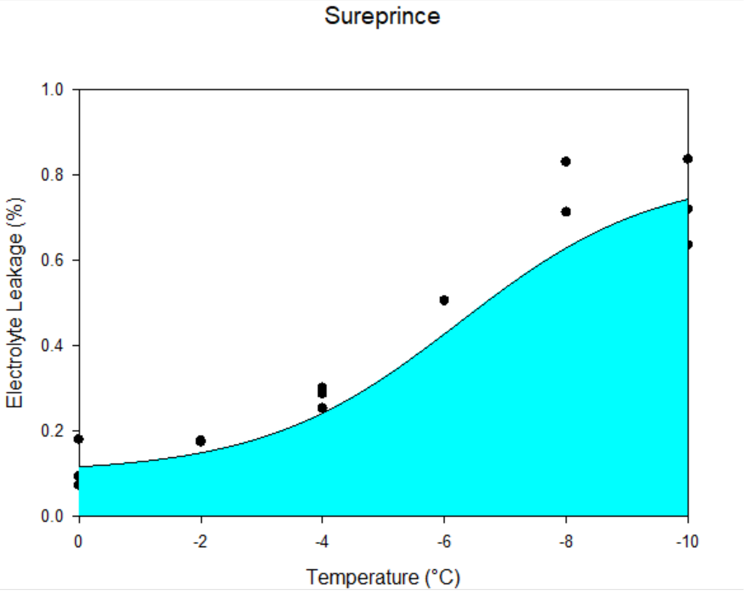

Snow Prince

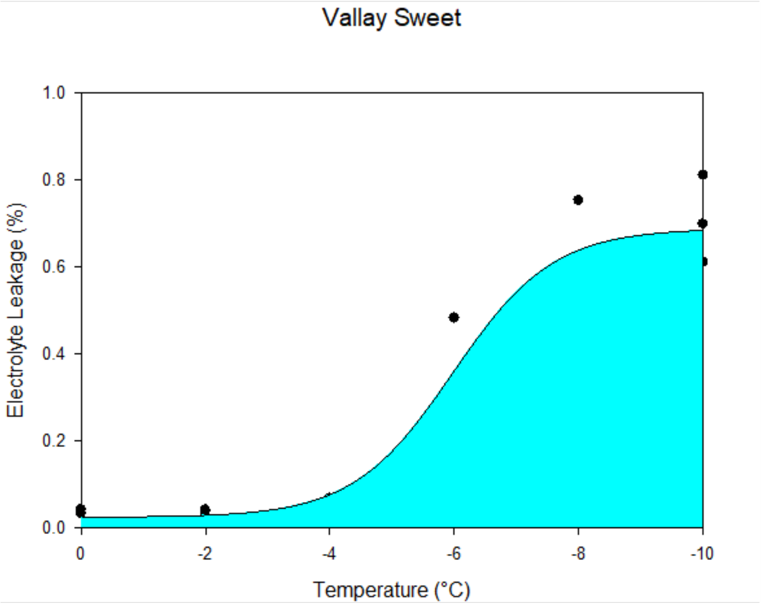

Rubyprince

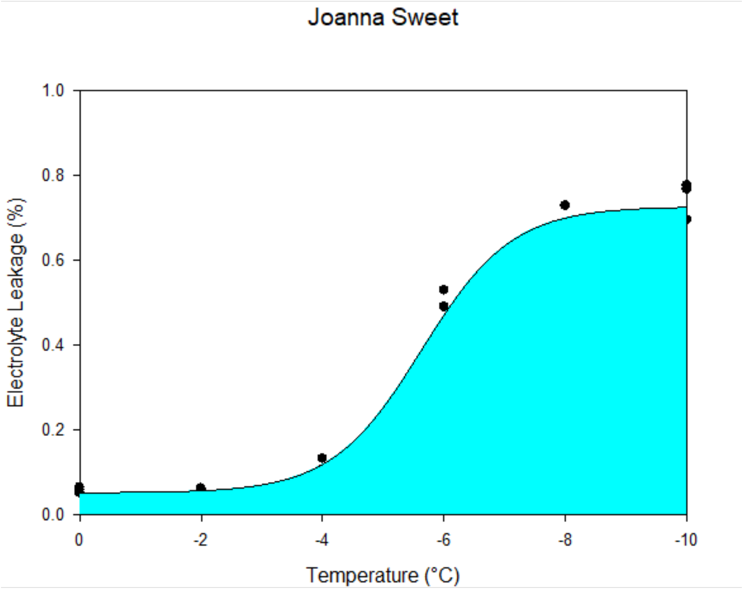

Juneprincess
Electrolyte Leakage (%)
1.0
0.8
0.6
0.4
0.2
0.0
0
-2
-4
-6
-8
-10
Temperature (°C)

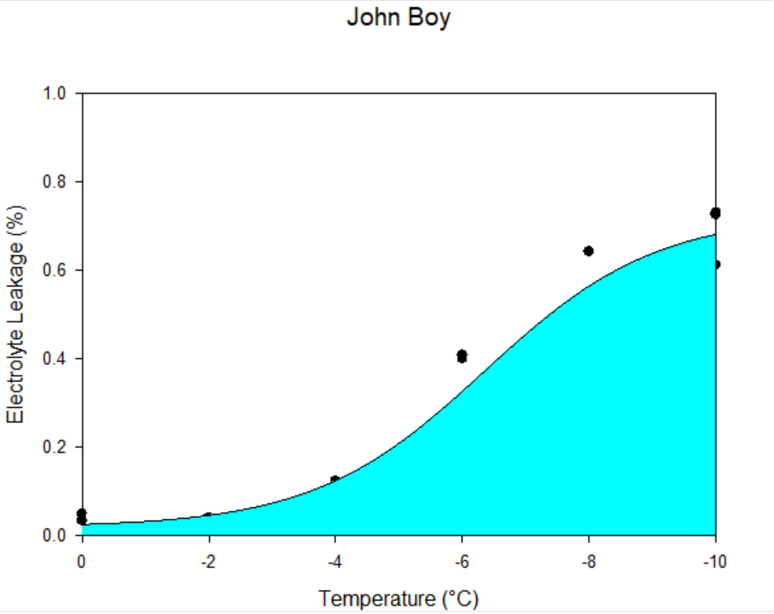

Arctic Belle
Electrolyte Leakage (%)
1.0
0.8
0.6
0.4
0.2
0.0
0
-2
-4
-6
-8
-10
Temperature (°C)

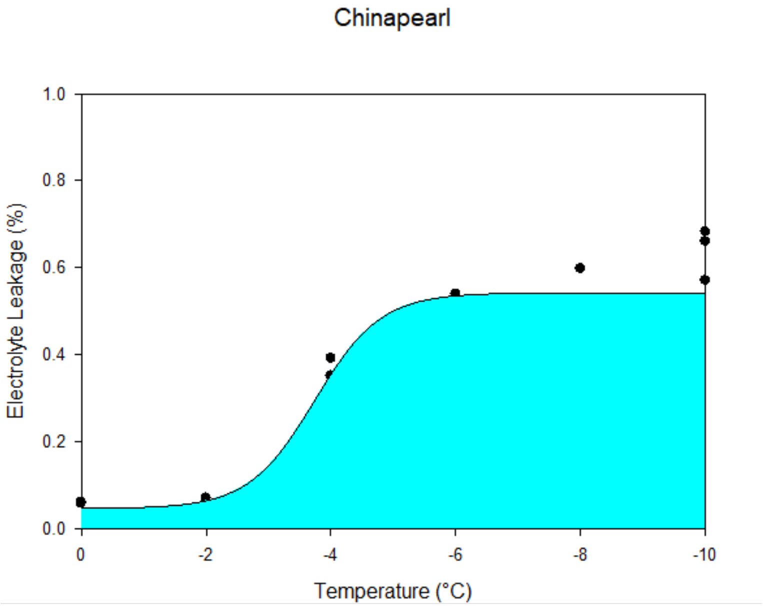

Arctic Blaze
Electrolyte Leakage (%)
1.0
0.8
0.6
0.4
0.2
0.0
0
-2
-4
-6
-8
-10
Temperature (°C)

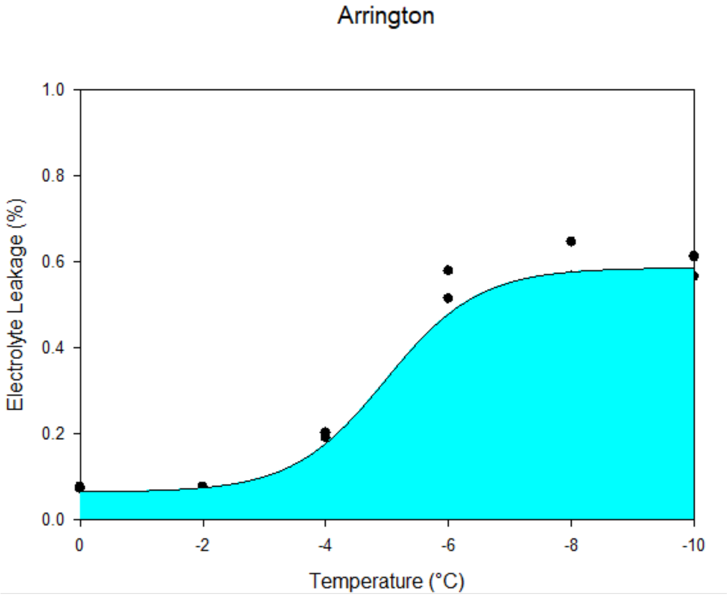

Sureprince
Electrolyte Leakage (%)
1.0
0.8
0.6
0.4
0.2
0.0
0
-2
-4
-6
-8
-10
Temperature (°C)

