DIPLOMA THESIS

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Analysis of dehydrin genes at the protein and at the mRNA levels in chilling-treated cucumbers

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1. Introduction

Cucumber (*Cucumis sativus* L.) Is an important vegetable crop belonging to the family Cucurbitaceae. Its immature fruit is consumed fresh, cooked, and processed in various culinary dishes. It is a tropical or subtropical plant grown in both greenhouses (parthenocarpic cucumber) and open fields under warm humid conditions (Pandey and Kujur, 2021). It is cultivated more extensively than any other cucurbit crop except watermelon. According to FAOSTAT (2022), Asia occupied 90% of the global production share of cucumbers and gherkins (tiny cucumbers for pickling), while Europe came second with 6% of the production share. China is the largest producer with a highly significant production share in the global cucumber market. In 2022, China produced ca. 77.3 million tonnes and the second largest producer which is Türkiye produced ca. 1.9 million tonnes of cucumber. In Europe, Ukraine and Spain are among the top ten producers of cucumbers and gherkins globally.

Abiotic stresses, such as salinity, high temperature and chilling were reported to inhibit plant growth and development. Cucumber as a chilling-susceptible plant; upon exposure to low temperatures, it shows significant chilling injury. Chilling stress causes disruption in cell membranes leading to an increase in permeability and solute leakage. It results in accumulation of reactive oxygen species (ROS) that serve as signaling molecules (secondary messengers) and damaging agents. The oxidative breakdown of the double bonds in the fatty acids of membrane lipids induced by ROS can be reflected negatively on the membrane integrity. Chilling stress also triggers alterations in the expression of stress-responsive genes (Wang, 1982; Hara et al., 2003; Mahajan and Tuteja 2005; Solanke and Sharma 2008). Various cellular mechanisms occur to mitigate negative effects of chilling stress on plants including changes in pathways regulating ion and water fluxes, osmo-adjustment, accumulation of cryoprotectants and antioxidants, synthesis of stress-responsive proteins (Shinozaki and Yamaguchi-Shinozaki, 1999). Many low-temperature-responsive genes encode dehydrin proteins as stress-responsive and protective proteins to improve plant tolerance to stress conditions.

Dehydrins are a group of hydrophilic intrinsically disordered proteins (IDPs) belonging to Late Embryogenesis Abundant (LEA) proteins. They are also known as group II or D11 LEA proteins. They display repeated sequence motifs. They are found in different plant tissues in the cytoplasm, chloroplasts, mitochondria and in the nucleus (Rorat, 2006). Their gene expression increases in response to various abiotic stresses such as drought, salinity, cold stress, and high temperature, indicating that they play a vital role in establishing and maintaining plant tolerance

to these stresses. They prime plant cells for acclimation to more severe stress conditions by activating stress response pathways and enhancing plant adaptation to future stress events. They stabilize cellular structures against reduced water potential, scavenge ROS and function as molecular chaperones preventing denaturation and aggregation of cellular proteins and enzymes under stress conditions (Vaseva et al., 2010; Wang, et al., 2020).

2. Objectives

The overarching objective of my thesis is to increase the understanding of chilling responses and acclimation in cucumber as a chilling-sensitive plant in general with a specific focus on:

- investigating the different sensitivities of greenhouse and open field-cultivated cucumber to chilling stress conditions.
- identifying the impacts of chilling temperatures on physiological responses such as leaf water content, and proline content in cucumber.
- examining malondialdehyde (MDA) content as a marker of oxidative stress in cells.
- measuring the electrolyte leakage to evaluate the damage inflicted on cellular membranes.
- surveying chilling conditions that induce the expression of dehydrin proteins in cucumber.
- analyzing the expression of dehydrin genes at both the protein and the mRNA levels.

3. Literature Review

3.1. Climate change

Although the global temperature is on the rise, cold extremes and other extreme climate events are predicted to be more frequent worldwide (Horton et al., 2015). Cold extremes refer to temperatures that fall below historical averages which can lead to sever damage to people, animals, agriculture, natural ecosystems and even infrastructure. Moreover, climate change can affect the severity of winter storms as the warmer the atmosphere, the more moisture it can hold resulting in increased intensity in rainfall or snowfall. Areas with a history of few freeze events could encounter more damaging winter events. These events can cause damage through freezing fog, freezing rain, icing, ice storms, heavy snowfall, and lake-effect snow events (U.S. Department of Homeland Security, n.d.).

In 2022, the IPCC sixth assessment report stated that "climate change impacts will increase the number of people at risk of hunger in 2050 ranging from 8 million people to 80 million people compared with a world with no climate change" (IPCC 2022). Climate change has negative effects on agriculture threatening our endeavors to end food crisis and achieve food security and sustainable agricultural production at a global scale. Climate change-induced abiotic stresses affect the distribution of suitable agricultural regions, disrupt water availability and decline crop yield and livestock production. In 2016, the Food and Agriculture Organization (FAO) has warned in its published data about continuing the current situation of climate change that by the year 2100 there could be a decline in the crop yield, for example, 20–45% decline in maize yields, 5–50% in wheat and 20–30% in rice (Arora, 2019).

As indicated by the Eurostat data, the 2022 crop year in the EU experienced extreme weather events in terms of both temperatures and rainfall. The most significant cold weather event during winter 2021-2022 was a sharp decrease in temperatures in most of the Balkan Peninsula and eastern Europe, when daily minimum temperatures plunged during by the end of January to as low as -24°C (like in Romania). This did not stick around long, only lasted a few days causing minor damage to crops. April was typically colder than usual in most regions. And because of the cold April conditions, some delays to the sowing of summer crops occurred. A distinct cold spell in the first half of April extended from central Spain to the Baltic Sea region. The impact on annual crops was limited, however, there was severe and irreversible damage to blooming fruit crops, particularly in Spain. This late-season cold snap resulted in reduced yields and economic losses for growers. In Romania and Hungary, winter

crops were negatively affected by the cold and dry conditions in March and April (Eurostat, 2023).

3.2. Plant responses to cold temperatures.

Temperature is one of the most important environmental factors that affects the growth and development of plants. According to the classification of Levitt (1980) and Źróbek-Sokolnik (2012), most higher plants are classified as mesophiles exhibiting their optimal growth within a temperature range from 10℃ to 30℃. Distribution of plants is mainly determined by the minimum temperature at which a species can survive. Plants, unlike animals, cannot regulate their cells and tissues to a constant optimal temperature, but they adjust their biology and metabolism to fit the temperature fluctuations. Plants encounter temperature stresses categorized into three types: (a) chilling stress (< 15℃), (b) freezing stress (< 0℃), and (c) high temperature stress (Raju et al., 2018; Lu et al., 2021).

Chilling stress occurs when plants are exposed to temperatures below their optimum range, but above freezing, typically ranging between 0°C and 15℃ depending on the plant species. Chilling stress causes various physiological and biochemical changes in plants, including alterations in cell membranes, changes in gene expression, increased production of reactive oxygen species (ROS), and disruption of essential processes, such as photosynthesis, respiration, enzyme activity, and protein synthesis (Fig. 3.1). The damage resulting from these physiological, biochemical and metabolic changes triggered by temperatures above freezing point and below 15°C is known as chilling injury (Reyes and Jennings, 1994). Chilling injury severity is determined by the temperature, the duration of the stress event, and the developmental stage of the plant. The lower the temperature and the longer the duration are, the greater the injury will be (Fig. 3.2). Crop losses caused by chilling damage represent a significant economic threat to the crop production industry (Mahajan and Tuteja 2005; Solanke and Sharma, 2008).

Plants detect the shifts in temperature of its surrounding, and translate that into biological signals via primary sensory mechanisms including decreasing membrane fluidity which in turn activates transmembrane proteins complex, also known as calcium channels causing calcium ions to enter the cell (Görlach et al., 2015; Lamers et al., 2020). Transient Ca^{+2} influx into the cytosol modulates the intracellular Ca^{+2} level. Alterations in the cytosolic Ca^{+2} concentration are sensed by calcium-binding proteins, or calcium sensors which then interact with other proteins to transmit the signal within the cell. These proteins initiate protein

phosphorylation cascades and regulate the expression of transcription factors and cold-regulated (COR) genes in stressed plants (Zhang et al., 2014; Ma et al., 2015; Guo et al.,2018; Ding et al., 2019; Wei et al., 2021).

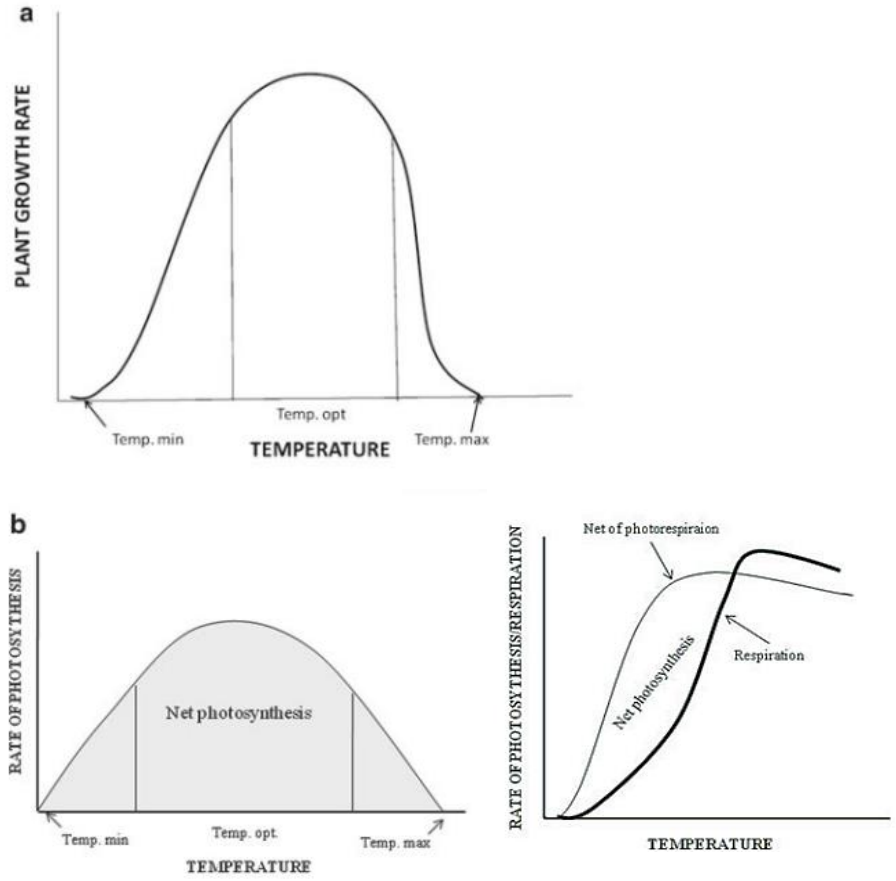


Fig. 3.1. Plant responses to temperature (Fitter and Hay 2002). **(a)** the effect of temperature on plant growth rate; **(b)** the effect of temperature on the rate photosynthesis and respiration.

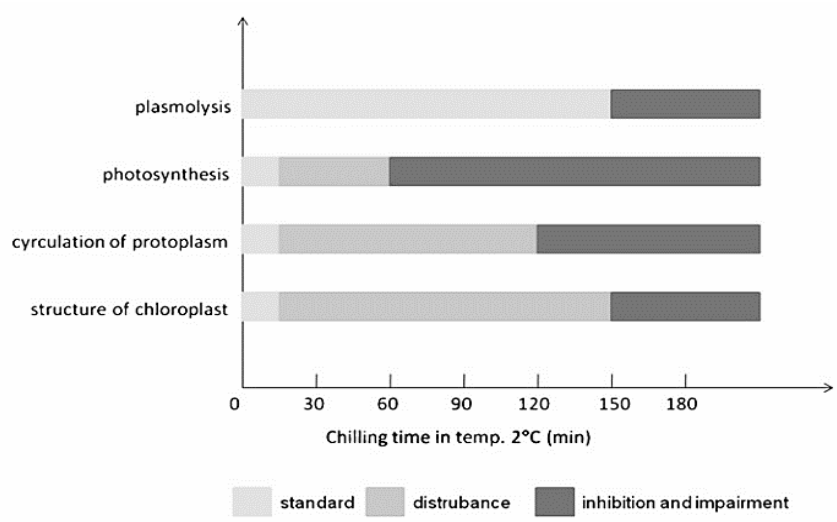


Fig. 3.2. Functional impairments manifest in chilling-sensitive plants, in response to stress duration (Źróbek-Sokolnik, 2012).

