Journal of Animal & Plant Sciences, 34(3): 2024, Page: 780-790
ISSN (print): 1018-7081; ISSN (online): 2309-8694
https://doi.org/10.36899/JAPS.2024.3.0764

EXPRESSION ANALYSIS OF *ML237*, *R2R3 MYB* AND *OIL_143* GENES THAT AFFECT THE COLD TOLERANCE DURING THE COLD-HARDENING STAGE ON SUCCESSFUL CRYOPRESERVATION OF *MENTHA* × *PIPERITA* L.

S. Galatali[1], T. Zengin[1] and E. Kaya[*1]

[1]Molecular Biology and Genetics Department, Faculty of Science, Mugla Sitki Kocman University, 48000, Kötekli, Muğla, Türkiye
[*]Corresponding author's e-mail address: ergunkaya@mu.edu.tr

ABSTRACT

Mentha × *piperita* L., one of the oldest known hybrid species, was formed by the derivation of *M. spicata* L. and *M. aquatica* L. Since the plant is a hybrid species, it has sterile seeds and can be propagated vegetatively. The fact that the plant grown in natural and field environments is affected by biotic and/or abiotic environmental conditions keeps the continuity of the species under pressure and this situation requires the germplasm of the plant to be protected as an alternative. The current study aimed to investigate the expressions of *ML237*, *R2R3 MYB* and *oil_143* genes determined by the bioinformatics blast studies that were effective against the cold tolerance in the *M.* × *piperita* at transcriptional level during the cold-hardening which is one of the cryopreservation treatment stages. With this study, it is aimed to obtain basic information that can be useful in cryopreservation studies for these and similar plants to be carried out in the future, and in biotechnological applications to increase viability and regeneration after cryo-storage. According to the T-Test result after the statistical evaluation of the results, a significant increases were observed the *ML237*, *R2R3 MYB* and *oil_143* genes.

Key words: cold stress, long-term conservation, gene expression, Real-Time PCR

Published first online April 25, 2024 Published final May 31, 2024

INTRODUCTION

There are more than sixty species in different regions of the world belonging to the genus *Mentha* (lamiaceae), which is one of the ten most traded plants in the world in terms of essential oil (Ahmadi *et al*., 2022). The seven species, namely *M. piperita* L., *M. spicata* L., *M. suaveolens* Ehrh., *M. pulegium* L., *M. aquatica* L., *M. arvensis* L., *M. longifolia* L. and fourteen taxa of this species show natural distribution in the Turkey. Some of the species belonging to the genus *Mentha* were formed by natural ways and some by artificial crossing. This situation plays a crucial role in the diversity of species and causes significant differences between species in terms of essential oil yield and quality. Especially in hybrid species, there may be changes in their chemical contents or the density of the secondary metabolites they produce, even if they belong to the same species, due to genetic expansion or environmental conditions (climate, soil structure, altitude, pH, etc.) (Khanam and Mohammad, 2016; Jafari-Sales and Pashazadeh, 2020; Hasan and Abd Manan, 2020; Nor Kamal *and* Abd Manan, 2020).

Mentha × *piperita* L., one of the oldest known hybrid species (Figure 1A), was formed by the derivation of *M. spicata* and *M. aquatica* (Anwar *et al*., 2019). In addition to the fact that this hybrid species has more than 200 components in its essential oil, previous studies on *M. piperita* have shown that its essential oil and menthanolic extracts have biological activities such as antiviral (Li *et al*., 2017), antibacterial (Sokovic *et al*., 2008; Kizil *et al*., 2010), antifungal (Kizil *et al*., 2010; Tyagi and Malik, 2010), antibiofilm formation (Rasooli *et al*., 2008; Sandasi *et al*., 2010) and antioxidant (Kizil *et al*., 2010) and due to these properties, they have great economic importance (Telci *et al*., 2011; Saharkhiz *et al*., 2012). Since the plant is a hybrid species, it has sterile seeds and can be propagated vegetatively. The fact that the plant grown in natural and field environments is affected by biotic and/or abiotic environmental conditions keeps the continuity of the species under pressure and this situation requires the germplasm of the plant to be protected as an alternative (Senula *et al*., 2007; Ozkaya *et al*., 2022).

