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Effect of Cold on Protein, Proline, Phenolic Compounds and Chlorophyll Content of Two Pepper (*Capsicum annuum* L.) Varieties

Esra KOÇ[1♠], Cemil İŞLEK[1], A.Sülün ÜSTÜN[1]

[1]*Ankara University, Science Faculty, Department of Biology, Ankara, Turkey*

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ABSTRACT

In this study, quantitative changes of total soluble protein in leaf and stem, apoplastic protein, total phenolic compounds, proline and chlorophyll content were determined in a the cold-susceptible KM-121 (Kahramanmaraş- hot) and cold-resistant hybrid pepper Mert (*Capsicum annuum* L.). The plants were raised in a growth room under a controlled environment of 25 ± 2 °C and 16 h light / 8 h dark photoperiod. Then, plants exposed to 4°C cold stress for three days and control groups were kept at 25°C. Our results show that cold treatment increased accumulation of apoplastic and total soluble protein, proline, phenolic compounds in pepper seedlings, while decreased the content of chlorophyll.

Key Words: *Capsicum annuum, Cold Stres, Protein, Phenolic, Proline.*

1. INTRODUCTION

Low temperature constitutes one of the major hazards to agriculture and is an important factor that limits the survival, productivity and geographical distribution of plants in large areas of the world [1]. Exposure to a low non-lethal temperature usually induces a variety of biochemical, physiological (soluble proteins, apoplastic protein (having antifreeze activity), enzymes, phenolics, etc.) and molecular changes in plants which can result in an acclimation response that is characterized by a greater ability to resist injury or survive an otherwise lethal low temperature stress [2]. This process is known as cold acclimation [3- 4].

In the present study, we examined whether cold-sensitive KM–121 and cold-resistant hybrid pepper MERT responded differently to cold treatment in terms of differential total soluble protein, apoplastic protein, proline, phenolic compound accumulation and changes in chlorophyll a, b and a+b in leaves and stems of pepper in a treatment comprising shifts from 25° to 4°C.

2. THE METHODOLOGY

2.1. Plant Material and Growth Condition

The seeds of two genotypes, the cold susceptible KM-121 (Turkey) and the cold resistant genotype Mert (Hybrid- Sivri pepper, Antalya - Turkey) belonging to the Capsicum annuum variety were used in this study.

The surface of the pepper (*Capsicum annuum* L.) seeds was sterilized by % 0.75 sodium hypochlorite for 2 min, and thoroughly washed with sterile distilled water. After germination, pepper seeds were sown in a plastic pot containing a steam-sterilized soil, fertilizer and sand mix (1:1:1 v/v/v). The plants were raised in a growth room under a controlled environment of 25 ± 2 °C and 16 h light / 8 h dark photoperiod. Then, plants at 4 and 5 leaf stages of each species were divided into two groups. One group of each species was maintained in the same chamber with 16 h light / 8 h dark photoperiod and at 25 ± 2 °C as the control. The other group of each species was transferred in a chamber for cold acclimation with: 16 h light / 8 h dark photoperiod and at 4 °C for 3 day. On the 3th day after treatment, seedlings were randomly taken from the control and cold- treated seedlings of each genotype and then seedlings were divided into leaves and stems, were ground with mortar and pestle using liquid nitrogen and stored at -70 °C until analysis.

2.2. Extraction of Apoplastic Proteins

Apoplastic proteins were extracted from pepper stems and leaves by vacuum infiltration with 20 mM ascorbate and 20 mM $CaCl_2$ (pH 3), followed by centrifugation at 900g to recover the apoplastic contents

Corresiponding author: ekoc@science.ankara.edu.tr

[5]. Protein concentrations were measured using a modified Bradford procedure with BSA as standart protein [6].

