Don Stresi Koşullarında Zeytinde Nişasta Metabolizmasında Meydana Gelen Değişimlerin Moleküler Düzeyde İncelenmesi

Deniz Susan Muti

YÜKSEK LİSANS TEZİ

Tarımsal Biyoteknoloji Anabilim Dalı

Nisan 2021

Investigation of Changes in Starch Metabolism of Olive Plant at Molecular Level under Frost Stress

Deniz Susan Muti

MASTER OF SCIENCE THESIS

Department of Agricultural Biotechnology

April 2021

Investigation of Changes in Starch Metabolism of Olive Plant at Molecular Level under Frost Stress

Deniz Susan Muti

This thesis was submitted to the Eskişehir Osmangazi University
Graduate School of of Natural and Applied Sciences in partial
fulfillment of the requirements for the degree of Master of Science
in Discipline of Plant Biotechnology of the
Department of Agricultural Biotechnology

.

Supervisor: Prof. Dr. Ece Turhan

This thesis was supported by TÜBİTAK within the framework of the project ‘‘215O811’’

April 2021

ETHICAL STATEMENT

I hereby declare that this thesis study titled ‘‘Investigation of Changes in Starch Metabolism of Olive Plant at Molecular Level under Frost Stress’’ has been prepared in accordance with the thesis writing rules of Eskişehir Osmangazi University Graduate School of Natural and Applied Sciences under the academic consultancy of my supervisor Prof. Dr. Ece Turhan. I hereby declare that the work presented in this thesis is original. I also declare that, all information and data presented in this thesis have been obtained within the scope of scientific and academic ethical principles and rules, all materials used in this thesis which are not original to this work have been fully cited and referenced, and all knowledge, documents and results have been presented in accordance with scientific ethical principles and rules. 13/04/2021

Deniz Susan Muti

ÖZET

Don stresi altındaki zeytin bitkisinin nişasta metabolizmasındaki değişimler iki çeşitte moleküler düzeyde incelenmiştir. Çelebi (İznik) çeşidi düşük don toleransına sahiptir ve Memeli çeşidi ise yüksek don toleransına sahiptir. 1003 öncelikli alan 215O811 nolu TÜBİTAK projesi kapsamında (ana poje no 215O797) desteklenen bu çalışmada Bornova Zeytincilik Araştırma Enstitüsü'nde yer alan diğer alt proje (proje no: 215O812) ekibi tarafından daha önce TAGEM/BBAD/13/A08/P06/02 projesi kapsamında belirlenen dona toleransı düşük olan Çelebi (İznik) ve yüksek olan Memeli çeşitlerinde fidan üretimi yapılarak ana projenin yapıldığı İstanbul Bilgi Üniversitesi Genetik ve Biyomühendislik Bölümü'ne gönderilmiştir. Tüplü fidanlar, kontrollü don testleri uygulaması için sıcaklık ve nem kontrollü olan kabinine yerleştirilmiştir. Kontrol bitkilerine ise don testleri uygulanmamıştır. Kabininin sıcaklığı +4 ºC'den itibaren 2ºC/sa hızla kademeli olarak -20 ºC'ye kadar düşürülmeye başlanmış ve +4, 0, -4, -8, -12, -16 ve -20ºC sıcaklık kademelerinde analizler için örneklemeler yapılmıştır.

Her iki çeşitte de düşük sıcaklıkta nişasta içeriği azalırken glikoz ve maltoz içeriği artmıştır. β-amilaz (BAM) enzim aktivitesi de düşük sıcaklıkta artmış olsa da, BAM aktivitesi dona dayanıklı Memeli çeşidinde dona duyarlı Çelebi (İznik) çeşidine göre daha yüksek bulunmuştur. BAM1 gen ekspresyonu düşük sıcaklıklarda Memeli çeşidinde yukarı regüle edilmiştir, ancak Çelebi (İznik) çeşidinde ya kontrol uygulamasına yakın ya da azaldığı görülmüş. Sonuç olarak, donma toleransının, BAM enzimine bağlı nişastanın parçalanmasının bir ürünleri olan glikoz ve maltoz içeriğindeki artışla ilişkili olduğu belirlenmiştir. Ayrıca, dona dayanıklı zeytin çeşitlerinin ıslahında BAM1 geninin bir işaretleyici gen olarak kabul edilebileceği sonucuna varılmıştır.

Anahtar Kelimeler: β-amilaz, don stresi, glikoz, maltoz, nişasta, *Olea europaea* L.

SUMMARY

The changes in the starch metabolism under frost stress in the olive plant was investigated at a molecular level in two cultivars. cv. Çelebi (İznik) has a low frost tolerance and cv. Memeli has a high frost tolerance. In this study which was supported within the TÜBİTAK project No. 215O811 (main project No. 215O797) with 1003 priority area, the other sub-project (project number: 215O812) team in Bornova Olive Research Institute produced seedlings in the cv. Çelebi (İznik) with a low frost tolerance and cv. Memeli with a high frost tolerance that was previously determined within the scope of TAGEM/BBAD/13/A08/P06/02 project and was sent to Istanbul Bilgi University Genetics and Bioengineering Department where the main project was carried out. Seedling frost tests were done in a controlled cabin. Frost tests were not applied to control plants. The temperature of the controlled cabin was gradually lowered to -20°C at 2°C/h starting from +4°C and sampling for analysis was done at +4, 0, -4, -8, -12, -16 and -20°C temperature levels and were kept at this temperature for 2 hours.

Glucose and maltose content increased while the starch content decreased under freezing temperatures in both cultivars. Although the β-amylase (BAM) enzyme activity also increased under freezing temperatures, BAM activity was higher in the frost-tolerant Memeli cultivar than in the frost-sensitive Çelebi (İznik) cultivar. The BAM1 gene expression upregulated under freezing temperatures in the cv. Memeli but was either close to the control treatment or decreased in the cv. Çelebi (İznik). As a result, it was determined that the freezing tolerance is associated with the increase in glucose and maltose content as a product of starch degradation due to the BAM enzyme. Besides, it was concluded that the BAM1 gene could be considered as a marker genes in breeding frost tolerant olive cultivars.

Keywords: β-amylase, frost stress, glucose, maltose, *Olea europaea* L., starch

LIST OF CONTENTS

Page

ÖZET ..... **vi**

SUMMARY ..... **vii**

LIST OF CONTENTS ..... **viii**

LIST OF FIGURES ..... **x**

LIST OF TABLES ..... **xi**

LIST OF ABBREVIATIONS AND SYMBOLS ..... **xii**

1. INTRODUCTION AND PURPOSE **1**

2. LITERATURE REVIEW **6**

3. MATERIALS AND METHODS **28**

3.1. Plant Material 28

3.1.1. Çelebi (İznik) 28

3.1.2. Memeli 29

3.2. Plant Stress Treatment 29

3.3. Method 30

3.3.1. Glucose (Glu) content determination 30

3.3.2. Maltose content determination 31

3.3.3. Starch content determination 31

3.3.4. β-amylase activity assay 32

3.3.5. Gene expression assay 32

3.3.5.1. Total RNA isolation 32

3.3.5.2. cDNA (Complementary DNA) synthesis 33

3.3.5.3. Quantitative Real Time PCR (qRT-PCR) applications 34

3.3.6. Analysis of gene expression data 36

3.4. The data evaluation.....37
4. RESULTS AND DISCUSSION 38
4.1. Glucose (Glu) Content 38
4.2. Maltose Content.....41
4.3. Starch Content 44
4.4. β- Amylase Activity.....48
4.5. BAM1 Gene Expression 51
5. CONCLUSION AND RECOMMENDATIONS 55
REFERENCES 56
APPENDIX 68
A.1.: Interaction table of Glc content in Memeli and İznik (Çelebi) cultivars 68
A.2.: Interaction table of maltose content in Memeli and İznik (Çelebi) cultivars.....68
A.3. : Interaction table of starch content in Memeli and İznik (Çelebi) cultivars 69
A.4. : Interaction table of BAM activity in Memeli and İznik (Çelebi) cultivars.....69
A.5. :Interaction table of the relative gene expression level of the BAM1 gene in Memeli and Çelebi (İznik) cultivars (The dependent variable $2^{-\Delta\Delta Ct}$).....70

LIST OF FIGURES

Figure **Page**

2.1. Some of the changes generally seen in plants exposed to low, non-freezing temperatures…….. 9

2.2. Starch synthesis in woody plants. Carbohydrates build up as starch in the amyloplasts of woody tissues and roots and as mono- or disaccharides in the vacuole…….... 16

3.1. A) The location of the Çelebi (İznik) olive cultivar in Turkey, B) The morphological image of the Çelebi (İznik) olive cultivar 28

3.2 A) The location of the Memeli olive cultivar in Turkey, B) The morphological image of the Memeli olive cultivar .....…… 29

4.1. The glucose content in the Çelebi (İznik) cultivars under frost stress. The vertical bars show the ±SE of the replicates 41

4.2. The maltose content in the Çelebi (İznik) cultivars under frost stress. The vertical bars show the ±SE of the replicates 44

4.3. The starch content in the Çelebi (İznik) cultivars under frost stress. The vertical bars show the ± SE of the replicates 48

4.4. The BAM activity in the Çelebi (İznik) cultivars under frost stress. The vertical bars show the ± SE of the replicates 51

4.5. The relative gene expression level of the BAM1 gene in leaf tissues of Çelebi (İznik) and Memeli olive cultivars under frost stress treatment. The vertical bars show the ± SE of the replicates 54

LIST OF TABLES

Table **Page**

3.1. Components and preparation of the RNA- Primer mixture …… 33
3.2. Components and preparation of mastermix …… 34
3.3. PCR program for cDNA synthesis ……..…… 34
3.4. Information on the primers and probs used in the Real Time PCR ……... 35
3.5. Optimization conditions of the primers used in the Real Time PCR program.... 35
3.6. Real Time PCR program ……..……..... 36
4.1. The maltose content in leaf tissues of Çelebi (İznik) and Memeli olive cultivars under frost stress treatment ……..... 40
4.2. The maltose content in leaf tissues of Çelebi (İznik) and Memeli olive cultivars under frost stress treatment ……..... 43
4.3. The starch content in leaf tissues of Çelebi (İznik) and Memeli olive cultivars under frost stress treatment ……..……..... 47
4.4. The BAM activity in leaf tissues of Çelebi (İznik) and Memeli olive cultivars frost stress treatment ……..……. 50
4.5. The relative gene expression level of the BAM1 gene in leaf tissues of Çelebi (İznik) and Memeli olive cultivars under frost stress treatment. The ± show the SE of the replicates ……..…… 53
A.1. Interaction table of maltose content in Memeli and İznik (Çelebi) cultivars……..... 68
A.2. Interaction table of starch content in Memeli and İznik (Çelebi) cultivars…… 68
A.3. Interaction table of BAM activity in Memeli and İznik (Çelebi) cultivars……..... 69
A.4. Interaction table of the relative gene expression level of the BAM gene in Memeli and İznik (Çelebi) cultivars (The dependent variable $2^{-\Delta\Delta Ct}$)……... 69

LIST OF ABBREVIATIONS AND SYMBOLS

Symbol	Explanation
α	Alpha
~	Approximately
β	Beta
<	Bigger than
°C	Centigrade
c.v.	Cultivar
°	Degree
Δ	Delta
e.g.	For example
∞	Infinity
-	Minus
%	Percentage
+	Plus
±	Plus–minus
i.e.	That is

Abbreviation	Explanation
AI	Acid invertase
ATP	Adenosine 5'-triphosphate
ADP	Adenosine di-phosphate
Adg1	ADPglucose pyrophosphorylase activity
ANOVA	Analysis of variance
APX	Ascorbate peroxidase
AsA	Ascorbic asid
bp	Base pair

LIST OF ABBREVIATIONS AND SYMBOLS (Continued)

Abbreviation	Explanation
BC	Before christ
CO_2	Carbon dioxide
cm	Centimetre
CA	Cold acclimation
COR	Cold responsive
cDNA	Complementary deoxyribonucleic acid
CRT	C-repeat
CBF	C-repeat binding factor
Ct	Cycle Threshold
DA	Deacclimation
DREB	Dehydration responsive element binding
dH_2O	Distilled water
ddH_2O	Double distilled water
et. al.	et alia/ meaning “and others.”
EtOH	Ethanol
FAOSTAT	Food and Agriculture Organization Corporate Statistical Database
Fru	Fructose
GWD	Glucan water dikinase
Glu	Glucose
GR	Glutathione reductase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
G	Gram
gFW	Gram fresh weight
ha	Hectars
HPLC	High performance liquid chromatography
h	Hour

LIST OF ABBREVIATIONS AND SYMBOLS (Continued)

Abbreviation	Explanation
HXK	Hexokinase
L	Litre
MEX1	Maltose Excess 1
DPE2	Maltose metabolising transglucosidase 2
mRNA	Messenger ribonucleic acid
µg	Microgram
µL	Microlitre
µm	Micrometer
µmol	Micromol
mg	Miligram
mL	Mililitre
mM	Milimol
min	Minute
M	Mol
nm	Nanometer
nmol	Nanomol
NCBI	National Center for Biotechnology Information
NI	Neutral invertase
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
N	Normal
ns	Not significant
PAHBAH	p-hydroxybenzoicacidhydrazide
Pgm	Plastidial phosphoglucomutase activity
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction

LIST OF ABBREVIATIONS AND SYMBOLS (Continued)

Abbreviation	Explanation
pH	Power of hydrogen
ROS	Reactive oxygen species
RT-PCR	Real time polymerase chain reaction
rcf	Relative centrifugal force
rpm	Revolutions per minute
RNA	Ribonucleic acid
s	Second
Na	Sodium
NaOH	Sodium hydroxide
SAI	Soluble acid invertase
SDH	Sorbitol dehydrogenase
SE	Standart Error
SPS	Sucrose phosphate synthase
SS	Sucrose synthase
Suc	Sucrose
SOD	Superoxide dismutase
Tris-HCL	Tris hydrochloride
WH	Winter hardening
BAM	β -Amylase

1. INTRODUCTION AND PURPOSE

The olive tree is a very valuable plant that has a historical and cultural value, a natural heritage feature and has been the leading role of many legends from the mythological times to the present day (Polat and Tunalıoğlu, 2012). It is thought to have been cultivated in the Bronze age in the East Mediterranean area (Wang et. al., 2018). The olive tree plays a crucial role in the Mediterranean civilization and is one of the oldest known cultivated trees in the world. It is mostly grown between the 30° and 45° latitudes in both hemispheres (Cansev et. al., 2009). Southern Turkey, Syria, Lebanon, Palestine and Israel located in the Mediterranean area are recognised as the primary areas of olive trees based on written tablets, olive pits and wood pieces found in age-old tombs. Olive trees can be found scattered, starting from the origin centers to the other parts of the Mediterranean area (Sakar, 2016). They are especially popular in the Mediterranean countries because table olives and olive oil are highly consumed (Kaya, 2016). According to the FAO (2019), which takes into account average production data for the last five years, Spain comes first with 7.072.879 tonnes, Italy second with 2.303.364 tonnes, and Greece third with 2.183.493 tonnes, while Turkey is the forth largest olive producer with of 1.711.093 tonnes of production in the world (FAOSTAT, 2021). Olive production takes place mainly in the Aegean, Marmara and Mediterranean areas. The oldest olive seed was found in Turkey (particularly in the Tarsus-Mersin area) dated back to the Neolithic age. On the other hand, archeological remains suggest that olives were grown and used in Anatolia for the first time in the third to second millennium BC (Yoruk and Taskin, 2014).

Besides being popular in the Mediterranean area, the cultivation of olive tree is spanning to other countries such as the United States, India, Peru, Argentina, Afghanistan, Pakistan and other Asian, African, South American and Middle Eastern countries. With people growing more aware of their health, the interest in this fruit has highly increased. In Mediterranean countries, wild olive trees (*Olea europaea* subsp. europaea var. sylvestris) are found in negative growing environments and help towards the natural landscape. On the other hand, the cultivated olive (*Olea europaea* subsp. europaea var. europaea) trees are generally grown in more convenient and appropriate environments (Sakar et. al., 2016).

Olive (*Olea europaea* L.) is a tropical and subtropical crop, which is frequently grown near the limits of its cold tolerance. The optimum temperature for olive tree growth is 20-30ºC. Nevertheless, the growing demand for olive oil has spread the cultivation of the tree outside of their adaptive area (Hashempour et. al., 2018).

Temperature is one of the main factors affecting plant development. Plant growth and development depend on the surrounding temperatures and each species has its range defined by minimum, maximum and optimum (Hatfeild and Prueger, 2015). Plants live in environments that frequently change and these changes are more often unfavorable or stressful conditions for growth, reproduction and development (Zhu, 2016). Plant stress has been described by Lichtenthaler (1996) as ‘‘any negative situation or material that affects or prevents a plant’s development, metabolism and/or growth’’, by Strasser as ‘‘a situation provoked by factors that change the balance’’, and by Larcher as ‘‘physiology changes that appear when species are exposed to exceptional negative conditions that do not show a threat to life but alert an alarm response’’ (Kranner et. al., 2010).

Elements that cause stress can be ‘‘biotic’’, which are caused by living organisms, like plant pathogens (fungi, bacteria and viruses), herbivorous animals and insects, or it can be ‘‘abiotic’’, which are caused by non-living factors, like salinity, drought, severe temperatures, and soil pH. Abiotic stresses also consist of mechanical stresses such as hail, wind, the struggle of root growth in compacted soils, and wounding, and also stresses related to toxic, synthetic chemicals, gaseous pollutants (ozone, sulfur dioxide), heavy metals and xenobiotics (Rhodes and Nadolska-Orczyk, 2001; Kranner et. al., 2010). As immobile organisms, plants are incapable of escaping abiotic and biotic factors. Low temperatures represent one of the worst abiotic stresses affecting temperate plants (Janská et. al., 2010). Out of the 350.000 plant species that are grown on Earth, more than half of them are grown in the tropics and the subtropics areas. Throughout evolution, these species could not improve their ability to tolerate low temperatures. The effects of low temperatures on plants that grow in warm climates can cause a decrease or total crop loss due to direct damage or late maturation (Lukatkin et. al., 2012). Most of the warm climate plants can cold acclimate to obtain tolerance against extracellular ice formation in the vegetative tissues.

Cold stress, which consists of chilling (< 20-8°C) and/or freezing (< 0-8°C) temperatures, negatively affects the growth and development of plants and restrains agricultural yield (Chinnusamy et. al., 2007).

Cold hardiness is a term used for the capability of plants to adapt and tolerate freezing temperatures. Generally, the mechanisms related to this capability are very different ranging from features that occur at a structural level (the whole plant) to cellular adaptations that involves unique metabolites and proteins and changes to the stucture of the membrane. Mechanisms that are in charge of cold hardiness usually vary within the same plant in a rather small proximity. For example, xylem tissues and bark tissues in woody plants, leaf tissues and the crown in cereal plants. They can all be very different in how the plants adapt and tolerate freezing temperatures. The mechanisms related to freezing tolerance have been identified as avoidance and tolerance. The mechanisms that are related to freezing avoidance have usually been associated with a plant's physical features to find out when and where ice formation happens in the plant, whereas the mechanisms related to freezing tolerance usually indicates the biochemical adaptions caused by a precise set of genes (Gusta and Wisniewski, 2013).

The freezing temperature is one of the most crucial environmental factors and it restricts the productivity and distribution of most plants (Hashempour et. al., 2018). Olive tree growth and productivity are defined by freezing temperatures in the winter and by frost damage in the spring and fall. Even though the olive tree is fairly durable to below zero temperatures, exceeding the threshold to freezing temperatures is likely to injure the plant. Temperatures below -7°C can cause injury to parts of the plant that are above ground and can negatively impact productivity, while at -12°C damage might be critical enough to endanger the life of the tree. The most unique symptoms of frost damage consist of; leaf chlorosis, tip burn of shoots tips, bark split into branches, defoliation and damages to the buds and fruits (Barranco et. al., 2005).

Plants have developed sensitive mechanisms to handle freezing stress by recognizing the stress signal, sending it through a range of signal transduction pathways and therefore adjusting their metabolic processes.

Plants under freezing temperatures show different degrees of freezing tolerance as a result of cold acclimation (Song et al., 2016). Cold acclimation is a complicated process that includes numerous changes in metabolism and physiology. For example, cold acclimation is followed by an increase in carbohydrate and protein levels, changes in the membrane properties and changes in gene expression (Ristic and Ashworth, 1992). Cold acclimation is an action by which plants gain freezing tolerance by being previously exposed to low non-freezing temperatures (Chinnusamy et al., 2007).