Due to the risk of damage to plant species and varieties as a result of biological, chemical and physical stresses, plant genetic resources are conserved by ensuring the continuity of the germplasms via short-medium and long-term preservation methods (Ozudogru *et al*., 2011; Ozudogru *et al*., 2013; Souza *et al*., 2017;

Kaya *et al*., 2020; Aksoy *et al.*, 2021). In *in vitro* preservation methods that provide short- or medium-term storage, the methods are not economical and requires large areas, and can also cause problems such as contamination and somaclonal variation (Guldag *et al.*, 2023). The cryopreservation technique, which is a long-term preservation method, allows the use of many tissues and organs of the living plant (seeds, shoot tips, nodal buds, synthetic seeds, somatic or zygotic embryos and calli) and ensures that they are kept in liquid nitrogen at very low temperatures (-196 °C) (Towill, 2002; Lambardi and de Carlo, 2003).

The cryopreservation technique is basically applied in two ways, traditional and improved. Traditional methods (two-stage freezing technique) are not preferred because they are both time consuming and the equipment used is very expensive. On the other hand, developed methods (single-stage freezing technique) are performed by rapid and single-stage freezing of tissues using a relatively low cost cryoprotective solution. Improved methods include vitrification, dehydration, encapsulation-vitrification, encapsulation-dehydration and droplet freezing techniques (Benson, 2008).

For a successful cryopreservation, the percentage of viability and regeneration of plant tissues or plantlets after cryotreatment is very crucial. Otherwise, the use of cryo-preservation, which results in low regeneration, for the preservation of germplasm will not be successful. After cryostorage, for the high viability and successful regeneration, the physiological state, age and size of the explant used, as well as the tolerance of the plant to the physical stresses encountered are very effective (Harding *et al*., 2009; Kim *et al*., 2009; Ozudogru *et al*., 2012; Souza *et al*., 2018; Maślanka and Szewczyk, 2021).

In order to increase viability and regeneration success, a treatment called "cold-hardening" is also performed before the cryopreservation process. During the cold-hardening period, each plant is incubated in a dark environment at ~ 4°C for the maximum time (two weeks - two months) suitable for it. During this process, the relevant signal transduction pathways are activated and some osmo-active compounds known to be effective in freezing tolerance are synthesized (Rosa *et al*., 2009; Maślanka and Szewczyk, 2021). Thus, together with the oxidative stress that may occur during cryopreservation, the synthesis of the relevant molecules against cold and freezing stress occurs and a molecular response is formed in order to provide tolerance. In a study by Chang *et al*. (2000), they reported an increase in the resistance of plants to frost stress after cold-hardening (Kami, 2012). Recently, Maślanka and Szewczyk (2021) showed that in addition to the sucrose preculture application in cryopreservation experiment, the regeneration rate of meristems with cold-hardening increased compared to those with only sucrose preculture after cryopreservation.

Plants create a tolerance response to low temperature stresses by regulating the expression levels of proteins with different functions and especially transcription factors and some genes. For example, for the expression of proteins responsible for the cold stress response, CBF/DREB transcription factors positively regulate the expression of interest genes by connecting to specific sequences containing highly conserved CCGAC bases, also known as DRE/CRT, in the promoter site of dehydration and cold sensitive genes (Chew and Halliday, 2011; Rihan *et al*., 2017; Vazquez-Hernandez *et al*., 2017; Mehrotra *et al*., 2020). Plants also manage to survive by regulating various signaling networks with the wide va-riety of transcription factors (such as MYBs) they possess in order to adapt to the changing environmental conditions in their environment. Studies have reported that *MYB* genes positively regulate the many protein expressions involved in the reaction to abiotic stress (Mehrotra *et al*., 2020; Dai *et al*., 2007; Ma *et al.,* 2009; Su *et al*., 2014; Lv *et al*., 2017). In this context, in the current study, during the cold-hardening, which is one of the cryopreservation treatment stages, the expressions of *ML237*, *R2R3 MYB* and *oil_143* genes determined by the bioinformatics blast studies that were effective against cold tolerance in the *M. × piperita* were analyzed at transcriptional level. With the current study, it is aimed to obtain basic information that can be useful in cryopreservation studies for these and similar plants to be carried out in the future, and in biotechnological applications to increase viability and regeneration after cryo-storage.