2.3. Extraction of Total Soluble Proteins

Total soluble proteins were extracted from leaves and stems [7]. This homogenization consisted of with a chilled mortar and pestle using a buffer containing ice-cold 50 mM Tris-HCl, pH 6.8; 1% (v/v) 2-β mercaptoethanol and (50 mgL^{-1} PMSF). The homogenate was centrifuged at 15,800g and 4˚C for 5 min. Supernatant stored at –20°C for analysis. Protein concentrations were measured using a modified Bradford procedure with BSA as standard protein.

2.4. Quantification of Protein Using the Bradford Assay

Protein extracts were thawed and their concentration determined by a colorimetric method. In the Bradford assay, protein concentration is determined by quantifying the binding of the dye, Coomassie Brilliant Blue G-250, to the unknown protein solution, as compared to known standards. Tubes containing 100 µl aliquots of known concentrations of Bovine Serum Albumin were prepared. One ml Coomassie Brilliant Blue solution was added to each tube and vortexed. The reactions were left at room temperature for 5 min. The absorbance at wavelength of 595 nm was determined against the blank and the standard curve of absorbance versus protein concentration plotted. Reactions containing dilutions of the soluble protein extracts (unknown concentrations) were set up as above and the absorbance at 595 nm determined. The proteins concentration of the extracts was determined from the Standard curve, using a spectrophotometer (CECİL 5000).

2.5. Proline Assay

Free proline content was estimated using the acid ninhydrin method [8]. Plant tissues (leaves and stems) were grounded in a mortar and pestle with % 3 (w/v) sulfosalicylic acid aqueous solutions and the homogenate was filtered through Whatman No. 1 filter paper, then 2 ml of filtered extract was taken for the analysis to which 2 ml acid ninhydrin and 2 ml glacial acetic acid were added. The reaction mixture was incubated in a boiling water bath for 1 h and the reaction was finished in an ice bath. Four milliliter of toluene was added to the reaction mixture and the organic phase was extracted, in which was read at 520 nm using toluene as blank by UV-visible spectrophotometer (CECİL 5000).

2.6. Total Phenolic Assay

A ground fresh sample of 0.1 g phenolic compounds were extracted with 500 µl 80 % aqueous methanol in a boiling water bath (80°C) for 15 min and extracts was ultracentrifugated for 10 min at 500g, then pellet was re-extracted with same procedure [9]. The total phenolic content of leaves and stems were determined by using the Folin–Ciocalteu assay [10]. 100 µl of extracts 750 µl of Folin–Ciocalteu's phenol reagent was added to the mixture and shaken. After 5 min, 750 µl of (6 %) Na_2CO_3 solution was added. After incubation for 90 min at room temperature, the absorbance against prepared reagent blank was determined at 765 nm with an UV-visible spectrophotometer (CECİL 5000). Total phenolic content of leaves and stems was expressed as mg. Gallic acid was used as standard.

2.7. Chlorophyll Assay

A ground fresh sample of 0.1 g was extracted with 80 % acetone. The absorbance of resulting supernatant was recorded at 664 and 647 nm using an UV-visible Spectrophotometer (CECİL 5000) [11].

2.8. Statistical Analysis

All results reported were the means of three replicates. Data were analyzed by two-factor variance analysis using SPSS (15.0).

3. FINDINGS

In the present study, transferred seedlings from 25 to 4°C for 3 d resulted in increased in leaf apoplastic and total soluble protein amounts in both cultivars during low temperature period compared to seedlings maintained at a constant temperature of 25°C. In the statistical analysis performed, two interactions between genotype x temperature control and cold-treated stems and leaves were found significant at $p < 0.01$. While apoplastic protein content was the highest in the cold-treated seedlings of Mert genotype, total soluble protein in the cold-treated seedlings of KM-121 is higher than the Mert variety. Also, it was determined that the total soluble protein content in the stem and leaves of cold-treated seedlings of KM-121 and Mert are higher than those of the control seedlings (Figure 3.1.-3.2.).