Sugars play numerous roles in freezing tolerance (Fernandez et. al., 2012). It is known that sugars can lower the freezing point and can also increase the intracellular osmotic ability. This reduces the rate of dehydration during extracellular freezing. Sugars also serve as cryoprotectants for the cell membranes and proteins by reducing electrolytes and toxic compounds that occur under freezing temperatures (Palonen, 1999). As osmolytes, they are responsible for the safeguard of water in plant cells, and therefore lowering the water availability for ice formation in the apoplast. Sugars also protect plant cell membranes during dehydration caused by the cold, altering water molecules in forming hydrogen bonds with lipid molecules. Sugar's glass-forming characteristics suggest further protection against cold damage, particularly in woody species that can tolerate temperatures down to –20°C. Sugars may also show the ability to clean reactive oxygen species (ROS) (Fernandez et. al., 2012). The accumulation of sugars during cold acclimation has been recorded in many species such as: *Opuntia ficus-indica* (L.) Miller (Goldstein and Nobel 1991), *Secale cereale* (Koster and Lynch 1992), *Triticum aestivum* L. (Perras and Sarham 1984) and *Populus x canadensis* (Sauter and Cleve 1991) and *Olea europaea* L. (Saadeti et. al., 2019). Sauter and Cleve (1991) also investigated the starch- sugar change in Populus x canadensis during seasonal cold acclimation. They found that the increased levels of carbohydrates were followed by a decreased level of starch (Ristic and Ashworth, 1992).

Typical proteins related to freezing temperatures consist of Cold responsive (COR) proteins and dehydrins. One of the main progress in freezing tolerance research is the discovery of the cold-inducable C-repeat binding factor (CBF) or Dehydration responsive element binding (DREB) transcriptional activators. They have been proved to control various genes related to freezing temperatures in plants.

The regulation of these genes is achieved through the binding of CBF proteins to a precise motif (C-repeat) that exists in the promotor area of the genes that form the CBF regulon (Gusta and Wisniewski, 2013). The accumulation of messenger RNA's (mRNA) in plants usually happens after a few hours of being in cold temperatures and stays at high levels until the plants are back at their normal growth temperature. The relationship between freezing tolerance and the expression time of these genes implies that the COR gene products could play a part in freezing tolerance (Wanner and Junttila, 1999).

Biotechnology proposes new strategies that might develop transgenic plants with enhanced tolerance to frost stress. Many studies have suggested that genes expressed under freezing temperatures are important in plants for cold acclimation. Transgenic techniques are being actively applied throughout the world to develop traits that include tolerance to abiotic and biotic stresses in many crops (Sanghera et. al., 2011).

Promising results have been made on the researches on tolerance to stress in different species and cultivars. It is important in terms of future product yield and in choosing which cultivars to be grown where. In this experiment, it was aimed to determine the change in the starch metabolism caused by frost stress between a frost sensitive olive cultivar [Çelebi (İznik)] and a more frost tolerant olive cultivar (Memeli). Thus, by explaining the starch mechanism developed by the olive cultivars under frost stress, eliminating problems that lead to production and yield loss and developing new production methods was aimed.

2. LITERATURE REVIEW

The olive tree is the sixth most valuable oil crop in the world (Guerra et. al., 2015). The cultivated species of the *Olea europaea* has economic importance for the therapeutic and nutritional characteristics of the oil produced from the fruit (D'Angeli et. al., 2003). There are many different cultivars of olive trees in the Mediterranean area and just this area alone produces 99% and consumes 87% of the world's olive oil (Loumou and Giourga, 2003). Turkey has 5% of the world's existing olive fields and 9% of the olive production. In Turkey, the olive production area constitutes 3,4% of the total cultivated land. The 7 most important cities that produce 78% of Turkey's olive are, respectively, Aydın, Izmir, Manisa, Muğla, Balıkesir, Bursa and Çanakkale (Polat and Tunalıoğlu, 2012).

Plants are sessile organisms that have to adapt to stressful environments. Abiotic stresses are accountable for almost 50% of the world's crop loss (Fernandez et. al., 2012). The reaction to a specific kind of stress is mostly made up of both stress specific adapive responses and general responses that are for primary protection (Baena-González, 2010). Whether a stress factor has a positive or a negative effect depends of the balance between sensitivity and tolerance. A plant's reaction to stress differs with an increasing period and/or the intensity of the stress (Kranner et. al., 2010).

One of the most harmful abiotic stresses that affect plants is freezing stress (Fernandez et. al., 2012). Worldwide climate change has caused changes in temperature patterns in temperate areas and is the primary cause of freezing injury in fruit trees under negative conditions (Yun et. al., 2014). Frost stress inhibits the expression of the full genetic potential of plants because it directly restricts the metabolic reactions and indirectly causes cold-induced osmotic, oxidative and other stresses (Chinnusamy et. al., 2007).

The harmful results of freezing temperatures are seen in the damage done to the cells membrane, which induces injury in the cell compartmentation. It is seen in the swelling and bursting of the plasmalemma, the demolition of the endoplasmic reticulum, and the alterations of the golgi apparatus.

However, the most distinct changes are in swelling and corruption of the mitochondria, the matrix increasing, the cristae shortening and a reduction in their numbers. Freezing temperature causes the swelling and structure alteration in the chloroplasts, especially in the corruption of the membranes, disruption of the grains, decreasing the number of ribosomes and the loss of starch grains (Lukatkin et. al., 2012). The accumulation of cryoprotective solutes and changes in the membrane structure make sure that the plant is capable to survive frost (Purdy et. al., 2013). However, examining frost tolerance in woody plants is difficult because freezing injury that happens in the field usually only becomes noticeable in the spring when growth begins (Turhan and Ergin, 2012).

Olea europaea is a warm climate, evergreen, long-living species that have a low tolerance to frost. Olive trees do not have a proper dormancy and its cultivation is delayed considerably due to not being able to survive at temperatures below -12°C and encounter serious leaf damage at -7°C. In the olive trees, the younger leaves are more sensitive to freezing temperatures than the older leaves (D'Angeli et. al., 2003). The crucial temperature of the olive tree is thought to be about -10°C, but because frost tolerance is associated with the acclimation process, the fatal temperature can change as a reaction to environmental conditions (Camerini, 1999). The freezing tolerance in olive cultivars might be different because of the different survival rates of certain organs and tissues. As an example, the leaves are more sensitive to freezing temperature than shoots. The sensitivity of the leaves may be because thinner cell walls and more exposure to the cold environment (Wang et. al., 2018).

When the fruit trees are exposed to freezing temperatures, physical injury occurs first, and water movement is usually reduced (Yun et. al., 2014). Under most situations, ice formation in the intercellular spaces and cell walls of the plant tissues is induced by freezing temperatures. This happens because the intercellular liquid has a greater freezing point than the cytoplasm. Once the extracellular ice forms, a quick drop in water potential outside the cell happens. Therefore, the water from the cytoplasm flows to the plasma membrane by osmosis thus leading to dehydration. The amount of water lost determines the water potential of the extracellular ice, which depends on the beginning soluble concentration of the cytoplasm and the degree of the freezing temperature (Xin et. al., 2000). Dehydration is thought to be the most significant stress experienced by frozen cells; therefore, frost-resistant genotypes are also resistant to drought (Campo, 2005).

Levitt (1980) has described that, ice development starts in the apoplast or the vascular tissues. Once it has started, the ice extends along with the columns of water very quickly. Ice growth in woody tissues of lemon (Lucas 1954), mulberry (Kitaura 1967), orange (Yelenosky 1991), and peach (Anderson and Smith 1989) have been reported at a speed between 7 and 74 cm/min (Gusta and Wisniewski, 2013).

Overwintering of perennial plants consists of three development stages; cold acclimation (CA), freezing tolerance, and deacclimation (DA) (Sun et. al., 2020). Many important crops, like, soybean, tomato, rice, cotton and maize are sensitive to freezing temperatures and they cannot tolerate the formation of ice in their tissues. However, the temperature limit for frost damage can be lowered even in frost sensitive plants by being previously subjected to below the optimal low temperatures, i.e. cold acclimation (Chinnumasy, 2007). Cold acclimation is an original mechanism that has been evolving throughout the natural evolution of plants (Yue et. al., 2015). In the natural environment, cold acclimation begins with decreasing temperatures in the late autumn or the early winter. It might take a few days to a few weeks to attain the maximum stage of freezing tolerance by cold acclimation, depending on the species of the plants. The tolerance attained can differ from –10°C to less than –60°C. Many physiological and biochemical changes are well-known to happen during cold acclimation as shown in Figure 2.1. (Xin et. al., 2000). Cold acclimation requires the remodeling of the tissue and cell structures and the reprogramming of the metabolism and gene expression (Chinnumasy, 2007). Cold acclimation is a complicated process that includes the accumulation of cryoprotectants, which cause the physical and biochemical restucturing of the cell membranes and ends in the anatomical changes in certain tissues. The leaf is the main place of the production of substances crucial for the promotion and/or inhibition of cold acclimation. (D'Angeli et. al., 2003).

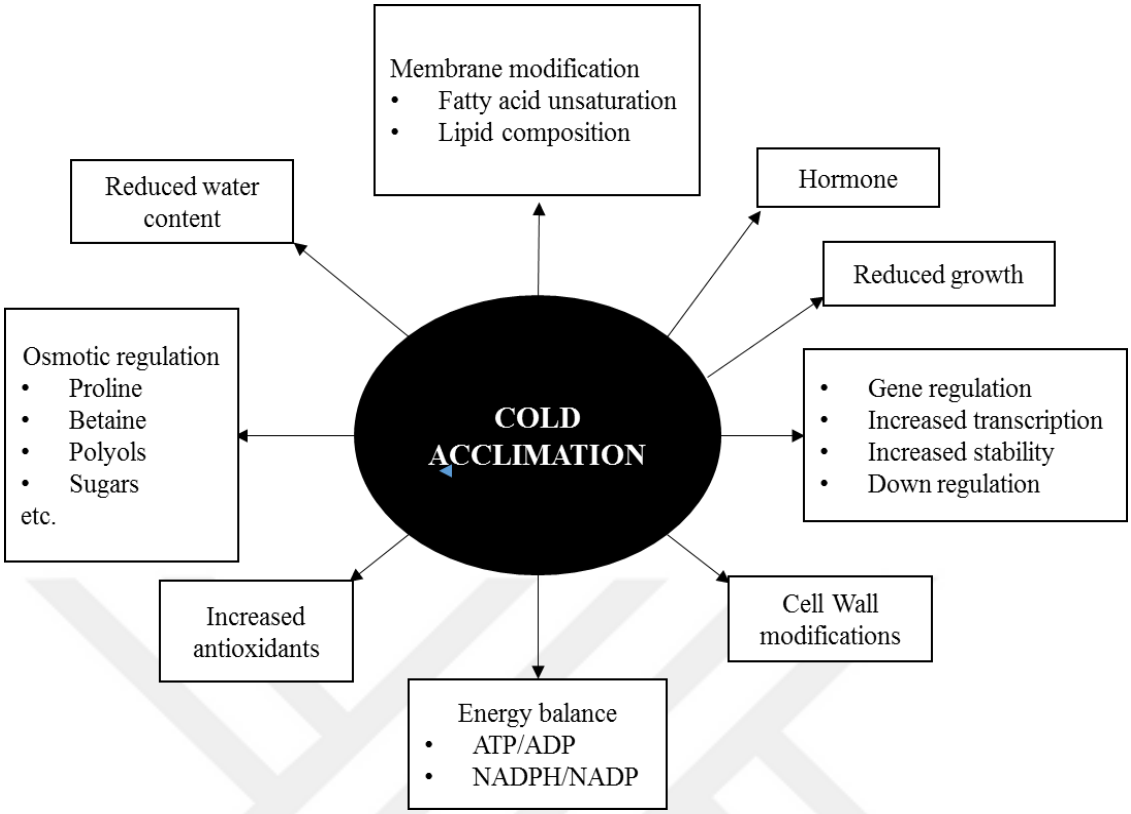

Figure 2.1. Some of the changes generaly seen in plants exposed to low, non-freezing temperatures (Xin et.al., 2000).

Cold hardiness development in trees is divided into five groups; the time of cold acclimation induction, the rate of acclimation, the rate of freezing tolerance obtained, the sustainabillty of the freezing tolerance during the winter months, and the loss rate of freezing tolerance once spring growth starts again. Scientists recorded that intracellular freezing was deadly to plant cells but this did not happen in cold acclimated plants (Wisniewski et. al., 2003).

Many physiological changes happen inside the leaf in reaction to freezing temperatures. When a plant was transferred to freezing temperatures, calcium ion changes were noticed within seconds, protein phosphorylation within minutes, changes in the gene expression along with changes in the mRNA accumulation and enzyme activity within a few hours and a decrease in the water composition and changes in the structure such as leaves thickening happening within days to weeks (Wanner and Junttila, 1999).

It was reported that a cold acclimated winter wheat, when cooled down to -1°C by 2°C/h could withstand temperatures down to -24°C, but when it was not acclimated it was killed at -12°C when kept at this temperature for 15 days. A cold acclimated spring wheat, when cooled down to -1°C by 2°C/h could tolerate temperatures down to -9°C, however when not acclimated it was killed when kept at -3°C for 48h. (Gusta and Wisniewski, 2013).

Mete et. al., (2016), investigated the cellular membrane damage under freezing temperatures between different olive cultivars. 45-year-old Ayvalık, Domat, Çilli, Erkence, Memeli, Memecik, Otur, Eşek and Uslu olive cultivars were used as the plant material. Leaves were collected from the middle parts of the one year old shoots. Samples were collected in October when the cold temperatures started, in January the coldest month of the year and in March when the flowering started. They were collected in each month's second week. A cooled water bath device was used for the frost tests. Temperatures were lowered from +4°C down to -2, -5, -8, -11, -14, -17 and -20°C at a rate of 2°C/h and were kept at this temperature for 2 hours. To determine the cellular membrane damage, the ion leakage method was used. The greatest damage in all the cultivars was determined in November when cold acclimation had not yet fully occurred. The least damage was determined in January varying between cultivars. The Memeli cultivar was identified as the least damaged cultivar in November. The same cultivar was determined that it had the lowest damage rate among all the cultivars in January. As a result, the Memeli and Otur cultivars were determined as the cultivars with the highest frost tolerance.

Many authors have found a positive connection between the soluble sugar level and the rate of tolerance to abiotic stresses (Vagufalvi et.al., 1999). Soluble sugar levels increase extremely once subjected to freezing temperatures (Fernandez et. al., 2012). Sugars play a crutial part in plant growth and development under abiotic stress by adjusting the carbohydrate metabolism (Shin et. al., 2015). The increase in freezing tolerance consists of the accumulation of soluble sugars and amino acids. These solutes are thought to protect the cell membranes and enzymes from permanent damage caused by freezing temperatures (Nagao et. al., 2004). The accumulation of soluble sugars helps the cryostability of cellular membranes increase, and increased membrane cryostability is necessary for freezing tolerance (Yuanyuan et.al., 2009).

Sugar not only serves as an energy and carbon source but also acts as a signaling molecule in many physiological processes and plays a role in the plants growth and development (Yue et. al., 2015). In Arabidopsis, changes in the levels of soluble sugars are a very quick reaction to low temperatures, appearing as quickly as 2 h after exposure to 1°C (Koch, 1996).

Freezing tolerance is attained through physiological changes lead by various molecular pathways. These changes cause an increase in the cells sugar levels, a change in the membranes lipid composition and the synthesis of antifreeze proteins (Schubert, 2019). Throughout cold acclimation, the accumulation of soluble sugars has been recorded in many plants and the process of sugar accumulation has a connection with freezing tolerance during cold acclimation (Xin et. al., 2000). The accumulation of soluble sugars is one of the foremost important metabolic responses seen in frost stressed plants (Sitnicka and Orzechowski, 2014). Freezing temperatures cause a quick accumulation of carbohydrates and this helps towards obtaining freezing tolerance during cold acclimation by decreasing cellular dehydration, helping to protect macromolecules directly, serving as a nutritional source during acclimation and supporting recovery (Rekarte-Cowie et. al., 2008; Trischuk et. al., 2014). Sugars can also act as cryoprotectants to the plasma membranes and proteins during freezing stress (Sun et. al., 2020). However, sugar accumulation might signal the cell that the flowering season is oncoming, which could affect the productivity of cultivated plants negatively (Sitnicka and Orzechowski, 2014). Schilling (2004) stated that proteins work together with sugars to start freezing tolerance (Gusta and Wisniewski, 2013). Sugars are the main products of photosynthesis and display many roles as energy and carbon transport molecules, hormone like signalling factors and a source for plants to make polysaccharides, proteins and lipids (Halford et. al., 2011). In plants, sugars mainly come from the metabolism of starch (Sun et. al., 2020). The most plentiful found sugars in plants are sucrose (Suc), maltose, glucose (Glc) and fructose (Fru) (Halford et. al., 2011).

Many genes, whose products participate in various metabolic pathways and cellular functions, can either be induced or repressed determined by the availability of sugars. Normally, sugars prefer to induce to the expression of enzymes related with the biosynthesis, use and storage of supplies, while repressing the expression of enzymes related with photosynthesis and supply mobilization (Ho et. al., 2001).

Studies on overwintering wheat varieties have shown that the ability to increase soluble carbohydrates has been severely connected to freezing tolerance. In a study done with 14 wheat varieties showing different rates of winter hardiness, the soluble sugar levels more than doubled during a 30 day freezing temperature period (Vagujfalvi et. al., 1999).

One of the most crucial metabolic changes that plants can achieve low temperature tolerance is the accumulation of carbohydrates (Fernandez et. al., 2012). The carbohydrate metabolism is a significant pathway of plants catching photosynthetic energy and supplying the necessary carbon for the production of new tissues (Qi et. al., 2011). The changes in the carbohydrate metabolism have two main roles: to balance carbon assimilation, storage, growth and respiration in reaction to the decreasing carbon request during the growth interruption and decreasing carbon resource as the photosynthesis is downregulated; and the upregulated cryoprotective and osmo- carbohydrates as a reaction to freezing temperatures (Chang et. al., 2020). Freezing tolerance usually means the resistance against osmotic stresses. Carbohydrate accumulation lowers the freezing point of the cytoplasm and protecting the protein and biomembrane against freezing injury, which helps to support the osmotic homeostasis of cells (Saadeti et. al., 2019). Carbohydrates are the main substrates of cellular respiration and for the synthesis of stress proteins and lipids and the repair of these macromolecules after frost stress, act as low molecular weight antioxidants and mediators in the transmission of freezing temperature signal (Borovik et. al., 2019). Rearranging the main carbohydrate metabolism, consisting of photosynthetic activity and sugar concentrations, was proved to play an important role throughout cold acclimation (Nägele and Heyer, 2013). The enzymes of carbohydrate metabolism are important for growth, development, and breaking down carbohydrates in the sink organs (Turhan and Ergin, 2012). The carbohydrate metabolism in plants is more complicated than it is in nearly all the other organisms (Keurentjes et. al., 2008). Carbohydrates are well-known for their crucial role in the plant's protection against freezing injury (Cai et. al., 2004).

Ristic and Ashworth (1992), studied the carbohydrate levels in the leaf tissue of *Arabidopsis thaliana* L. (Heyn) cv. Columbia during cold acclimation. Leaves from 26 day old plants were used to determine freezing tolerance after 0, 6, 12, 18, 24, 36, 48 h, 5 days and 10 days exposure to +4°C. At just 48 h of cold treatment, the lethal freezing temperature

decreased from - 5.7°C to - 9.4°C. The levels of sugars and of starch content were analyzed to determine the effect of cold acclimation. Cold acclimation of *Arabidopsis thaliana* was followed by an increase in the level of sugars, which doubled after only 6h of exposure to +4°C, increasing from 30.6 to 63.9 mg/g dry weight. The levels of sugars reached a maximum after 5 days (148.6 mg/g dry weight), then started to decrease untill the 10^{th} day. Accompanying the increase in sugar levels, the starch content increase during cold acclimation by doubling after 6 h of exposure to +4°C. It continued to increase and reached a maximum at 10 days with the value of 218.2 mg/g dry weight. These results suggest that cold acclimation causes changes in the carbohydrate composition.

It has been found that various tissues in the same tree, like twigs, leaf buds and flower buds, react differently to freezing temperature. Studies done by Bassett et. al. (2006) and Renaut et. al. (2008) found that protein and carbohydrate levels in twigs were changed under freezing temperatures. For example, in apricot trees, Suc levels increased in January and decreased in March, whereas in walnut trees starch decreased during the winter months. In poplar trees, Glc and Fru decreased in the dormant periods, Glc increased and starch decreased with bud burst (Yun et. al., 2014).