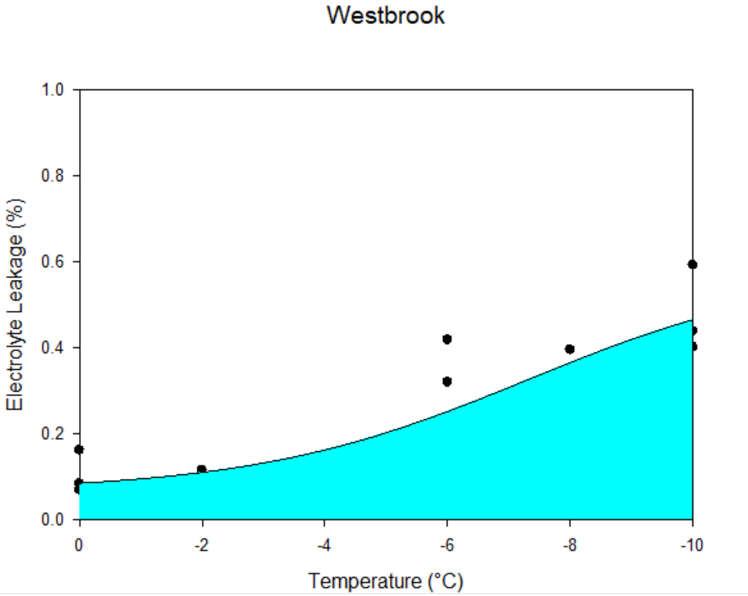

Vallay Sweet
1.0
0.8
0.6
0.4
0.2
0.0
Electrolyte Leakage (%)
0
-2
-4
-6
-8
-10
Temperature (°C)

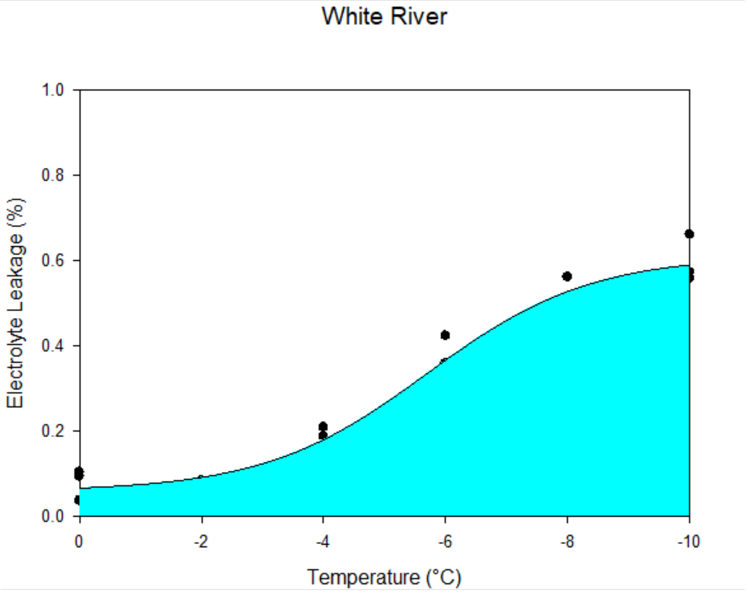

Joanna Sweet
1.0
0.8
0.6
0.4
0.2
0.0
Electrolyte Leakage (%)
0
-2
-4
-6
-8
-10
Temperature (°C)

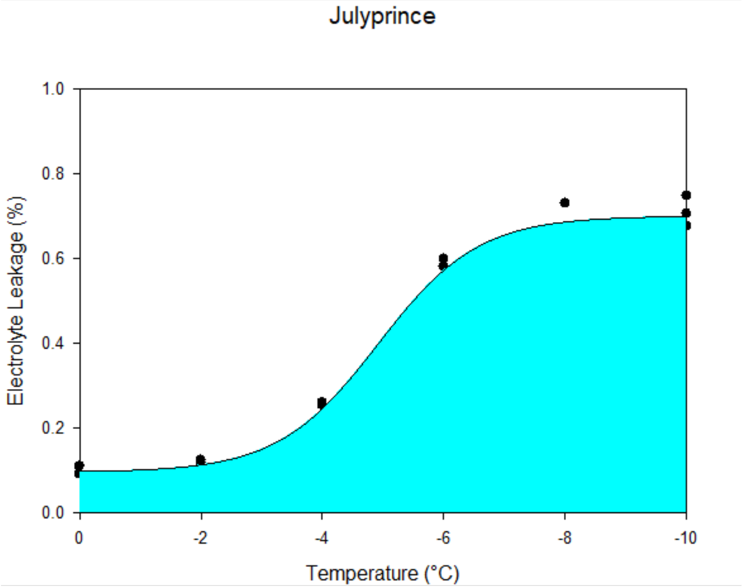

John Boy
1.0
0.8
0.6
0.4
0.2
0.0
Electrolyte Leakage (%)
0
-2
-4
-6
-8
-10
Temperature (°C)

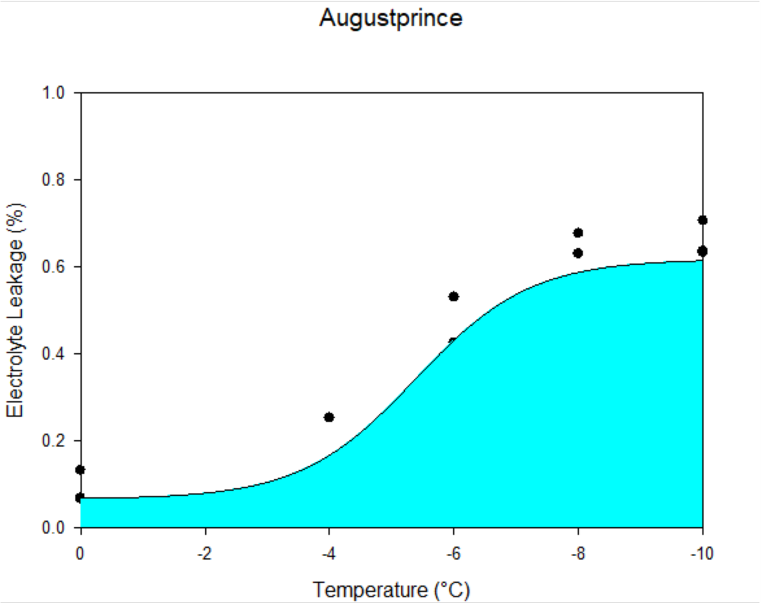

Chinapearl
1.0
0.8
0.6
0.4
0.2
0.0
Electrolyte Leakage (%)
0
-2
-4
-6
-8
-10
Temperature (°C)