One critical aspect of chilling stress in plants is its impact on membrane structure and function. Cell membranes have been considered as the primary sites of cold injury in plants (Fig. 3.3). Two factors are thought to contribute to the membrane damage during cold stress. The first is the degree of saturation of the membrane lipids. Chilling-sensitive plants contain high proportions of saturated fatty acids within their membranes, while cold-tolerant plants have higher concentrations of unsaturated fatty acids. Lyons (1973) stated that low temperatures induce the phase transition of cell membranes from a liquid-crystal structure to a gel state causing membrane dysfunction as it solidifies and becomes rigid. However, high levels of unsaturated fatty acids in membranes would preclude phase transition under chilling stress conditions. The higher the degree of unsaturation of fatty acids, the lower the temperature at which phase transition occurs. Tobacco plants engineered with a chloroplast ω-3 fatty acid desaturase gene (the fad7 gene) isolated from A. thaliana, contain increased levels of trienoic fatty acids that were found to enhance cold tolerance (Kodama et al., 1994). The decrease in membrane fluidity and loss of function induced by lipid peroxidation is the second factor causing membrane damage. Membrane fluidity describes the ease with which lipids and proteins can move within the lipid bilayer and is crucial for membrane function. Transgenic tobacco plants expressing citrus dehydrin CuCOR19 showed lower malondialdehyde (MDA) content acting as a radical-scavenging protein to protect membrane systems under chilling stress conditions (Hara et al., 2003).

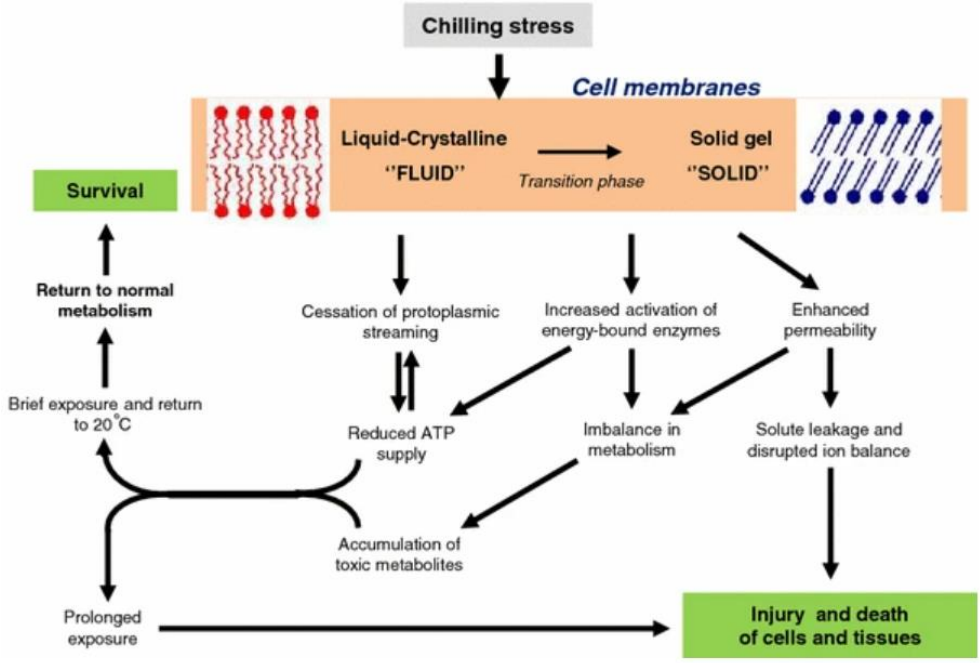


Fig. 3.3. Symptoms of chilling injury in chilling-sensitive plants. Cell membrane is the primary site of chilling-induced injury leading to a cascade of cellular processes with different effects on the plant (Lyons 1973; Raison and Lyons 1986).

Tissue dehydration, a common effect of various abiotic stresses, occurs due to either excessive water loss or inadequate root water uptake. In response to chilling stress, the high concentration of calcium ions in the cytosol induces the opening of stomata causing transpiration to significantly exceed water uptake (Liang et al., 2009). Excessive loss of water can be regulated by inducing morphological changes in the plants structures to reduce the rate of transpiration. In several chilling-sensitive plants, the initial sign of cold stress manifests as wilting of the leaves even with sufficient water supply in the soil (Mahajan and Tuteja 2005; Solanke and Sharma, 2008). Interestingly, wilting helps minimize the loss of water as the drooping leaves expose less surface area. At low temperature, the water potential lowers because of the accumulation of solutes in the cell sap and hydrophilic proteins in the cytoplasm causing decreased osmotic potential of the cell that fosters water uptake and turgor maintenance (Liang et al., 2009; Shinozaki and Yamaguchi-Shinozaki, 1999).

Photosynthesis is significantly reduced in plants when exposed to chilling temperatures. Low temperature induces inhibition of (i) photosynthetic capacity, (ii) repair mechanisms, such as D1 protein repair cycle, (iii) xanthophyll cycle-mediated energy dissipation, and (iv) antioxidant enzymes. The damage induced in chilling-sensitive plants includes reversible decrease in photosynthetic capacity and efficiency, known as photoinhibition and irreversible oxygen-dependent degradation of photosynthetic apparatus including photosynthetic pigments, referred to as photooxidation (Venema et al., 2000). The damage is exacerbated when chilling occurs in the presence of light implying that greater inhibition of photosynthesis and greater chlorophyll bleaching occurs. This damage can be partly attributed to stomatal closure induced by water stress caused by chilling as stomatal closure slow down CO2 assimilation and consequently reduce the photosynthetic rate (Chaves et al., 2003). Chilling stress inhibited biosynthesis of chlorophyll in rice seedlings because of the inhibited synthesis of δ-aminolevulinic acid (ALA). Also at 12℃, the conversion efficiency of protochlorophyllide (Pchlide) into chlorophylls (Chls) lowered. Dry weight significantly decreased in the chilling-stressed seedlings. Moreover, chilling stress inhibited plastid development and chloroplast biogenesis. After 48h, seedlings chilled at 12℃ found having no functional thylakoid structure (Zhao et al., 2020).

At low temperature, the balance between the efficiency to capture light energy and the capacity to dissipate this energy is disrupted. This disturbance results in increased excitation pressure on photosystem II (PSII), consequently inhibiting the photosynthetic activity which is known as photoinhibition (Ivanov et al., 2012). Photoinhibition of photosystem I and II

destroys the photosynthetic apparatus and reduces photosynthetic efficiency which represents the optimal conversion of captured light energy into biomass (Yang et al., 2017). Decreased photosynthetic efficiency in response to chilling stress leads to a surplus of absorbed light energy beyond what is needed for photosynthesis. This excess of energy causes increased production of reactive oxygen species (ROS) within the cells. ROS play a dual role acting as signaling molecules and damaging agents. Chloroplasts, mitochondria, peroxisomes, and plasma membranes are the primary sites of cellular ROS generation (Singh et al., 2019). ROS cause the destruction of P-680, a chlorophyll molecule in the reaction-center of PSII, as well as the degradation of D1 protein, one of the PSII reaction-center subunits, and the repair cycle of PSII starts. They also can oxidize biomolecules, such as nucleic acids, lipids, and proteins, disrupt membrane, deactivate enzymes, and modulate gene expression.

Chilling stress suppress the activity of several oxidoreductive enzymes, such as catalase, causing hydrogen peroxide to accumulation and free radicals to be increasingly produced (Suzuki and Mittler 2006 ; Liang et al 2009 ; Sun et al 2010). Also, it leads to a decline in protein turnover, and alterations in post-translational modifications of proteins. Cell dehydration diminishes the hydrophobic effect, potentially resulting in enzyme inactivation, conformational changes in proteins, and protein denaturation and aggregation (Mahajan and Tuteja 2005 ; Solanke and Sharma 2008).

Plants grow all over the planet under adverse conditions of temperature, light intensity, and water and nutrient availability. Plants have evolved mechanisms not only to adapt to these wide variations in their local habitats, but also to withstand unfavorable changes that may arise and hinder their growth and development. This implies that adaptation is crucial for plants, and plants are preadapted to manage adverse changes and stress conditions. However, they often activate a protective mechanism in response to the stress condition itself. They detect stress signals, and subsequently undergo a series of signal transduction processes which result in changes in gene expression to cope with the stress imposed (Shinozaki and Yamaguchi-Shinozaki, 1999). Low temperatures affect all agricultural sectors with negative consequences for food security, govern the geographical distribution of plants across our planet and alter the timing of key biological events, such as flowering impacting harvest stability (Zhu et al., 2007; Seo et al., 2009; Theocharis et al., 2012). Two strategies plants adopt to cope with the damage induced by abiotic stresses and to prevent more detrimental effects on their cellular structures: tolerance and avoidance. Tolerance mechanisms encompass acclimation promoting gradual buildup of plant resistance. Cold acclimation is an

effective countermeasure against exposure to low temperatures. Several physiological and molecular changes occur during cold acclimation in order to help the plant survive and adapt to stress conditions. While avoidance mechanisms count on accumulation of cryoprotectant molecules, such as proline, sugars and glycerol, and synthesis of stress-responsive proteins to mitigate stress damage within the plant cell (Jahed et al., 2023).

Osmoprotectants include a variety of low molecular weight compounds, such as amino acids (proline), amines (glycine betaine), sugars and sugar alcohols (mannitol) and proteins (dehydrins) that improves stress tolerance. They mainly accumulate in the cytosol, chloroplast and other organelles within the cell. They synthesize to protect the membrane and cell structures from damage or dehydration, modulate intracellular solute concentration, maintain plasma membrane fluidity, prevent protein denaturation, and scavenge ROS. They protect the cell from dehydration by establishing extensive hydrogen binds with water molecules limiting intercellular water movement (Liang et al., 2009; Jahed et al., 2023). Shen et al (1997) demonstrated that phosphoribulokinase (PRK) can be protected by mannitol against oxidative inactivation by hydroxyl radicals in plants.

A general increase in soluble proteins was documented in plants at low temperatures (Graham and Patterson, 1982; Chen et al.,1983). Also, the level of phenylalanine ammonia-lyase (PAL) increases at low temperature leading to an increase in the phenolics synthesis which contributes to improving chilling tolerance. According to Lafuente et al (2004), PAL contributes to reduction of chilling damage in citrus fruits stored at 1.5 degrees after it was effectively enhanced by ethylene treatment. An increase in abscisic acid (ABA) levels in the potato leaves was observed when exposed to low temperature. ABA accumulation triggers the activation of cold-responsive genes and the synthesis of protective compounds, ultimately enhancing the cold tolerance of potato plants. Chilling damage can be slowed down if water loss from tissues is prevented, implying that ABA protects against such damage through promoting stomatal closure.

ROS involving superoxide ($O_2^{\cdot -}$), hydroxyl radicals ($OH^{\cdot}$), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) act as signaling molecules in intracellular signaling and cell-to-cell communication that modulate the expression of various genes, including those encoding antioxidant enzymes and modulators of H_2O_2 production. Under normal conditions, ROS are neutralized through diverse enzymatic and non-enzymatic mechanisms. Non-enzymatic mechanisms, such as ascorbic acid and glutathione, and ROS-scavenging enzymes, such as

superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), and glutathione peroxidase (GPX) scavenge ROS or protect ROS targets from damage. However when plants experience stress, the balance between ROS generation and scavenging is disrupted causing mitochondrial membrane dysfunction, DNA damage (Murata et al., 2007; Nishiyama et al., 2011).