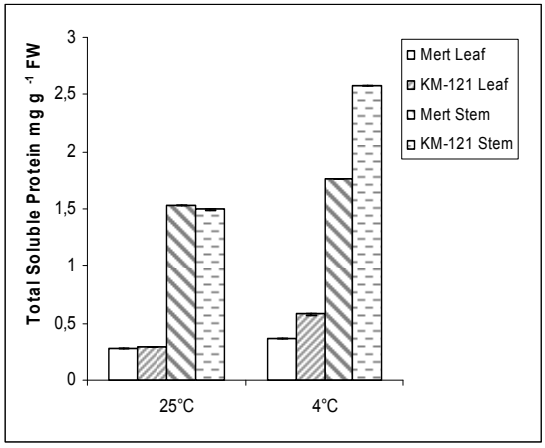


Figure 3.1. Changes in the content of total soluble protein in the stems and leaves of pepper subjected to low temperature (4°C) ($p < 0.01$). * FW: Fresh Weight.

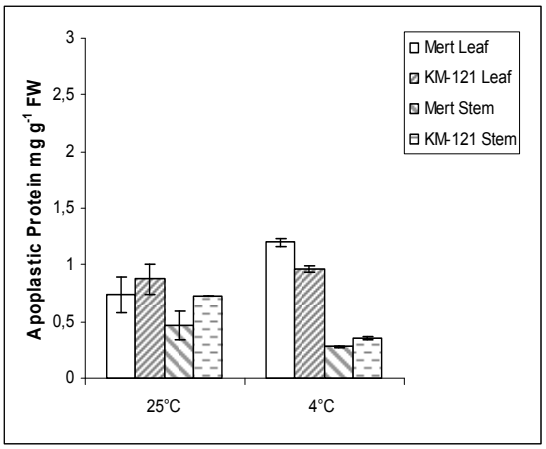


Figure 3.2. Changes in the content of apoplastic protein in the stems and leaves of pepper subjected to low temperature (4°C) ($p < 0.01$). * FW: Fresh Weight.

When proline content in both stems and leaves of the two pepper genotypes are compared, it can be concluded that the proline content in leaves of KM-121 genotype was less than Mert genotype, but proline content in stems of KM-121 genotype was higher than Mert genotype. Proline accumulation was the highest in the cold-treated leaves of Mert genotype. In the two-factor variance analysis, both cold and control treatments have significantly ($p < 0.01$) increased free proline content in leaves and stems of two genotype. The change in the proline belonging to the stems of cold-treated and control seedlings of the two pepper genoytpes are shown in Figure 3.

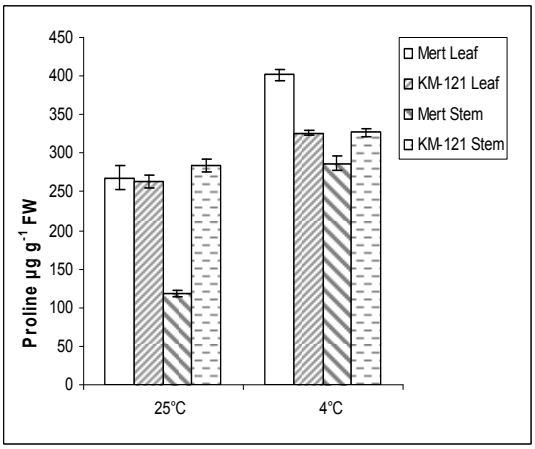


Figure 3.3. Proline accumulation in the stems and leaves of pepper subjected to low temperature (4°C) ($p < 0.01$). * FW: Fresh Weight.

When the stems and leaves of the two pepper genotypes are compared in terms of phenolic compounds, our results show that the differences in cold tolerance between the control and cold-treated leaves were reflected by differences in their abilities to accumulate phenolic compounds under cold stress conditions ($p < 0.01$). Gallic acid accumulation in leaves of Mert genotype was higher than both KM-121 genotype and control seedlings. Moreover, it was established that gallic acid accumulation in leaves of KM-121 genotype subject to low temperature was higher than control seedlings. In the other hand, in the statistical analysis performed, significant differences between KM-121 and Mert cold-treated stems were not found at $p > 0.01$ (Figure 3.4.).