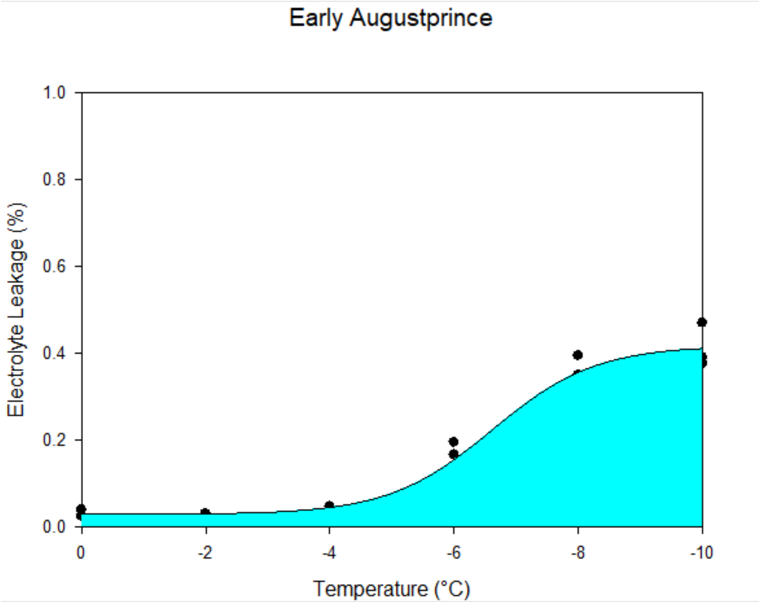

Arrington

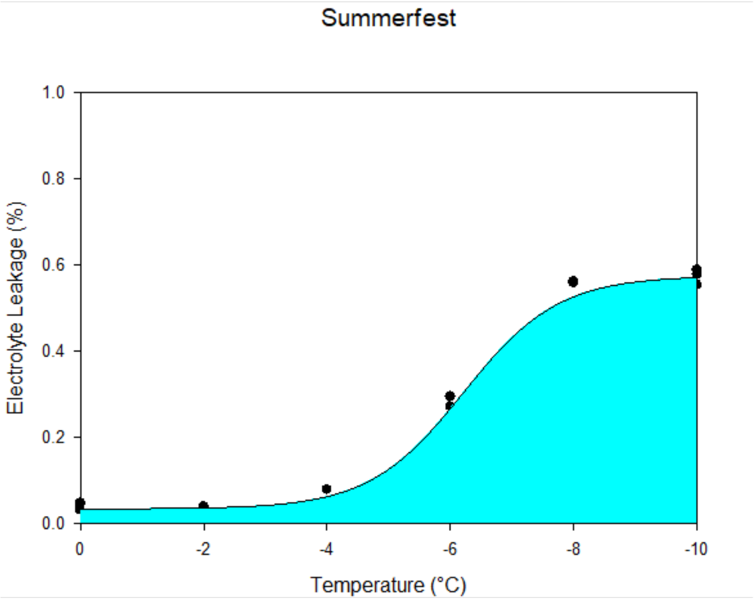

Westbrook

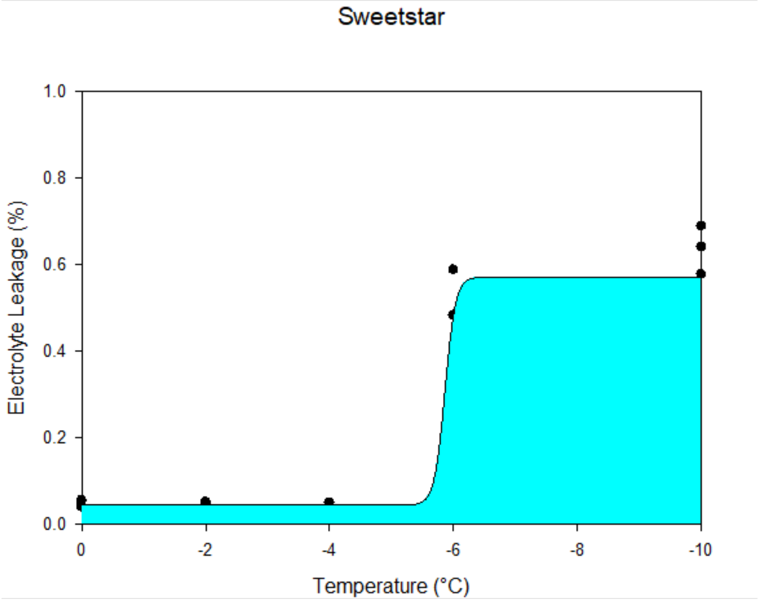

White River

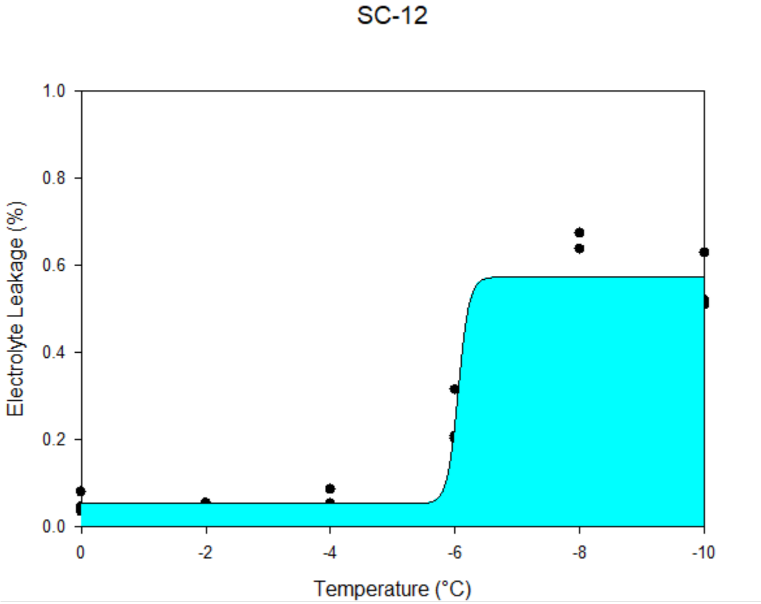

Julyprince

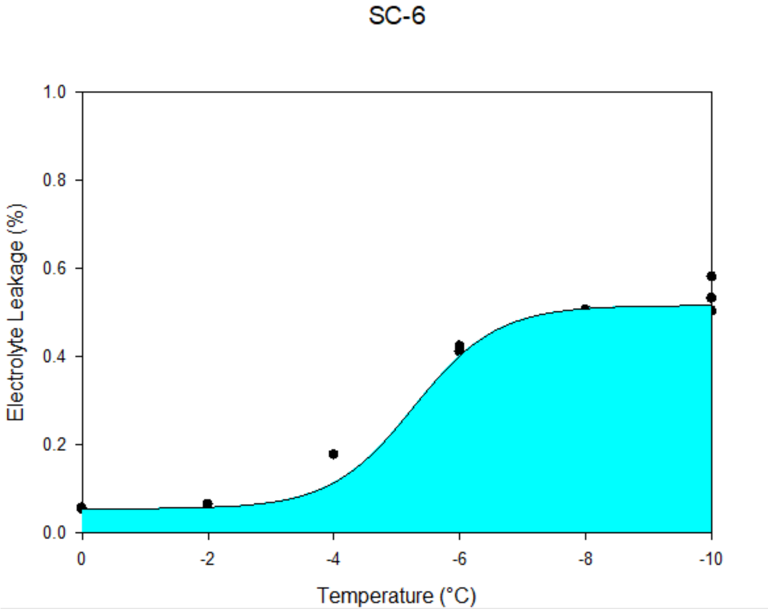

Augustprince

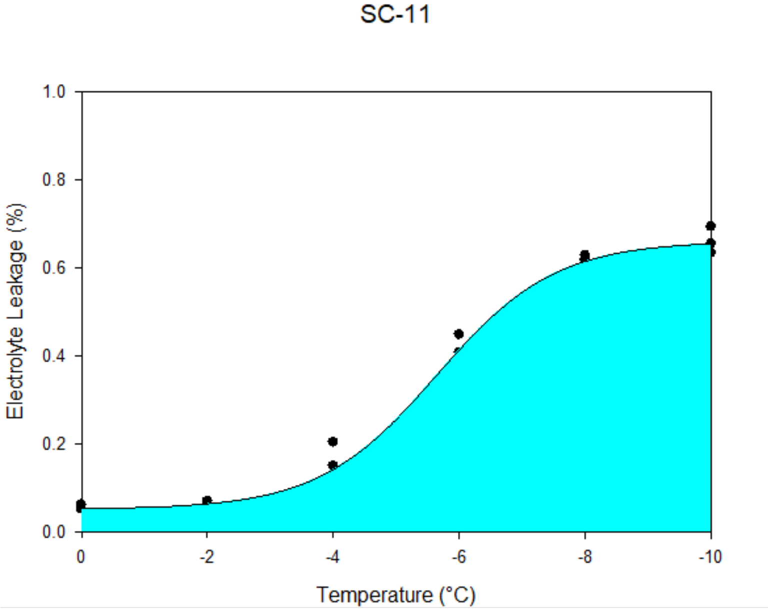

Early Augustprince

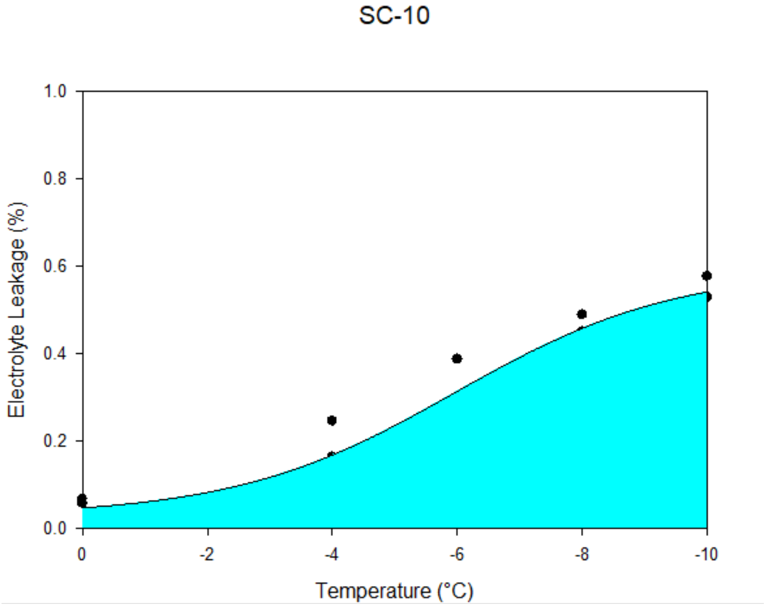

Summerfest

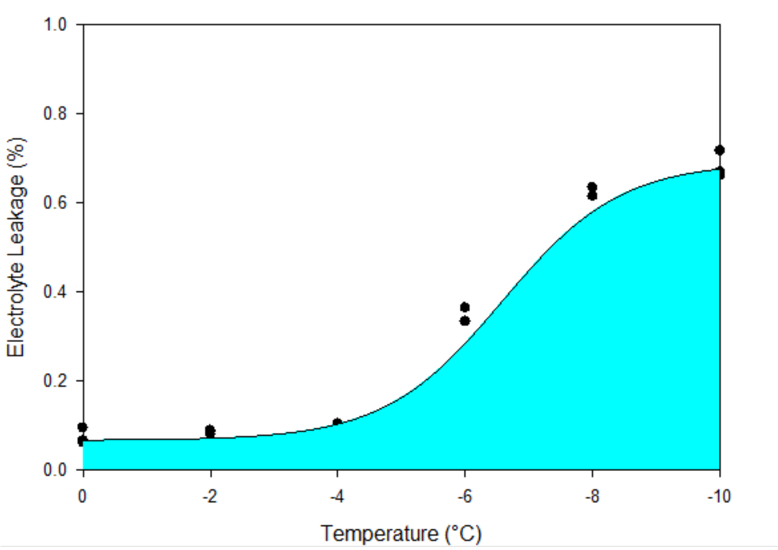

Sweetstar


SC-12

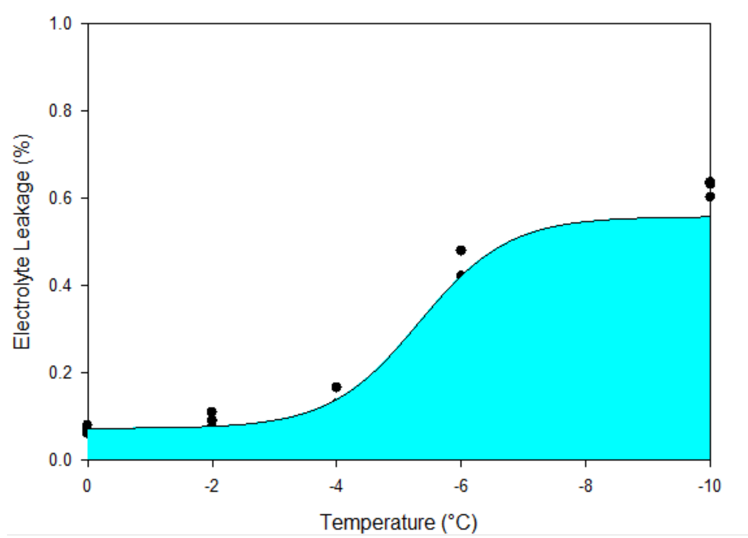

SC-6


SC-11

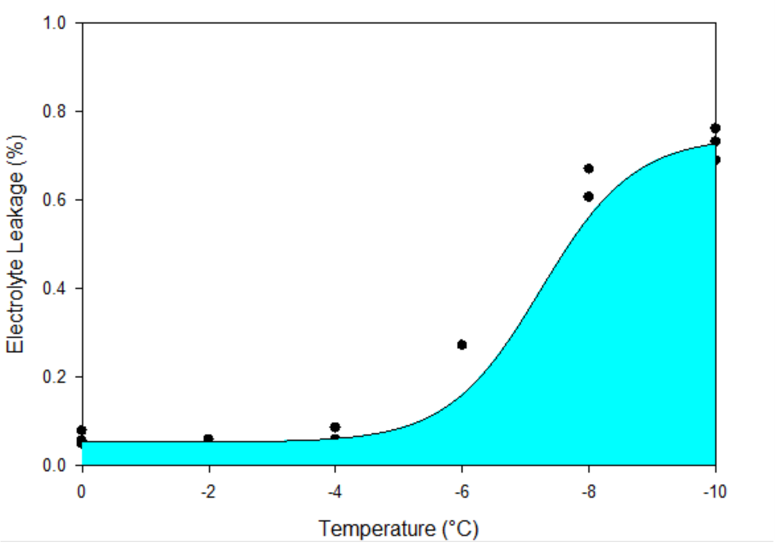

SC-10



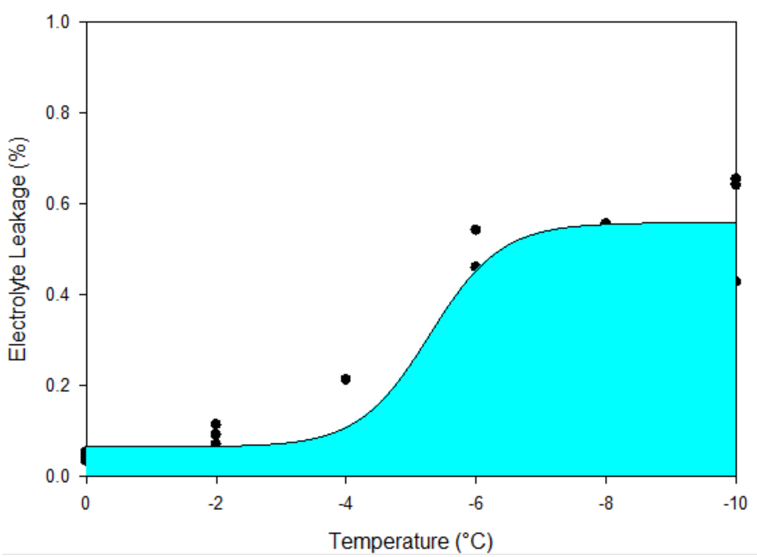
SC-13



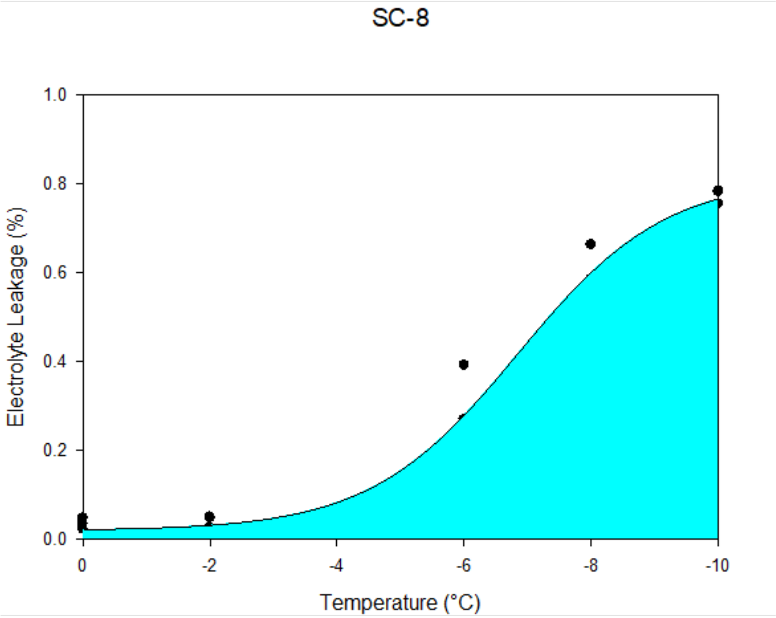
SC-2

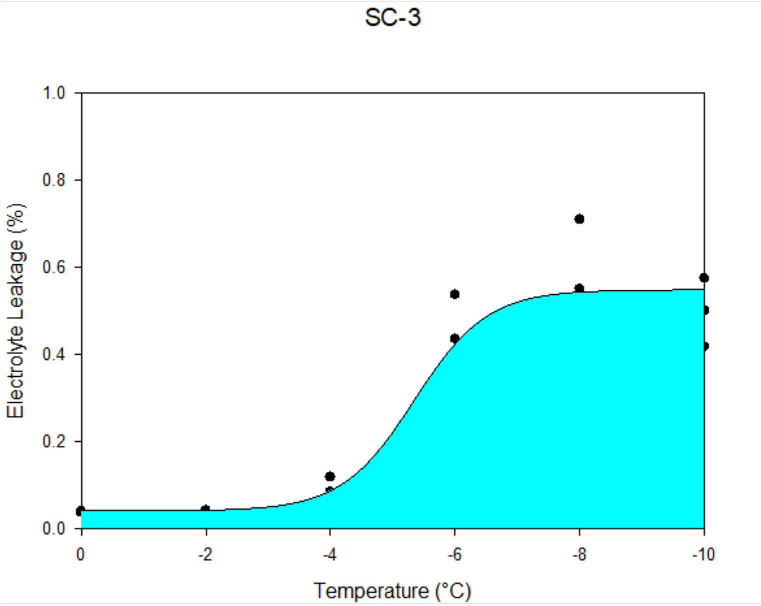


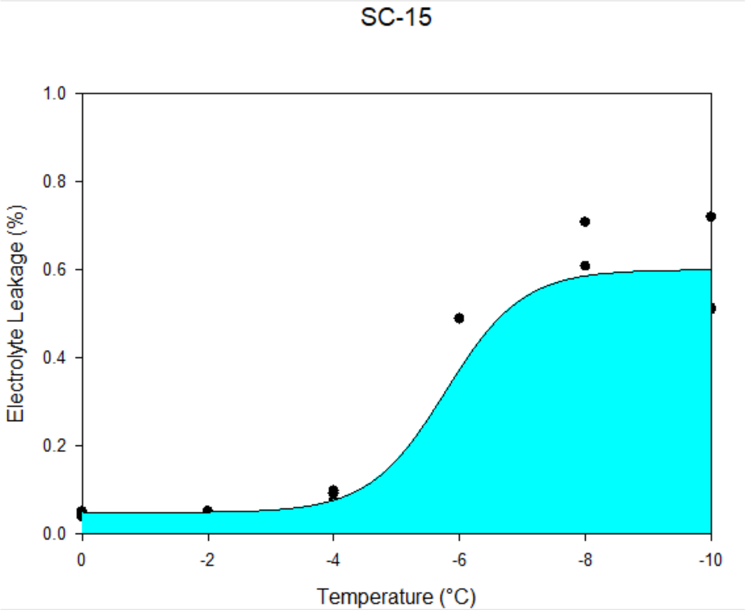
SC-7

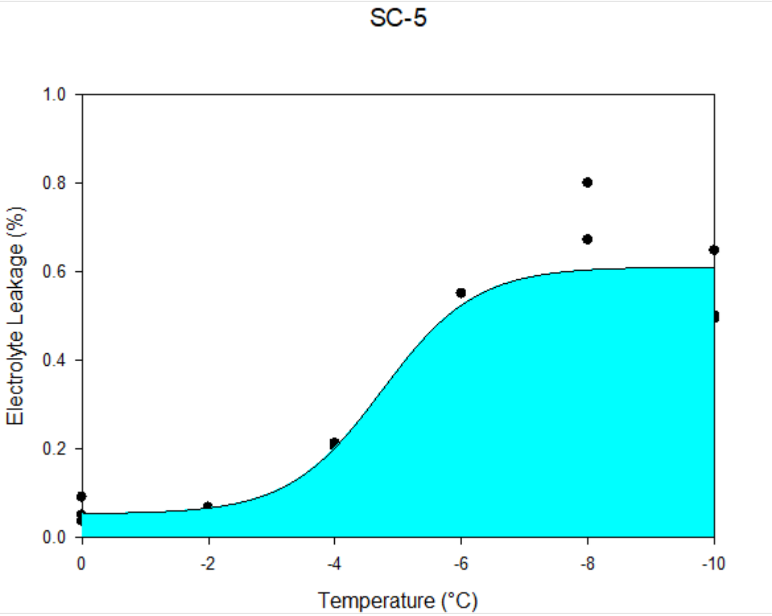


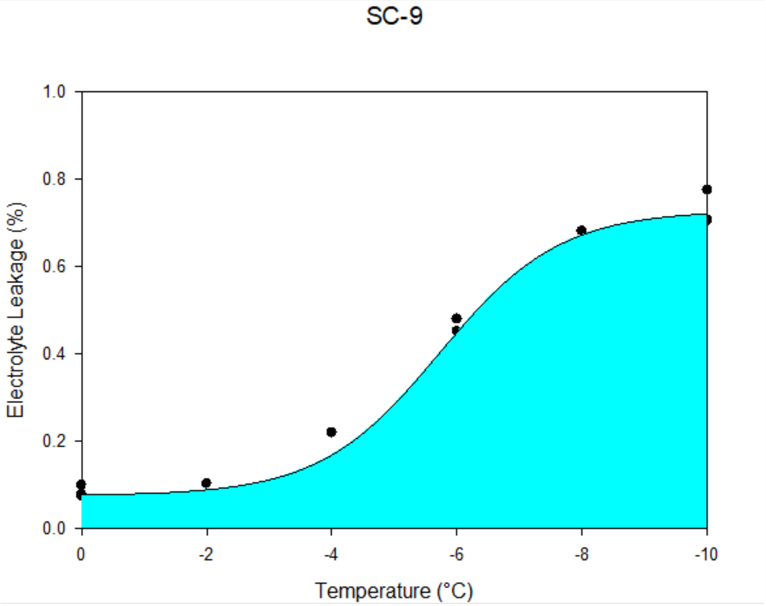
SC-14

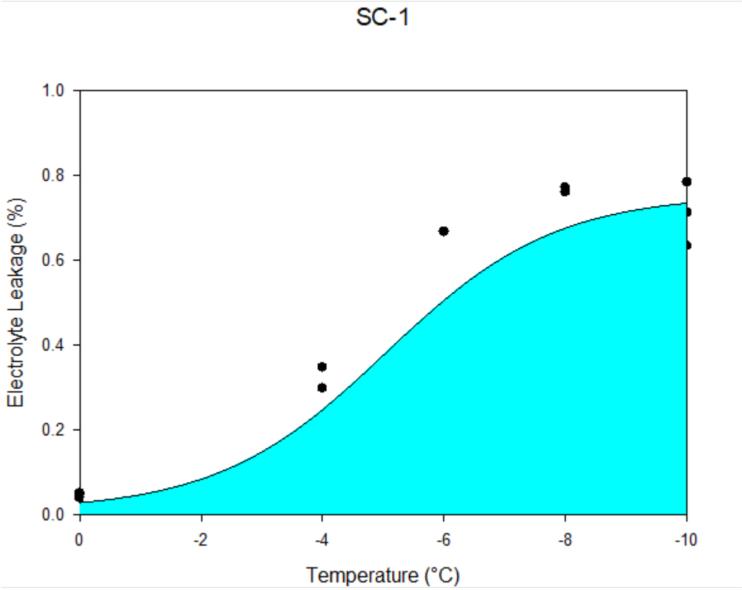

SC-8
Electrolyte Leakage (%)
1.0
0.8
0.6
0.4
0.2
0.0
0
-2
-4
-6
-8
-10
Temperature (°C)

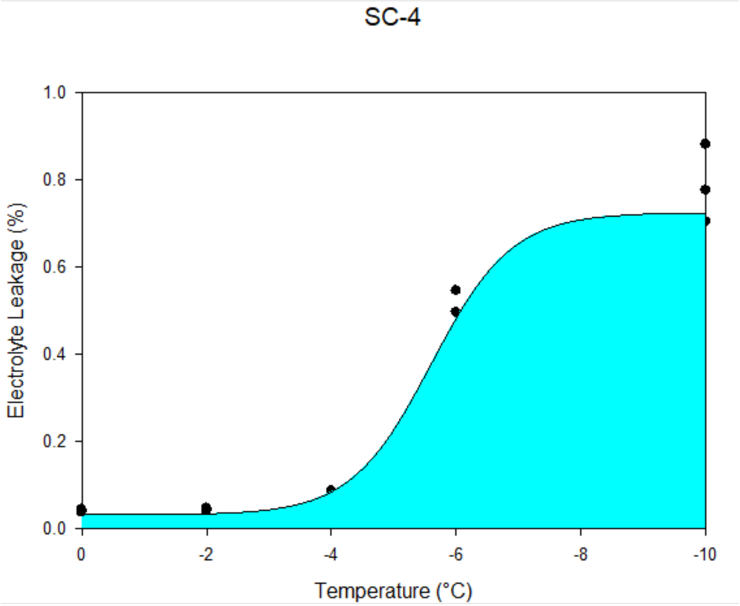

SC-3
Electrolyte Leakage (%)
1.0
0.8
0.6
0.4
0.2
0.0
0
-2
-4
-6
-8
-10
Temperature (°C)


SC-15
Electrolyte Leakage (%)
1.0
0.8
0.6
0.4
0.2
0.0
0
-2
-4
-6
-8
-10
Temperature (°C)


SC-5
Electrolyte Leakage (%)
1.0
0.8
0.6
0.4
0.2
0.0
0
-2
-4
-6
-8
-10
Temperature (°C)


SC-9
Electrolyte Leakage (%)