3.3. Cucumber (*Cucumis sativus* L.)

Cucumber (*Cucumis sativus* L. 2n=14 chromosomes) belongs to the Cucurbitaceae family that encompasses various cultivated species of economic importance, such as melon, watermelon squash and pumpkin. Cucumbers are indigenous to India (Dash, 2017) and have spread to other parts of the world over time. The domesticated cucumbers have been extensively bred for diverse characteristics, such as less bitter flesh, fewer seeds, larger fruit size, shape, and pest resistance. The bitter taste found in plants of the Cucurbitaceae family results from cucurbitacins that are toxic secondary metabolites produced only in this family. Today, cucumbers are eaten fresh, pickled, and incorporated into salads, sandwiches, and smoothies. They offer a rich source of vitamins, minerals, and fibers. Cucumber is considered by researchers as a model plant for flower sex determination studies, genome evolution and genetic variation due to: (i) its relative short life cycle, from planting to harvest, the growth cycle is between 50-70 days, depending on variety, (ii) being the first Cucurbitaceae crop genome sequenced (Yang et al., 2012; Pandey and Kujur, 2021).

Cucumbers can be easily cultivated whether from seeds or purchased as young plants or grafted plants during the spring or early summer. There are many cucumber cultivars suitable for greenhouse and open-field cultivation. Greenhouse cultivars produce a crop for a longer season from mid-summer through to early autumn, while open-field cultivars are weather dependent, usually start fruiting later and end earlier. Small or mini cucumber cultivars produce lots of fruits, which are ready for harvest sooner than longer ones. There are also cultivars with tiny fruits (gherkins), ~ 4-8 cm in length, for pickling, and Germany is both the largest consumer and supplier of pickled cucumber in Europe. F_1 hybrid varieties are generally more vigorous and productive, but seeds are more expensive. Cucumbers, whether grown outdoors or in a greenhouse, need regular watering and warm weather to crop well. Some cucumber cultivars are cross-compatible, but parthenocarpic cultivars are often used in greenhouses since they do not require pollination to develop fruit (Guan, 2018). Cucumber is a creeping plant that grows horizontally along the ground as it has a fragile and thin stem, but

it also can grow vertically using other structures, such as fences for support (Fig. 3.4). Vertically grown cucumber is more common to prevent fruit and foliar diseases by keeping leaves dry during watering, and to improve air circulation, and sun exposure. Cucumber plants are monoecious having both male and female yellow-coloured flowers on the same plant, but separate flowers (Fig. 3.4b). Within the past 40 to 50 years, several gynoecious varieties were developed that are mostly bear female flowers and require some monoecious pollinator plants to be in the field to for pollination (Brandenberger et al., 2021). They are annual plants that complete their life cycle in one year. Cucumber is a pepo fruit developed from one flower having a single inferior ovary that is comprised of three fused carpels. It is fleshy crispy several-seeded fruit that is eaten at immature stage of development (Britannica, 2024).

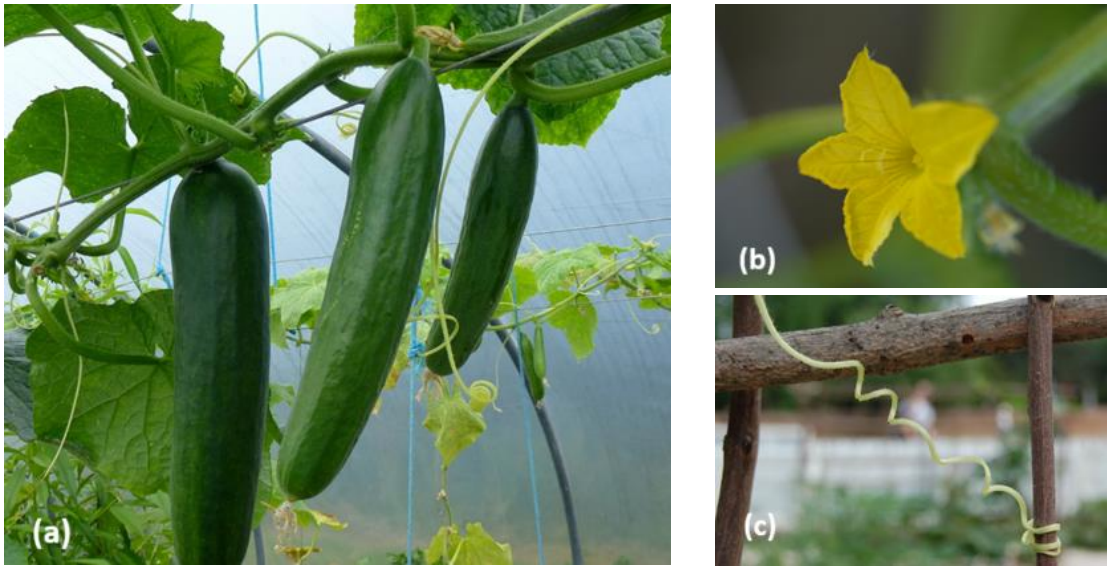


Fig. 3.4. (a) Vertically grown cucumber plants; **(b)** cucumber yellow-coloured flower; and **(c)** tendril used for climbing (North Carolina State University, n.d.).

Both the cultivated *Cucumis sativus* var. *sativus* L. and the wild *C. sativus* var. *hardwickii* Alef. represent the primary gene pool of cucumber. The wild variety can be utilized as a promising source of several valuable genes for the improvement of cultivated cucumber. Considering the cross-compatibility feature of both the wild and cultivated cucumber, desirable traits like resistance can be easily transferred to cultivated cultivars through backcross breeding. Cucumber was the first Cucurbitaceae crop to have its genome sequenced. Since 2009, four genome assemblies of cucumber have been released, featuring one wild accession (*C. sativus* var. *hardwickii* line PI 183967), and three cultivated accessions (i) Chinese long inbred line 9930, (ii) North European Borszczagowski cultivar line B10, and (iii) US pickling cucumber inbred line Gy14. These assemblies were primarily constructed

using Illumina short sequences (Huang et al., 2009; Wóycicki et al., 2011; Yang et al., 2012). Chinese long inbred line 9930 was the first cucumber material used for genome sequencing. Chinese long cucumber was sequenced using Sanger and next-generation Illumina sequencing technologies and released in 2009. Li et al (2019) reassembled Chinese Long genome using PacBio long reads,10X Genomics and high-throughput chromosome conformation capture (Hi-C) data with estimated genome size 367 Mbp. Version 2.1 of the Gy14 genome was assembled using the PacBio long reads.

The Köppen-Geiger climate classification stands as one of the most comprehensive and widely used classification systems. It distinguishes five main climate zones based on the natural vegetation of the region: A (tropical), B (arid), C (temperate), D (continental), and E (polar). According to Köppen-Geiger classification, tropical plants require warmer temperatures, an average annual temperature above 18℃, and high humidity resemble the tropics, the region surrounding the equator (Paiva and Beckmann-Cavalcante, 2023). While subtropical plants in the regions that considered within the temperate climate can endure heavy rainfall, intense heat, dense humidity and prolonged drought resembling the temperate plants in their hardiness.

Cucumber is a tropical plant requiring warm and humid conditions. It also prefers full sun exposure, but because of the large leaf surface area, low humidity in this case increases evaporation. Considering that cucumber cannot withstand waterlogging, more attention is given to the irrigation. It grows in warm, moist, and well-drained soil. cold soils early in the season can result in blossom end rot (BER) which is a physiological condition due to reduced calcium availability. Cucumber is tolerant to a wide range of soil pH, but slightly alkaline soil with a pH between 6.0 and 6.8 is preferred. The optimal temperature for cucumber growth is approximately 30°C, with an optimal night temperature ranging between 18°C to 21°C. A minimum temperature of 15°C is required for good development (Pandey and Kujur, 2021).

3.4. Cucumber and cold temperatures

Plants that are native to temperate climates, may have developed adaptations to withstand colder temperatures, while species of tropical and subtropical origin may be more susceptible to chilling injury when exposed to lower temperatures. Several economically important crops, including cucumber, squash, tomato, cotton, and corn, are highly susceptible to chilling stress during both vegetative and reproductive growth stages. Cucumber lacks the ability to tolerate and survive even moderately cold conditions. Cucumber growth is inhibited when

temperatures exceed 35℃ or drop below 12℃. According to Reyes and Jennings (1994), seedlings of 'Encore' cucumber displayed more sensitivity to chilling at 2℃ and 6℃ than 10℃ and 15℃, as indicated by their reduced ability to resume growth upon returning to 26℃. Also, root growth declined after 48 h of stress exposure, however, seedlings stressed at 10℃ and 15℃ could maintain their root growth at these temperatures and when returned to 26℃. Electrolyte leakage from cucumber roots increased at 2℃. After 96 h, seedlings chilled at 2℃ and 6℃ were irreversibly damaged and unable to recover.

Plant age is one of the crucial growth conditions since different developmental stages and tissue types exhibit distinct levels of tolerance to low temperature (Hodges et al.,1997; Ye et al., 2009). Amin et al (2021) highlighted the physiological responses of cucumber plants to low temperature and high humidity across different leaf stages. Low-temperature stress decreased plant height, root length, leaf area, dry mass and had unfavorable effects on chlorophyll contents, photosynthesis, phytohormones, and enzymatic activities. The results showed that plants at 6 leaf stage experienced less damage compared to the 2 and 4 leaf stages when subjected to low temperature and high humidity. At all leaf stages in cucumber plants, cold stress significantly inhibited PSI and PSII activity. The photoinhibition induced was less in the 6-leaf stage plants and consequently the photosynthetic activity is higher than at 2 and 4 leaf stages. Moreover, lipid peroxidation and ROS production were less at 6 leaf-stage plants. Stomatal closure reduced CO_2 assimilation causing decreased photosynthetic activity. Antioxidant enzymes were upregulated in response to low temperature, however, they were higher in 6 leaf-stage plants than at 2 and 4 leaf stages.

Terashima et al (1994) observed decreased photosynthetic activity in cucumber under chilling treatment at 4℃ for 5 h and demonstrated that PSI was readily photoinhibited in response to chilling conditions compared to PSII. Sonoike (1994) reported after that iron-sulfur centers that function as electron acceptors within PSI were destructed under the same chilling treatment as above confirming that PSI is the main site of photoinhibition in cucumber leaves.

Cucumber plants chilled at 5 °C and 85% relative humidity experienced chilling injury encompassing alterations in water balance, photosynthesis, respiration and other physiological and biochemical processes. They experienced water loss causing dehydration symptoms and wilting leaves. They also displayed significantly reduced photosynthetic activity and chlorophyll bleaching especially in the light. However in the dark, they experienced slight

reduction in photosynthetic activity, but no chlorophyll loss. Rapid leakage of electrolytes, and reduceb respiration rate were detected as well (Wright and Simon, 1973).

3.5. Plant Dehydrins (DHNs)

Late embryogenesis abundant (LEA) proteins are termed by Galau et al., 1986 after their RNAs found abundant in the embryo cotyledons of cotton (*Gossypium hirsutum* L.) during late embryogenesis and rapidly disappeared during germination. LEA proteins are mainly found in plants seeds and in other plant tissues. They are hydrophilic, thus usually called hydrophilins, and intrinsically unstructured proteins (IUPs) involved in plant growth and development as well as in stress response and adaptation to unfavorable environmental conditions (Kosová et al., 2014). They are classified into seven groups LEA1, LEA2, LEA3, LEA4, LEA5, dehydrins (DHNs) and seed maturation protein (SMPs).