Seven 15-year-old olive cultivars (Amphisis, Conservallia, Koroneiki, Gorgan, Manzanilla, Rashid, and Shengeh) were selected in the early winter of 2016 and their freezing tolerance was investigated. Samples were kept at freezing temperatures (0, −5, −10, −15, −20, or −25°C) for 12h and the temperature was lowered to 0°C at 1.5°C/h and to -25°C at 5°C/h. The highest freezing tolerance was found in Amphisis (− 13.1°C), while Rashid (−6.9°C) was the most freezing-sensitive. Amphisis and Shengeh showed the highest contents of leaf carbohydrate (73.29 and 66.68 mg/gFW, respectively), which supports the freezing tolerance results, while the lowest content was found in Rashid (39.44mg/ gFW). The level of leaf carbohydrate in all cultivars was higher than the level of bark carbohydrate. Amphisis and Shengeh were found to be the most freezing tolerant cultivars of all (Saadeti et. al., 2019).

A study was done to understand how low night temperatures affect the carbohydrate content and enzyme activities that take part in the sugar metabolism in the leaves of tomato seedlings (*Lycopersicon esculentum* Mill.). The tomatoes were grown in controlled day/night temperatures of 25/15, 25/9 and 25/6°C for 9 days and then recovered at 25/15°C

for an additional 9 days. Leaves were sampled at 0, 1, 3, 5, 7 and 9 days from the low temperature plants and 3, 6 and 9 days from the recovery plants. Fructose, Glc and Suc levels and starch levels were higher at 9°C than that of the control. Only the starch levels decreased at 6°C and there was an important difference in the Fru levels between 6°C and 9°C. The activity of SPS increased in the tomato leaves. Neutral invertase (NI) and SS activity decreased after 3 days and soluble acid invertase (SAI) activity increased after 5 days. After the 9 days recovery, the starch and Glc levels did not return to the level of the control (Qi et al. 2011).

Soluble sugars that increase during cold acclimation may come from starch degradation. (Fernandez et. al., 2012). In perennial plants, the starch to sugar change is an essential parameter for studying freezing stress. Changes in the sugar levels are typically followed by changes in the starch content (Sun et. al., 2020). During increasing freezing temperatures the increase in soluble sugars and the decrease in starch has been noticed within many species (Jones et. al.,1999). Sicher (2011) explained that the synthesis and accumulation of the two most induced sugars during frost stress, maltose and raffinose, absolutely relies on the existence of starch (Tarkowski and Ende, 2015).

Patton et. al. (2007), examined thirteen genotypes of zoysiagrass with different winter hardiness levels. The plants were grown for 4 weeks at 8°C/2°C day/night temperatures with a 10 h photoperiod. Samples were taken from rhizomes and stolons from the non-acclimated and cold-acclimated plants. Carbohydrate and proline analyses were done. The levels of soluble sugars and proline increased with cold acclimation whereas the starch levels decreased. This connection shows that high levels of total reducing sugars and proline are positively related to zoysiagrass freezing tolerance, while high levels of starch had a negative effect on freezing tolerance

Starch is the most extensive and commonly found storage polysaccharide that exissts in most plants and is stored as granules (Yue et. al., 2018). Starch granules are semicrystalline particles made up of long polymers of Glc, especially amylopectin (Sparla et. al., 2006). In most plants, the biosynthesis, breakdown and storage of starch takes place in plastids, such as the chloroplasts of photosynthetic organs (e.g. leaves) or amyloplasts of non-photosynthetic organs (e.g. stems and seeds) (Jacobs, 2018). Starch accumulates in the chloroplasts of the leaves throughout the day and is degraded at night when the photosynthesis is not active, to provide sugars for local use and export (Monroe et. al., 2014). The amount of carbohydrate produced by the nighttime starch degradation is crucial for the normal growth of the plant (Smith et. al., 2005). The starch synthesis pathways in woody plants are shown in Figure 2.2. In storage organs, starch is synthesized from Suc and can be stored over many seasons or even years, fueling regrowth or seedling formation at the start of the growing period. In photosynthetic cells, starch is synthesized mainly using a part of the CO_2-fixed carbon and is stored briefly in the chloroplasts. This starch is called "transitory" because it is synthesized and broke-down within 24-hours (Thalmann et. al., 2017). Transitory starch accumulation in photosynthetic organs is present in land plants (Lu et. al., 2006). Transitory starch is broke-down throughout the night, or when the photosynthesis level is low, to produce substrates for respiration and the synthesis of Suc and alternative metabolites (Zeeman and Rees, 1999).

Starch metabolism is best understood in the leaves, where starch increases throughout the day and is broken down at night to provide excess carbon that is photosynthetically stable in the leaves to make Suc and to provide a source of reduced carbon at night when photosynthesis is not possible (Lu et. al., 2006). The amount of starch that accumulates in leaves vary between species. In *Arabidopsis thaliana*, starch is the main carbohydrate accumulated and is synthesized during the photoperiod. It has been known that the starchless Arabidopsis lines pgm (missing plastidial phosphoglucomutase activity) and adg1 (ADPglucose pyrophosphorylase activity) mutants grow more slowly in the day/night circumstances than the wild type, but yet under continuous illumination the growth ratio is equal to the wild type. Starchless and reduced starch mutants that are grown under day/night conditions flower a later development stage than the wild type. The shoot to root percentage of the starchless mutants is decreased compared to the wild type. (Zeeman and Rees, 1999).

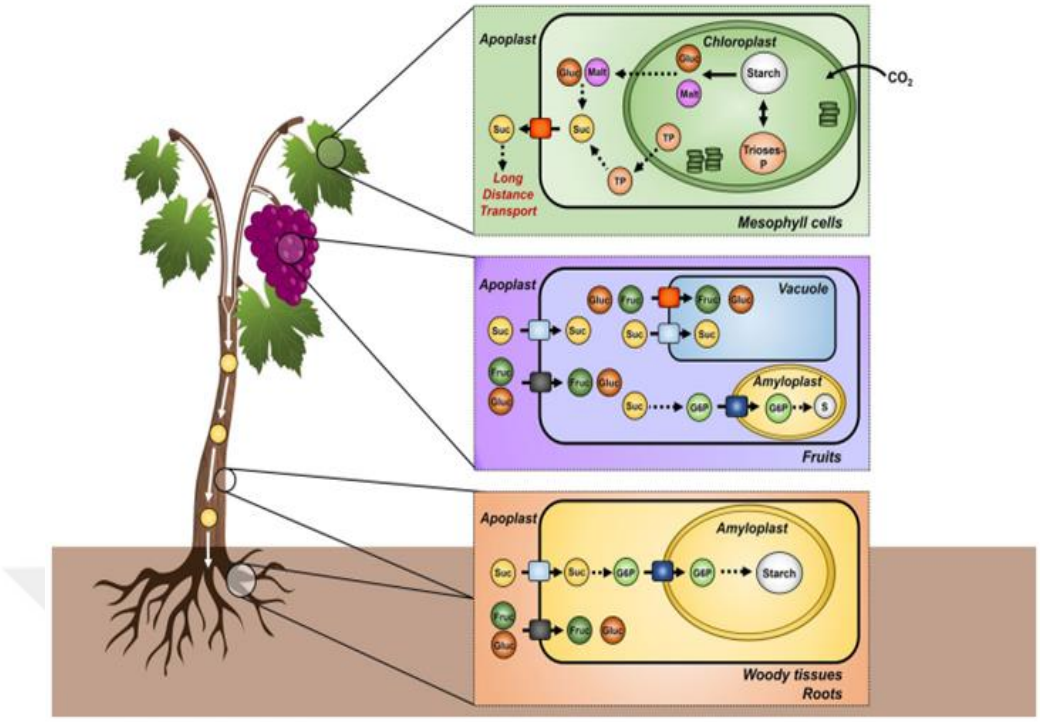

Figure 2.2. Starch synthesis in woody plants. Carbohydrates build up as starch in the amyloplasts of woody tissues and roots and as mono- or disaccharides in the vacuole (Noronha et. al., 2018).

Starch, which is a significant nutrient in the plant growth and development, is not only used for important life activities but is also connected to the response to abiotic stresses (Sun et. al., 2020). Temperature and season changes play a crucial role in starch accumulation in plants. Seasonal changes in starch levels are usually the highest in the spring and autumn seasons, when plants are metabolically active and are the lowest in the winter because of the increase in sugar levels, which is related to the cold acclimation processes (Zhao et. al., 2018). The starch content of leaves can also react to environmental stresses and in recent studies, it has been shown that freezing tolerance has been connected to starch degradation (Lu et. al., 2006). The first step of the starch degradation process is the breakdown of starch into alpha 1,4-glucans and alpha 1,6-glucans through pruning enzymes. Next, amylases act on glucan molecules to produce maltose. Amiloglycosidases are involved in breaking down glucans, where as alpha glucosidases are involved in breaking down maltose and maltotrioses into Glu units (Witt and Sauter, 1995).

Glucose is a versatile metabolite that is involved in the regulation of various physiological functions, including plant growth and development, energy source, being precursors for many metabolic pathways, components of structural building blocks and osmoprotectants under stress conditions. In the day, leaves store carbon in the form of starch and a portion of sugars are transported to the sink organs. On the other hand, at night time, the stored starch is degraded into Glu or maltose and is transported from the chloroplast to the cytoplasm (Saddhe et. al., 2020). There are three different Glu signalling pathways known in plants; the first is hexokinase-dependent, the second is glycolysis-dependen and the third is hexokinase-independent. Hexokinase acts as a intracellular Glu sensor (Janska et. al., 2010). Glucose is a variable metabolite and signaling molecule related to the regulation of various functions through a glucose hexokinase (HXK) sensor. HXK1 is thought to be an intracellular Glc sensor that has a sihnaling role and independent catalytic activity (Saddhe et. al., 2020).

Glucose is a monosaccharide and normally accumulates during the day in plants leaves, used the following night time and usually increase extensively once subjected to abiotic stress such as freezing temperatures (Pommerrenig et. al., 2018). Changes in the levels of sugars, such as Glc and maltose have been shown to affect the freezing tolerance in plants.

These sugars can affect osmotic potentials and function as signaling molecules (Yuanyuan et. al., 2009). It has been stated that Glu levels are crutial for frost tolerance in Arabidopsis supported by independent analyses (Pommerrenig et. al., 2018). It has been stated that the accumulation of Glu can protect the plants cells from freezing temperatures (Saddhe et. al., 2020). It has been reported that this sugar serves as an osmoprotectant and also plays a role in protecting cellular membranes from damage caused by freezing by reacting with the lipid bilayer (Yuanyuan et. al., 2009).

The connection between freezing tolerance and sugar levels in cabbage seedlings was studied. Three week old seedlings were subjected to temperatures from +5°C to -6°C during a 10 day period. Glucose, Suc and Fru were identified as soluble sugars and all of the soluble sugars except starch increased positively with the degree of freezing tolerance. Sugar levels increased considerably under cold acclimation for 3 days; Glu levels increased about six

folds and reached a plateau after 7 days of freezing treatment. The Glu levels showed a increase with freezing tolerance at -6°C, indicating that the increase in sugar levels under freezing temperatures could be connected to obtaining freezing tolerance. However, the freezing tolerance was lost after 1 day once brought back to control temperatures and this was related to the decrease in sugar content (Sasaki et. al., 1996).

An experiment was done in three annual bluegrass ecotypes Western Pennsylvania (OK), Coastal Maryland (CO), and Central Que´bec (CR). Annual bluegrass was grown in a heated greenhouse (22°C day/ 18°C night) for 6 weeks. The freezing tests were performed in controlled freezers. Temperature was lowered at 3°C/90 mins and kept at this temperature for 60 mins at each test temperature. The non-acclimated plants were tested between -17°C and -5°C and cold-acclimated plants between -29°C and -14°C. At the end of each temperature plateau each ecotype were removed from the freezers and thawed at 28°C for 24 h. Total carbohydrate levels almost doubled in annual bluegrass acclimated to low, nonfreezing temperatures. Glucose levels increased after exposure to freezing and was accumulated remarkably higher in the cold sensitive ecotype CR than the CO and OK ecotypes (Dionne et. al., 2001).

Hjihashemil et. al. (2018) examined nine cultivars of *Stevia rebaudina* labeled as accessions 1–9. The plants were incubated in a growth chamber set at 25±1°C for 2 months. Four of each cultivar were maintained in a growth chamber set at 25±1°C while the other four were exposed to cold stress for 1 month in a growth chamber set at 5±1°C. At the end of the cold stress, all plants were moved to a growth chamber set at 25±1°C for 4 h before further analysis. Cold stress increased carbohydrate and Glu accumulation in the nine Stevia cultivars, especially in cultivars 5, 6, 8, and 9. Glucose increased substantially in all cultivars in response to cold stress more so in cultivars 5, 6, 8, and 9 (~300-400 %) than cultivars 1, 2, 3, 4, and 7 (by ~100-200 %). Stevia leaves are used as a natural sweetener, so higher leaf mass and carbohydrate levels in cold-stressed Stevia cultivars can show higher yields under freezing temperatures.

Many studies have shown that low temperatures promote starch breakdown and that genes encoding essential enzymes such as glucan water dikinase (GWD), β-amylase (BAM, EC 3.2.1.2) are regulated differently and facilitate plant frost stress responses. (Kaplan and

Guy, 2004, 2005; Yano et. al., 2005; Li et. al., 2011; Purdy et. al., 2013). Various genes involved in starch metabolism have been noticed to increase in expression in reaction to frost. This reaction is followed by the breakdown of starch and then the accumulation of maltose (Purdy et. al., 2013).

Maltose, the product of BAM, is the main plastid export as a result of starch degradation (Grennan, 2006; Kaplan and Guy, 2005). Maltose as a product of starch degradation was first reported by Levi and Gibbs (1976) (Lu et.al., 2006). It plays an important role in photosynthetic protection and functions as an osmoprotectant under cold shock (Kaplan et al., 2006; Valerio et al., 2011). The disaccharide maltose is built up of two Glu units. It is also a reducing sugar due to one of its units remaining open in a chain form (Halford et. al., 2010). Many studies were done in *Arabidopsis thaliana* and other species suggest that maltose is the main product of starch degradation and at night, it is transported from the chloroplast. First, the maltose levels increase at night when the starch is degraded. Then the chloroplast envelope is permeable to maltose and so maltose is transported from the chloroplasts by a chloroplast envelope maltose transporter (MEX1) (Kaplan, 2006).

MEX1 is a maltose transporter that is in charge of the export of maltose, that is produced during the starch degradation in the chloroplast at night, to the cytosol (Smith et. al., 2005). Maltose can be catabolized by maltose metabolizing transglucosidase (DPE2) by being exported into the cytosol with the MEX1. Interestingly, studies on this subject have shown that both MEX1 and DPE2 affect plant frost tolerance by regulating maltose distribution (Li et. al., 2011; Lloyd et. al., 2004; Purdy et. al., 2013).

When maltose is transported to the cytosol during transitory starch degradation it is metabolized to Glu and/or Suc and maltodextrins by the activity of cytosolic glucosyltransferases (Kaplan, 2006). The loss of MEX1 and DPE2 causes maltose levels to increase extremely in *Arabidopsis thaliana* and potato (*Solanum tuberosum*) (Fulton et. al., 2008). An Arabidopsis mutant without a MEX1, accumulates extreme levels of maltose in the leaves compared to the wild type (Purdy et. al., 2013).

Studies in Arabidopsis done by Kaplan and Guy (2005), show that maltose assists the protection of the photochemical efficiency of PSII against cold shock. Maltose

accumulation caused by stress provides support to the accumulation of osmoprotectants and cryoprotectants (Monroe et. al., 2014). Maltose can protect proteins, membranes and the photosynthetic electron transport chain at physiologically suitable levels under freezing stress. Proteins in living organisms are generally surrounded by water molecules and under freezing stress, these water molecules form ice and denature proteins. Maltose molecules have hydrophilic groups and changing these water molecules with maltose may decrease the damage caused by freezing temperatures (Lu et. al. 2006).

A study was done with three different kale types (Scotch type, Lacinato type and Feral type). The plants were grown in a growth chamber at 25°C for 59 days. Plants were then randomly divided into either 2°C or 25°C groups and continued to be grown. Leaf samples were collected at 0, 3, 7 and 11 days after acclimation from two individual plants of each condition. High-performance liquid chromatography (HPLC) was used for carbohydrate quantification. Black Tuscany showed the highest content of maltose however over time a decrease was detected. The increase in maltose content between days 0 and 3 of cold acclimation is explained by the maltose production in plants and the plant's ability to accumulate starch within the chloroplast stroma (Megías-Pérez et. al., 2020).

Amylases break down starch and increase soluble sugar concentrations in plants that act as osmolytes. Studies have shown that amylases are effective in freezing tolerance, which is an important property in polar plants (Łopieńska Biernat et. al., 2017). The β-amylase is the main enzyme to hydrolyze starch. Furthermore, the BAM has a sugar-binding 14 domain at the N-terminus and is a member of glycosyl hydrolase family 14 (Sun et. al., 2020).

The BAM structure is made up of a standard $(\beta\alpha)_8$ barrel core and a C-terminal long loop. The active site is found buried in the $(\beta\alpha)_8$ barrel pocket (Vajravijayan et. al., 2018). It is a major enzyme in the Glu metabolism pathway and it can degrade starch into maltose to increase carbohydrate levels in plants (Sun et. al., 2020). Out of all the enzymes and genes associated with carbohydrate metabolism, BAM is induced by freezing temperatures and its main role in plants is to break down starch (Shin et. al., 2015). In starch degradation α-amylases do not have an important role whereas β- amylases seem to be crucial (Sparla et. al., 2006).

The main pathway of transitory starch degradation consists of the hydrolysis of starch to maltose by BAM. BAM3 is the main isoform in the nighttime leaf starch metabolism. Arabidopsis mutants that have excess BAM3 have higher levels of starch and lower levels of maltose at night compared to the wild type (Thalmann et. al., 2016). Because of its exceptional role in connecting starch degradation and the accumulation of different sugars, BAM is thought to play a special role in the accumulation of soluble sugars under freezing stress (Peng, 2014). The proof of the important role of BAM in potato and Arabidopsis leaves has come from RNAi lines and mutants missing certain BAM isoforms that have a starch excess phenotype (Grennan, 2006).

The activity of starch degrading enzymes was studied in two potato cultivars (cvs. "Desiree" and "Russet Burbank"). The potato cultivars were exposed to 2°C for 12h. The activity of BAM and the levels of reducing sugars were increased in cv. Desiree. There was no meaningful difference detected in the level of reducing sugars and the activity of BAM in cv. Russet Burbank. However, an increased activity of α-amylase was noticed. These results show that Desiree and Russet Burbank potato cultivars under cold stress might degrade starch through different pathways (Sitnicka and Orzechowski 2014).

Experiments to investigate the effects of cold acclimation on tea plant (*Camellia sinensis*) carbohydrate metabolism were performed using a 15-year-old tea plant clone, "Longjing43". Samples were collected from early November 2013 to March 2014. Three stages were defined as CA (November 26), WH (Winter hardening) (December 30) and DC (Deacclimation) (February 20). The starch content decreased in the cold acclimation (CA) stage and continued at a low level throughout the WH stage, then the starch content increased after DC. Accordingly, maltose slowly increased ecspecially in the WH stage. The BAM activity showed a sudden increase in the CA stage and decreased after December 19. The expression and activity of BAM were found to be majorly induced after only 6 hours of cold shock. Its product maltose, accumulated high levels at the WH stage, which showed it might assist tea plant freezing tolerance (Yue et. al., 2015).

β-amylase's are localized in the stroma, in the vacuole and the cytosol. Plants with decreased chloroplastic BAM activity could only degrade 8–30% of their total leaf starch in the dark, while wild-type plants could degrade 50% of leaf starch (Kaplan et. al. 2006). In

the Arabidopsis genome, nine genes are encoding BAM. Within the nine isoforms, BAM1 (At3g23920), BAM2 (At4g00490), BAM3 (or CT-BMY, At4g17090) and BAM4 (At5g55700) are possibly plastid localized (Thalmann et. al., 2017). These four BAM play different roles in starch degradation in chloroplasts (Valerio et. al., 2011). An Arabidopsis line missing BAM1 has a normal level of leaf starch, implying that BAM1 is not necessary for transitory starch degradation. The expression of the BAM3 gene is induced under freezing stress (Lu et. al.2006). BAM3 works in harmony with debranching enzymes, which are in charge of the hydrolysis of the α-1,6 branches of starch into soluble malto-oligosaccharides (Thalmann et. al., 2017).