1.0
0.8
0.6
0.4
0.2
0.0
0
-2
-4
-6
-8
-10
Temperature (°C)


SC-1
Electrolyte Leakage (%)
1.0
0.8
0.6
0.4
0.2
0.0
0
-2
-4
-6
-8
-10
Temperature (°C)


SC-4
Electrolyte Leakage (%)
1.0
0.8
0.6
0.4
0.2
0.0
0
-2
-4
-6
-8
-10
Temperature (°C)

Appendix C

Genetic diversity analysis of peach and nectarine population using FastStructure. Index – Numbers of accessions; POP – population groups; analysis of origin of the material suggested K=4 (Q1 - Q4).

Index	POP	Accessions	Origin	Q1	Q2	Q3	Q4
1	pop4	6820040	Italy	0.00001	0.01006	0.169111	0.820818
7	pop4	Admiral Dewey	US (GA)	0.000011	0.000011	0.000011	0.999966
14	pop4	Bolinha	Brazil	0.000011	0.00001	0.000011	0.999968
9	pop4	BR-2	Brazil	0.00001	0.00001	0.00001	0.99997
30	pop4	Criollo	Guatemala	0.000011	0.000011	0.000011	0.999968
35	pop4	De Wet	South Africa	0.00001	0.00001	0.00001	0.99997
42	pop4	Fortyniner	US (CA)	0.00001	0.00001	0.00001	0.99997
48	pop4	Golden Queen Improved	New Zealand	0.00001	0.00001	0.00001	0.999969
67	pop4	Miller'sLate	US (CA)	0.000011	0.000011	0.000011	0.999968
68	pop4	Muir Peach	US (CA)	0.00001	0.00001	0.00001	0.99997
75	pop4	Peregrine	UK	0.00001	0.00001	0.00001	0.99997
r87	pop4	Sanguine Tardeva	US (CA)	0.00001	0.00001	0.00001	0.99997
99	pop4	Tebana	Italy	0.00001	0.00001	0.00001	0.99997