Dehydrins' nomenclature is derived from dehydration proteins due to their overexpression under water deficit conditions (Close et al., 1989). Dehydrins have intrinsically disordered structures (IDPs) implying that they lack a defined 3-deminsional structure. Many disordered proteins are able to switch between non-functional disordered states to a functional state by a disorder-to-order transition. This transition is frequently triggered by binding to various cellular targets, such as membranes or other proteins. Dehydrins are boiling- stable proteins ranging from 9 to 70 kDa in size. They are divided into six subgroups, K_n, SK_n, K_nS, Y_nK_m, Y_nSK_m, and F_nSK_m, according to the presence of conserved sequence motifs named K-, Y-, S-, and F- segments where the subscripts n and m describe the number of copies of that motif. The K-segment is rich in lysine, present in most DHNs and consists of 15 amino acids with the consensus sequence (XKXGXXD/EKIKD/EKXPG). Serine-rich (S-) segment occurs in one copy within the protein and generally thought to influence the localization or function of DHNs by enhancing phosphorylation, while the Y-segment is composed of conserved tyrosine residues located in the N-terminus of the protein in one or several tandem copies whose function remains unclear. The F-segment consists of 11-amino acid sequence, DRGLFDFLGKK, with a pair of hydrophobic F residues at the core of the sequence. The amino acids that lie between the conserved segments are termed as φ-segments and they are rich in glycine (G) and polar amino acids (Richard Strimbeck, 2017; Wei et al., 2019; Ohkubo et al., 2020; Wang, et al., 2020).

K-segments function in maintaining membrane structure and enzyme cryoprotection. φ-segments play a crucial role in maintaining the disordered structure of dehydrins, acting as

molecular shields to prevent partially denatured proteins under stress conditions from aggregation. Histidine rich domains of citrus dehydrin *CuCOR15* was able to bind metals, such as Fe^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+}, and Zn^{2+} implying that metal-binding dehydrins might reduce metal toxicity under water deficit conditions (Hara et al., 2005). φ-segments are highly flexible allowing the optimal positioning of K-segments that optimize the interaction with their targets. K-segments facilitate positioning of dehydrins near enzyme surfaces, potentially enabling interactions that safeguard or regulate enzyme activity under stress conditions (Hughes and Graether, 2011; Malik et al., 2017).

Dehydrin loci are multigenic and found in clusters on different chromosomes. DHNs are widely distributed in various compartments of the plant cell, such as cytoplasm, chloroplasts, and mitochondria and in different plant tissues. DHNs containing the Y-segment, such as Y_nSK_m and Y_nK_m, are commonly detected within the nucleus. The presence of these proteins in the nucleus suggests a potential protective function therein. The expression of DHN genes is induced when plants are exposed to environmental stress conditions such as salinity, water deficit, and low temperature (Kosová et al., 2014). Consequently, it is proposed that dehydrins play a crucial role in establishing and enhancing plant tolerance to various abiotic stresses. Proteins and membrane phospholipids are surrounded by a thin layer of highly ordered water molecules bound via hydrogen bonds to the protein or membrane surface. This water envelope maintains their stability and integrity and prevents direct interaction with each other. In nonpolar environments, K-segments of dehydrins adopt an amphipathic α-helix structure and bind membranes and other protein preventing further loss of water envelope (Fig. 3.5). Therefore, it is suggested that dehydrins safeguard membranes, enzymes, and other macromolecules from dehydration stress. They also have functions to stabilize enzymes and prevent conformational changes that lead to protein denaturation and aggregation (Koag et al., 2003).

It was observed that different dehydrin structural types show distinct functions. Maize YSK_2 *DHN1* can bind to lipid vesicles, suggesting that it has a potential role in maintaining the structure and function of membranes. K_3S-type *CuCOR19* dehydrin from Citrus displays higher radical scavenging activity against both hydroxyl and peroxyl radicals than mannitol in vitro. SK_n- and K_n-type DHNs represented by the wheat K_6 *WCS120* and barley K_9 *DHN5*, may contribute to plant acclimation to low temperature through protecting the transport systems of water of and solutes upon stress (Rorat, 2006, Hara et al., 2004). According to Palmer et al., 2019, the amino acid composition of the dehydrin protein is a major factor

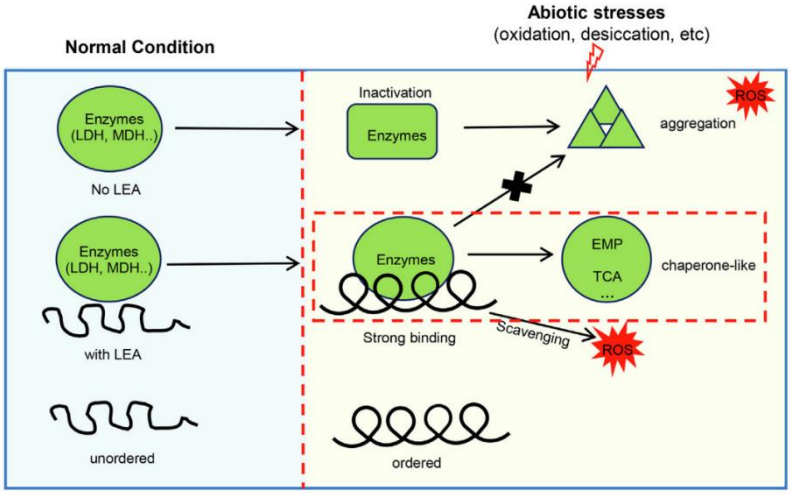


Fig. 3.5. Chaperone-like function of LEA proteins. LEA proteins interact with enzymes preventing their inactivation under stress conditions (Źróbek-Sokolnik, 2012).

preventing protein aggregation under heat and cold stress implying that the polar nature of the dehydrin contributes to its cryoprotection feature. The scrambled sequences of the wild-type K_2 and YSK_2 dehydrins, from *Vitis riparia* (wild grape), resulted in repositioning lysine residues that form K-segments. K-segments are thought to play an important role in enzyme cryoprotection. Despite the scrambling of the sequence, scrambled YSK_2 dehydrins exhibit comparable effectiveness to the wild-type YSK_2 in protecting LDH from freeze and heat damage. In agreement with Kazuoka and Oeda (1994) who credited the cryoprotective activity of spinach *COR85* dehydrin to its high content of polar acidic amino acids, such as Asp and Glu. Moreover, *COR85* dehydrin was found to accumulate in leaves, but not in roots of non-stressed spinach plants during normal developmental stages, indicating that it might have potential role maintaining leaf cell homeostasis.

3.6. Dehydrins confer abiotic resistance to cucumber.

LEA protein sequences belonging to 14 different plant species, derived from LEAP database, were utilized for determination of homolog peptides within cucumber genome. There have been 79 LEA genes identified in the cucumber genome named from *CsLEA-01* to *CsLEA-79*. Cucumber possessed the highest number of LEA genes compared to LEA genes found in other plants, such as tomato, potato and soybean as identified LEA proteins were 27,29, and 36 respectively. Chromosome 6 harbored the highest number of *CsLEA* genes among cucumber chromosomes, which was sixteen *CsLEA* genes. Chromosome 3 contained 15 *CsLEA* genes, while Chromosome 7 contained 14 *CsLEA* genes. But chromosome 2 only contained 5 *CsLEA* genes which was the lowest number of *CsLEA* genes. *CsLEA-10*, *CsLEA-11*, *CsLEA-31*, and *CsLEA-54* belonged to dehydrin group proteins, and they had no conserved

motifs. RNA-seq data of *CsLEA* genes were analysed for expression analysis and the dehydrin gene *CsLEA-54* and other eight *CsLEA* genes showed higher expression profiles in all tissues of cucumber. qRT-PCR was implemented to explore LEA genes that response under drought stress conditions in leaf and root tissues of cucumber. LEA genes which have high expression levels in all tissues, and which are highly expressed in only leaf or in root tissues were selected. The expression of the dehydrin gene *CsLEA-54* was upregulated in drought-stressed cucumber plants in root and leaf tissues after stress application (Fig. 3.6). For this reason, it might enhance the cucumber tolerance to water deficit stress. *CsLEA-09*, *CsLEA-32*, and *CsLEA-57* genes showed a response to drought conditions 3 hours later and considered as early response genes (Celik et al., 2016).

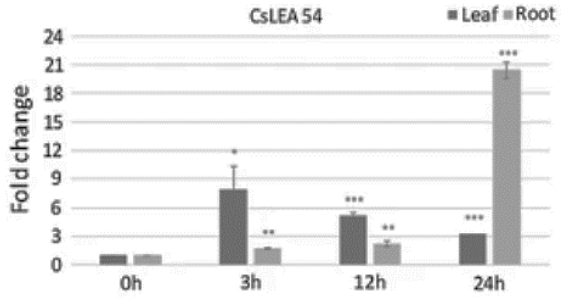


Fig. 3.6. Expression profile of *CsLEA-54* gene by qRT-PCR analysis in 0 (control) and 3, 12 and 24 h in leaf and root cucumber tissues under drought stress conditions (Celik et al., 2016).

According to Lee et al., 2017, Genome-wide analysis detected 32 genes encoding DHNs in eight genome sequences of five Cucurbitaceae species, *Cucumis sativus* var. *Sativus*, *Cucumis sativus* var. *Hardwickii*, *Cucumis melo*, *Citrullus lanatus*, and *Momordica charantia*. The study included Chinese long Inbred line 9930, Gy14 gynoecious inbred line, and Borszczagowski line B10 under the species *Cucumis sativus* var. *Sativus* and identified four, three, and four DHNs encoding genes respectively, and four in wild cucumber. K_n, SK_n and Y_nSK_m-dehydrin structural types were found in the in the deduced protein sequences of cucumber dehydrin genes. Only SK_3-type DHN genes were strongly expressed in cucumber. K_n and SK_n-type DHNs are closely linked to plant acclimation to low temperature. SK_n-type DHNs are mainly localized in the vascular system and apical meristems suggesting that they may protect the vascular system and apical meristems from dehydration and maintain the transport of water and solutes for plant growth and survival. Therefore, the association of SK_3-type DHN genes to cold tolerance suggests their potential role in cucumber adaptation to low temperature (Rorat 2006; Yin et al 2006; Xing et al 2011). The dehydrin gene *CsLEA-11*, identified in *Cucumis sativus*, belongs to Y_3SK_2-type DHNs and is rich in hydrophilic amino

acids. Its expression was induced in response to heat and cold stress revealing that it might help cucumber withstand heat and cold stress conditions. Also, *CsLEA-11* protects lactate dehydrogenase (LDH) activity which has been found to aggregate and unfold below 4°C and lose more than 75% of its activity at 65°C (Zhou et al., 2017).

Classical breeding of cucumber to improve tolerance to low temperature has been limited due to the lack of a cold-tolerant cucumber germplasm. Therefore, Yin et al (2004, 2006) used transgenic approaches to express *Dhn10* and *Dhn24* genes encoding dehydrins in cucumber to enhance its chilling tolerance. These two genes were found in cold-acclimated potato species *Solanum sogarandinum* L. The 1633 bp promoter region of the *S. sogarandinum* glucosyl transferase (GT) gene was used since it can be induced by various abiotic stresses. The fusion constructs were introduced into cucumber plants using Agrobacterium-mediated transformation. The resultant transgenic cucumber lines were used to assess the potential role of these dehydrins in improving cold tolerance. In case of *DHN10*, among three T1 transgenic cucumber lines, one exhibited chilling tolerance at 6°C for 24 h without any noticeable chilling injury compared to the non-transgenic line (Yin et al., 2004). While three *DHN24* trasgenic lines showed reduced chilling damage and another one showed increased freezing tolerance that tested by asessing the electrolyte leakag (Yin et al., 2006).

3.7. Low temperature- induced dehydrins and their functions

Both ABA-dependent and ABA-independent pathways regulate the expression of dehydrin genes under cold and drought stress. In ABA-dependent pathways, ABA-responsive *cis*-elements (ABRE) in the promoter regions of dehydrin genes serve as binding sites for ABF transcription factors (ABRE-binding factors) to induce upregulation of dehydrin gene expression. C-repeat binding factor/Dehydration responsive element binding 1 (CBF/DREB1) pathway represents the main ABA-independent cold-inducible pathway. *CBF/DREB1* genes, first discovered in *A. Thaliana*, encode transcription factors contain a conserved AP2 DNA-binding domain flanked by two unique sequence motifs called signature motifs. These TFs can bind to CRT/DRE sequences in the promoters of dehydrin and other COR genes (Lee et al., 2012; Kosová et al., 2019).