MATERIALS AND METHODS

Plant material and *in vitro* culture establishement: *M. × piperita* seeds were provided by Mugla Metropolitan Municipality, Agricultural Department, Local Medicinal and Aromatic Plants Seed Center (Mugla, Turkey, 37.17117620659824, 28.38721630972173). The seeds were surface sterilized according to protocol of Kaya *et al.* (2017). The seeds were treated with 70 % ethyl alcohol for five minutes, 10 % H_2O_2 for five minutes and two times 20 % commercial bleach for tem minutes (Kaya *et al.*, 2021). Finally, the seeds were rinsed thoroughly with sterile distilled water and they were incubated under standard growth room terms (16 hr light / 8 hr dark photoperiod, 50 $\mu molm^{-2}s^{-1}$, 25 ± 2 °C) in MS (Murashige and Skoog, 1962) nutrient medium without plant growth regulator (Kaya *et al.*, 2021).

Cold-hardening treatments: For cold-hardening treatments, the *M. × piperita* micro-shoots (Figure 1B), which were *in vitro* subcultured to obtain sufficient plant material before, were covered in an opaque manner for 24, 72 and 168 hours and incubated at +4°C in the dark and (This step was optimized for *Thymus vulgaris* L.

cryopreservation (Ozudogru *et al*., 2011). This stage is thought to be effective in terms of viability (Sakai and Nishiyama, 1978) and regeneration of plants living in temperate climates after cryopreservation (Reed, 1988) (Figure 1C).

Molecular analyzes - Primer design: Because there is no work on cold stress arrangement in *M. × piperita*, initially, genes related in cold stress regulation were determined in *Arabidopsis thaliana* (L.) Heynh.. after that, target genes were identified in *M. × piperita* by providing BLAST over the accession code of the relevant FASTA or gene sequence in *A. thaliana* reaching est and nr/nt databases in NCBI. 18S ribosomal RNA (JX444508.1), ML237 (AW255239.1), MYB (KY081780.1) and oil_143 (EL342294.1) predicted to be related in cold stress regulation, in the NCBI Primary BLAST database for later use in Q-PCR. Primers were created from the mRNA sequences of the genes (Table 1).

RNA isolation: The genomic RNA was obtained via the GeneJET Plant RNA Purification Kit (Thermo Scientific™, catalog number: K0801). In order to determine the quantitative purity of the total RNAs was performed spectrophotometric analysis by considering the A_{260}/A_{280} and A_{260}/A_{230} values. Agarose gel electrophoresis was performed to determine the qualitative purity and amount of total RNAs. The band profiles of 28S, 18S and 5.8S rRNA subunites formed by the total RNAs obtained from both the control (rootstock) and the plants subjected to three different periods of cold-hardening (24, 72 and 168 hours), were observed under the UV transmitter (Figure 1D).