Arabidopsis was cold stressed at +4°C for 12 h, BAM 3 transcript levels increased and induction was found to happen as early as 2 h of exposure to cold stress. The increased BAM 3 transcript level was related to maltose accumulation (Kaplan and Guy, 2004). Likewise when reducing the potato storage temperature from 20°C to 5°C/3°C for 10 days, it resulted in increased BAM activity, followed by maltose accumulation (Nielsen et al. 1997).

Many analyses have shown that the soluble carbohydrates and different gene expression changes throughout cold acclimation and deacclimation in woody and herbaceous plants (Shin et. al., 2015). The carbohydrate metabolism genes, including BAM genes, C-repeat binding factor (CBF) regulons and other genes induced by cold stress, determine the sugar level changes that support stress response. First, the sugar acts as a main osmoprotectant and supplies resources in terms of energy and substrates for many physiological activities, then the high sugar level increases cold tolerance (Yue et. al., 2015). Plant genes reaction to the changing carbohydrate conditions can differ. Some genes are induced, some are repressed and some are only slightly affected. The sensitivity of carbohydrate- responsive gene expression to environmental factors also increases its ability to support acclimation (Koch, 1996).

The BAM enzymes are known to play a crucial part in both the starch degradation and gene regulation (Monroe and Storm, 2018). The expression and activity of BAM are controlled by abiotic stresses such as salt and cold stresses (Shin et. al., 2015). β-amylase gene is upregulated during cold acclimation and has been related to cold hardiness because

it catalyzes the degradation of starch to carbohydrates and they supposedly act as membrane stabilizers and osmoprotectants (Lee et. al., 2012). Information about the expression of BAM in fruit plants is limited, but in *Poncirus trifoliata,* researchers suggest that the relative expression level of BAM genes increases during cold acclimation and has a positive connection with freezing tolerance (Sun et. al., 2020). Guerra et. al. (2015) reported that starch degradation in the chloroplast in the olive plant is associated with transcriptional changes and that the BAM gene expression is promoted during cold acclimation.

Lee et. al., (2012), examined the changes in the carbohydrate content and the BAM gene expression in ten-year-old blueberry (*V. corymbosum* cvs. "Sharpblue" and "Jersey") that were grown in an experimental orchard in Korea from September 2010 to March 2011. As a target gene blueberry β-amylase (VcBAM) was selected because of its association with the carbohydrate metabolism. To analyze the VcBAM expression, shoots were collected monthly from October 2010 to February 2011. The expression of VcBAM was measured by quantitative real-time polymerase chain reaction (qRT-PCR). The expression levels of VcBAM that was normalized to the MET housekeeping gene, increased considerably in both cultivars during cold acclimation.

The sugar levels in the shoots of the two blueberry cultivars quickly increased during CA and reached a maximum level in December 2010 and then started decreasing. The sugar levels were considerably higher in "Jersey" than in "Sharpblue". The VcBAM expression became evident in November 2010. In January 2011, the relative expression of VcBAM was approximately two-fold higher in "Jersey" than in "Sharpblue". During this period, the higher VcBAM expression in "Jersey" than in Sharpblue" which complies with a larger decrease of starch content in "Jersey" than in "Sharpblue", represents higher metabolic activities in "Jersey". Between February and March the sudden decrease in sugar levels and increase in starch levels maybe related to the sugars transforming into starch to get ready for growth renewal.

In a study, three-month-old *Poncirus trifoliata* seedlings were subjected to +4°C for 0 h, 6 h, 1 d, 3 d and 6 d. Leaf samples were collected at each time point, instantly frozen in liquid nitrogen and stored at −80°C until use. The expression of *Poncirus trifoliata* β-amylase 1 (PtrBAM1) levels was analysed by RT-PCR. Under low temperature, the transcript levels of PtrBAM1 were quickly up-regulated within 6 h and increased continuously. The function of PtrBAM1 in cold tolerance was also evaluated in the PtrBAM1 overexpressing transgenic tobacco plants that were subjected to freezing temperature (−4°C). The PtrBAM1 overexpressing tobacco plants resulted in higher BAM activity, lower starch accumulation and more maltose content than the wild type revealing that the overexpression of PtrBAM1 enhanced freezing tolerance in the transgenic plants. The PtrBAM1 overexpressing tobacco plants that contained higher levels of soluble sugars than the wild type after cold treatment, revealed the importance of sugars produced from PtrBAM1 starch degradation in cold stress tolerance (Peng et. al., 2014).

In an experiment, two peach cultivars (*Prunus persica* 'Daewol' and 'Kiraranokiwami') grown in a peach orchard were selected and the carbohydrate changes under freezing temperatures were analysed. Samples from the two cultivars, were collected at the end of each month from August 2011 to April 2012. Samples were incubated in a refrigerating bath for the freezing test. The starting temperature was 5°C for the freezing test and cooled at a 5°C/h until the target temperature was reached. Five target temperatures were selected as followed: 5, −5 to −20, and −80°C. The target temperature was maintained for 2 h, then they were removed and thawed at +4°C. Cold tolerance was determined by the electrolyte leakage method. The minimum cold tolerance in both cultivars was seen at the end of August 2011. During cold acclimation, the cold tolerance greatly increased in both cultivars. The maximum cold tolerance was seen at the end of December 2011. Sugars were analyzed using a HPLC connected to a detector (2420-ELSD). At the beginning of the experiment, the level of starch in both cultivars briefly increased, then decreased until January 2012. Total carbohydrate levels in the two peach cultivars increased during cold acclimation, coinciding with the decrease in starch content, and decreased during deacclimation. For gene expression, BAM was used as a target gene and RNA polymerase II was used as a housekeeping gene. Differences in the relative expression of BAM were confirmed by quantitative real-time RT-PCR. Relative expression levels of BAM increased during cold acclimation and decreased during deacclimation. Relative expression of BAM

was strongly correlated with cold tolerance. The increase in carbohydrate content during cold acclimation is thought to have been a result of starch degradation. During DA the carbohydrate level decreased quickly while the starch content increased (Shin et. al., 2015).

Sun et. al., (2020), studied the carbohydrate metabolism and freezing tolerance under CA and DA stages in two kiwi fruit trees (*Actinidia arguta* cvs. "CJ-1" and "RB-3"). Shoots were sampled from October 2017 to March 2018. The materials were exposed to temperatures of −5, −10, −15, −20, −25, and −30°C for 8 h each. Changes in the starch content from the October 23 to March 5 showed a decrease in both cultivars. The soluble sugar levels showed a dome shaped trend in both cultivars however, "CJ-1" accumulated soluble sugars earlier than "RB-3".The BAM activity increased in both cultivars but was higher in "CJ-1" than "RB-3" over the seasonal changes. The relative expression of *Actinidia arguta* β-amylase 3 (AaBAM3) was upregulated 3-fold in "CJ-1" than in "RB-3" at January 20, which indicates that the AaBAM3 gene was the main gene accountable for the different freezing tolerance of the two cultivars

The functional and biochemical properties of certain cell types are determined by their specific gene expression profiles. Global gene expression profiles can be described by a transcriptome, which shows the identity and the level of expression of each expressed gene in a certain population of cells (Lee and Lee, 2003).

In the model plant Arabidopsis the genes encoding transcription factors make up 6-10% of their genome (Wisniewski et. al., 2018). Cold responsive genes that are thought to be involved with the progress of cold tolerance consist of encoding enzymes in charge of the synthesis of sugars or sugar by-products and appropriate solutes and proteins (Wisniewski et. al., 2003). Many genes related to cold hardiness in woody perennial trees have been determined using expressed sequence tag sequencing, Complementary Deoxyribonucleic acid (cDNA) microarrays and subtractive hybridization. The cold responsive genes that have been identified so far are divided into two specific groups. In the first group are genes that encode enzymes or structural components of the cell that take a part in the protection of the cells under freezing temperatures. The second group is genes that encode transcriptor factors and other regulatory proteins that regulate reactions either transcriptionally or posttranscriptionally under freezing temperatures (Hirt and Shinozaki, 2003).

It has been determined that the CBF transcripton factors play an important role in the gene regulation that happens during cold acclimation in various plant species, both sensitive and tolerant to the frost, showing that the CBF expression level is usually related to the degree of frost tolerance (Guerra et. al., 2015). It was also proved that the transcriptional induction CBF genes under frost stress were regulated differently by photoperiod or between various tissues. Different CBF genes demonstrated various levels and patterns of accumulation within the same species, thus the role of each CBF gene might be different during certain development stages (Barros et. al., 2012).

Kreps et. al. (2002) have proved that out of the ~8000 Arabidopsis genes analyzed, 2086 responded to freezing temperatures and 42% of those genes were induced. When the change in the expression level was compared to the control (untreated) plants, it was at least 2 fold. This suggests that almost 25% of the Arabidopsis transcriptome could respond to cold temperatures.

Arabidopsis plants that were exposed to freezing temperatures lead to the fast induction of CBF genes. CBFs activate many cold-inducible genes expression by binding to their C-repeat (CRT)/DRE regulatory element in the promoters. Worldwide transcriptome analyzes show that in Arabidopsis almost 12% of cold-regulated genes are controlled by the CBFs (Liu et. al., 2012).

In Arabidopsis, most of the cold responsive genes are noticable within 1-2 hours of being under cold temperatures and the transcript levels stay high as long as the plants are kept at cold temperatures, and immediately turns to it basal level once returned to optimal growth temperatures. Yet, most of the cold responsive genes are only temporarily induced in the early and middle phases of cold acclimation (Hirt and Shinozaki, 2003).

Stockinger et al. (1997) first identified a transcription factor binding to the dehydration-responsive element (DRE) element and activating cold- induced gene expression. They were able to isolate CBF1, an Arabidopsis gene encoding a DRE/CRT binding protein. Five other genes encoding CBF1 homologs, called DREBs (dehydration-responsive element binding proteins) and two other CBFs, called CBF2, and CBF3 was later

cloned from Arabidopsis (Hirt and Shinozaki, 2003). The accumulation of carbohydrates can be caused by the expression of cold-inducible genes. Therefore, the cold-responsive transcription factor CBF3's over- expression induces both freezing tolerance and the accumulation of carbohydrates (Rekarte-Cowie et. al., 2008). The increased levels of sugars in Arabidopsis plants overexpressing CBF3/DREB1A have been put forward that there is a link between the CBF/DREB1 pathway and the carbohydrate accumulation induced by freezing temperatures (Yano et. al., 2005). The DREB genes are divided into two different groups by their response frost stress and drought. The DREB1 genes, respond to low temperature but not to drought, whereas the DREB2 genes, respond to drought but not too frost stress (Hirt and Shinozaki, 2003).

It has been stated that the expression of key regulators of abiotic stress responses such has CBF3 and COR15A was induced by Glu. The expression of CBF3 in transgenic Arabidopsis plants induces the expression of COR genes to increase feezing tolerance in non acclimated plants. The expression of COR15A is regulated by CBF3, indicating that glucose may assist the regulation of cold stress tolerance (Yuanyuan et. al., 2009).

3. MATERIALS AND METHODS

This study investigating the changes in starch metabolism under frost stress in İznik (Çelebi) and Memeli olive cultivars was carried out in Eskişehir Osmangazi University Faculty of Agriculture, Department of Agricultural Biotechnology laboratory between the years of 2018 - 2020.

3.1. Plant Material

3.1.1. Çelebi (İznik)

Its origin is the Bursa province, İznik district. With around 400.000 trees, it constitues 5% of the Marmara region. It is considered a green table olive. The tree is of medium strength and usually forms a small, flat and drooping crown. The leaves are medium length, wide and elliptical. The fruit is large, long and cylindrical shaped, and contains 20.8 % of oil. Some fruits have a small nipple at the tip. It partially shows perioidicity and is moderately productive. It has a low frost tolerance (Kıvrak, 2019a). The location in Turkey and the morphological image of the Çelebi (İznik) cultivar are shown in Figure 3.1.

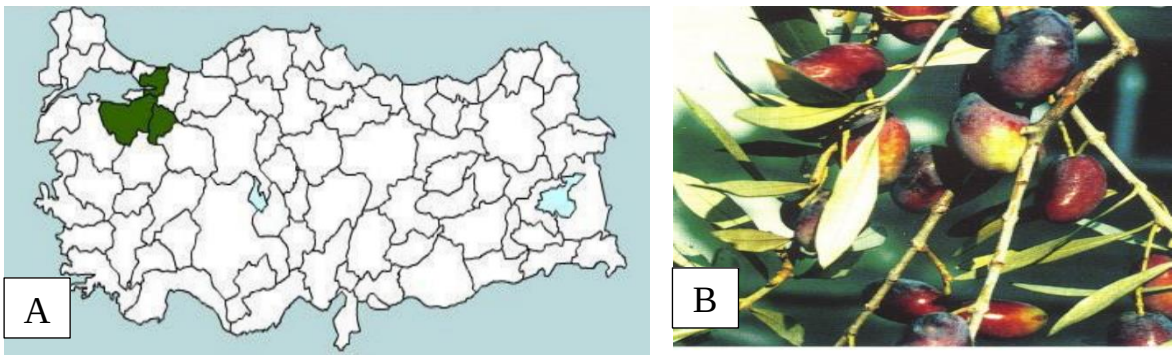

Figure 3.1. A) The location of the Çelebi (İznik) olive cultivar in Turkey (Kıvrak, 2019a), B) The morphological image of the Çelebi (İznik) olive cultivar (Kaya, 2017).

3.1.2. Memeli

Its origin is the İzmir province, Menemen district. There are around 80,000 trees in the Aegean region. It is considered as both oil and table olive. The tree grows strong and upright. The leaves are medium, medium-long, broad and elliptical. The fruit is large and oval with a distinctive nipple at the tip of fruit. The fruit contains 20% of oil. The oil is moderate and of high quailty. It shows periodicity and its productivity is good. It has a higher frost tolerance that the Çelebi (İznik) cultivar (Kıvrak, 2019b). The location in Turkey and the morphological image of the Memeli cultivar are shown in Figure 3.2.

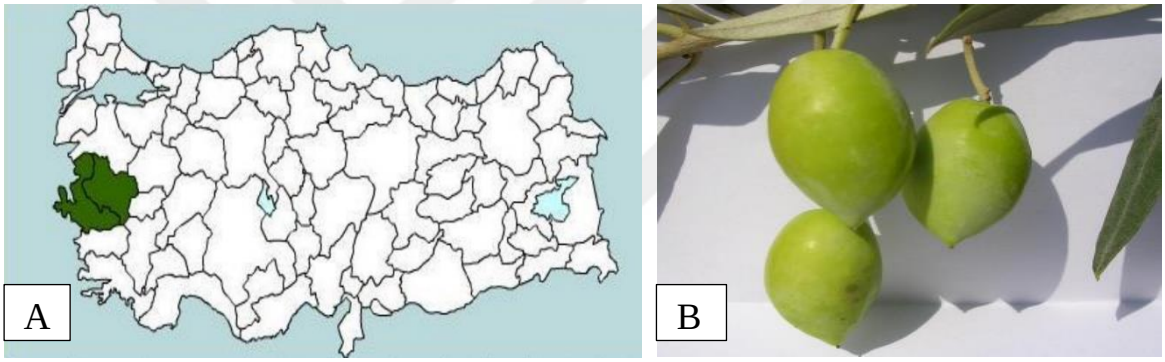

Figure 3.2. A) The location of the Memeli olive cultivar in Turkey (Kıvrak, 2019b), B) The morphological image of the Memeli olive cultivar (Kaya, 2017).

3.2. Plant Stress Treatment

In this study which was supported within the TÜBİTAK project No. 215O811 (main project No. 215O797) with 1003 priority area, the other sub-project (project number: 215O812) team in Bornova Olive Research Institute produced seedlings in the Çelebi (İznik) cultivar with a low frost tolerance and Memeli cultivar with a high frost tolerance that was previously determined within the scope of the TAGEM / BBAD / 13 A08 / P06 / 02 project and was sent to İstanbul Bilgi University Genetics and Bioengineering Department where the main project was carried out. Frost tests done on the seedlings were carried out based on Arora et. al. (1992) method with modifications according to Eris et al. (2007) and Cansev et. al. (2009) proposed for the olive tree. Tubed seedlings were placed in a temperature and

humidity controlled cabin for the application of controlled frost tests. Frost tests were not applied to control plants. The temperature of the cabin was gradually lowered to -20°C at 2 °C/h starting from +4°C and sampling for analysis was done at 0, -4, -8, -12, -16 and -20°C temperature levels. When each temperature level was reached, sampling was done after the plants were kept at this temperature for 2 hours. Homogeneous leaves that have completed their development in the middle part of the shoots were collected. Some of these leaves were preserved for use in molecular analysis. After the leaf samples were quickly frozen in liquid nitrogen, they were kept at -80ºC until the start of the analysis. Besides, for some carbohydrate analysis, discs were taken from the leaf samples and were fixed in tubes that contained 80% ethyl alcohol and preserved at -20°C. The samples were sent to our department by the cold chain for analysis.

3.3. Method

3.3.1. Glucose (Glu) content determination

Glucose content extraction was done according to Turhan et. al. (2006) with some modifications. 5 mL of 80% ethanol (EtOH) was added to 250 mg of whole leaf tissues and was incubated in a water bath (Memmert WNB22, Turkey) at 85°C for 1 hour, and the suspension from the tissue was taken out and added to a new tube. This process was done 4 times including 1 hour, 30 mins, 15 mins and 15 mins. The EtOH solutions were combined and evaporated until dry at 55°C with the aid of continuous ventilation. The dry precipitaions were used for soluble sugar analysis. The whole leaves that were left after the solution was taken were used for starch content determination. The dried precipitates were dissolved in 1 mL distilled water and centrifuged (BECKMAN COULTER Allegra 64R, USA) at 10.000 g, 4°C for 5 mins (Turhan et. al, 2006) The extract was then filtered through 0.45-µm membrane filters. 20µL of the filtrate was injected in to the HPLC (SHIMADZU LC20A, Japan) using Carbo Sep Coregel 87C column kept at 80°C and ddH_2O was used as a solvent at a flow rate of 0.6 mL min^{-1}. Also standard solutions ranging from 0-3000 mg/L of Glu were dissolved in ddH_2O and used as standards for the concentration calculation.

3.3.2. Maltose content determination

The maltose content extraction method was the same as Glu content extraction method that was done according to Turhan et. al. (2006) with some modifications. The dried precipitates were dissolved in 2.5 mL distilled water and centrifuged (BECKMAN COULTER Allegra 64R, USA) at 10.000 g, 4°C for 5 mins. The extract was then filtered through Sep-pak C18 cartridges that were first washed with 5 mL methanol and 5 mL distilled water and 0.45-µm membrane filters (Tajoddin et. al., 2012). 20µL of the filtrate was injected in to the HPLC (SHIMADZU LC20A, Japan) using Carbo Sep Coregel 87C column kept at 80°C and ddH_2O was used as a solvent at a flow rate of 0.6 mL min^{-1}. Also standard solutions ranging from 0-3000 mg/L of maltose were dissolved in ddH_2O and used as standards for the concentration calculation.

3.3.3. Starch content determination

The starch content extraction method was the same as Glu content extraction method that was done according to Turhan et. al. (2006) with some modifications and the analyze was carried out according to Dinar et. al. (1982). The whole leaves that were left after the solution was taken were used for starch content determination. After the leaves were totally dry 3 mL of distilled water was added and vortexed (Phoenix Instruments RS-VA 10, Belgium). A blank was made for every treatment. The tubes were then autoclaved (ALP CLG-32L, Japan) at 121°C for 60 mins. After the samples had been autoclaved, 2 mL of buffer [50 mL Amyloglucosidase + 125 mL dH_2O + 25 mL 2M Acetate (pH: 4.8)] was added to each sample and each blank. They were then incubated in a water bath (Memmert WNB22, Turkey) at 55°C overnight. Standards were made using Glu. 1 mL of the sample mix was taken and 1 mL of samner solution was added to each sample and blank. All of the tubes were boiled for 5 mins. After cooling, quantification was done using a spectrophotometer (Perkin Elmer Lambda 25, USA) at 550 nm.