Accumulation of dehydrin transcripts and/or proteins has been documented in various plants during cold acclimation. Kazuoka and Oeda (1994) purified a dehydrin protein named as *COR85* from the leaves of cold-acclimated spinach (8-week-old-seedlings exposed to 4°C for 14 days). *COR85* was found to have a cryoprotective activity against freezing inactivation

of LDH, it was much more effective than bovine serum albumin (BSA) as a known cryoprotectant. A 35-kD dehydrin protein was found to confer chilling tolerance during emergence of cowpea (*Vigna unguiculata*) seedlings. Y_2K cowpea DHN can adopt an amphipathic α-helix suggesting that it serves in stabilizing proteins in the endomembrane environment (Ismail et al.,1999a, b). Under chilling stress conditions, Tobacco plants expressing *CuCOR19* dehydrin displayed lower malondialdehyde (MDA) content, and a lower level of electrolyte leakage than the control indicating a potential function in membrane protection (Hara et al., 2003). *CuCOR19* dehydrin as previously mentioned, showed in vitro binding activity to metal ions, such as Cu^{2+} and Fe^{2+}, radical-scavenging activity, and inhibitory activity against peroxidation of soybean liposomes (Hara et al., 2003, 2004, 2005; Rorat, 2006).

4. Materials and Methods

4.1. Plant materials and growth conditions.

4.1.1. Plant materials

Four cucumber (*Cucumis sativus* L.) F1 cultivars: two open-field-grown cultivars "Joker and Dirigent" and two greenhouse-grown cultivars "Grafito and Diapason" were used as plant materials in this research. Seeds of cultivars were obtained from Rijk Zwaan Kft (De Lier, The Netherlands), except for the "Joker" hybrid, which was purchased from ZKI-Vetőmag KfT (Kecskemét, Hungary).

4.1.2. Preparation and growing conditions of seeds

Seeds soaked up in 100 ml distilled water for 16 h at room temperature. Rockwool cubes used to germinate the cucumber seeds were cut into pieces (L3.5 cm x W3.5 cm x H7 cm) and then 4-cm-depth holes were made into the cubes. One cucumber seed was placed in the hole of each rockwool cube in a pot (9x9x10 cm) filled with perlite. Cucumber seedlings were grown in a growth chamber under a 16/8 h photo/dark period and the temperature were maintained at 26/20℃ ± 1 (day/night). They were watered with distilled water until the appearance of the first leaf, then 50 ml of ½ Hoagland nutrient solution (pH = 5.8) was used for irrigating each plant every other day (Taiz et al., 2015).

4.2. Cucumber chilling treatments (stress application)

When plants reached three weeks old, plants of each cultivar were divided into two groups. One group was kept at standard conditions as a control. The second group (treated) was exposed to chilling. Three different chilling stress treatments were carried out as follows:

Treatment	Conditions	Used cultivars
Control	standard conditions	
Treat. 1	8°C daytime for 4 h	Dirigent, and Diapason
Treat. 2	8°C day/5°C night for 24 h	Grafito, Diapason, Dirigent, and Joker
Treat. 3	4°C for 24 h in darkness	Grafito, and Joker

Table 4.1. Description of treatments used in the study on three-week-old cucumber plants.

Fully developed leaves of plants grown at standard conditions and those subjected to chilling treatment were collected and stored at -80℃ until use.

4.3. Determination of Relative Water Content (RWC)

Three leaf discs of 2.5 cm diameter from four different plants per treatment were punched out by a cork borer yielding four biological replicates per treatment. The fresh mass (FM) of the three discs of each plant was measured gravimetrically using a laboratory scale. Then, the three discs of each plant were incubated in a petri dish with 30 ml distilled water at 4℃ for 24 h in darkness after which the turgid mass (TM) was recorded. All samples were then kept in an oven at 104℃ for 24 h to obtain the dry leaf mass (DM). The RWC expresses the water content in percentage as related to the water content at full turgidity. It is calculated with the following formula according to Barrs (1968):

RWC (%) = [(FM-DM)/(TM-DM)]*100

where FM is the discs'fresh mass measured immediately after cutting, DM is the dry mass and TM is the turgid mass (Vaseva et al., 2010).

4.4. Electrolyte leakage Assay

The percentage of the electrolyte leakage was measured as described by Lutts et al (1996) in both cucumber control and chilled plants to evaluate the degree of chilling injury. Four randomly chosen plants per treatment (one leaf per plant) were taken and cut using a cork borer on paper towels into five discs of 1 cm diameter per leaf which were then placed in autoclavable pots containing 15 ml distilled water. These yielded four biological replicates per treatment. The first conductivity reading (EC1) was performed after 30 min of sampling at room temperature using a conductometer (HI8633, Hanna Instruments Inc., Woonsocket, USA). Then, all samples were placed at room temperature on a rotary shaker (100 rpm) for 24 h and after the second measurement of the leakage of electrolytes (EC2) was carried out. Samples were then autoclaved at 120℃ for 20 min to inflict the maximum possible damage on plant samples. The third conductivity reading (EC3) was performed after reaching room temperature as an index of maximum leakage. The electrolyte leakage was defined mathematically as the ratio of the initial conductivity (EC2) to the conductivity at its maximum (EC3) since the EC1 represents the electrolytes leaked from damaged cells on the edges of the leaf disks (Hatsugai and Katagiri 2018) and expressed as a percentage as follows:

EL (%) = (EC2 – EC1)/EC3)*100

4.5. TBARS assay of malondialdehyde (MDA) determination

TBARS assay is referred to as thiobarbituric acid reactive substances assay as MDA is condensed with thiobarbituric acid (TBA) forming TBA-MDA complex which can be easily assessed with a spectrophotometer at 532 nm. According to Hodges et al (1999), 0.2 g leaf sample was homogenized in 2 ml of 0.1% (w/v) cold trichloroacetic acid (TCA) in a chilled mortar and pestle. The homogenate was centrifuged at 13,000×g for 15 min at 4℃ to remove insoluble components. 0.75 ml of the supernatant was added to 0.75ml of TBA/TCA solution. In addition, 0.75ml of the supernatant was added to 0.75ml of 20% TCA and used as a blank in order to correct for interfering compounds which absorb at 532 nm by subtracting the absorbance. Positive control was prepared by adding 745 µL of 0.1% TCA and 5 µL MDA to 0.75ml of TBA/TCA solution. All mixtures were heated at 95-100℃ for 30 min, and then cooled in an ice-bath for 10 min. Then they centrifuged at 13,000×g for 10 min at 4℃ at to clarify the solution. 365 µL of each was transferred into wells of ELISA plate and absorbances were measured with microplate reader at 440, 532 and 600nm. The value for non-specific absorption at 440 and 600 nm was subtracted. All measurements were made in duplicate. MDA concentration was calculated using an extinction coefficient of 157 $mM^{-1}cm^{-1}$ as follows:

1) $[(Ab_{532+TBA} - Ab_{600+TBA}) - (Ab_{532-TBA} - Ab_{600-TBA})] = A$

2) $[(Ab_{400+TBA} - Ab_{600+TBA}) * 0.0571] = B$

3) MDA equivalents (nmol. ml^{-1}) = $(A - B/157\,000) * 10^6$

* TBA/TCA solution was freshly prepared by dissolving 0.125 g TBA in 25 ml 20% (w/v) TCA. Heatin plate can be used to help dissolve the TBA in the solution.

4.6. Determination of proline content

Proline content was estimated according to Mehrabi et al (2024).1 g of leaf sample was homogenized in 0.5 ml of 3% aqueous sulfosalicylic acid and the homogenate was centrifuged at 13,000×g for 10 min at 4℃ to remove insoluble components. To 100 µL of the supernatant, 200 µL acidic ninhydrin and 200 µL of glacial acetic acid were added in a test tube and incubated for 1 h at 100°C, and then the reaction was terminated in an ice bath. The reaction mixture was extracted with 0.5 ml toluene, mixed vigorously with a stirrer and then centrifuged at 13,000×g for 2 min. The mixture separated into two phases: chromophore-containing toluene and the aqueous phase, and the absorbance of the toluene phase was

measured on the spectrophotometer at 520 nm using toluene as a blank. The proline concentration was determined from a standard curve of purified proline and calculated as follows:

Proline content (μmoles/g FW) = [(μg proline/ml × ml toluene) / 115.5] / [(g sample) /5]

*Acidic ninhydrin was prepared by heating 0.625 grams of ninhydrin in a mixture of 15 ml of glacial acetic acid and 10 ml of 6 M phosphoric acid with agitation, until fully dissolved. The reagent remains stable for 24 h when stored at 4°C.

4.7. Isolation of total cellular RNA, cDNA synthesis and RT-PCR

4.7.1. Isolation of total cellular RNA and gDNA removal.

Total RNA of leaf samples was isolated using TRI Reagent (Sigma-Aldrich Co., MO, USA) according to the protocol described by the manufacturer. In short, 0.1 g leaf sample was ground in liquid nitrogen and then placed in an Eppendorf tube containing 1 ml of TRI reagent and kept waiting at room temperature for 5 min. The sample was centrifuged at 13000 x g for 15 min at 4℃. 200 μL chloroform was added to the supernatant in a sterile RNase-free tube and let stand at room temperature for 10 min and centrifuged at 4℃, 12000 x g for 15 min. The aqueous phase on the top was carefully transferred into a new Eppendorf tube and 0.5 ml isopropanol was added. The sample was kept waiting at room temperature for 10 min and centrifuged under the same conditions. The precipitated RNA was washed twice with 1 ml 75% cold ethanol for each and then centrifuged at 4℃, 7500 x g for 5 min. The pellet was air-dried and redissolved in 50 μL RNase-free water at 55℃ for 10 min in a heating block. RNA integrity was visualized on a 1% agarose gel and purity and concentration of total RNA were detected with a NanoDrop spectrophotometer at 260 nm and 280 nm (NanoDropND-1000, NanoDrop Technologies, USA). The purified RNA was kept at -80℃ until use.

Isolated RNA was exposed to DNase I enzyme (Thermo Fisher Scientific, USA) to remove genomic DNA and Incubated at 37°C for 15 min. Then, inactivation by heating at 65°C for 10 min in the presence of EDTA occurred according to the manufacturer's guidelines. Quality and durability of the DNA-free RNA was checked by ethidium bromide-stained agarose gel in 1× TBE buffer.

4.7.2. cDNA synthesis

Complimentary DNA (cDNA) was synthesized using Thermo Scientific Maxima H Minus First Strand cDNA Synthesis Kit in a 63.5 µL reaction volume containing 48 µL DNA-free RNA, 10 µL 5X RT buffer, 2 µL water, 1 µL oligo $(dT)_{18}$ primers, 1 µL dNTPs, 0.5 µL Riboluck RNase inhibitor, and 1 µL RT enzyme. The reaction mixture was incubated for 1 h at 42°C followed by 10 min at 70°C as described by the manufacturer. The cDNAs were then stored at −80 °C until be used in PCR amplification.

4.7.3. RT-PCR

The syntheszied cDNA was amplified using gene-specific primers (Table 4.2) designed according of Szegő et al (2019). PCR amplification was conducted using Thermo Scientific DreamTaq DNA Polymerase to amplify the coding seyuence of *DHN1* and *LEA D-11* genes and a control actin (*CsaAct-7*) housekeeping gene. PCR mixture of 25 µL total volume per tube was made up of 1 µL cDNA used as a template, 16.4 µL dH_2O, 5 µL DreamTaq Green buffer, 1 µL primer forward-reverse, 0.5 µL dNTPs, and 0.125 µL Taq-Polymerase. PCR thermal cycling was set under the following conditions: initial denaturation at 95 °C for 3 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s, 72°C for 30 s, and a final extension at 72 °C for 5 min. PCR products with expected sizes were visualized on 1% ethidium bromide-stained agarose gel (Szegő et al., 2019).

Gene	Accession number	Protein	Primer sequences (5′–3′)	Product length
DHN1	XP_011653150	dehydrin Rab18 (Cucumis sativus)	ATGGTGGCATACTTCATCGC GTCGTGGTTGACACTGCTTG	154 bp
LEA D-11	XM_011656037.1	late embryogenesis abundant protein D-11-like	CTATCCAATTCGCCAGACCG GATGTCCACTCCCATCCTCT	191 bp
CsaAct-7	XM_004147305	Cucumis sativus actin-7	TCGTGCTTGACTCTGGTGATGG TTTCCCGTTCGGCAGTGGTTGT	171 bp

Table 4.2. Primer sequences for PCR amplification.