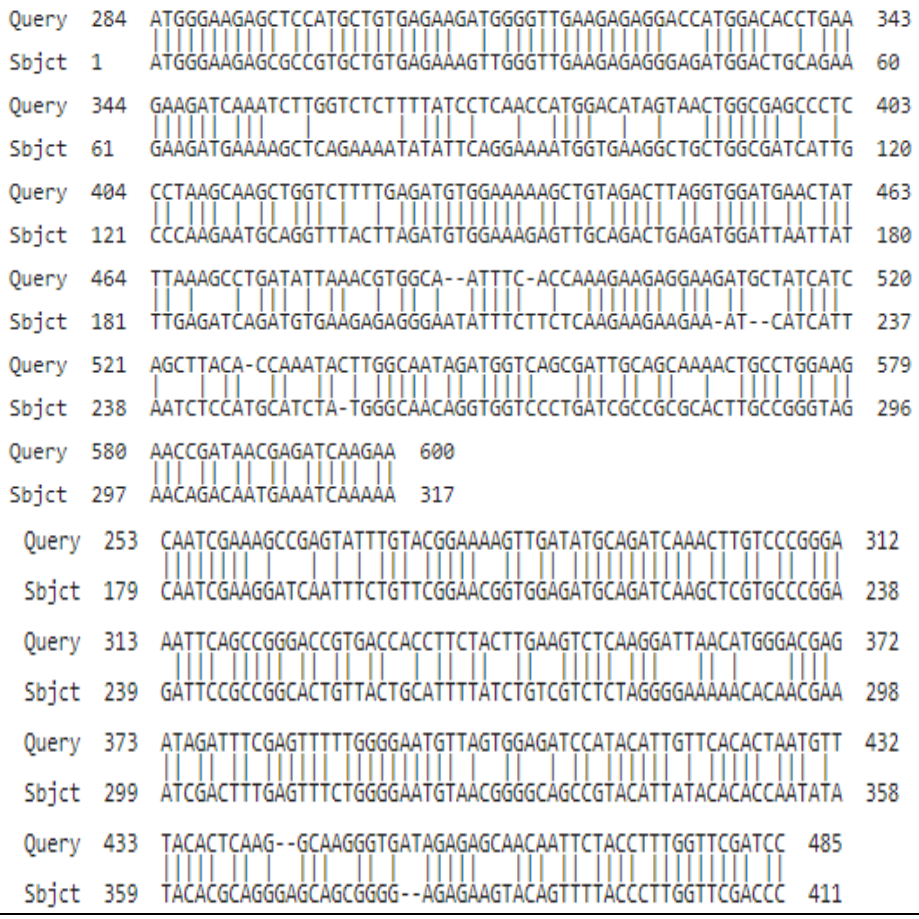
cDNA synthesis: cDNA was synthesized by using total RNAs with the OneScript® Plus Reverse Tran-scriptase OneScript® cDNA Synthesis kit. Synthesis reaction mixture in 14.5 µL total vol-ume containing final concentrations of 500 ng/µL total RNA, 7.5 µL nuclease-free H_2O, 0.5 µM oligonucleotide and 500 µM dNTP and was incubated at 65 °C for 5 minutes, and after it was put on ice, 1× (4µL) of 5× RT Buffer, 20U/rxn of RNaseOFF Ribonuclease Inhibitor, and 1 µL of OneScript® RTase (200 U/µL) were added and then the final mixture in a total volume of 20µl was incubated for 30 minutes at 50 °C and then at 85 °C for 5 minutes to stop the reaction.

Table 1. Sequence matches as a outcome of BLAST between *M. piperita* and *A. thaliana*.