3.3.4. β-amylase activity assay

β-Amylase activity was measured according to Yue et. al. (2015) with some modifications. The leaf tissues were previously ground in liquid nitrogen. 0,1 g of tissue was extracted using 1,5 mL 50 mM Tris-HCL (pH: 8.0) extraction buffer. The samples were then centrifuged (BECKMAN COULTER Allegra 64R, USA)at 13.000g for 20 mins at +4°C. The supernatant was taken and the pellet was thrown. 85µL supernatant was mixed with 75µL of 20 mg/mL soluble starch and 150 µL of 0.1 N NaAcetate (pH: 4.6). 95µL of this mix was taken and put aside (Sample 1) and the remainder mix was incubated (Thermo Scientific 4334, USA) at 37°C for 60 mins (Sample 2). As the incubation was about to finish sample 1 was boiled for 3 mins. To stop the reaction, sample 1 was added to sample 2 (sample 3). 100µL of sample 3 was taken and added to 150 µL of ddH_2O and 750 µL of p-hydroxybenzoicacidhydrazide (PAHBAH) / NaOH solution and boiled at 100°C for 5 mins. After boiling, it was cooled to room temperature and quantification was done spectrophotometerically (Perkin Elmer Lambda 25, USA) at 410 nm, 37°C with a maltose standard curve. One unit of BAM activity was defined as the amount of BAM required to produce 1 nmol maltose per min via the degradation of amylopectin at 37°C. The total soluble protein content of the crude enzyme extract was determined using the Bradford assay method (Bradford, 1976).

3.3.5. Gene expression assay

3.3.5.1. Total RNA isolation

RNA isolation was performed according to MacRae (2017) with some modifications. The leaf samples were ground in sterile mortars using liquid nitrogen. 50 mg of the ground sample was added to TRIzols. The TRIzols were then homogenated (Omni International Bead Ruptor 12, USA) for 45 seconds and then kept on ice for 2 mins, then incubated in room temperature for 5 mins. The TRIzols were then centrifuged (BECKMAN COULTER Allegra 64R, USA) at 12000 rcf for 5 mins at +4°C. The supernatant was transferred into a new sterile tube and 200 µL of chloroform was added and mixed. The tubes were incubated at room temperature for 5 mins. They were then centrifuged at 12000 rcf for 20 mins at 4°C. The supernatant was transferred into a new steril tube and 500 µL of isopropanol was added

and incubated at room temperature for 10 mins. They were then centrifuged at 12000 rcf for 10 mins at +4°C. After centrifugation, the supernatant was thrown and the pellet was observed at the bottom of the tube. 1 mL of 70% EtOH was added to the pellet and centrifuged at 7500 rcf for 5 mins at +4°C. The EtOH was thrown and the EtOH remaining in the tube was evaporated using a heating block (Eppendorf AG 5355, Germany) at 57°C for 30 mins. 50 µL of RNase-free water was added to each dry pellet and dissolved by pipetting. The isolated RNAs amounts and purity were quantified spectrophotometrically (Thermo Scientific, NanoDrop ND–1000, USA). The RNAs were stored at -20ºC.

3.3.5.2. cDNA (Complementary DNA) synthesis

The Transcriptor First Strand cDNA Synthesis Kit (Roche, Cat No. 04379012001) was used for cDNA synthesis. The components given in Table 3. 1. were mixed thoroughly and the samples were centrifuged to ensure it was all collected at the bottom of the tube. The tubes were placed in the thermocycler (Thermo Scientific 5020, Finland) at 65ºC for 10 mins. While the samples were in the thermocycler the mastermix was prepared as shown in Table 3. 2. Once the thermocycler had finished the samples were put on ice for 5 min to bind the primers to the RNA then 7 µL of the mastermix was added to each tube and vortexed (Phoenix Instruments RS-VA 10, Belgium). The tubes were then placed in the thermocycler for a second time and were set to the program shown in Table 3. 3. After reverse transcription, aliquots were stored at -20°C until use.

Table 3. 1. Components and preparation of the RNA-Primer mixture (for 1 reaction)

Component	**Volume**	**Final Concentration**
Water (PCR grade)	Variable	-
Reaction Buffer (5X)	4.0 µL	1X
RNA Sample	Variable	1pg- 2.5 µg
Final Volume	18.0 µL	

Table 3. 2. Components and preparation of mastermix (for 1 reaction)

Component	**Volume**
Reaction Buffer	4.0 µL
Protector RNase Inhibitor	0.5 µL
Deoxynucleotide Mix	2.0 µL
Transcriptor Reverse Transcriptase	0.5 µL
Final Volume	7.0 µL

Table 3. 3. PCR program for cDNA sythesis

Temperature (°C)	**Hold (hh:mm:ss)**
25ºC	00:10:00
50ºC	01:00:00
85ºC	00:05:00
+4ºC	02:00:00 or ∞

3.3.5.3. Quantitative Real Time PCR (qRT-PCR) applications

Primer Design

Target genes were obtained by using Olea BAM1 encoding the BAM enzyme and the mRNA and cDNA sequences belonging to the housekeeping gene region Olea-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using the "National Center for Biotechnology Information (NCBI)" database. The primer and probe design for BAM1 "beta-amylase1, chloroplastic [*Olea europaea* var. sylvestris] (LOC111379737)" known with accession No. XM_023003175 and for GAPDH "glyceraldehyde-3-phosphate dehydrogenase A, chloroplastic [*Olea europaea* var. sylvestris] (LOC111405940)" known with accession No. XM_023035062 was made using the sequence of their gene regions. The sequences of the determined primer pairs and probes, the binding temperature of the primers and the PCR product size base pair (bp) are shown in Table 3.4.

Table 3.4. Information on the primers and probs used in the Real Time PCR

Name		**Sequence (5'-3')**	**Tm (°C)**	**PCR product size (bp)**
BAM1	Forward	GAGAGGGAGATGTGTGCATTTA	54.4	113
	Reverse	ACATTSTTCCSTTCCCTCAT	51.6	
	Prob	6FAM-TGSAGGCGGTTTGTTGCATTTGTA-TMR		
GAPDH	Forward	GAGCCTATTATCAGCAATGCTTC	55.0	121
	Reverse	CTCCAGTGTAGGAGTGAGTAGT	50.1	
	Prob	6FAM-AAGTCCTCGACCAGAAATTGGGCA-TMR		

Primer optimization

In this study, 1 primer pair was optimized using LightCycler 480 Probes Master Kit (Roche) contents. The content optimized according to the primers is given in Table 3.5. The binding temperature of all primer were optimized to 58°C.

Table 3.5. Optimization conditions of the primers used in the Real Time PCR program

Components	**Volume**
cDNA	Variable
Forward Primer (10µmol)	0.5 µL
Reverse Primer (10µmol)	0.5 µL
Prob	0.2 µL
LightCycler 480 Probes Mastermix (2X)	10 µL
Water (PCR grade)	Variable
Total Volume	20 µL

Loading samples to qRT-PCR device

The mixture of cDNA's containing samples belonging to each primer and the mastermix Transcriptor One-Step RT-PCR Kit (Roche: 04655877001) content were placed into 96 well plates and covered with ABI Sealing Foil. After sealing the plates were centrifuged at 5600 rpm for 1 min. The reaction was started by selecting the optimized temperatures in the RT-PCR instrument (Applied Biosystems, 7500 real time PCR system, USA). The PCR program used is shown in Table 3.6.

Table 3.6. Real Time PCR Program

	Temperature (°C)	**Number of Cycles**	**Hold (hh:mm:ss)**
Pre- Denaturation	95°C	60	00:10:00
Denaturation	95°C		00:00:10
Annealing	58°C		00:01:00
Extension	40°C		00:00:30

3.3.6. Analysis of gene expression data

After the Real Time PCR process, for the BAM1 gene analyzed in all samples under all applied temperatures, the peak profiles of the samples and controls were determined. Ct (Cycle Threshold) values were created by using the peak profiles. Relative expression values were calculated accroding to the $2^{-\Delta\Delta Ct}$ method (Pfaffl, 2006) using the Ct values from the RT-PCR results. The results of the expression levels of the Olea-BAM1 genes were obtained by the $2^{-\Delta\Delta Ct}$ method and normalized according to the housekeeping Olea-GAPDH gene. The changes in the relative expression levels were calculated by proportioning the $2^{-\Delta\Delta Ct}$ values of 3 replicates and the $2^{-\Delta\Delta Ct}$ values of the control samples for every cultivar and temperature treatment.

3.4. The data evaluation

The "IBM SPSS Statistics 22" program was used to evaluate the results for maltose, starch and BAM activity. The difference between the treatments was revealed at a 0.05 significant level using the “Duncan” test.

4. RESULTS AND DISCUSSION

When evaluating the results; the frost tolerance levels of Çelebi (İznik) and Memeli were compared between themselves and between each other.

4.1. Glucose (Glu) Content

The changes in the Glu content in the leaf tissues of the Çelebi (İznik) and Memeli olive cultivars under frost stress treatments are shown in Table 4.1. and Figure 4.1. The difference between cultivars, treatments and the interaction of cultivar * treatment were found to be statistically significant at the level of 0.05 in terms of the Glu content. The interaction table of Glu content in Memeli and Çelebi (İznik) cultivars is shown in Table A.1. It was determined that the Glu content in the Memeli cultivar, which has a high frost tolerance, was higher than the Çelebi (İznik) cultivar. When the cultivars were evaluated; It was determined that in terms of the average Glu content, the Memeli cultivar (2544.31 mg/L) was higher than the Çelebi (İznik) cultivar (2194.43 mg/L). Overall the highest content was seen in the Memeli cultivar under the -8°C treatment (3002.62 mg/L) and the lowest content was in the Çelebi (İznik) cultivar also under the -8°C treatment (1518.12 mg/L). Although the Glu content in the Memeli cultivar was higher than in the Çelebi (İznik) cultivar, the Glu content in the Memeli cultivar was higher than the Çelebi (İznik) cultivar under the 0°C and -4°C treatments. When the Glu content of just the Memeli cultivar was examined; the -8°C treatment was slightly higher whereas all the other treatment were lower than the Control treatments Glu content (2846.80 mg/L). However when the Glu content of just the Çelebi (İznik) cultivar was examined, the 0°C , -4°C, -16 and -20°C treatmants were higher, 4°C and -12°C treatments were slightly lower and -8°C treatment was significantly lower than the Control treatments Glu content (2054.75 mg/L). The average Glu content was determined as 2450.77 mg/L in the Control treatment, 1986.92 mg/L at +4°C, 2567.81 mg/L at 0°C, 2490.75 mg/L at -4°C 2260.37 mg/L at -8°C, 2205.56 mg/L at -12°C, 2427.00 mg/L at -16°C and 2565.81 mg/L at -20°C.

Glucose is a versatile metabolite that is involved in the regulation of various physiological functions, including plant growth and development, energy source, being precursors for many metabolic pathways, components of structural building blocks and osmoprotectants under stress conditions (Saddhe et. al., 2020). Glucose is a monosaccharide and normally increases extensively once subjected to abiotic stress such as freezing temperatures. It has been stated that the accumulation of Glu can protect the plants cells from freezing temperatures (Pommerrenig et. al., 2018). Glucose serves as an osmoprotectant and also plays a role in protecting cellular membranes from damage caused by freezing by reacting with the lipid bilayer (Yuanyuan et. al., 2009). In this study to determine the changes in the Glu content under frost stress, it was found that the Glu content in the Memeli cultivar, which has a high frost tolerance, was higher than the Çelebi (İznik) cultivar.

Similar to our observations in the olive plant, the Glu content was found to increase positively with the degree of freezing tolerance in cabbage seedlings (Sasaki et. al., 1996). Dionne et. al. (2001), found that the Glc levels increase in three annual blugrass ecotypes once exposed to freezing temperatures. When Hjihashemil et. al. (2018) investigated Glc levels in nine *Stevia rebaudina* under freezing temperatures, they found that Glu levels increased substantially in all cultivars.

Table 4.1. The Glc content in leaf tissues of Çelebi (İznik) and Memeli olive cultivars under frost stress treatment.

Cultivar	**mg/L**
Çelebi (İznik)	2194.43
Memeli	2544.31
Treatment	
Control	2450.77[a]
+4°C	1986.92[d]
0°C	2567.81[a]
-4°C	2490.75[a]
-8°C	2260.37[bc]
-12°C	2205.56[c]
-16°C	2427.00[b]
-20°C	2565.81[a]
ANOVA	
Cultivar	*
Treatment	*
Cultivar * Treatment	*

* Significant at the level of 0.05

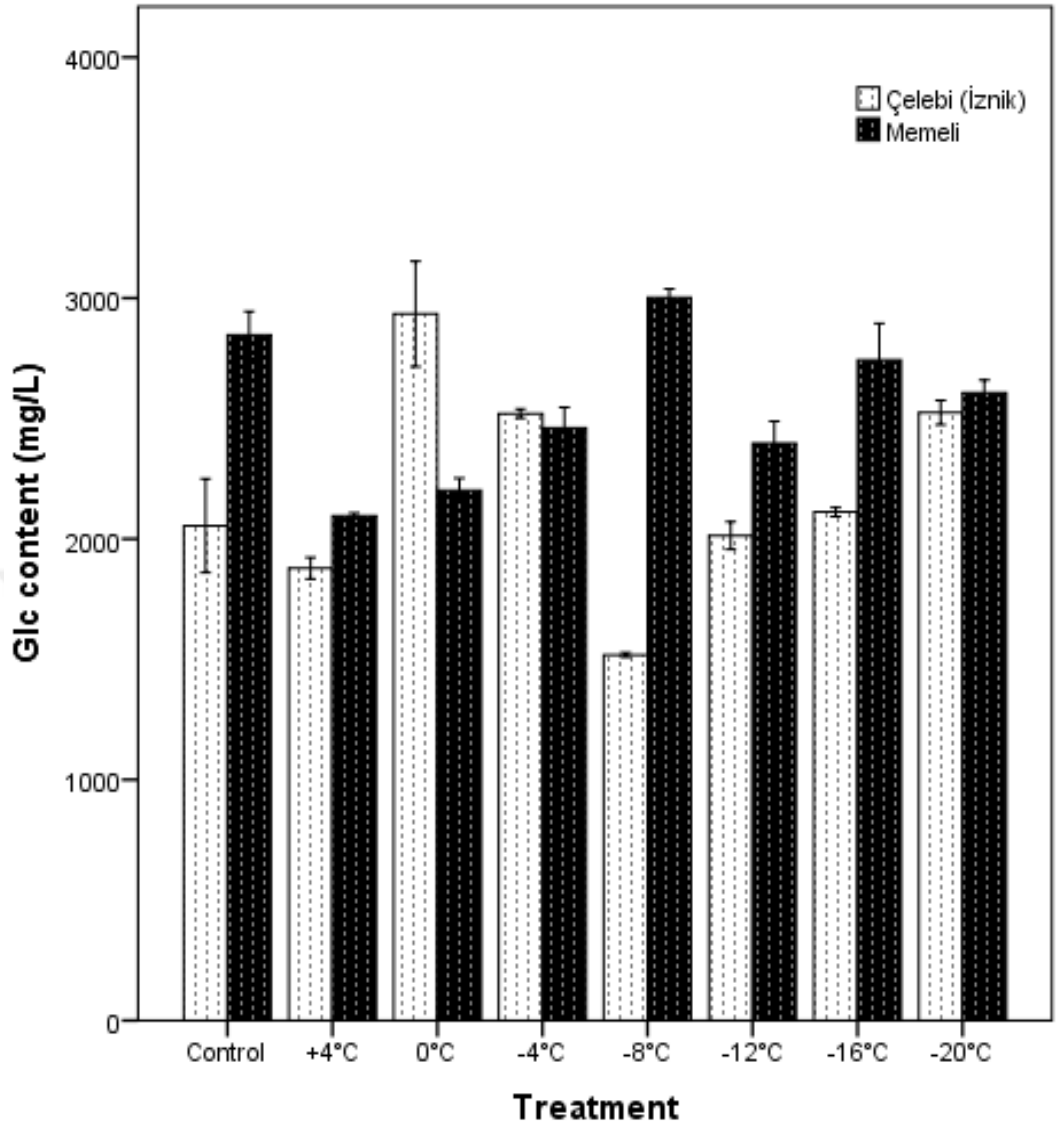

Figure 4.1. The Glu content in the Çelebi (İznik) and Memeli cultivars under frost stress. The vertical bars show the ± SE of the replicates.

4.2. Maltose Content

The changes in the maltose content in the leaf tissues of the Çelebi (İznik) and Memeli olive cultivars under frost stress treatments are shown in Table 4.2. and Figure 4.2. The difference between cultivars, treatments and the interaction of cultivar * treatment were found to be statistically significant at the level of 0.05 in terms of the maltose content. The interaction table of maltose content in Memeli and Çelebi (İznik) cultivars is shown in Table A.2.

It was determined that the maltose content in the Çelebi (İznik) cultivar, which has a low frost tolerance, was higher than the Memeli cultivar from -12°C. When the cultivars were evaluated; It was determined that in terms of the average maltose content, the Çelebi (İznik) cultivar (40.17 mg/L) was higher than the Memeli cultivar (37.30 mg/L). Overall the highest content was seen in the Çelebi (İznik) cultivar under the -16°C treatment (73.82 mg/L) and the lowest content was also in the Çelebi (İznik) cultivar under the + 4°C treatment (14.98 mg/L). Although the maltose content in the Çelebi (İznik) cultivar was higher than in the Memeli cultivar, the maltose content in the Memeli cultivar was higher than the Çelebi (İznik) cultivar until the -12°C treatment. When the maltose content of just the Çelebi (İznik) cultivar was examined; 0°C, -4°C and -8°C treatments were slightly higher, -12°C, -16°C and -20°C treatments were significantly higher and +4°C treatment was lower than the Control treatments maltose content (16.37 mg/L). However when the maltose content of just the Memeli cultivar was examined, the highest content was in the -20°C treatment (50.19 mg/gFW) and the lowest content was in the Control treatment. Memeli cultivar's treatments from +4°C to -20°C were all higher than the Control treatment's maltose content, where it continued to increase until the -8°C treatment and then decreased slightly untill the -16°C treatment and then increased again at the -20°C treatment. The average maltose content was determined as 19.05 mg/L in the Control treatment, 23.97 mg/L at +4°C, 25.33 mg/L at 0°C, 32.70 mg/L at -4°C 40.18 mg/L at -8°C, 49.49 mg/L at -12°C, 56.95 mg/L at -16°C and 62.29 mg/L at -20°C. When the treatments were examined in the terms of the average maltose content it showed that frost stress treatments increased the amount of maltose.

Maltose is the main product of starch degradation (Monroe et. al., 2014). It plays an important role in photosynthetic protection and functions as an osmoprotectant under cold shock (Kaplan et. al., 2006; Valerio et. al., 2011). Maltose can protect proteins, membranes, and the photosynthetic electron transport chain at physiologically suitable levels under freezing stress (Lu et. al. 2006). In this study to determine the changes in the maltose content under frost stress, it was found that the overall maltose content increased in both cultivars but was higher in the Çelebi (İznik) cultivar, which has a low frost tolerance, than the Memeli cultivar.

Similar to our observations in the olive plant, it has been found that maltose accumulates at high levels in the winter period, which may promote the frost tolerance of the tea plant (*Camellia sinensis*) (Yue et. al., 2015). Megías-Pérez et. al. (2020) also reported that the maltose content increased under freezing temperatures in the kale plants and that it is explained by the plant's ability to accumulate starch within the chloroplast stroma. Nielson et. al. (1997) stated that reducing potato storage temperatures resulted in an increased maltose accumulation. When Peng et. al. (2014) investigated the sugar levels in the PtrBAM1 overexpressing transgenic tobacco plants, they found that it resulted in a higher maltose accumulation under freezing temperatures when compared with the wild type.

Table 4.2. The maltose content in leaf tissues of Çelebi (İznik) and Memeli olive cultivars under frost stress treatment.