103	pop4	Tuscan Cling	US (MS)	0.00001	0.011644	0.00001	0.988336
105	pop4	Villa Ada	Italy	0.00001	0.00001	0.080732	0.919248

Index	POP	Trait	Origin	Q1	Q2	Q3	Q4
11	pop3	Bai Mang Pen Tao	China	0.17577	0.00001	0.824209	0.00001
31	pop3	Cumberland	US (NJ)	0.066996	0.00001	0.932984	0.00001
60	pop3	Kawanakajima Hakutou	Japan	0.009881	0.00001	0.990099	0.00001
107	pop3	Yumyeong	South Korea	0.134332	0.000011	0.865646	0.000011
21	pop2	Carored	US (SC)	0.00001	0.827438	0.036421	0.136131
28	pop2	Coronet	US (GA)	0.020034	0.8121	0.00001	0.167855
41	pop2	FlavBurst®	US (WV)	0.00001	0.836388	0.163592	0.00001
55	pop2	Joanna Sweet	US (CA)	0.137099	0.80609	0.00001	0.0568
56	pop2	John Boy	US (PA)	0.00001	0.832898	0.00001	0.167082
64	pop2	Loring	US (MO)	0.00001	0.832442	0.00001	0.167538
70	pop2	O'Henry	US (CA)	0.00001	0.99997	0.00001	0.00001
94	pop2	Sweet Breeze	US (CA)	0.00001	0.818485	0.00001	0.181495
96	pop2	Sweet Scarlet	US (CA)	0.00001	0.99997	0.00001	0.00001
101	pop2	Tra-Zee	US (CA)	0.00001	0.99997	0.00001	0.00001
104	pop2	Valley Sweet	US (CA)	0.028022	0.971957	0.00001	0.00001
5	pop1	880332	Pakistan	0.999968	0.000011	0.000011	0.000011
6	pop1	AR-233	Uzbekistan	0.999969	0.00001	0.00001	0.00001
26	pop1	Chugdar	Pakistan	0.999968	0.000011	0.000011	0.000011
27	pop1	Churko	Pakistan	0.999969	0.00001	0.00001	0.00001