4.8. Protein extraction and isolation of heat-stable proteins

According to Vaseva et al (2010), 0.5 g leaf sample was ground with liquid nitrogen in a prechilled mortar and pestle and extracted with 2 ml cold extraction buffer containing 200 µL 1 M Tris–HCl (pH 7.6), 8 µL 0.5 M EDTA (pH 8.0), and 40 µL 100 mM phenylmethylsulfonyl fluoride (PMSF). The homogenate was kept ice-cold for 5 min then centrifuged at 13,000×g for 15 min at 4 °C. The supernatant was carefully transferred to a new Eppendorf tube and

considered as total soluble proteins. Total protein concentration was determined by Bradford method employing bovine serum albumin as a standard (Bradford, 1976). 50 µL supernatant was added to 5 ml Bradford reagent and mixed by vortex. The absorbance was measured at 595 nm after 5 min against a blank prepared by adding 50 µL water to 5 ml Bradford reagent.

To isolate heat-stable protein fraction, the supernatant containing total soluble proteins was heated at 95-100°C for 10 min then cooled down for 5 min on ice. After, It was centrifuged at 13,000×g for 20 min at 4 °C to remove coagulated proteins. The supernatant that contains heat- stable proteins was pipetted into a new Eppendorf tube. Heat- stable proteins were precipitated with 20% cold TCA/acetone (1:1, v/v). The mixture was kept on ice for 5 min then centrifuged at 13,000×g for 3 min at 4 °C and the supernatant was discarded. The precipitate was rinsed with 80% acetone followed by centrifugation as above. The protein pellet was air-dried and then dissolved in 50 µL 50 mM Tris–HCl (pH 8.0) and stored at -80°C until use (Niu et al., 2018).

The heat-stable protein fraction was separated on Mini-PROTEAN TGX Precast Gel (Bio-Rad Laboratories Inc., USA) at 70V in a Bio-Rad dual slab cell. The gel was stained with GelCode Blue Safe Protein Stain (Thermo Scientific, Rockford, USA) according to the manufacturer's protocol.

* A 100 mM PMSF solution was prepared by adding 17.4 mg PMSF to 1 ml isopropanol and stored at −20°C.

* Bradford reagent preparation: 10 mg Coomassie Brilliant Blue G-250 was dissolved in 5 ml 95% ethanol. Then 10 ml 85% (w/v) phosphoric acid was added. Water was added to a final volume of 100 ml.

4.9. Western blot analysis

The heat-stable protein fraction was separated as described above. After electrophoresis, proteins were transferred from the gel onto a nitrocellulose membrane by electroblotting at 20 V overnight using a Bio-Rad Mini Trans-Blot. Then, the membrane was washed with TBS buffer (pH=7.6) for 10 min and blocked with 5% milk powder dissolve in TBS containing 0.1% Tween-20 (TBST) for 1 h and then washed with TBST for 10 min twice. Dehydrin protein was detected with 3 µL anti-dehydrin rabbit antibody (Anti-dehydrin affinity purified IgG, Agrisera AB, Vännäs, Sweden) as a primary antibody in 15 ml blocking buffer for 1 h then washed with TBST for 10 min three times. Goat anti-rabbit IgG, conjugated with alkaline

phosphatase (AffiniPure Goat Anti Rabbit IgG, Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) was used as a secondary antibody with the same dilution ratio as the primary antibody for 1 h then washed with TBST for 10 min three times. Bound antibodies were detected by a developer solution containing NBT/ BCIP (nitro-blue tetrazolium chloride/ bromochloroindolyphosphate) as a substrate (Szegő et al., 2019).

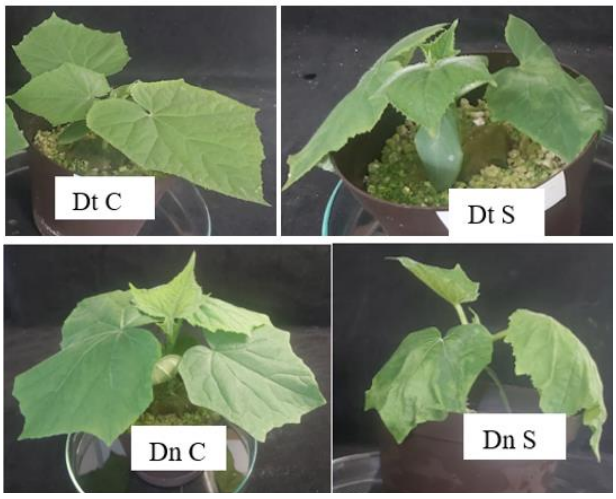
4.10. Statistical analysis

The data obtained from this study for different parameters were analysed by their mean and standard deviation. Experimental data were subjected to Analysis of variance (One-Way ANOVA) and differences among means were evaluated by Duncan's multiple comparison test. The post hoc analysis was performed by Tukey's b test. The evaluation was performed using IBM SPSS Statistics v29.0.1.0 (Chicago, IL, USA). The results are presented as mean values ± standard errors. Statistical significance was established at $p < 0.05$.

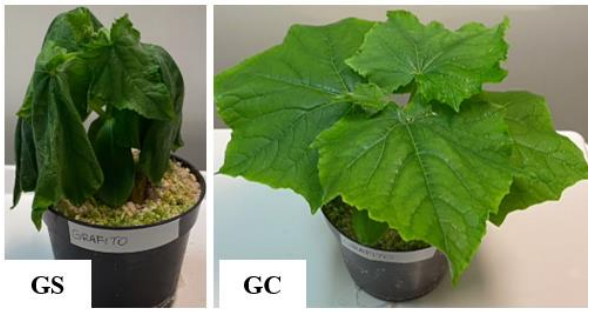
5. Results and Discussion

5.1. The effect of chilling stress on the morphology of cucumber plants.

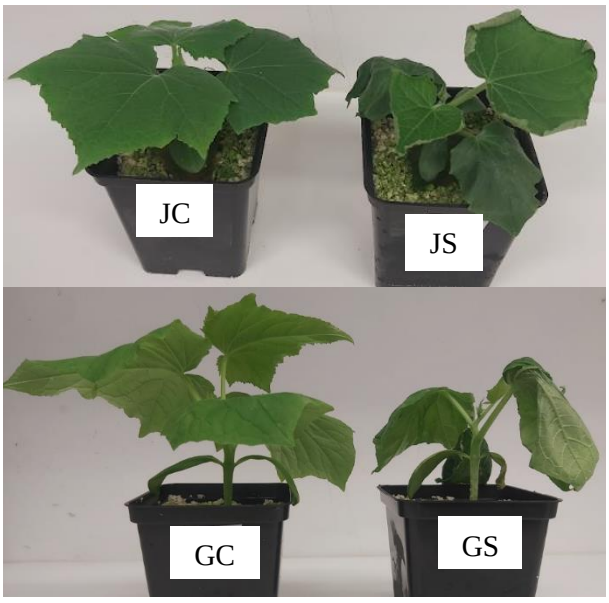
Leaf wilting is the earliest visible sign of chilling stress in cucumber (Wright and Simon, 1973). Drooping leaves and dehydration symptoms had been observed in all chilled cucumber cultivars under the three treatments of chilling compared to control plants grown under standard conditions (Fig. 5.1). Chilled Diapason plants experienced some chlorosis. Chilling-induced wilting was more severe in the presence of light in 2nd chilling treatment in agreement with McWilliam et al (1982).



1st chilling treat. 8°C daytime for 4 h



2nd chilling treat. 8°C day/5°C night for 24 h



3rd chilling treat. 4°C for 24 h in darkness

Fig. 5.1. shows the effect of chilling on cucumber plants compared to those grown under standard conditions (control plants); C: Control plants, S: plants under chilling stress, Dt: Dirigent, Dn: Diapason, G: Grafito, and J: Joker.

5.2. The effect of chilling on leaf relative water content (RWC).

RWC indicates the water status of the plant (Qian et al., 2008). In chilling sensitive plants such as cucumber, chilling temperatures induce water stress leading to dehydration symptoms in plant leaves. Under 3rd chilling treatment, RWC dramatically decreased in both chilled Grafito and Joker seedlings compared to non-chilled seedlings (Fig. 5.2) and in accordance with McWilliam et al (1982), the reduced water uptake through the roots and continued transpiration due to slow closure of stomata could cause decrease in leaf RWC and wilting

symptoms. Price (1990) and Young et al (2006) reported that transient elevation in cytosolic calcium ions in response to stress conditions induces the opening of stomata causing transpiration rate to significantly exceed water uptake.

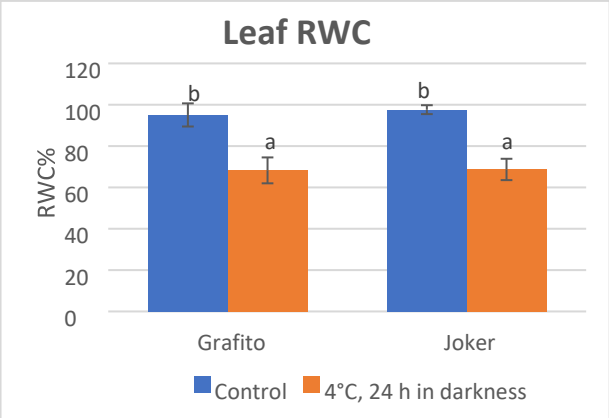


Fig. 5.2. Relative water content (RWC) of Grafito and Joker cultivars under control and 3rd chilling treatment. The data are the means with standard errors shown by vertical bars. Different letters above columns indicate significant differences between control and chilled plants at $p < 0.05$.

5.3. Electrolyte leakage and MDA content

Electrolyte leakage (EL) and MDA content were quantified to examine the lipid peroxidation and the damage inflicted on cellular membranes when cucumber seedlings were exposed to chilling temperatures (Feng et al., 2003; Zong Hui et al., 2003; Ding et al., 2023). The electrolytes leaked from a plant tissue result in an increase in electrolytic conductivity of water that contains the tissue and indicate increased membrane permeability. Chilled Grafito and Joker seedlings showed more electrolyte leakage than controls, noting that Grafito as a greenhouse-grown cultivar showed a little more leakage than Joker as an open field-grown cultivar under 3rd chilling treatment (Fig. 5.3). Accordingly chilling stress exhibited slight but not significant increase in the electrolytes leaked from cucumber leaf tissues.

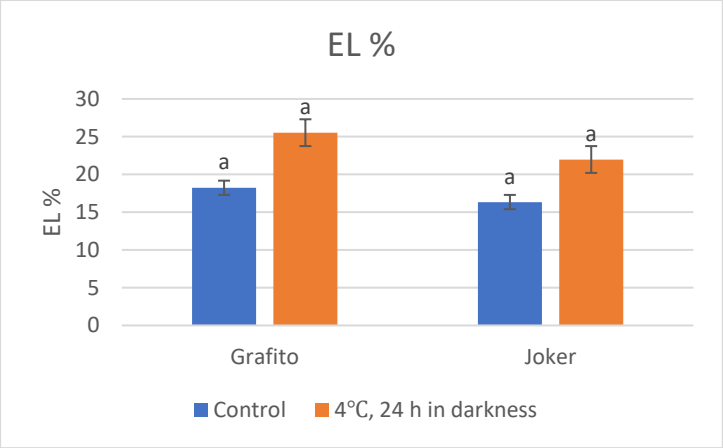
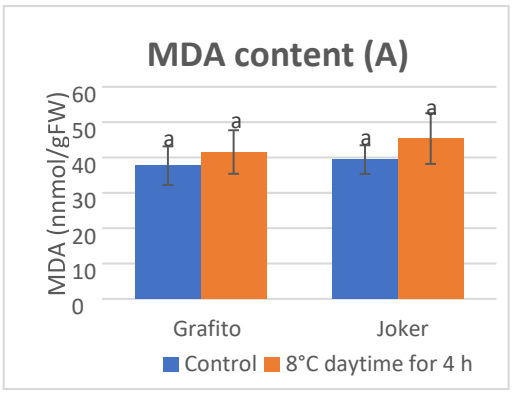
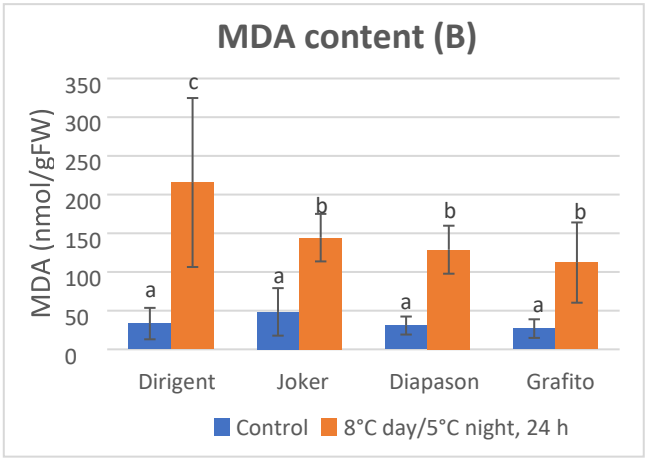


Fig. 5.3. Electrolyte leakage from cucumber leaf tissues chilled at 4°C in the dark for 24 h. The experiment was replicated 4 times using 5 leaf disks per replicate. The data are the means with standard errors shown by vertical bars. The same letters above columns indicate no significant difference between control and chilled plants at $p < 0.05$.