Cultivar	**mg/L**
Çelebi (İznik)	40.17
Memeli	37.29
Treatment	
Control	19.05^{g}
+4°C	23.97^{f}
0°C	25.33^{f}
-4°C	32.70^{e}
-8°C	40.18^{d}
-12°C	49.49^{c}
-16°C	56.95^{b}
-20°C	62.29^{a}
ANOVA	
Cultivar	*
Treatment	*
Cultivar * Treatment	*

* Significant at the level of 0.05

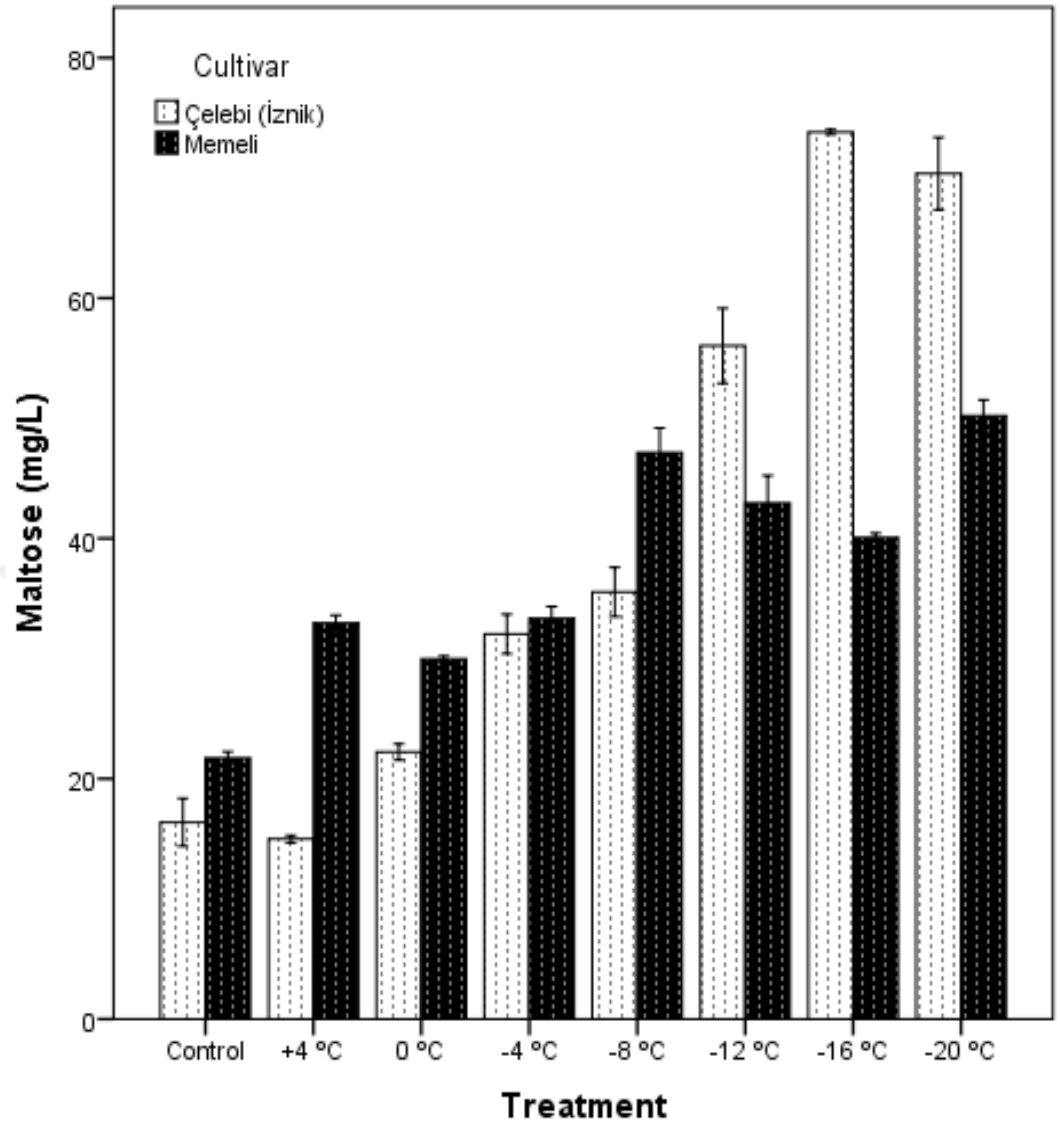

Figure 4.2. The maltose content in the Çelebi (İznik) and Memeli cultivars under frost stress. The vertical bars show the ± SE of the replicates.

4.3. Starch Content

The results of the changes in the starch content in the tissues of the Çelebi (İznik) and Memeli olive cultivars under frost stress treatments are shown in Table 4.3. and Figure 4.3. The difference between Cultivar, Treatment and the interaction of Cultivar * Treatment was found to be statistically significant at the level of 0.05 in terms of starch content.

The interaction table of starch content in Memeli and Çelebi (İznik) cultivars are shown in Table A.3. It was determined that the starch content in the Çelebi (İznik) cultivar which has a low frost tolerance, was lower than the Memeli cultivar in all treatments other than in the -8°C treatment. When the cultivars were evaluated; It was determined that in terms of the average starch content, the Çelebi (İznik) cultivar (4.17 mg/gFW) was lower than the Memeli cultivar(4.87 mg/gFW). When the treatments were examined the Control, +4°C, 0°C, -4°C and -8°C treatments were found to be in the same group. The overall highest starch content was seen in the Memeli cultivar under the Control treatment (5.94 mg/gFW) and the lowest content was seen in the Çelebi (İznik) cultivar under the -16°C treatment (2.17 mg/gFW). Although the starch content in all treatments was generally lower than the Control treatment in the Memeli cultivar, it was very close to the Control tratment untill the -8°C treatment and then a significant decrease was observed. When the starch content of just the Çelebi (İznik) cultivar was examined, the highest content was under the -8°C treatment (5.498 mg/gFW) and the lowest content was under the -16°C treatment (2.17 mg/gFW). Çelebi (İznik) cultivar's 0°C and -4°C treatments were slightly lower, -12°C, -16°C and -20°C treatment were significantly lower and the +4°C and -8°C treatments were higher than the Control treatment's starch content (5.18 mg/gFW). However when the starch content of just the Memeli cultivar was examined, the highest content was in the Control treatment (5.94 mg/gFW) and the lowest content was under the -20°C treatment (3.72 mg/gFW). The average starch content was determined as 5.56 mg/gFW in the Control treatment, 5.35 mg/gFW at +4°C, 4.93 mg/gFW at 0°C, 5.31 mg/gFW at -4°C 4.99 mg/gFW at -8°C, 3.84 mg/gFW at -12°C, 3.04 mg/gFW at -16°C and 3.27 mg/gFW at -20°C. When the treatments were examined in the terms of the average starch content it showed that frost stress treatments decreased the amount of starch.

Starch is the most extensive and commonly found storage polysaccharide that exists in most plants (Yue et. al., 2018).The starch content of leaves can react to environmental stresses and it has been shown that freezing tolerance has been connected to starch degredation (Lu et. al., 2006). Frost stress is widely known to trigger starch degradation (Dong et al., 2019). While frost stress causes starch accumulation in leaves in some studies (Kaplan & Guy, 2005), it reduced starch accumulation in others (Sicher, 2011; Lee et al., 2012; Yue et al., 2015).

Indeed, it has been reported that there is a negative correlation between plant growth and starch accumulation in plant leaves (de Freitas Lima et al., 2017). In this study to determine the changes in the starch content under frost stress, it was found that the overall starch content decreased in both cultivars but was lower in the Çelebi (İznik) cultivar, which has a low frost tolerance, than the Memeli cultivar.

Similar to our observations in the olive plant, the starch content decreased with cold acclimation in zoysiagrass (Patton et. al., 2007), peach trees (*Prunus persica*) (Shin et. al., 2015) and kiwi fruit trees (*Actinidia arguta*) (Sun et. al., 2020). Yun et. al. (2014) reported that the starch content decreased in the winter months in poplar trees and walnut trees. Qi et. al. (2011) also reported that the starch level in the leaves of tomato seedlings decreased at 6°C. Lee et. al. (2012), stated that there was a larger decrease of starch content in the frost tolerant cultivar "Jersey" than in the frost sensitive cultivar "Sharpblue". It was reported that in the tea plant (*Camellia sinensis*) the starch was dramatically degraded during CA in the early winter season, whereas the content of total sugars was constantly elevated (Yue et. al., 2015). Peng et. al. (2014) reported that the starch content in the PtrBAM1 overexpressing transgenic tobacco plants decreased under freezing temperatures when compared to the wild type. The results of many researchers correlate with the results obtained from this study.

Table 4.3. The starch content in leaf tissues of Çelebi (İznik) and Memeli olive cultivars under frost stress treatment.

Cultivar	**mg/gFW**
Çelebi (İznik)	4.17
Memeli	4.87
Treatment	
Control	5.56[a]
+4°C	5.35[a]
0°C	4.93[a]
-4°C	5.31[a]
-8°C	4.99[a]
-12°C	3.84[b]
-16°C	3.04[c]
-20°C	3.27[bc]
ANOVA	
Cultivar	*
Treatment	*
Cultivar * Treatment	*

* Significant at the level of 0.05

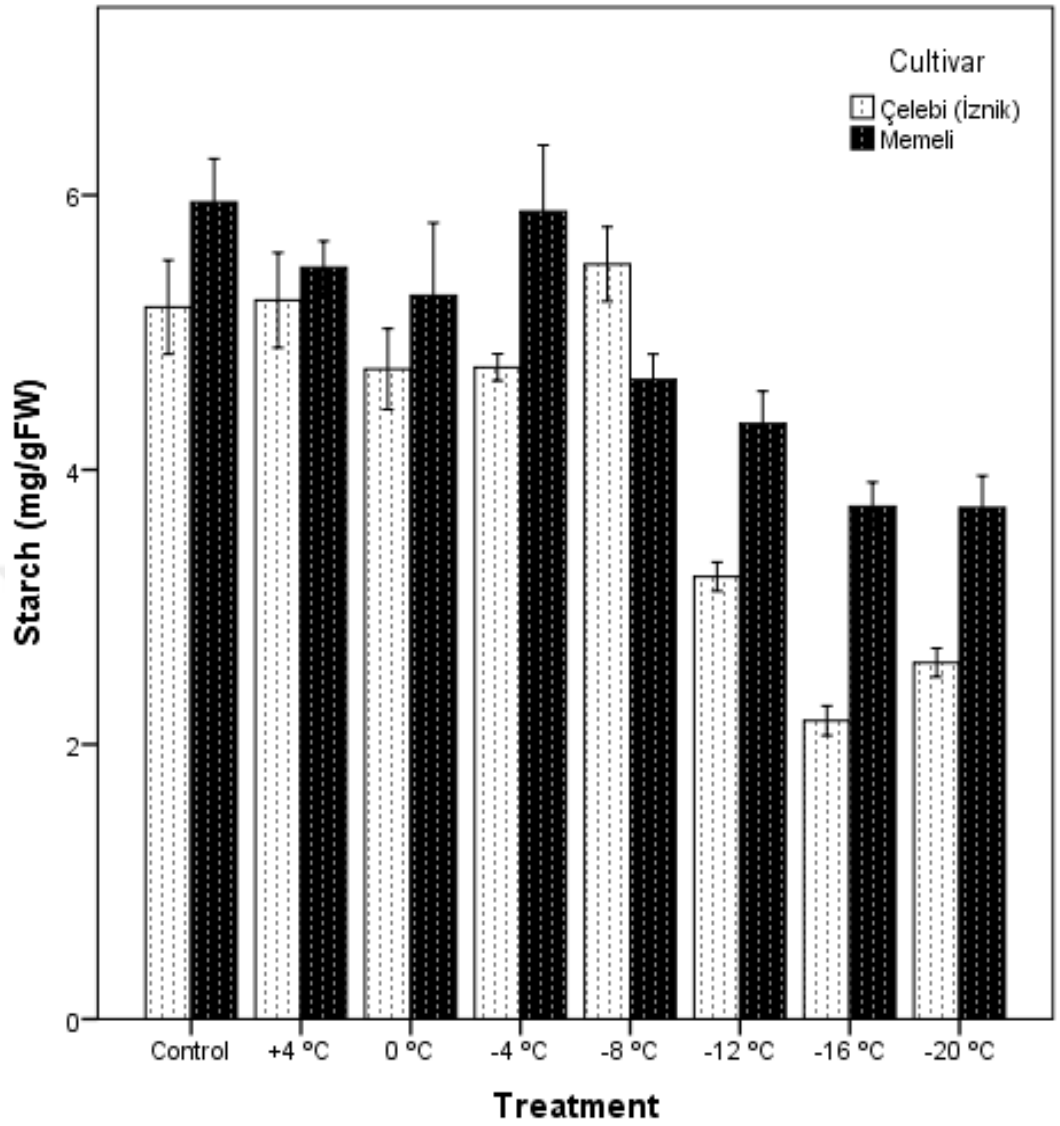

Figure 4.3. The starch content in the Çelebi (İznik) and Memeli cultivars under frost stress. The vertical bars show the ± SE of the replicates.

4.4. β- Amylase Activity

The results of the changes in the BAM activity in leaf tissues of the Çelebi (İznik) and Memeli olive cultivars under frost stress treatments are shown in Table 4.4. and Figure 4.4. While the difference between Cultivar and Treatment in terms of BAM activity were found statistically significant, the interaction of the Cultivar * Treatment was found to not be statistically significant at the level of 0.05. The interaction table of BAM activity in Memeli and Çelebi (İznik) cultivars are shown in Table A.4..

It was determined that the BAM activity in the Çelebi (İznik) cultivar, which has a low frost tolerance, was lower than the Memeli cultivar in all treatments other than in the -8°C treatment. When the cultivars were evaluated; It was determined that in terms of the average BAM activity, the Çelebi (İznik) cultivar (170.17 units/ mg protein) was lower than the Memeli cultivar (183.90 units/ mg protein). Overall the highest BAM activity was seen in the Memeli cultivar under the 0°C treatment (203.00 units/ mg protein) and the lowest content was seen in the Çelebi (İznik) cultivar under the +4°C treatment (160.30 units/ mg protein). When the BAM activity of just the Çelebi (İznik) cultivar was examined, the highest content was under the -4°C treatment (178.793 units/mg protein) and the lowest content was under the +4°C treatment (160.303 units/mg protein). The BAM activity of the Çelebi (İznik) cultivar was lower than the Control treatment in all treatments other than the -4°C and -20°C treatments. However when the BAM activity of just the Memeli cultivar was examined, the highest content was in the -4°C treatment and the lowest content was in the -8°C treatment (161.704 units/mg protein). The BAM activity of the Memeli cultivar increased until the -8°C treatment, decreased at -8°C and then started to increase again after the -12°C treatment. The average BAM activity was determined as 178.09 units/mg protein in the Control treatment, 170.57 units/mg protein at +4°C, 189.58 units/mg protein at 0°C, 190.90 units/mg protein at -4°C, 167.24 units/mg protein at -8°C, 167.32 units/mg protein at -12°C, 174.92 units/mg protein at -16°C and 181.40 units/mg protein at -20°C. The BAM activity was found to be higher in the Memeli cultivar than Çelebi (İznik) cultivar, however, frost stress treatments did not cause a dramatic decrease or increase in BAM activity in either cultivar.

The β- amylase is the main enzyme to hydrolyze starch (Sun et. al., 2020). β- amylase seem to have an important role in starch degradation (Sparla et. al., 2006). The expression and activity of BAM are controlled by abiotic stress (Shin et. al., 2015). BAM is thought to play a special role in the accumulation of soluble sugars under freezing stress (Peng, 2014). In this study to determine the changes in the BAM activity under frost stress, it was found that the overall BAM activity was lower in the Çelebi (İznik) cultivar, which has a low frost tolerance, than the Memeli cultivar.

Sitnicka and Orzechowski (2014) reported that in two potato cultivars exposed to low temperatures, the BAM enzyme activity increased in one cultivar but not the other suggesting that they might degrade starch throw different pathways. Peng et. al. (2014), stated that the BAM activity increase in the PtrBAM1 overexpressing transgenic tobacco plants under freezing temperatures when compared to the wild type. When Yue et. al. (2015) investigated the BAM activity in the tea plant (*Camellia sinensis*), they found that the BAM activity increased majorly in the CA stage. Sun et. al. (2020) also reported that the BAM activity increased in two kiwi fruit tree cultivars (*Actinidia arguta* cvs. "CJ-1" and "RB-3") under freezing temperatures.

Table 4.4. The BAM activity in leaf tissues of Çelebi (İznik) and Memeli olive cultivars under frost stress treatment.

Cultivar	**units/mg protein**
Çelebi (İznik)	170.17
Memeli	183.90
Treatment	
Control	178.09^{ab}
+4°C	170.57^{a}
0°C	189.58^{a}
-4°C	190.90^{a}
-8°C	167.24^{b}
-12°C	167.32^{b}
-16°C	174.92^{ab}
-20°C	181.40^{ab}
ANOVA	
Cultivar	*
Treatment	*
Cultivar * Treatment	ns

*Significant at the level of 0.05

ns: Not significant

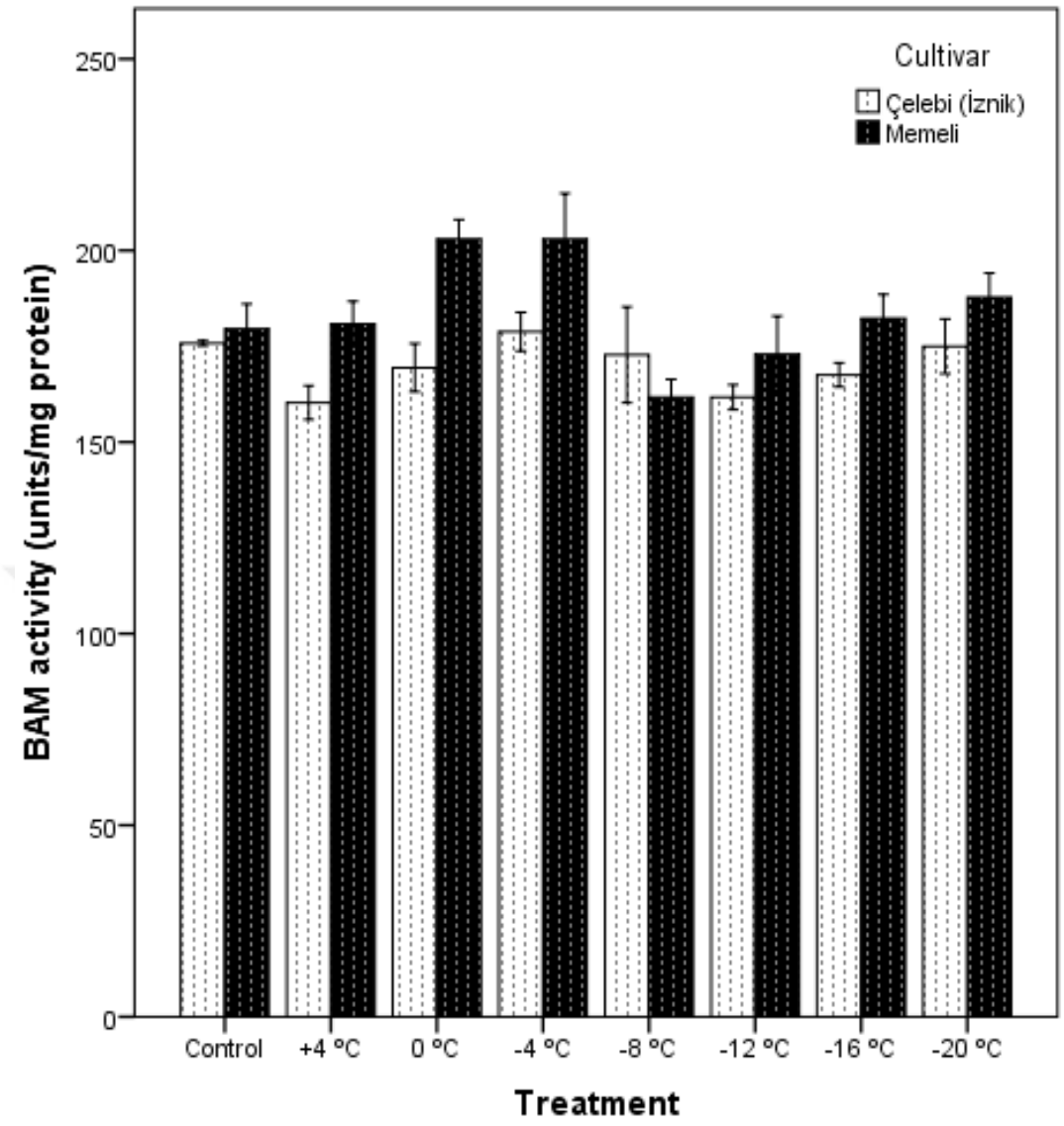

Figure 4.4. The BAM activity in the Çelebi (İznik) and Memeli cultivars under frost stress. The vertical bars show the ± SE of the replicates.