Gene Characteristics	Sequence matches
ML237* (*Hsp70*) • ***ACN: AW255239.1 • Match: 425/522 (%81) • Gap: 1/522 (%0) Length: 611bp	Query 1419 GGTTTGGAAACTGCCGGTGGTGTTATGACTGTTTTGATTCCGAGGAACACCACAATTCCG 1478 Sbjct 36 GGTTTGGAAACTGCTGGAGGAGTCATGACTATCTTGATCCCGAGAAACACTACCATTCCC 95 Query 1479 ACCAAGAAAGAGCAGATATTCTCTACCTATTCAGACAACCAGCCCGGTGTACTGATCCAG 1538 Sbjct 96 ACCAAGAAAGAGCAAGTCTTCTCGACCTATTCTGACAATCAGCCGGGAGTCCTGATCCAG 155 Query 1539 GTCTACGAAGGAGAGAGGGCACGAACAAAGGACAACAACCTTTTGGGAAAGTTCGAGCTC 1598 Sbjct 156 GTTTATGAAGGTGAAAGGACAAGAACAAGGGACAACAATTTACTCGGAAAGTTTGAACTC 215 Query 1599 AGTGGTATACCACCTGCTCCACGAGGTGTACCGCAGATTACTGTCTGTTTCGACATCGAC 1658 Sbjct 216 TCCGGAATCCCACCTGCACCCAGAGGAGTCCCTCAAATCACTGTCTGCTTTGATATTGAT 275 Query 1659 GCCAATGGTATCCTGAATGTGTCGGCTGAGGACAAGACGACTGGTCAGAAGAACAAGATC 1718 Sbjct 276 GCAAACGGTATCCTGAACGTTTCTGCGGAGGACAAGACCACGGGACAGAAGAACAAGATC 335 Query 1719 ACAATCACAAACGACAAGGGAAGGTTATCAAAGGAAGAGATCGAGAAGATGGTACAAGAG 1778 Sbjct 336 ACGATCACCAATGACAAGGGAAGACTCTCCAAGGATGATATCGAGAAGATGGTTCAAGAA 395 Query 1779 GCAGAGAAGTACAAGGCTGAGGATGAAGAACACAAGAAGAAGGTGGATGCAAAGAACGCT 1838 Sbjct 396 GCCGAGAAGTACAAATCGGAAGACGAAGAGCACAAGAAGAAGGTGGAAGCAAAGAACGCG 455 Query 1839 CTCGAGAACTATGCATACAACATGAGGAACACGATCAA-GGACGAGAAGATCGCATCTAA 1897 Sbjct 456 TTGGAGAACTACGCCTACAACATGAGGAACACCATCAAGGGATGAGAAGATCGCCTCCAA 515 Query 1898 GCTTGACGCAGCTGACAAGAAGAAGATTGAGGATGCAATCGA 1939 Sbjct 516 GCTGCCAGCTGCTGACAAGAAGAAGATTGAGGATGCGATTGA 557

* *R2R3-MYB* (***MYB15*)
- ***ACN: KY081780.1
- Match: 215/321 (%67)
- Gap: 8/321 (%2)

Length: 813bp



* *OIL_143* (***ATXTH21*)
- ***ACN: EL342294.1
- Match: 164/235 (%70)
- Gap: 4/235 (%1)

Length: 425bp

*The gene has been characterized in *Mentha* × *piperita.*
**The gene has been characterized in *A. thaliana*
***Accesion number

Quantitative polymerase chain reaction (QPCR): Real-Time PCR was realized via AmpliqonRealQ Plus 2× Master MixGreen kit as indicated in Table 2, using primers. The primers were designed using mRNA sequences in the NCBI Primary BLAST database in *M.* × *piperita* L. plant. Identification of gene ex-pression levels considered to be related in cold stress tolerance was made in the control of reference genes and 18 S rRNA housekeeping genes was used as positive control. PCR amplification was achieved with a 15 µl total volume of reaction mixture including 20 ng cDNA, 0.1 µM of each primers (forward and reverse), RealQ Plus 2× Master Mix and dH_2O. Reaction cycles were performed for 15 minutes at 95 °C for preincubation, 40 cycles for amplification for 30 seconds at 95 °C – 55 °C – 72 °C, and for cooling at 37 °C for 30 seconds.