Index	POP	Trait	Origin	Q1	Q2	Q3	Q4
33	pop1	DPRU.2495	China	0.99997	0.00001	0.00001	0.00001
52	pop1	Hunshu	Pakistan	0.999969	0.00001	0.00001	0.00001
61	pop1	Khanda	Pakistan	0.999969	0.00001	0.00001	0.00001
65	pop1	Lutkoo	Pakistan	0.999969	0.00001	0.00001	0.00001
84	pop1	Rogany Goy	Russia	0.962493	0.00001	0.00001	0.037487
98	pop1	Takashur I	Pakistan	0.999969	0.00001	0.00001	0.00001
2	admixed	745	Poland	0.489716	0.190824	0.00001	0.31945
3	admixed	880329	Pakistan	0.78135	0.00001	0.00001	0.21863
4	admixed	880330	Pakistan	0.624434	0.00001	0.00001	0.375546
8	admixed	Allstar®	US (MI)	0.00001	0.183471	0.446063	0.370456
10	admixed	Baby Crawford	US (CA)	0.202382	0.00001	0.238884	0.558725
12	admixed	Big Red	US (FL)	0.00001	0.661895	0.00001	0.338086
13	admixed	Biscoe	US (NC)	0.00001	0.00001	0.683488	0.316493
15	admixed	Brightstar	UC (CA)	0.00001	0.042397	0.308845	0.648748
16	admixed	Calmar	US (CA)	0.095318	0.00001	0.45935	0.445322
17	admixed	Candor	US (NC)	0.00001	0.514012	0.00001	0.485968
18	admixed	Carmen	US (CA)	0.00001	0.00001	0.693885	0.306095
19	admixed	Carogem	US (SC)	0.00001	0.260955	0.373333	0.365702
20	admixed	Caroking	US (SC)	0.00001	0.536927	0.00001	0.463053

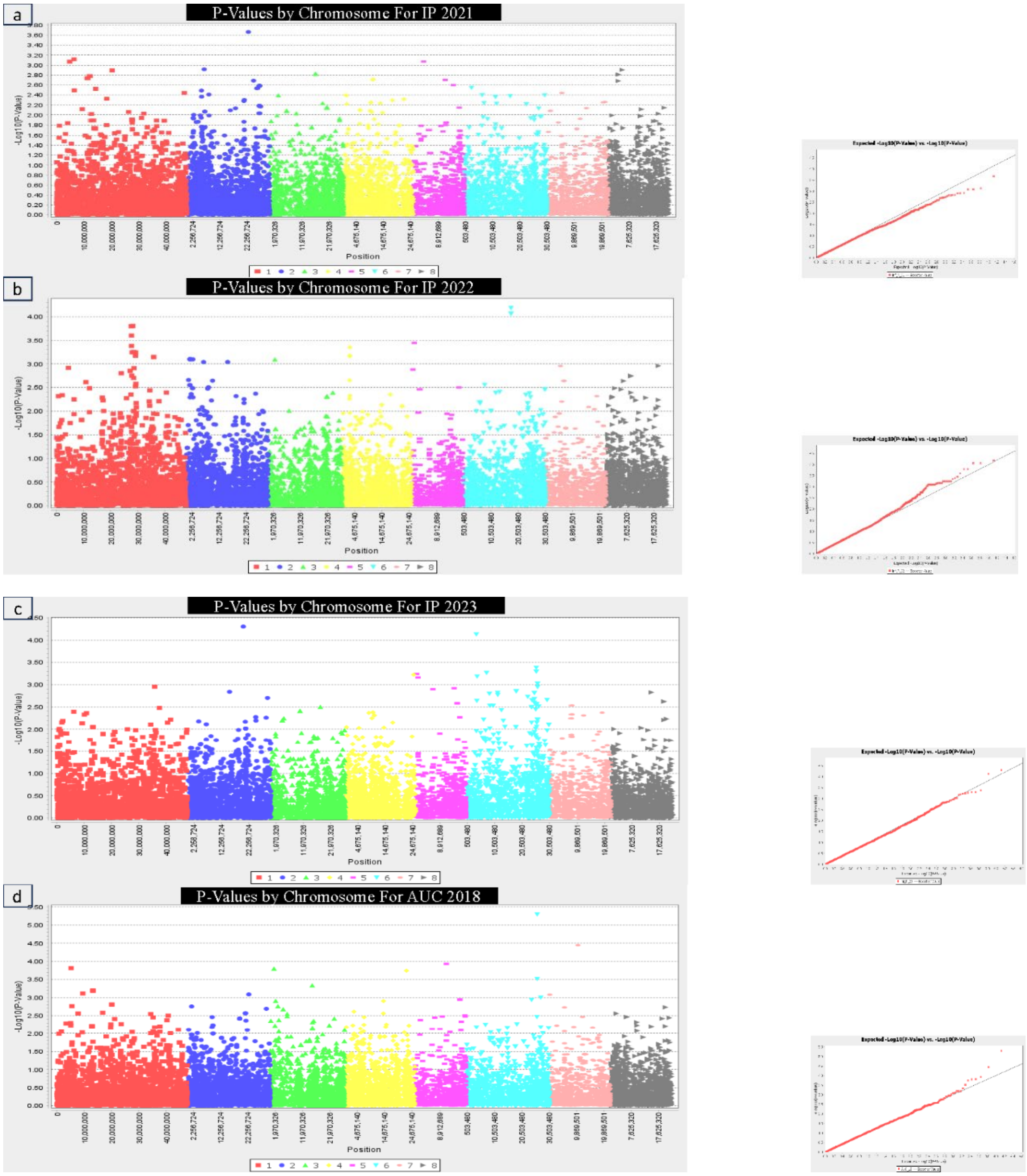
Index	POP	Trait	Origin	Q1	Q2	Q3	Q4
22	admixed	Cascata332	Brazil	0.380429	0.348695	0.00001	0.270866
23	admixed	Cascata519	Brazil	0.219025	0.320238	0.357776	0.102961
24	admixed	Cerrito	Brazil	0.404672	0.00001	0.082697	0.51262
25	admixed	China Pearl	US (NC)	0.343922	0.00001	0.280914	0.375153
29	admixed	Cresthaven	US (CA)	0.00001	0.505393	0.00001	0.494586
34	admixed	Demeur	US (CA)	0.280985	0.207513	0.130018	0.381484
32	admixed	DPRU.2228.20B	China	0.54417	0.000011	0.117244	0.338575
36	admixed	Early Augustprince	US (GA)	0.00001	0.37862	0.460891	0.160479
37	admixed	EarlyStar®	US (MI)	0.000011	0.253844	0.267247	0.478898
38	admixed	Elberta	US (GA)	0.00001	0.398843	0.601137	0.00001
39	admixed	Empress	US (CA)	0.146693	0.059596	0.582939	0.210772
40	admixed	Federica	Italy	0.00001	0.00001	0.328365	0.671614
43	admixed	Garnet Beauty	Canada	0.00001	0.52666	0.00001	0.47332
44	admixed	Gilgalo	Pakistan	0.441162	0.00001	0.00001	0.558817
45	admixed	Glenglo	US (WV)	0.00001	0.59456	0.00001	0.405419
46	admixed	Glory	US (GA)	0.00001	0.428584	0.499135	0.072271
47	admixed	Glowingstar®	US (MI)	0.00001	0.206663	0.351881	0.441447
49	admixed	Harrow Beauty	Canada	0.00001	0.318406	0.279751	0.401833
50	admixed	Harrow Diamond	Canada	0.00001	0.00001	0.587484	0.412495