4.5. BAM1 Gene Expression

The results of the BAM1 expression in leaf tissues of the Çelebi (İznik) and Memeli olive cultivars under frost stress treatments are shown in Table 4.5. and Figure 4.5. The interaction table of the relative gene expression level of the BAM1 gene in Memeli and Çelebi (İznik) cultivars are shown in Table A.5. When the average gene expression levels were examined; It was determined that the BAM1 gene was expressed 9.60 folds more in the Memeli cultivar than in the Çelebi (İznik) cultivar, which has low frost tolerance.

While the expression of the BAM1 gene in Çelebi (İznik) cultivar in the +4°C and 0°C applications was close to the Control treatment, it decreased in the other treatments. In the Memeli cultivar however, it was determined that BAM1 gene expression increased in parallel with the frost stress treatments. The highest increase was seen in the -16°C treatment (12.33 folds), which was followed by -20°C (10.51 folds), -12°C (9.29 folds), -8°C (8.63 folds), +4°C (7.81 folds), -4°C (5.58 folds) and 0°C (5.54 folds) respectively. The SE values of both cultivars are shown in Table 4.4.

It was determined that frost stress treatments increased the BAM1 gene expression in the Memeli cultivar and that it was expressed more in the Memeli cultivar than the Çelebi (İznik) cultivar. Kaplan and Guy (2004) stated that the transcript levels of BAM increased in *Arabidopsis Thaliana* when exposed to cold stress. Guerra et. al. (2015) reported that starch degradation is associated with the changes in the BAM1 gene expression promoted by cold acclimation. Similar to our observations in the olive plant, the VcBAM gene profile in the blueberry plant (Lee et. al., 2012) and in the CsBAM gene profile in the tea plant (Yue et. al., 2015) upregulated with the plant's natural adaptation to freezing temperatures. Peng et. al. (2014) stated that PtrBAM1 can be directly regulated by the C-repeat binding factor (CBF), which plays a role in frost tolerance by regulating the sugar content in *Poncirus trifoliata.* They also stated that the PtrBAM1 gene in the PtrBAM1 overexpressing tobacco plants enhanced freezing tolerance when compaired to the wild type. Shin et. al. (2015) reported that the BAM gene expression in two peach cultivars (*Prunus persica* cvs.'Daewol' and 'Kiraranokiwami') increased during cold acclimation. The relative expression of AaBAM in two kiwi fruit cultivars (*Actinidia arguta* cvs. "CJ-1" and "RB-3") were shown to increase in the CA stage which correlates with the decrease in starch content and the increase in soluble sugar levels (Sun et. al., 2020).

Table 4.5. The relative gene expression level of the BAM1 gene in leaf tissues of Çelebi (İznik) and Memeli olive cultivars under frost stress treatment. The ± show the ± SE of the replicates.

Cultivar	**Treatment**	**Relative Gene Expression Level ($2^{-\Delta\Delta Ct}$)**
İznik (Çelebi)	+4 °C	1.53±0.35
İznik (Çelebi)	0°C	1.52±0.43
İznik (Çelebi)	-4°C	0.55±0.43
İznik (Çelebi)	-8°C	0.29±0.35
İznik (Çelebi)	-12°C	0.97±0.43
İznik (Çelebi)	-16°C	0.57±0.43
İznik (Çelebi)	-20°C	0.79±0.43
Memeli	+4°C	7.81±0.43
Memeli	0°C	5.54±0.43
Memeli	-4°C	5.58±0.35
Memeli	-8°C	8.63±0.35
Memeli	-12°C	9.29±0.43
Memeli	-16°C	12.33±0.43
Memeli	-20°C	10.51±0.43
ANOVA		
Cultivar		*
Treatment		*
Cultivar * Treatment		*

* Significant at the level of 0.05

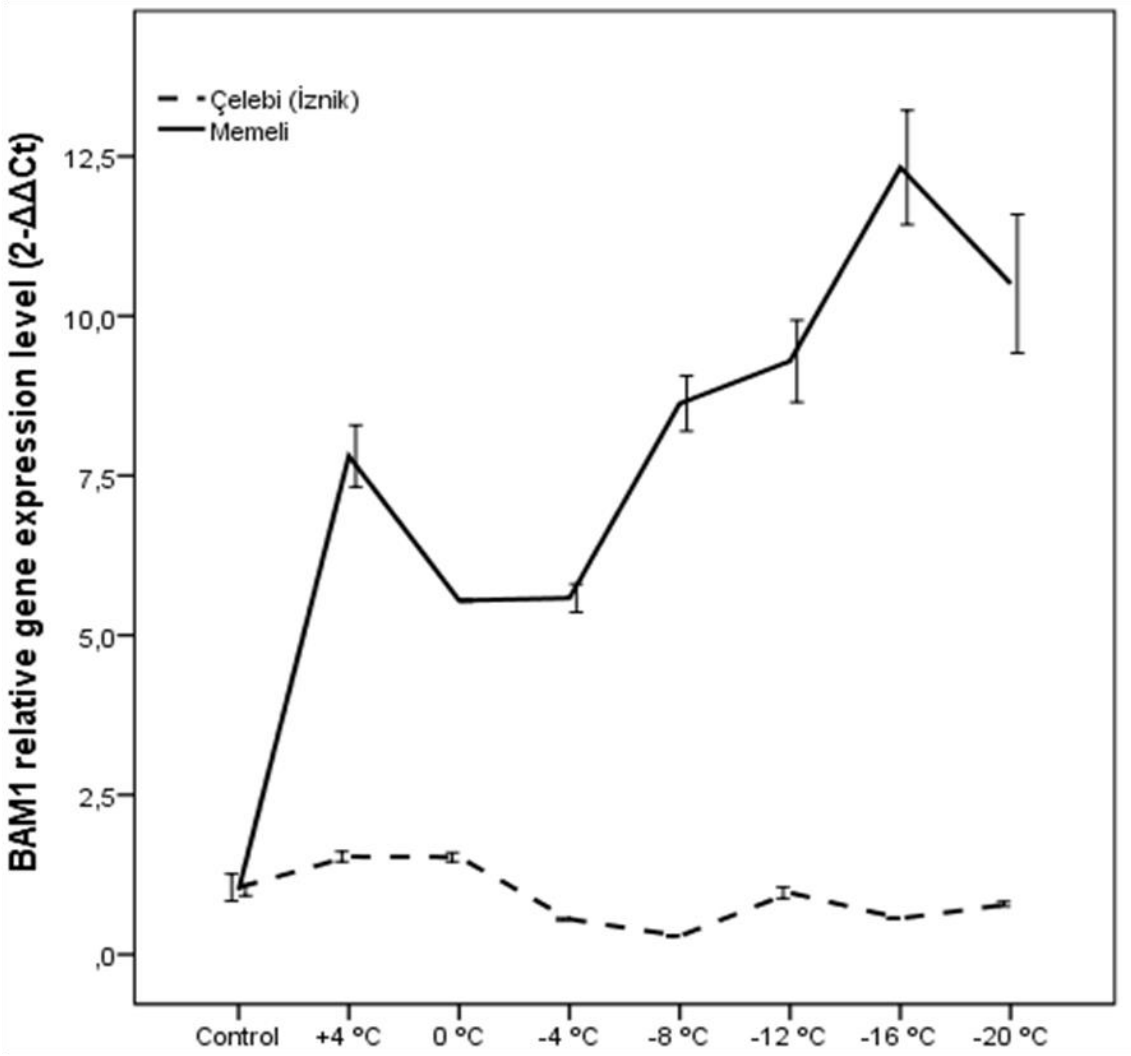

Figure 4.5. The relative gene expression level of the BAM1 gene in leaf tissues of Çelebi (İznik) and Memeli olive cultivars under frost stress treatment. The vertical bars show the ± SE of the replicates.

5. CONCLUSION AND RECOMMENDATIONS

As a result of starch metabolism and effective gene expression analysis carried out on Çelebi (İznik), which is relatively sensitive to frost stress, and tolerant Memeli olive cultivars;

- Maltose accumulation was much more pronounced in the frost-tolerant Memeli cultivar, however it started to accumulate in both cultivars after the +4°C application and Glc accumulation was seen in both cultivars, however was more distinct in the frost-tolerant Memeli cultivar after the -8°C suggesting that these sugars plays an important role in providing freezing tolerance due to accumulation in the early stages of stress in response to freezing temperatures.
- It was determined that frost stress triggers starch degradation in olive plants and partly due to its effect on increasing maltose content, the starch breakdown is important for frost tolerance in the olive plant.
- According to enzyme activity results; Since BAM1 activity was higher in the frost-tolerant Memeli cultivar than in the frost-sensitive Çelebi (İznik) cultivar, this enzyme was found to be effective in frost tolerance in the olive plant.
- It was determined that starch degradation in the chloroplast in the olive plant was associated with transcriptional changes and the BAM1 gene expression was promoted during cold acclimation.
- Gene expression analysis results showed that the expression of the gene (BAM1) encoding the enzyme studied in the cv. Çelebi (İznik), which is more sensitive to frost stress, decreased. In the cv. Memeli, gene expression was found to increase dramatically, especially at -16°C. Besides, it was concluded that the BAM1 gene could be considered as marker gene in breeding frost tolerant olive cultivars.

In line with the results outlined above; It is suggested that studies should be conducted to investigate the potential of the BAM1 gene, as well as other genes related to starch metabolism, to be used as marker genes in breeding frost-tolerant olive cultivars in a large population.

REFERENCES

Anderson, J. A., Smith, M. W., 1989, Ice propagation in peach shots and flowers, Hort Science, 24: pp. 450–452.

Arora, R., Wisniewski, M. E., Scorza, R., 1992, Cold acclimation in genetically related (sibling) deciduous and evergreen peach (*Prunus persica* [L.] Batsch), Plant Physiology, 99: pp. 1562-1568.

Baena-González, E., 2010, Energy signaling in the regulation of gene expression during stress, Molecular Plant, Vol: 3 No: 2: pp. 300–313.

Barranco, D., Ruiz, N., Campo, M. G., 2005, Frost tolerance of eight olive cultivars, 2005, Horticulture Science, 40(3): pp. 558-560.

Barros, P. M., Gonçalves, N., Saibo, N. J. M., Oliveria, M. M., 2012, Cold acclimation and floral development in almond bud break: insights into the regulatory pathways, Journal of Experimental Botany, Vol. 63, No. 2, pp. 695–709.

Bassett, C. L., Wisniewski, M. E., Artlip, T. S., Norelli, J. L., Renaut, J. and Farrell, R. E., 2006, Global analysis of genes regulated by low temperature and photoperiod in peach bark, Journal of the American Society for Horticultural Science, 131: pp. 551-563.

Borovik, O. A., Pomortsev, A. V., Korsukova, A. V., Polyakova, E. A., Fomina, E. A., et. al., 2019, Effect of cold acclimation and deacclimation on the content of soluble carbohydrates and dehydrins in the leaves of winter wheat, Journal of Stress Physiology and Biochemistry, Vol. 15, No. 2: pp. 62-67.

Bradford, M. M., 1976, A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Analytical Biochemistry, 72: pp. 248-254.

Cai, Q., Wang, S., Cui, Z., Sun, J., Ishii, Y., 2004, Changes in freezing tolerance and its relationship with the contents of carbohydrates and proline in overwintering centipedegrass (*Eremochloa ophiuroides* (Munro) Hack.), Plant Production Science, 7 (4): pp. 421- 426.

Camerini, F., 1999, Changes of biochemical parameters in olive (Olea europaea L.) leaves during an entire vegetative season, and their correlation with frost resistance, Acta horticulturae, 474: pp. 435-440.

Campo, M. G., Barranco D., 2005, Field evaluation of frost tolerance in 10 olive cultivars, 2005, Plant Genetic Resources, 3(3); pp. 385–390.

REFERENCES (Continued)

Cansev, A., Gülen, H., Eris, A., 2009, Cold-hardiness of olive (*Olea europaea L.*) cultivars in cold-acclimated and non-acclimated stages: seasonal alteration of antioxidative enzymes and dehydrin-like proteins, Journal of Agricultural Science, 147: pp. 51–61.

Chang, C. Y., Bräutigam, K., Hüner, N. P. A., Ensminger, I., 2020, Champions of winter survival: cold acclimation and molecular regulation of cold hardiness in evergreen conifers, New Phytologist.

Chinnusamy, V., Zhu, J., Zhu, J., 2007, Cold stress regulation of gene expression in plants, TRENDS in Plant Science Vol.12 No.10: pp. 444-451.

D'Angeli, S., Malhó, R., Altamura, M. M., 2003, Low-temperature sensing in olive tree: calcium signalling and cold acclimation, Plant Science, 165: pp. 1303–1313.

de Freitas Lima, M., Eloy, N. B., de Siqueira, J. A. B., Inzé, D., Hemerly, A. S., Ferreira, P. C. G., 2017, Molecular mechanisms of biomass increase in plants, Biotechnology Research and Innovation, 1: pp.14-25.

Dinar, M., Rudich, J., Zamski, E., 1983, Effects of heat stress on carbon transport from tomato leaves, Annals of Botany, Vol. 51, No. 1, pp. 97-103.

Dionne, J., Castonguay, Y., Nadeau, P., Desjardins, Y., 2001, Freezing Tolerance and Carbohydrate Changes during Cold Acclimation of Green-Type Annual Bluegrass (Poa annua L.) Ecotypes, Crop Science, 41, pp. 443–451.

Dong, S., Beckles, D. M., 2019, Dynamic changes in the starch-sugar interconversion within plant source and sink tissues promote a better abiotic stress response, Journal of Plant Physiology, 234–235: pp. 80–93.

Eriş, A., Gulen, H., Barut, E., Cansev A., 2007, Annual patterns of total soluble sugars and proteins related to coldhardiness in olive (*Olea europaea* L. 'Gemlik'), Journal of Horticultural Science & Biotechnology, 82 (4): pp. 597–604.

FAOSTAT, 2021, http://www.fao.org/faostat/en/data

Fernandez, O., Theocharis, A., Bordiec, S., Feil, R., Jacquens, L., et. al., 2012, *Burkholderia phytofirmans* PsJN acclimates grapevine to cold by modulating carbohydrate metabolism, MPMI, Vol. 25, No. 4: pp. 496–504.

Fulton, D. C., Stettler, M., Mettler, T., Vaughan, C. K., Li, J., et. al., 2008, β-AMYLASE4, a noncatalytic protein required for starch breakdown, acts upstream of three active β-Amylases in arabidopsis chloroplasts, The Plant Cell, Vol. 20: pp. 1040–1058.

REFERENCES (Continued)

Gharechahi, J., Alizadeh, H., Naghavi, M. R., Sharif, G., 2014, A proteomic analysis to identify cold acclimation associated proteins in wild wheat (*Triticum urartu L.)*, Molecular Biology Reports, 41: pp. 3897–3905.

Goldstein, G., Nobel, P. S., 1991, changes in osmotic pressure and mucilage during low-temperature acclimation of Opuntia fieusindiea, Plant Physiology, 97: pp. 954-961.

Grennan, A. K., 2006, Regulation of starch metabolism in arabidopsis leaves, Plant Physiology, Vol. 142: pp. 1343–1345.

Guerra, D., Lamontanara, A., Bagnaresi, P., Orrù, L., Rizza, F., et. al., 2015, Transcriptome changes associated with cold acclimation in leaves of olive tree (*Olea europaea* L.), Tree Genetics & Genomes, 11: 113.

Gusta, L. V., Wisniewski, M., 2013, Understanding plant cold hardiness: an opinion, Physiologia Plantarum, 147: pp. 4–14.

Hajihashemi, S., Noedoost, F., Geuns, J.M.C., Djalovic, I., Siddique, K.H.M. 2018. "Effect of cold stress on photosynthetic traits, carbohydrates, morphology, and anatomy in nine cultivars of Stevia rebaudiana". Frontiers in Plant Science, 9, 1430.

Halford, N. G., Curtis, T. Y., Muttucumaru, N., Postles, J., Mottram, D. S., 2011, Sugars in crop plants, Annals of Applied Biology, 158: pp. 1–25.

Hashempour, A., Ghasemnezhad, M., Sohani, M. M., Ghazvini, R. F., Abedi, A., 2018, Effects of freezing stress on the expression of fatty acid desaturase (FAD2, FAD6 and FAD7) and beta-glucosidase (BGLC) genes in tolerant and sensitive olive cultivars, Russian Journal of Plant Physiology, Vol. 66, No. 2, pp. 214–222.

Hatfield, J. L., Prueger, J. H., 2015, Temperature extremes: effect on plant growth and development, Weather and Climate Extremes, Vol. 10 Part A: pp. 4-10.

Hirt, H., Shinozaki, K., 2003, Plant responses to abiotic stress, Topics in Current Genetics, Vol. 4, pp: 151-175.

Ho, S. L., Chao, Y. C., Tong, W. F., Yu, S. M., 2001, Sugar coordinately and differentially regulates growth- and stress-related gene expression via a complex signal transduction network and multiple control mechanisms, Plant Physiology, Vol. 125: pp. 877- 890.

Jacobs, I., 2018, Analysis of aspects of the starch metabolic pathway in lower plants, Master of Science Thesis, Stellenbosch University, pp. 1-2.

REFERENCES (Continued)

Janská, A., Maršık, P., Zelenková, S., Ovesná, J., 2010, Cold stress and acclimation – what is important for metabolic adjustment?, Plant Biology, pp. 395–405.

Jones, K. S., Paroschy, J., McKersie, B. D., Bowley, S. R., 1999, Carbohydrate composition and freezing tolerance of canes and buds in *Vitis vinifera,* Journal of Plant Physiology, Vol. 155: pp. 101-106.

Kaplan, F., Guy, C. L., 2005, RNA interference of Arabidopsis beta-amylase8 prevents maltose accumulation upon cold shock and increases sensitivity of PSII photochemical efficiency to freezing stress, The Plant Journal, 44: pp. 730–743.

Kaplan, F., Guy, C. L., 2004, β-Amylase induction and the protective role of maltose during temperature shock, Plant Physiology, Vol. 135: pp. 1674–1684.

Kaplan, F., Sung, D. Y., Guy, C. L., 2006, Roles of β-amylase and starch breakdown during temperatures stress, Physiologia Plantarum, 126: pp. 120–128.

Kaya, H. B., Cetin, O., Kaya, H. S., Sahin, M., Sefer, F., Tanyolac, B., 2016, Association mapping in turkish olive cultivars revealed significant markers related to some important agronomic traits, Biochemical Genetics, 54: pp. 506–533.

Kaya, H., 2017, Zeytinde çeşit tanımlama ve çeşitlerimiz, Zeytincilik Araştırma Enstitüsü, Date of Access: 22.12.2019, http://tarimorman.gov.tr

Keurentjes, J. J. B., Sulpice, R., Gibon, Y., Steinhauser, M. C., Fu, J., et. al., 2008, Integrative analyses of genetic variation in enzyme activities of primary carbohydrate metabolism reveal distinct modes of regulation in *Arabidopsis thaliana*, Genome Biology, Volume 9, Issue 8, Article R129.

Kitaura, K., 1967, Freezing and injury of mulberry trees by late spring frost, Bulletin Sericulture Experiment Station., Tokyo, 22: pp. 202–323.

Kıvrak, M., 2019a, Zeytincilik ve zeytin işleme teknolojisi programı, Marmara Bölgesi, Balıkesir Üniversitesi, Date of access: 20.12.2019, http://edremit.balikesir.edu.tr/?pID=bolum&bID=5

Kıvrak, M., 2019b, Zeytincilik ve zeytin işleme teknolojisi programı, Ege Bölgesi, Balıkesir Üniversitesi, Date of access: 20.12.2019, http://edremit.balikesir.edu.tr/?pID=bolum&bID=5

Koch, K. E., 1996, Carbohydrate-modulated gene expression in plants, Annual Review of Plant Physiology and Plant Molecular Biology, 47: pp. 509–540.

REFERENCES (Continued)

Koster, K. L, Lynch, D. V., 1992, Solute accumulation and compartmentation during the cold acclimation of puma rye, Plant Physiology, 98: pp. 108-113.

Kranner, I., Minibayeva, F. V., Beckett, R. B., Seal, C. E., 2010, What is stress? Concepts, definitions and applications in seed science, New Phytologist, 188: pp. 655–673.

Kreps, J. A., Wu, Y. J., Chang, U. S., Zhu, T., Wang, X., and Harper, J. F., 2002, Transcriptome changes for Arabidopsis in response to salt, osmotic, and cold stress. Plant Physiology, 130: pp. 2129-2141.

Lee, J. H., Yu, D. J., Kim, S. J., Choi, D., Lee, H. J., 2012, Intraspecies differences in cold hardiness, carbohydrate content and β-amylase gene expression of *Vaccinium corymbosum* during cold acclimation and deacclimation, Tree Physiology, 32, pp. 1533–1540.