MDA is a low-molecular-weight end product of the enzymatic and radical-induced decomposition of unsaturated fatty acids and is measured as an index of lipid peroxidation.

MDA content had no significant increase when exposed to chilling for 4 h. However, MDA content under 2nd chilling treatment had a significant increase in all the studied cultivars compared to control plants, noting that this increase was highly remarkable in chilled Dirigent plants, while MDA content increased significantly just in chilled Joker plants under 3rd chilling treatment, indicating that lipid peroxidation in cucumber plants increased under chilling temperatures. The increase in MDA content in Joker plants chilled at 8°C/5°C day/night is highly significant compared to those chilled at 4°C in darkness. This is in accord with the theory that light could exacerbate the damage induced by chilling stress in cucumber (Heath and Packer, 1968; Wise and Naylor, 1987; Terashima et al., 1994; Barth and Krause, 1999; Xu, et al., 2008). According to Kudo and Sonoike (2002), chilling-sensitive plants such as cucumber experienced PSI and PSII photoinhibition upon exposure to moderate or high light at chilling temperatures (4°C). Photoinhibition causes reduction in the photosynthetic rate. The excess of absorbed light energy due to decreased photosynthetic activity results in overproduction of cellular ROS (Murata et al., 2007; Lukatkin, 2002). ROS induce lipid peroxidation (Lukatkin, 2003). Hodgson and Raison (1991) reported that chilling of cucumber for a few hours at moderate light induced peroxidation of membrane lipids. These results indicate that chilling stress induced ROS accumulation and caused lipid peroxidation in cucumber leaves.





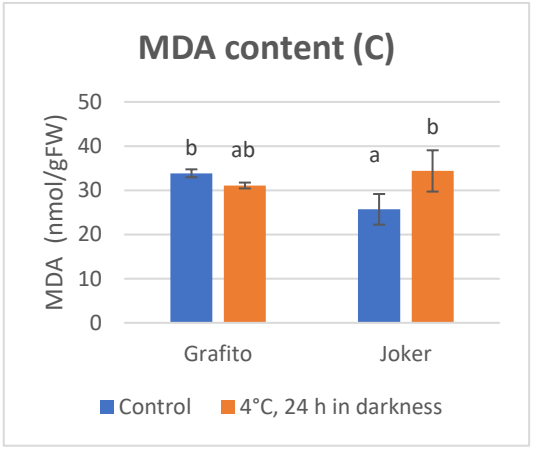


Fig. 5.4. MDA content in leaves of cucumber seedlings under control and 8°C daytime for 4 h (A), 8°C day/5°C night for 24 h (B) and 4°C for 24 h in darkness (C) . The data are the means with standard errors shown by vertical bars. Different letters above columns indicate significant differences between control and chilled plants, whereas the same letters show no significant difference at $p < 0.05$.

5.4. Proline content

Under 2nd chilling treatment, proline content increased significantly in both chilled Grafito and Joker plants relative to the control. In comparison, leaves of chilled Joker plants as an open field- grown cultivar had significantly higher proline content than chilled Grafito as a greenhouse-grown cultivar under the 2nd chillin treament. The results are consistent with the notion that chilling significantly induced the accumulation of proline in the chilling-treated plants and this increase was mostly due to the positive role of proline in osmoregulation in cucumber seedlings in response to chilling stress conditions (Feng et al., 2003 Zong Hui et al., 2003). Alagoz et al (2023) showed that proline as an osmoprotectant helps to mitigate stress through adjusting and maintaining the osmotic potential of the cell at reasonable levels and scavenging ROS. It also functions as a signaling molecule triggering changes in gene expression.

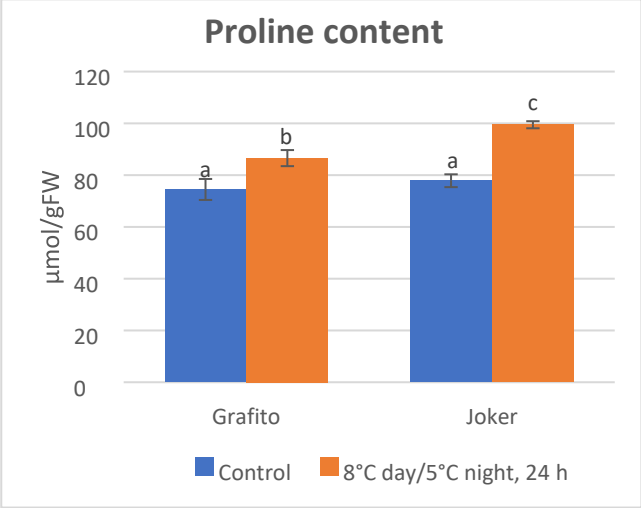


Fig. 5.5. Effects of chilling on proline content in cucumber leaves. The data are the means with standard errors shown by vertical bars. Different letters indicate significant differences between chilled and control treatments at $p < 0.05$.

5.5. RNA isolation, cDNA synthesis and RT-PCR

The concentration of RNA isolated from control and chilled plants is shown in Table 5.1. and the intergrity of RNA and synhesized cDNA visualized on ethidium bromide-stained agarose gel are shown in Fig. 5.6.

Table 5.1. Total RNA concentrations in (ng/µL) G: Grafito, C:control, S:stressed and J:Joker.

	RNA Concentrations (ng/µL)	
	Control and Chilled at 8°C day/5°C night, 24 h	Control and Chilled at 4°C night, 24 h in darkness
GC	1184.5	1063.3
GS	1433.9	2199.8
JC	1676.7	589.86
JS	2515.6	894

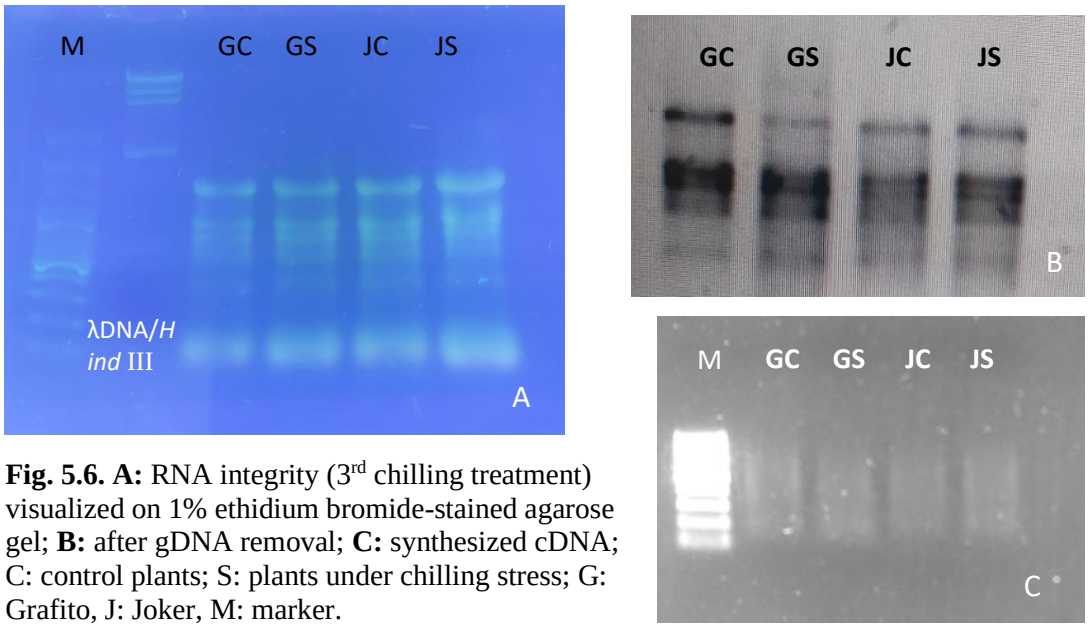


Fig. 5.6. A: RNA integrity (3rd chilling treatment) visualized on 1% ethidium bromide-stained agarose gel; **B:** after gDNA removal; **C:** synthesized cDNA; C: control plants; S: plants under chilling stress; G: Grafito, J: Joker, M: marker.

Transcription of *DHN1* and *LEA D-11* genes was studied by RT-PCR using actin (*CsaAct-7*) gene as an internal control to ensure equal loading of cDNA templates. RT-PCR analysis showed that under 2nd chilling treatment, both *DHN1* and *LEA D-11* were expressed in control and chilled seedlings, although at different levels. The expression of *DHN1* was significantly upregulated in chilled Grafito compared to control which suggested that it might be involved in the response to chilling conditions in the presence of light. In addition, *LEA D-11* was more strongly expressed in both stressed cultivar. RT-PCR analysis also showed that under 3rd chilling treatment, *DHN1* was expressed in both control and chilled Grafito seedling, while it was only expressed in chilled Joker seedlings suggesting that it might be induced by chilling treatment in this cultivar. In case of *LEA D-11*, its expression was upregulated in

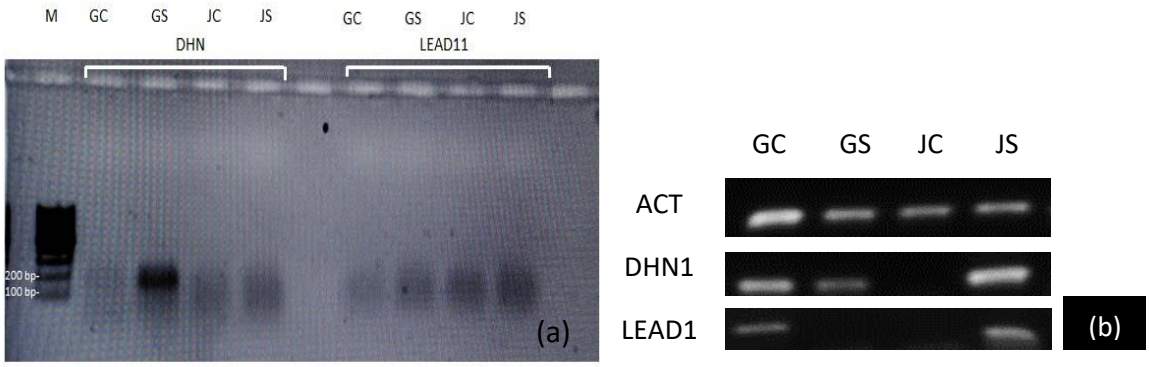


Fig. 5.7. RT-PCR amplification products of *DHN1*, *LEA D-11* and *ACT* (*CsaAct-7*) genes in the leaves of cucumber plants under 2nd (a) and 3rd (b and c) chilling treatments; C: control plants; S: plants under chilling stress; G: Grafito, J: Joker, M: 100 bp marker.

chilled Joker seedlins as well. Open-field-cultivated cultivar (Joker) exhibited more significant induction of DHN proteins under 4°C chilling treatment for 24 h in the dark.