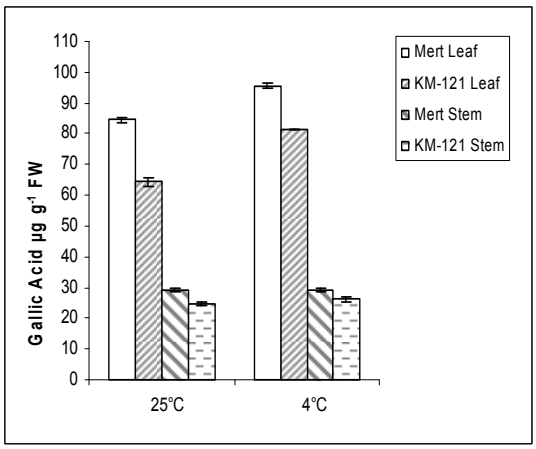
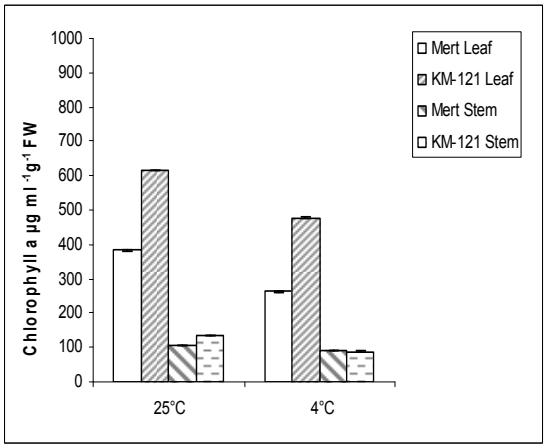


Figure 3.4. Change in the content of phenolic compounds in stems ($p > 0.01$) and leaves ($p < 0.01$) of pepper subjected to low temperature (4°C) . * FW: Fresh Weight.

In this study, the amount of chlorophyll a,b and total chlorophyll were decreased in chilling phase (Figure 3.5). When subjected to chilling, the content of chlorophyll a and total chlorophyll of both Mert and KM-121 genotype significantly ($p < 0.01$) decreased, while the change in the content of chlorophyll b were not found significant at $p > 0.01$ (Figure 5.).



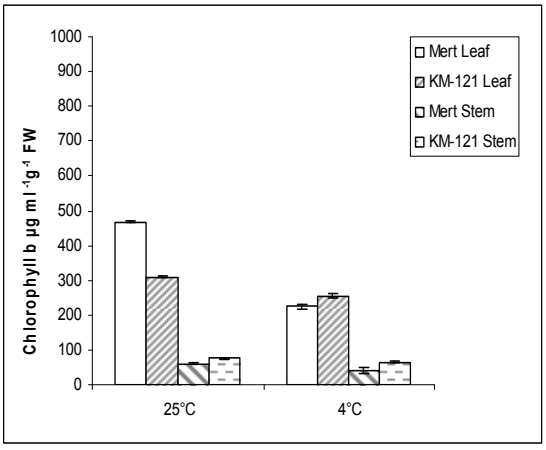


Figure 3.5. Changes in the content of chlorophyll a ($p < 0.01$) and b ($p > 0.01$) in the stems and leaves of peppers subjected to low temperature (4°C) ($p < 0.01$). * FW: Fresh Weight.

4. DISCUSSION

Each plant species has its unique set of temperature requirements, which are optimum for proper growth and development. Low temperature is one of the abiotic stresses that are principal cause of crop failure world wide, dipping average yields for most major crops [12]. Many plants, especially native to warm habitat, exhibit symptoms of injury when exposed to low non-freezing temperatures. These plants include maize (*Zea mays*), cotton (*Gossypium hirsurum*), tomato (*Lycopersicum esculentum*) which are particularly sensitive to temperatures below 10°C exhibit signs of injury [13-14]. The symptoms of stress induced injury in these plants appear of the 48 to 72h, later, However, this varies from plant to plant and also depend upon the sensitivity of individual plant to cold stress [15].