Lee, J. Y., Lee, D. H., 2003, Use of serial analysis of gene expression technology to reveal changes in gene expression in arabidopsis pollen undergoing cold stress, Plant Physiology, Vol. 132, pp. 517–529.

Levi C., Gibbs M., 1976, Starch degradation in isolated spinach chloroplasts, Plant Physiology, 57, pp. 933–935.

Levitt, J., 1980, Responses of plants to environmental stresses Vol.1. Chilling, freezing and high temperature stresses, 2nd Edn., Academic Press, pp. 497– refs.

Li, T., Xu, S. L., Oses-Prieto, J. A., Putil, S., Xu, P., et. al., 2011, Proteomics analysis reveals post-translational mechanisms for coldinduced metabolic changes in Arabidopsis, Molecular Plant, 4: pp. 361–374.

Lichtenthaler, H. K., 1996, Vegetation stress: an introduction to the stress concept in plants, Plant Physiology, 148: pp. 4–14.

Liu, H., Ouyang, B., Zhang, J., Wang, T., Li, H., et al., 2012, Differential modulation of photosynthesis, signaling, and transcriptional regulation between tolerant and sensitive tomato genotypes under cold stress, PLOS ONE, 7(11): e50785.

Lloyd, J., Blennow, A., Burhenne, K., Kossmann, J., 2004, Repression of a novel isoform of disproportionating enzyme (stDPE2) in potato leads to inhibition of starch degradation in leaves but not tubers stored at low temperature, Plant Physiology, 134, pp. 1347–1354.

REFERENCES (Continued)

Łopieńska-Biernat, Pastorczyk, M., Giełwanowska, I., Żółtowska, K., Stryiński, R., Zaobidna, E., 2017, The influence of short-term cold stress on the metabolism of non-structural carbohydrates in polar grasses, Polish Polar Research, 38(2), pp. 187-204.

Loumou, A., Giourga, C., 2003, Olive groves: "The life and identity of the Mediterranean", Agriculture and Human Values, 20: pp. 87–95.

Lu, Y., Sharkey, T. D., 2006, The importance of maltose in transitory starch breakdown, Plant, Cell and Environment, 29, pp. 353–366.

Lucas, J. W., 1954, Subcooling and ice nucleation in lemons, Plant Physiology, 29: pp. 245–251.

Lukatkin, A. S., Brazaitytė, A., Bobinas, Č., Duchovskis, P., 2012, Chilling injury in chilling-sensitive plants: a review, Žemdirbystė=Agriculture, vol. 99, No. 2: pp. 111–124.

MacRae, E., 2017, Protocols for nucleic acid analysis by nonradioactive probes, Second edition edited by: Hilarioand, E. and Mackay, J., Methods in Molecular Biology, vol. 353: Humana Press Inc., Totowa.

Megías-Pérez, R., Hahn, C., Ruiz-Matute, A. I., Behrends, B., Albach, D. C., Kuhnert, N., 2020, Changes in low molecular weight carbohydrates in kale during development and acclimation to cold temperatures determined by chromatographic techniques coupled to mass spectrometry, Food Research International, 127, 108727.

Mete, N., Şahin, M., Çetin, Ö., Hakan, M., Güloğlu, U., et. al., 2016, Bazı zeytin çeşitlerinde don toleransının dönemsel değişimi, Zeytin Bilimi, 6(1): pp. 25-31.

Monroe, J. D. and Storm, A. R., 2018, Review: The Arabidopsis β-amylase (BAM) gene family: Diversity of form and function, Plant Science, 276: pp. 163–170.

Monroe, J. D., Storm, A. R., Badley, E. M., Lehman, M. D., Platt, S. M., et. al., 2014, β-Amylase1 and β-Amylase3 are plastidic starch hydrolases in Arabidopsis that seem to be adapted for different thermal, pH, and stress conditions, Plant Physiology, Vol. 166, pp. 1748–1763.

Nagao, M., Minami, A., Arakawa, K., Fujikawa, S., Takezawa, D., 2005, Rapid degradation of starch in chloroplasts and concomitant accumulation of soluble sugars associated with ABA-induced freezing tolerance in the moss *Physcomitrella patens*, Journal of Plant Physiology, 162: pp. 169-180.

REFERENCES (Continued)

Nägele, T., Heyer, A. G., 2013, Approximating subcellular organisation of carbohydrate metabolism during cold acclimation in different natural accessions of *Arabidopsis thaliana*, New Phytologist, 198: pp. 777–787.

Nielsen, T. M., Deiting, U, Stitt, M., 1997, A beta-amylase inpotato tubers is induced by storage at low temperature.Plant Physiology, 113: pp. 503–510.

Noronha, H., Silva, A., Dai, Z., Gallusci, P., Rombolà, A. D., Delrot, S., Gerós, H., 2018, A molecular perspective on starch metabolism in woody tissues, Planta, 248: pp. 559–568.

Palonen, P., 1999, Relationship of seasonal changes in carbohydrates and cold hardiness in canes and buds of three red raspberry cultivars, Journal of the American Society for Horticultural Science, 124(5): pp.507–513.

Patton, A. J., Cunningham, S. M., Volenec, J. J., Reicher, Z. J., 2007, Differences in freeze tolerance of Zoysiagrasses: II. Carbohydrate and proline accumulation, Crop Science, Vol. 47, pp. 2170–2181.

Peng, T., Zhu, X., Duan, N., Liu., J. H., 2014, PtrBAM1, a β-amylase-coding gene of *Poncirus trifoliata*, is a CBF regulon member with function in cold tolerance by modulating soluble sugar levels, Plant, Cell and Environment, 37, pp. 2754–2767.

Perras, M., Sarham, F., 1984, Energy state of spring and winter wheat during cold hardening. Soluble sugars and adenine nucteotides, Physiology Plant, 60: pp. 129-132.

Polat, Z. Tunalıoğlu, R., 2012, Zeytin'in (Olea Europaea L.) ekolojik, ekonomik ve görsel değerlerinin bir bütün olarak görebilmektedir, Zeytin Bilimi, 3 (2) pp. 115-122.

Pommerrenig, B., Ludewig, F., Cvetkovic, J., Trentmann, O., Klemens, P.A.W., Neuhaus, H.E. 2018. "In concert: Orchestrated changes in carbohydrate homeostasis are critical for plant abiotic stress tolerance". Plant Cell Physiology., 59(7), pp. 1290–1299.

Pfaffl, M. W., 2006, Relative quantification, Real-time PCR, edited by: Dorak, M. T., pp. 63-82.

Purdy, S. J., Bussell, J. D., Nunn, C. P., Smith, S. M., 2013, A mex1 null mutant accumulates very high levels of maltose in the leaves compared to wild type (WT) and has a starch-excess phenotype, PLOS ONE, Volume 8, Issue 11.

REFERENCES (Continued)

Qi, H., Hua, L., Zhao, L., Zhao, L., 2011, Carbohydrate metabolism in tomato (*Lycopersicon esculentum* Mill.) seedlings and yield and fruit quality as affected by low night temperature and subsequent recovery, African Journal of Biotechnology, Vol. 10(30): pp. 5743-5749.

Rekarte-Cowie, I., Ebshish, O. S., Mohamed, K. S., Pearce, R. S., 2008, Sucrose helps regulate cold acclimation of Arabidopsis, Journal of Experimental Botany, Vol. 59, No. 15: pp. 4205–4217.

Renaut, J., Hausman, J. F., Bassett, C., Artlip, T., Cauchie, H. M., et. al., 2008, Quantitative proteomic analysis of short photoperiod and low-temperature responses in bark tissues of Peach (*Prunus persica* L. Batsch), Tree Genetics & Genomes, 4: pp. 589-600.

Rhodes, D., Nadolska-Orczyk, A., Plant stress physiology, 2001, Encyclopedia Of Life Sciences, pp 1-7.

Ristic, Z., Ashworth, E. N., 1992, Changes in leaf ultrastructure and carbohydrates in *Arabidopsis thaliana* L. (Heyn) cv. Columbia during rapid cold acclimation, Protoplasma, 172: pp. 111-123.

Saadeti, S., Baninasab, B., Mobli, M. and Gholami, M., 2019, Measurements of freezing tolerance and their relationship with some biochemical and physiological parameters in seven olive cultivars, Acta Physiologiae Plantarum, pp. 41:51.

Saddhe, A., A., Manuka, R., Penna, S., 2020, Plant sugars: Homeostasis and transport under abiotic stress in plants, Physiologia Plantarum, 171, pp. 739–755.

Sahin, S., Bilgin, M., 2017, Olive tree (*Olea europaea L.*) leaf as a waste by-product of table olive and olive oil industry: a review, Journal of the Science of Food and Agriculture, 98: pp. 1271–1279.

Sakar, E., Unver, H., Bakir, M., Ulas, M., Sakar, Z. M., 2016, Genetic relationships among Olive (*Olea europaea L.)* cultivars native to turkey, Biochemical Genetics, 54: pp. 348–359.

Sanghera, G. S., Wani, S. H., Hussain, W., Singh, N. B., 2011, Engineering cold stress tolerance in crop plants, Current Genomics, 12, pp. 30-43.

Sasaki, H., Ichimura, K., Oda, M., 1996, Changes in Sugar Content during Cold Acclimation and Deacclimation of Cabbage Seedlings, Annals of Botany 78: pp. 365–369.

REFERENCES (Continued)

Sauter, J. J., Cleve, B. V., 1991, Biochemical and ultrastructural results during starch-sugar-conversion in ray parenchyma cells of *Populus* during cold adaptation, Journal of Plant Physiology, 139: pp. 19-26.

Schilling, B. S., 2004, Physiological and molecular responses of spring and winter canola (*Brassica napus*) during cold acclimation, PhD Thesis, Univ Saskatchewan, Saskatoon, SK, Canada.

Schubert, M., Grønvold, L., Sandve, S. R., Hvidsten, T. R., Fjellheima, S., 2019, Evolution of cold acclimation and its role in niche transition in the temperate main grass subfamily pooideae, Plant Physiology, Vol. 180: pp. 404–419.

Shin, H., Kim, K., Oh, Y., Yun, S. K., Oh, S- II, et. al., 2015, Carbohydrate changes in peach shoot tissues and their relationship to cold acclimation and deacclimation, The Horticulture Journal, 84 (1): pp. 21–29.

Sicher, R., 2011, Carbon partitioning and the impact of starch deficiency on the initial response of Arabidopsis to chilling temperatures, Plant Science, 181: pp. 167–176.

Sitnicka, D., Orzechowski, S., 2014, Cold-induced starch degradation in potato leaves – intercultivar differences in the gene expression and activity of key enzymes, Biologia Plantarum, 58 (4): pp. 659-666.

Smith, A. M., Zeeman, S. C., Smith, S. M., 2005, Starch degradation, Annual Review Plant Biology, 56: pp. 73–98.

Song, J. B., Gao, S., Wang, Y., Li, B. W., Zhang, Y. L., Yang, Z. M., 2016, miR394 and its target gene LCR are involved in cold stress response in *Arabidopsis*, Plant Gene, 5 pp. 56–64.

Sparla, F., Costa, A., Schiavo, F. L., Pupillo, P., Trost, P., 2006, Redox regulation of a novel plastid-targeted β-amylase of *Arabidopsis*, Plant Physiology, Vol. 141, pp. 840–850.

Stockinger, E. J., Gilmour, S. J., Thomashow, M. F., 1997, *Arabidopsis thaliana* CBF1 encodes an AP2 domain-containing transcription activator that binds to the C-repeat/DRE, a cisacting DNA regulatory element that stimulates transcription in response to low temperature and water deficit, Proceedings of the National Academy of Sciences USA, 94: pp. 1035-1040.

Sun, S., Fang, J., Lin, M., Qi, X., Chen, J, et. al., 2020, Freezing tolerance and expression of β-amylase gene in two *Actinidia arguta* cultivars with seasonal changes, Plants, 9, 515.

REFERENCES (Continued)

Tajoddin, M., Manohar, S., Lalitha, J., 2012, Laboratory experiments illustrating evaluation of raffinose family oligosaccharides of mung bean (*Phaseolus aureus* L.) cultivars, Indian Journal of Innovations and Developments, 1(5): pp. 390-394.

Tarkowski, Ł. P., Ende, W. V., 2015, Cold tolerance triggered by soluble sugars: a multifaceted countermeasure, Frontiers in Plant Science, Vol. 6, Article 203.

Thalmann, M., Santelia, D., 2017, Starch as a determinant of plant fitness under abiotic stress, New Phytologist, 214: pp. 943–951.

Thalmann, M., Pazmino, D., Seung, D., Horrer, D., Nigro, A., et. al., 2016, Regulation of leaf starch degradation by abscisic acid is important for osmotic stress tolerance in plants, The Plant Cell, Vol. 28, pp. 1860–1878.

Trischuk, R. S., Schilling, B. S., Low, N. H., Gray, G. R., 2014, Cold acclimation, de-acclimation and re-acclimation of spring canola, winter canola and winter wheat: The role of carbohydrates, cold-induced stress proteins and vernalization, Environmental and Experimental Botany, 106: pp. 156–163.

Turhan, E., Ergin, S., 2012, Soluble Sugars and Sucrose-Metabolizing Enzymes Related to Cold Acclimation of Sweet Cherry Cultivars Grafted on Different Rootstocks, The Scientific World Journal, Volume 2012, Article ID 979682.

Turhan, E., Aktas, H., Deventurero, G., Karni, L. Bar-Tal, A., Aloni, B., 2006, Blossom-end rot is associated with impairment of sugar metabolism and growth of pepper (*Capsicum annuum* L.) fruits, Journal of Horticultural Science & Biotechnology, 81 (5): pp. 921–927.

Vágújfalvi, A., Kerepesi, I., Galiba, G., Tischner, T., Sutka, J., 1999, Frost hardiness depending on carbohydrate changes during cold acclimation in wheat, Plant Science, 144, pp. 85–92.

Vajravijayan, S., Pletnev, S., Mani, N., Pletneva, N., Nandhagopal, N., Gunasekaran, K., 2018, Structural insights on starch hydrolysis by plant β-amylase and its evolutionary relationship with bacterial enzymes, International Journal of Biological Macromolecules, 113: pp. 329–337.

Valerio, C., Costa, A., Marri, L., Issakidis-Bourguet, E., Pupillo, P., et. al., 2011, Thioredoxin-regulated β-amylase (BAM1) triggers diurnal starch degradation in guard cells, and in mesophyll cells under osmotic stress, Journal of Experimental Botany, Vol. 62, No. 2, pp. 545–555.

REFERENCES (Continued)

Wanner, L. A., Junttila, O., 1999, Cold-induced freezing tolerance in Arabidopsis, Plant Physiology, Vol. 120, pp. 391–399.

Wisniewski, M., Bassett, C., Gusta, L. V., 2003, An overview of cold hardiness in woody plants: Seeing the forest through the trees, Horticultural Science, Vol. 85 (5): pp. 952-959.

Wisniewski, M., Nassuth, A., Arora, R., 2018, Cold hardiness in trees: A mini-review, Frontiers in Plant Science, Vol. 9, Article 1394.

Witt, W., Sauter, J. J., 1995, *In vitro* degradation of starch grains by phosphorylases and amylases from poplar wood", Plant Physiology, 146, pp. 35-40.

Xin, Z., Browse, J., 2000, Cold comfort farm: the acclimation of plants to freezing temperatures, Plant, Cell and Environment, 23: pp. 893–902.

Yano, R., Nakamura, M., Yoneyama, T., Nishida, I., 2005, Starch-related α-glucan/water dikinase is involved in the cold-induced development of freezing tolerance in *Arabidopsis*, Plant Physiology, Vol. 138, pp. 837–846.

Yelenosky, G., 1991, Supercooling and freezing in the main stem of Valencia orange trees, Cryobiology, 28: pp. 382–390.

Yoruk, B., Taskin V., 2014, Genetic diversity and relationships of wild and cultivated olives in Turkey, Plant Systematics and Evolution, 300: pp. 1247–1258.

Yuanyuan, M., Yali, Z., Jiang, L., Hongbo, S., 2009, Roles of plant soluble sugars and their responses to plant cold stress, African Journal of Biotechnology, Vol. 8 (10): pp. 2004-2010.

Yue, C., Cao, H. L., Wang, L., Zhou, Y. H., Huang, Y. T., et. al., 2015, Effects of cold acclimation on sugar metabolism and sugar-related gene expression in tea plant during the winter season, Plant Molecular Biology, 88: pp. 591–608.

Yue, C., Cao, H., Lin, H., Hu, J., Ye, Y., et. al., 2019, Expression patterns of alpha-amylase and beta-amylase genes provide insights into the molecular mechanisms underlying the responses of tea plants (*Camellia sinensis*) to stress and postharvest processing treatments, Planta, 250: pp. 281–298.

REFERENCES (Continued)

Yun, S. K., Bae, H., Chung, K. H., Yoon, I. K., Nam, E. Y., et. al., 2014, Sugar, starch, and proline in peach trees exposed to freezing temperatures during dehardening, Agricultural Sciences, 5: pp. 913-921.

Zeeman, S. C., Rees, T. A., 1999, Changes in carbohydrate metabolism and assimilate export in starch-excess mutants of *Arabidopsis*, Plant, Cell and Environment, 22, pp. 1445–1453.

. Zhao, X., Guo, P., Hu, L., Hui, B., Cheng, Q., Via, B. K., 2018, Fast measurement of starch content in cold-treated seedlings by infrared spectra, Scandinavian Journal Of Forest Research, Vol. 33, No. 8, pp. 729–734.

Zhu, J. K., 2016, Abiotic stress signaling and responses in plants, Cell, 167.

APPENDIX

A.1.: Interaction table of Glc content in Memeli and İznik (Çelebi) cultivars

Dependent Variable: mg/L

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	19329278.563[a]	15	1288618.571	21.056	.000
Intercept	647998987.569	1	647998987.569	10588.200	.000
Cultivar	3532455.188	1	3532455.188	57.720	.000
Treatment	4398715.237	7	628387.891	10.268	.000
Cultivar * Treatment	11593599.838	7	1656228.548	27.063	.000
Error	6364811.229	104	61200.108		
Total	699840595.000	120			
Corrected Total	25694089.792	119			

Sig: Significant level (5%)

A.2.: Interaction table of maltose content in Memeli and İznik (Çelebi) cultivars

Dependent Variable: mg/L

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	12152.149[a]	15	810.143	93.100	.000
Intercept	62281.827	1	62281.827	7157.280	.000
Cultivar	85.934	1	85.934	9.875	.004
Treatment	8840.596	7	1262.942	145.134	.000
Cultivar * Treatment	2617.852	7	373.979	42.977	.000
Error	234.951	27	8.702		
Total	73269.279	43			
Corrected Total	12387.099	42			

Sig: Significant level (5%)

A.3. : Interaction table of starch content in Memeli and İznik (Çelebi) cultivars

Dependent Variable: mg/gFW

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	70.236[a]	15	4.682	14.277	.000
Intercept	1147.912	1	1147.912	3500.054	.000
Cultivar	6.940	1	6.940	21.159	.000
Treatment	60.377	7	8.625	26.299	.000
Cultivar * Treatment	5.925	7	.846	2.581	.025
Error	15.087	46	.328		
Total	1384.326	62			
Corrected Total	85.322	61			

Sig: Significant level (5%)

A.4. : Interaction table of BAM activity in Memeli and İznik (Çelebi) cultivars

Dependent Variable: units/mg protein

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	7307.317[a]	15	487.154	3.338	.002
Intercept	1415892.545	1	1415892.545	9701.131	.000
Cultivar	2131.743	1	2131.743	14.606	.001
Treatment	3090.567	7	441.510	3.025	.016
Cultivar * Treatment	1759.527	7	251.361	1.722	.141
Error	4378.538	30	145.951		
Total	1456519.037	46			
Corrected Total	11685.856	45			

Sig: Significant level (5%)

A.5. :Interaction table of the relative gene expression level of the BAM1 gene in Memeli and Çelebi (İznik) cultivars (The dependent variable $2^{-\Delta\Delta Ct}$)

Dependent Variable: units/mg protein

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	530.796[a]	13	40.830	108.863	.000
Intercept	685.653	1	685.653	1828.104	.000
Cultivar	451.373	1	451.373	1203.462	.000
Treatment	35.041	6	5.840	15.571	.000
Cultivar * Treatment	45.998	6	7.666	20.440	.000
Error	6.751	18	.375		
Total	1220.434	32			
Corrected Total	537.547	31			

Sig: Signifcant level (5%)