Table 2. Primary sequences, Tm values and accession numbers of genes considered to be associated with cold stress designed in the NCBI database

Gene	Primer Sequences	Tm (°C)	Accession numbers
18S Ribosomal RNA	F: 5'-CGAGACCTCAGCCTGCTAAC-3' R: 5'-CGGCCCAGAACATCTAAGGG-3'	60.18 60.18	JX444508.1
ML237	F: 5'-TTTCTGCGGAGGACAAGACC-3' R: 5'-TAGGCGTAGTTCTCCAACGC-3'	59.97 59.83	AW255239.1
MYB	F: 5'-CCATGGTGGGAGAGTTGGAC-3' R: 5'-ACTTGTCGTCCTCGTCGTTC-3'	60.04 60.04	KY081780.1
oil_143	F: 5'-CTCGATTCCGTCTCAGGCTC-3' R: 5'-ATCTGCATCTCCACCGTTCC-3'	59.97 59.82	EL342294.1

Statistical analysis of data: The statistical analyzes of data obtained via Q-RT PCR were realized by T-Test method. A $p \leq 0.05$ value was thought as statistically significant.

Figure 1. The shoots of the economically valuable *M.* × *piperita* plant growing under *ex vitro* (A) and *in vitro* (B) conditions. *In vitro* shoots after 168 hours of cold-hardening (C). The gel image of total RNA isolated from *M.* × *piperita* plant, M: RNA marker Invitrogen™ (D).

RESULTS

Investigation of *ML237* gene expression level during cold stress tolerance: RT PCR was realized with five repeats to determine the expression level of *ML237* gene in four experimental groups, one of which was a control. The mean, standard deviation and standard errors of the obtained CT values were calculated (Table 3).

After Real-Time PCR, The experimental and the control groups of the all the melting curve graph peaks of the *ML237* gene showed the different expression levels in the same time interval, and there was no foreign DNA contamination and primer dimers (Figure 2A). The values of CT derived after 18S rRNA standardization via with $2^{-\Delta CT}$ method were rationed to the control group and a graph was structured. As a result of the T-Test achieved to understand if the results of expression were meaningful, a significant increase was observed in the *ML237* gene after 24, 72 and 168 hours of cold stress applications compared to the control group. A meaningful increase was also seen between the treat-ment of 24 hours compared with 72 and 168 hours treatments. However, the significant increase between 72 hours and 168 hours treatments were not observed (Figure 2B).

Investigation of *R2R3 MYB* gene expression level during cold stress tolerance: Similarly, RT PCR was realized with 5 replications to determine the expression level of *R2R3 MYB* gene in four experimental groups, one of which was a control. The mean, standard deviation and standard errors of the obtained CT values were calculated (Table 4).

After Real-Time PCR, in the control and experimental groups, the all peaks of the melting curve graph of the *R2R3 MYB* gene were varied expression levels in the same time interval, and also foreign DNA contamination or primer dimer were not observed (Figure 2C). The CT values derived after 18S rRNA normalization with $2^{-\Delta CT}$ method were ra-tioned to the control group and a graph was structured. In the graph, an increase was ob-served in 72 and 168 hours of cold stress applications compared to the 24-hour cold stress application, but this increase is not significant according to the T-Test result (Figure 2D).

Investigation of *oil_143* gene expression level during cold stress tolerance: RT PCR was realized with 5 repeats to determine the *oil_143* gene expression levels in four experimental groups, one of which was a control. The mean, standard deviation and standard errors of the obtained CT values were calculated (Table 5).

Table 3. The results of T-Test with mean, standard error and standard deviation values of *ML237* gene after normalization of CT values with 18S rRNA.

Repeats	0 hr	24 hr	72 hr	168 hr
1st	100	1889,588	6311,889	4952,208
2nd	100	942,149	8078,167	5791,865
3rd	100	3134,145	10112,529	7558,353
4th	100	529,636	3059,025	2148,116
5th	100	964,646	2011,221	2599,208
ORT	0,00	1492,033	5914,566	4609,950
SDV	0,00	1044,186	3385,710	2253,378
SEM	0,00	466,9742665	1514,135701	1007,741402
T-TEST		**0,04**	**0,02**	**0,01**
T-TEST		**0,02**		
T-TEST			**0,01**	
T-TEST				**0,08**

Table 4. The T-Test results with mean, standard error and standard deviation values of *R2R3 MYB* gene after normalization of CT values with 18S rRNA.