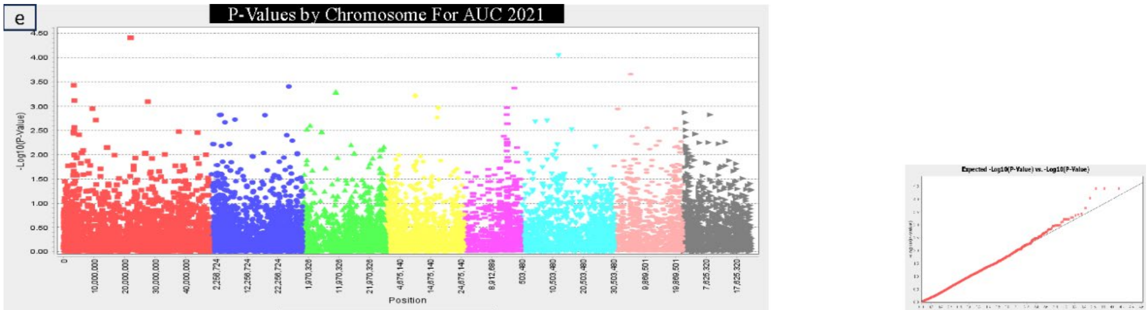
Index	POP	Trait	Origin	Q1	Q2	Q3	Q4
51	admixed	Hiley	US (GA)	0.00001	0.331813	0.133364	0.534813
53	admixed	Jade™	France	0.071487	0.694315	0.00001	0.234188
54	admixed	Jefferson	US (VA)	0.00001	0.450946	0.549034	0.00001
57	admixed	Julyprince	US (GA)	0.00001	0.101023	0.696967	0.202
58	admixed	Juneprince	US (GA)	0.00001	0.657867	0.259969	0.082154
59	admixed	Juneprincess	US (GA)	0.00001	0.659279	0.00001	0.340701
62	admixed	Kijewska Zapala	Poland	0.306308	0.017191	0.050812	0.625689
63	admixed	Lola	Russia	0.645667	0.00001	0.031794	0.322528
66	admixed	Majestic	US (LA)	0.00001	0.00001	0.745947	0.254033
69	admixed	Nata	n/a	0.00001	0.377797	0.622183	0.00001
74	admixed	Pallas	US (GA)	0.467279	0.036005	0.146924	0.349793
71	admixed	PF15A	US (MI)	0.00001	0.669271	0.330709	0.00001
72	admixed	PF17	US (MI)	0.00001	0.499574	0.193222	0.307194
73	admixed	PF23	US (MI)	0.00001	0.395872	0.343273	0.260845
76	admixed	Prarie Schooner	US (IL)	0.000011	0.408377	0.48132	0.110292
77	admixed	Premier	Brazil	0.403232	0.315459	0.176985	0.104323
78	admixed	Ranniaya	Russia	0.00001	0.361618	0.638362	0.00001
79	admixed	Raritan Rose	US (CA)	0.00001	0.144729	0.500889	0.354372
80	admixed	Red Gold	US (MO)	0.00001	0.318649	0.303456	0.377885

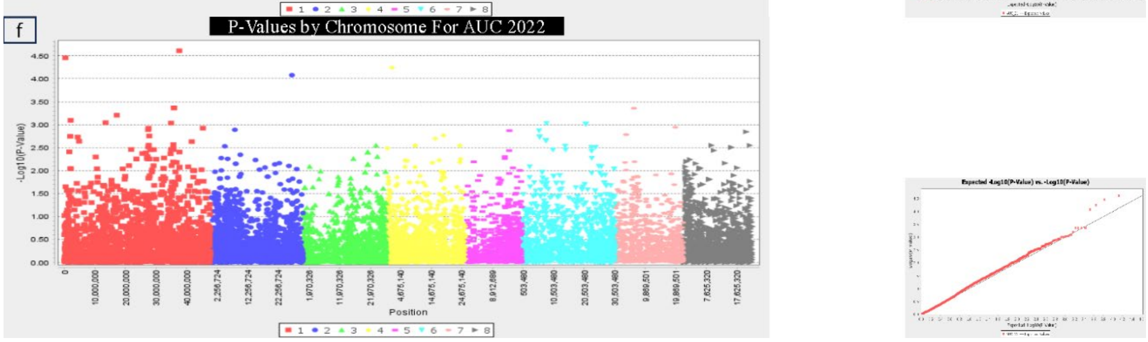
Index	POP	Trait	Origin	Q1	Q2	Q3	Q4
81	admixed	Redglobe	US (MD)	0.024315	0.766516	0.000011	0.209159
82	admixed	Redhaven	US (MI)	0.00001	0.56474	0.00001	0.43524
83	admixed	Rich May	US (CA)	0.087769	0.473081	0.131442	0.307707
85	admixed	Ruston Red	US (LA)	0.000011	0.310089	0.52613	0.16377
86	admixed	SC5	US (SC)	0.023176	0.236998	0.379742	0.360083
88	admixed	Scarlet Pearl	US (GA)	0.00001	0.55796	0.00001	0.442021
89	admixed	Scarletprince	US (GA)	0.471275	0.115603	0.264223	0.148899
90	admixed	Sulina	Brazil	0.305706	0.283072	0.115046	0.296176
91	admixed	Summerprince	US (GA)	0.00001	0.628646	0.00001	0.371333
92	admixed	Sunprince	US (GA)	0.00001	0.00001	0.792963	0.207017
93	admixed	Sureprince	US (GA)	0.00001	0.263846	0.518773	0.217371
95	admixed	Sweet Dream	US (CA)	0.171091	0.620696	0.00001	0.208203
97	admixed	Sweetstar	France	0.00001	0.196455	0.369104	0.434431
100	admixed	Tho Muang	Thailand	0.617976	0.00001	0.370581	0.011433
102	admixed	Turquesa	Brazil	0.286573	0.00001	0.001998	0.711419
106	admixed	Winblo Sport	US (NC)	0.000011	0.236796	0.376461	0.386731

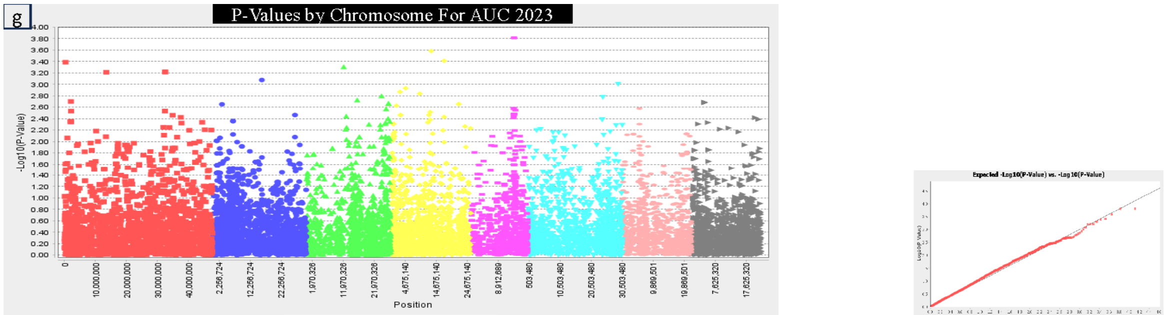
Appendix D

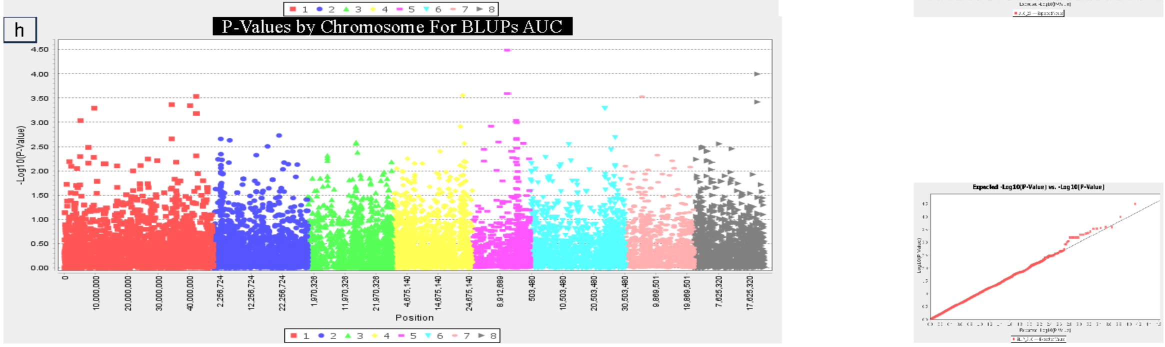
Manhattan plot showing genome-wide association (GWAS) using a general linear model (GLM) for all traits.




e
P-Values by Chromosome For AUC 2021
-Log10(P-Value)
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
0
10,000,000
20,000,000
30,000,000
40,000,000
2,256,724
12,256,724
22,256,724
1,970,326
11,970,326
21,970,326
4,675,140
14,675,140
24,675,140
8,912,689
503,480
10,503,480
20,503,480
30,503,480
9,869,501
19,869,501
7,625,320
17,625,320
Position
1 2 3 4 5 6 7 8
Expected -Log10(P-Value) vs. -Log10(P-Value)


f
P-Values by Chromosome For AUC 2022
-Log10(P-Value)
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
0
10,000,000
20,000,000
30,000,000
40,000,000
2,256,724
12,256,724
22,256,724
1,970,326
11,970,326
21,970,326
4,675,140
14,675,140
24,675,140
8,912,689
503,480
10,503,480
20,503,480
30,503,480
9,869,501
19,869,501
7,625,320
17,625,320
Position
1 2 3 4 5 6 7 8
Expected -Log10(P-Value) vs. -Log10(P-Value)


g
P-Values by Chromosome For AUC 2023
-Log10(P-Value)
4.00
3.80
3.60
3.40
3.20
3.00
2.80
2.60
2.40
2.20
2.00
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0
10,000,000
20,000,000
30,000,000
40,000,000
2,256,724
12,256,724
22,256,724
1,970,326
11,970,326
21,970,326
4,675,140
14,675,140
24,675,140
8,912,689
503,480
10,503,480
20,503,480
30,503,480
9,869,501
19,869,501
7,625,320
17,625,320
Position
1 2 3 4 5 6 7 8
Expected -Log10(P-Value) vs. -Log10(P-Value)


h
P-Values by Chromosome For BLUPs AUC
-Log10(P-Value)
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
0
10,000,000
20,000,000
30,000,000
40,000,000
2,256,724
12,256,724
22,256,724
1,970,326
11,970,326
21,970,326
4,675,140
14,675,140
24,675,140
8,912,689
503,480
10,503,480
20,503,480
30,503,480
9,869,501
19,869,501
7,625,320
17,625,320
Position
1 2 3 4 5 6 7 8
Expected -Log10(P-Value) vs. -Log10(P-Value)

Appendix E

This table header includes columns for the SNP identifier, the total number of genes identified in the 100kb flanking region of each SNP, and the size of the flanking region in kilobases.