5.6. The expression of dehydrins at the protein level

DHN transcripts respond quickly to environmental changes whether by accumulation or degradation, whereas the proteins are more stable and conservative; therefore, it is necessary to study DHN role in the plant response to chilling stress at protein level. Western bloting and mmunoblot analysis against the K-segment consensus sequence were performed to characterise DHN proteins expressed under chilling conditions. Immunoblot analysis of DHNs under 2nd chilling treatmemt detected a band at ~55kDa in size in chilled Grafito cultivar indicating a higher vulnerability of Grafito cultivar to chilling stress in the presence of light that leads to stronger response. This finding matched with the expression pattern of

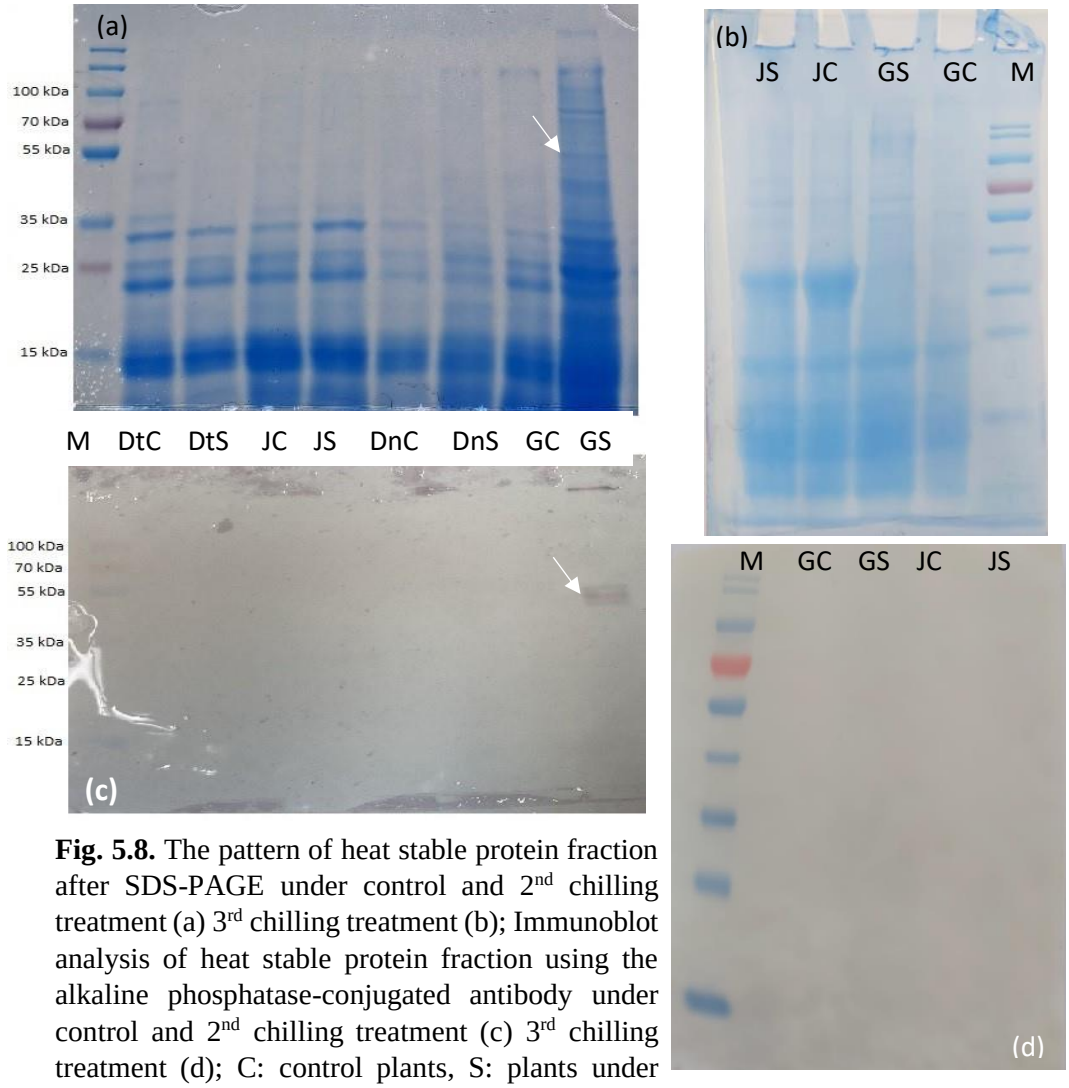


Fig. 5.8. The pattern of heat stable protein fraction after SDS-PAGE under control and 2nd chilling treatment (a) 3rd chilling treatment (b); Immunoblot analysis of heat stable protein fraction using the alkaline phosphatase-conjugated antibody under control and 2nd chilling treatment (c) 3rd chilling treatment (d); C: control plants, S: plants under chilling stress, Dt: Dirigent, Dn: Diapason, G: Grafito, J: Joker and M: size marker (kDa).

DHN1 and *LEA D-11* genes in Grafito under the same treatment since Kosová et al (2010) demonstrated a positive correlation between the level of accumulation of DHN transcripts or proteins and plant acquired tolerance. Under the 3rd chilling treatment, DHNs could be detected at the transcriptional level. However, no DHN protein was detected by the alkaline phosphatase-conjugated antibody. This was explained by Yin et al (2004) as the expression level of dehydrins might be too low to be detected by the alkaline phosphatase-conjugated antibody. Kosová et al (2014) suggested that regulation through post-translational modifications, such as phosphorylation might occur controlling the localization and the level at which the protein accumulate and be detectable. Previous studies suggested that DHNs could be involved in the tolerance of cucumber to chilling stress (Yin et al., 2004, 2006; Lee et al., 2017; Zhou et al., 2017, 2018). Zhou et al (2018) identified four DHNs in cucumber genome using bioinformatics-based methods, including 1 Y_nK_n-type, 2 Y_nSK_n-type, and 1 SK_n-type DHNs according to the conserved motifs named K-, Y- and S-segments. SK_n-type DHNs are induced by both low temperature and dehydration. K_n-type DHNs are strongly induced by low temperature.

6. Conclusion

Leaf water stress, leakage of electrolytes, and lipid peroxidation are negative effects induced in response to chilling stress in cucumber plants. Chilling- induced injury is influenced by the time and temperature of exposure and how the chilling treatment has been administered in the presence of light or in the dark. Adaptive changes occur when plants are exposed to chilling stress in order to reduce stress effects on chilling-sensitive plants, such as cucumber and ensure their survival and continued growth. Plants commonly accumulate small molecules such as proline in response to abiotic stress factors due to their vital role in osmotic adjustment process.

In addition, chilling stress significantly impacts dehydrin proteins prompting their accumulation in plant tissues which is consistent with our findings. Chilling conditions induced the accumulation of both *DHN1* and *LEA D-11* transcripts in Joker cultivar while the accumulation of *DHN1* transcripts significantly increased in Grafito cultivar the presence of light during chilling treatment. It is suggested that dehydrins play a vital role to safeguard the cell from damage and enhance the tolerance of the plant to stress conditions. DHNs are key proteins for the structure and function of cellular membranes implying that they might be involved in the membrane protection and reduction of electrolyte leakage. DHNs generally accumulate in plants in response to various abiotic stresses so it is important to avoid the interference in plant responses when chilling treatment is applied in the presence of light.

7. Summary

Chilling stress causes serious physiological and biochemical changes negatively impacting plant growth and crop yield. Chilling injury occurs when sensitive plants such as cucumber are subjected to low, but non-freezing temperatures ranging between 0℃ and 15 ℃. Cucumber is an important vegetable crop; however, it shows growth inhibition when temperatures exceed 35℃ or drop below 12℃. The present work is an attempt to clarify the relationship between dehydrin proteins and chilling tolerance in both greenhouse- and open field-cultivated cucumbers.

Seeds of four cucumber cultivars: “Joker and Dirigent” open-field-grown cultivars and “Grafito and Diapason” greenhouse-grown cultivars, were imbibed in distilled water and then grown in a growth chamber with a light/dark temperature of 26/20 °C and a photoperiod of 16 h. Three-week-old seedlings were exposed to three different chilling treatments: (i) at 8°C daytime for 4 h, (ii) at 8°C day/5°C night for 24 h, and (iii) at 4°C for 24 h in darkness. Then series of experiments were performed to monitor changes and injuries induced during chilling including leaf relative water content, leakage of electrolytes, malondialdehyde (MDA) content, proline content, and the expression of dehydrins (DHNs) at both the protein level and mRNA level.

Wilting leaves and dehydration symptoms manifested on chilling-stressed seedlings under all three treatments compared to control seedlings. Under the 3rd chilling treatment, chilled Grafito and Joker seedlings showed approx. 30% decrease in leaf relative water content (RWC) relative to control suggesting that chilling exposure resulted in water stress in the leaves. The electrolyte leakage (EL) and MDA content increased in chilled seedling. This increase could be an indication of lipid peroxidation and membrane damage upon chilling exposure. In addition, Grafito and Joker seedlings subjected to 2nd chilling treatment showed a remarkable increase in the proline content compared to control. The accumulation of proline, as an osmoprotectant, is important to reduce chilling-induced damage in cucumber seedlings. The expression of *DHN1* and *LEA D-11* genes were upregulated at the mRNA level under chilling treatments. DHNs were also detected at the protein level under the 2nd chilling treatment in chilled Grafito seedlings, although no DHN was detected under the 3rd chilling treatment. These results suggest that dehydrins are significant to counteract the negative effects of chilling stress on cucumber seedlings.

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List of Abbreviations

ABA	Abscisic acid
APX	Ascorbate peroxidase
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BSA	Bovine serum albumin
CAT	Catalase
CBF	C-repeat binding factor
cDNA	Complementary DNA
COR genes	Cold-regulated genes
CRT	C-repeat
C. sativus	*Cucumis sativus*
CsLEA	*Cucumis sativus* Late embryogenesis abundant
DHN	Dehydrin
DM	Dry mass
DNA	Deoxyribonucleic acid
DREB	Dehydration responsive element binding
EC	electrolytic conductivity
EDTA	Ethylene diamine tetraacetic acid
FM	Fresh mass
GPX	Glutathione peroxidase
IDPs	Intrinsically disordered proteins
IUPs	Intrinsically unstructured proteins
LDH	Lactate dehydrogenase
LEA	Late embryogenesis abundant
MDA	Malondialdehyde
NBT	4-nitro blue tetrazolium chloride
OD	Optical density
PMSF	Phenylmethylsulfonyl fluoride
PSI	Photosystem I
PSII	Photosystem II
REL	Relative electrolyte leakage
RNA	Ribonucleic acid
RT	Reverse Transcriptase

RT-PCR	Reverse transcription polymerase chain reaction
RWC	Relative Water Content
SMP	Seed maturation protein
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
TBS	TRIS-buffered saline
TBST	TRIS-buffered saline containing 0.1% Tween 20
TFs	Transcription factors
Treat.	Treatment
TCA	Trichloroacetic acid
TM	Turgid mass

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	treatments; C: control plants; S: plants under chilling stress; G: Grafito, J: Joker, M: 100 bp marker.
Fig. 5.8.	The pattern of heat stable protein fraction after SDS-PAGE under control and 2nd chilling treatment (a) 3rd chilling treatment (b); Immunoblot analysis of heat stable protein fraction using the alkaline phosphatase-conjugated antibody under control and 2nd chilling treatment (c) 3rd chilling treatment (d); C: control plants, S: plants under chilling stress, Dt: Dirigent, Dn: Diapason, G: Grafito, J: Joker and M: size marker (kDa).

DECLARATION

the public access and authenticity of the thesis

Student's name:	Amany Mohamed Ragab
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Title of thesis:	Analysis of dehydrin genes at the protein and at the mRNA levels in chilling-treated cucumbers
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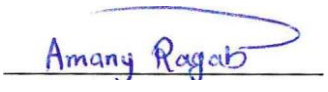

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