Cold stress induced an accumulation in soluble protein content in *Medicago sativa* [16]. Synthesis of specific proteins is an important mechanism involved in increasing freezing tolerance during cold acclimation [17]. Low temperature can result in the synthesis of proteins [4].

Two possible functions of apoplastic proteins have been proposed [17, 18]. One potential role of apoplastic protein is to prevent the formation of large ice crystals. The inhibition of ice recrystallization may be the primary role of AFPs in freezing-tolerance organisms [19].

It has been reported that winter rye AFPs inhibit ice recrystallization at very low concentrations. Therefore, even the presence of low concentrations of rye AFPs in the apoplast may be effective in maintaining the small size of extracellular ice crystals. This enhances freezing tolerance by preventing physical damage caused by larger ice crystals. AFPs may also function as a barrier to inhibit ice formation [18]. In order to tolerate the stresses that they face, plants have to adapt their metabolism. Several cellular and metabolic functions are changed by low temperatures or freezing. This process requires the accumulation of proteins such as soluble protein and apoplastic protein whose synthesis increases at low temperature. Our results show that total protein and apoplastic protein have protective role in peppers subject to low temperature.

Proline accumulates in higher plants in response to serious abiotic and biotic stresses such as water stress and chilling stress [20]. It has been reported that proline content was increased in potato hybrids when plants subjected to cold treatment [21]. In present research proline accumulated when the plants were transferred to chilling temperatures (4°C) [22]. In cold acclimated plants proline content was higher than non-acclimated plants and cold acclimated plants recovered faster than non-acclimated ones. In this work, we conclude that proline accumulation, typical plant cold stress response. Our results demonstrate that proline content increased in stem and leaf of pepper is activated by cold stress. We found a significant correlation between freezing tolerance and an increase of proline concentration in stems and leaves of pepper after exposure to low temperatures. In this study, low temperature significantly ($p < 0.01$) increased proline accumulation in pepper upon chilling.

Abiotic stress leads to a series of molecular, biochemical, physiological and morphological changes that adversely affect plant growth and productivity. A low temperature is a major factor limiting the productivity and geographical distribution of many species, including important agricultural crops [23]. Higher plants manifest a unique capability of the synthesis of a large amount of diverse molecules so-called secondary metabolites, such as phenolic compounds or polyphenols. Phenolics are low molecular compounds in all tissues of higher plants with great significance in plant development. The synthesis and release of phenolics are induced by various biotic and abiotic factors [24]. The effect of the composition of phenolic compounds in winter wheat (*Triticum aestivum* L.) leaves cause the hardening was studied and increasing level of phenolic compounds in winter wheat leaves with a cold stress were detected [25]. Cold acclimation induced accumulation of total phenolics, which was positively related to antioxidant capacity. Low temperature stress-induced phenolic compounds may increase the antioxidant activity. Antioxidants are vital substances which possess the ability to protect body from damage by free radical induced oxidative stress (with low temperature, salinity, pathogen etc.). Phenolic compounds are natural antioxidant [26]. In this research, the content of phenolic compounds increased in leaves of KM-121 and Mert pepper genotypes. So, we could say, phenolic compounds accumulates in pepper in response to cold stress

Low temperature is one of the most important factors that may limit photosynthetic activity. Decrease of photosynthesis induced by low temperatures is a well-known response of chilling-sensitive plants [22]. It has been reported that chlorophyll a and b content was decreased in plants when plants subjected to cold

treatment [27]. The observed decline of chlorophyll content induced by low temperature confirms our results. Relative chlorophyll content of the cultivars decreased under cold stress conditions in comparison to controlled conditions. A decrease in the chlorophyll content could be a typical symptom of oxidative stress.

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