SNPs	LOCUS NAME	ANNOTATIONS
S2_18060792	Prupe.2G123200	Integrase-type DNA-binding superfamily protein
S2_18025478	Prupe.2G123300	0
S2_9245044	Prupe.2G123400	Transcription factor jumonji (jmjC) domain-containing protein
	Prupe.2G123500	ribosomal protein S19
	Prupe.2G123700	DNAJ homologue 2
	Prupe.2G123800	TRAF-like family protein
	Prupe.2G123900	Copper transport protein family
	Prupe.2G124000	Phosphoglycerate mutase family protein
	Prupe.2G124200	zinc ion binding
	Prupe.2G124300	0
	Prupe.2G124400	0
	Prupe.2G124500	0
	Prupe.2G124600	Plant protein of unknown function (DUF247)
	Prupe.2G124700	0
	Prupe.2G124800	Plant protein of unknown function (DUF247)

SNPs	LOCUS NAME	ANNOTATIONS
	Prupe.2G124900	Plant protein of unknown function (DUF247)
	Prupe.2G125000	0
	Prupe.2G125100	Plant protein of unknown function (DUF247)
	Prupe.2G123600	RING/U-box superfamily protein
	Prupe.2G124100	myb-like HTH transcriptional regulator family protein
	Prupe.2G122600	Chalcone and stilbene synthase family protein
	Prupe.2G122700	alpha/beta-Hydrolases superfamily protein
	Prupe.2G122800	0
	Prupe.2G123000	autophagy 3 (APG3)
	Prupe.2G123200	Integrase-type DNA-binding superfamily protein
	Prupe.2G123300	0
	Prupe.2G123400	Transcription factor jumonji (jmjC) domain-containing protein
	Prupe.2G123500	ribosomal protein S19
	Prupe.2G123700	DNAJ homologue 2
	Prupe.2G123800	TRAF-like family protein
	Prupe.2G123900	Copper transport protein family
	Prupe.2G124000	Phosphoglycerate mutase family protein
	Prupe.2G124200	zinc ion binding
	Prupe.2G124300	0

SNPs	LOCUS NAME	ANNOTATIONS
	Prupe.2G124400	0
	Prupe.2G124500	0
	Prupe.2G124600	Plant protein of unknown function (DUF247)
	Prupe.2G124700	0
	Prupe.2G124800	Plant protein of unknown function (DUF247)
	Prupe.2G124900	Plant protein of unknown function (DUF247)
	Prupe.2G125000	0
	Prupe.2G122500	Tetratricopeptide repeat (TPR)-like superfamily protein
	Prupe.2G122900	histone mono-ubiquitination 1
	Prupe.2G123600	RING/U-box superfamily protein
	Prupe.2G124100	myb-like HTH transcriptional regulator family protein
	Prupe.2G123100	ATP binding;leucine-tRNA ligases;aminoacyl-tRNA ligases;nucleotide binding;ATP binding;aminoacyl-tRNA ligases
	Prupe.2G066300	3-oxo-5-alpha-steroid 4-dehydrogenase family protein
	Prupe.2G066500	ribonucleotide reductase 2A
	Prupe.2G066600	disease resistance protein (TIR-NBS-LRR class), putative
	Prupe.2G066800	YELLOW STRIPE like 7
	Prupe.2G066900	0
	Prupe.2G067000	Protein kinase superfamily protein

SNPs	LOCUS NAME	ANNOTATIONS
	Prupe.2G066400	3-oxo-5-alpha-steroid 4-dehydrogenase family protein
	Prupe.2G066700	Adaptin family protein
S5_7720068	Prupe.5G063000	cullin 1
	Prupe.5G063100	cullin 1
	Prupe.5G063300	cullin 1
	Prupe.5G063400	RING/U-box superfamily protein
	Prupe.5G063500	cullin 1
	Prupe.5G063600	0
	Prupe.5G063700	cullin 1
	Prupe.5G063800	alpha/beta-Hydrolases superfamily protein
	Prupe.5G063900	Phosphotyrosine protein phosphatases superfamily protein
	Prupe.5G064000	0
	Prupe.5G064100	hydroxyproline-rich glycoprotein family protein
	Prupe.5G063200	cullin 1
	Prupe.5G064200	0
S6_11447678	Prupe.6G140100	Pentatricopeptide repeat (PPR) superfamily protein
	Prupe.6G140300	cysteine-rich RLK (RECEPTOR-like protein kinase) 10
	Prupe.6G140400	cysteine-rich RLK (RECEPTOR-like protein kinase) 10
	Prupe.6G140500	0

SNPs	LOCUS NAME	ANNOTATIONS
	Prupe.6G140600	cyclin p4;1
	Prupe.6G140700	crooked neck protein, putative / cell cycle protein, putative
	Prupe.6G140800	Eukaryotic aspartyl protease family protein
	Prupe.6G141000	0
	Prupe.6G141100	pathogenesis-related 4
	Prupe.6G141200	5\'-3\' exoribonuclease 3
	Prupe.6G141300	NAD(P)-linked oxidoreductase superfamily protein
	Prupe.6G141400	NDR1/HIN1-like 1
	Prupe.6G141500	Polynucleotidyl transferase, ribonuclease H-like superfamily protein
	Prupe.6G141600	Protein kinase superfamily protein
	Prupe.6G141700	phosphoenolpyruvate carboxylase kinase 1
	Prupe.6G141800	F-box family protein
	Prupe.6G141900	PR5-like receptor kinase
	Prupe.6G142000	RING/U-box superfamily protein
	Prupe.6G142100	suppressor of npr1-1 constitutive 4
	Prupe.6G142200	suppressor of npr1-1 constitutive 4
	Prupe.6G142400	Late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein family
	Prupe.6G142500	0

SNPs	LOCUS NAME	ANNOTATIONS
	Prupe.6G142600	Late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein family
	Prupe.6G140200	0
	Prupe.6G140900	0
	Prupe.6G142300	Protein kinase superfamily protein
S7_11673726	Prupe.7G080200	DNA-3-methyladenine glycosylase (MAG)
S7_12386756	Prupe.7G080300	0
	Prupe.7G080400	spindle pole body component 98
	Prupe.7G080600	0
	Prupe.7G080700	beta glucosidase 13
	Prupe.7G080900	Protein kinase superfamily protein
	Prupe.7G081100	0
	Prupe.7G081200	Cation efflux family protein
	Prupe.7G081300	Cupredoxin superfamily protein
	Prupe.7G081400	0
	Prupe.7G081600	TRAF-like family protein
	Prupe.7G081700	TRAF-like family protein
	Prupe.7G081800	Cupredoxin superfamily protein
	Prupe.7G081900	Cysteine/Histidine-rich C1 domain family protein

SNPs	LOCUS NAME	ANNOTATIONS
	Prupe.7G082000	0
	Prupe.7G082100	Cupredoxin superfamily protein
	Prupe.7G082200	Protein of unknown function DUF106, transmembrane
	Prupe.7G082300	C2H2 and C2HC zinc fingers superfamily protein
	Prupe.7G082500	0
	Prupe.7G080500	beta glucosidase 15
	Prupe.7G080800	seven in absentia of Arabidopsis 2
	Prupe.7G081000	0
	Prupe.7G082400	P-loop containing nucleoside triphosphate hydrolases superfamily protein
	Prupe.7G081500	TRAF-like family protein
	Prupe.7G089300	Sucrose-6F-phosphate phosphohydrolase family protein
	Prupe.7G089500	ABC-2 type transporter family protein
	Prupe.7G089600	calcium-dependent protein kinase 20
	Prupe.7G089700	0
	Prupe.7G089800	0
	Prupe.7G089900	cellulose synthase like E1
	Prupe.7G090000	Protein of unknown function, DUF593
	Prupe.7G090200	0
	Prupe.7G090300	0

SNPs	LOCUS NAME	ANNOTATIONS
	Prupe.7G090400	thioredoxin O1
	Prupe.7G090500	RING-H2 group F2A
	Prupe.7G090600	FASCICLIN-like arabinogalactan protein 17 precursor
	Prupe.7G090700	TPX2 (targeting protein for Xklp2) protein family
	Prupe.7G090800	calmodulin-domain protein kinase cdpk isoform 2
	Prupe.7G090900	0
	Prupe.7G091000	Pentatricopeptide repeat (PPR) superfamily protein
	Prupe.7G091200	0
	Prupe.7G091300	0
	Prupe.7G091400	AGAMOUS-like 62
	Prupe.7G091600	Dihydroneopterin aldolase
	Prupe.7G091700	ubiquitin-specific protease 8
	Prupe.7G091800	0
	Prupe.7G091900	0
	Prupe.7G089400	carotenoid isomerase
	Prupe.7G090100	0
	Prupe.7G091100	DNAJ heat shock N-terminal domain-containing protein
	Prupe.7G091500	Ribosomal protein L22p/L17e family protein