Repeats	0 hr	24 hr	72 hr	168 hr
1st	100	39,777	137,554	397,237
2nd	100	12,414	63,288	69,737
3rd	100	11,423	67,830	19,479
4th	100	61,985	239,496	130,134
5th	100	180,250	86,454	158,008
ORT	0,00	61,170	118,924	154,919
SDV	0,00	69,804	73,561	145,724
SEM	0,00	31,21726843	32,89749644	65,16968133
T-TEST		**0,28**	**0,59**	**0,45**
T-TEST		**0,26**		
T-TEST			**0,24**	
T-TEST				**0,6**

Table 5. The results of T-Test with mean, standard error and standard deviation and values of *oil_143* gene after normalization of CT values with 18S rRNA.

Repeats	0 hr	24 hr	72 hr	168 hr
1st	100	176,5406	573,5821	736,1501
2nd	100	103,5265	482,3231	360,5002
3rd	100	50	147,4269	63,72803
4th	100	3,257706	21,46414	102,8114
5th	100	149,4849	37,11309	13,21273
ORT	0,00	96,562	252,382	255,280
SDV	0,00	70,922	258,226	300,351
SEM	0,00	31,717	115,482	134,321
T-TEST		**0,9**	**0,25**	**0,3**
T-TEST		**0,19**		
T-TEST			**0,25**	
T-TEST				**0,95**

Real Time PCR results showed that in the experimental and control groups, all the melting curve graph peaks of the *oil_143* gene were also varied expression levels in the same time interval, and also foreign DNA contamination or primer dimer were not observed too (Figure 2E). CT values derived from 18S rRNA normalization with $2^{-\Delta CT}$ method were rationed to the control group and a graph was structured. In the

graph, an increase was observed in 72 and 168 hours of cold stress applications compared to the 24-hour cold stress application, but this increase is not significant according to the T-Test result (Figure 2F).

DISCUSSION

The genetic resources of *Mentha* spp. should be noticed as an crucial species for the preservation of aromatic and medicinal plant species. The introduction and collection of the new genetic material of Mentha's is essential to acquiring a sufficient genetic pool for further work in plant breeding for the selection of superior quality genotypes. Peppermint germplasm needs to be protected clonally under field conditions or as pot plants, as many cultivars are sterile. One of the most important commercial peppermint species, *M. × piperita*, are sterile hybrids and the persistence of specific genotypes is very important to the peppermint oil industry. Although mint species have internal bacterial contamination, they can be easily cultured *in vitro* (Reed, 1999).

The expression of *18S ribosomal RNA* and *ML237*, *MYB* and *oil_143* genes, which are considered to be related in cold stress regulation, were evaluated in the transcriptional determination of some genes of the plant *Mentha x piperita*, which are considered to be effective in cold stress adaptation, before the application of cryopreservation, which is the main objective of the current thesis study. In the thesis study, the expressions of the related genes were determined quite significantly during the cold acclimation period, which is one of the pre-cryo applications.

In the current work, the cold-hardening step of *M. × piperita* plant before cryopreservation has been beneficial in obtaining very successful results on plant viability and regeneration after thawing. Cold-hardening is related with many biochemical and physiological differentiations, including the membrane protein content alterations, the sugar content increases, the protein composition and the plant hormone concentrations variations, and the expression of associated genes changes (Crowe *et al*., 1990; Guy, 1990; Lang *et al*., 1994; Thomashow, 1998). In the current work, as a comparing the expression results of cold stress associated genes in *M. × piperita* plant with the control group (before cold preculture) and the cold hardening periods at +4 °C for 24, 72 and 168 hours of *ML237* and *MYB* gene expressions increased after treatment with 72 hours, and decreased with 168 hours of cold hardening (Figure 2).

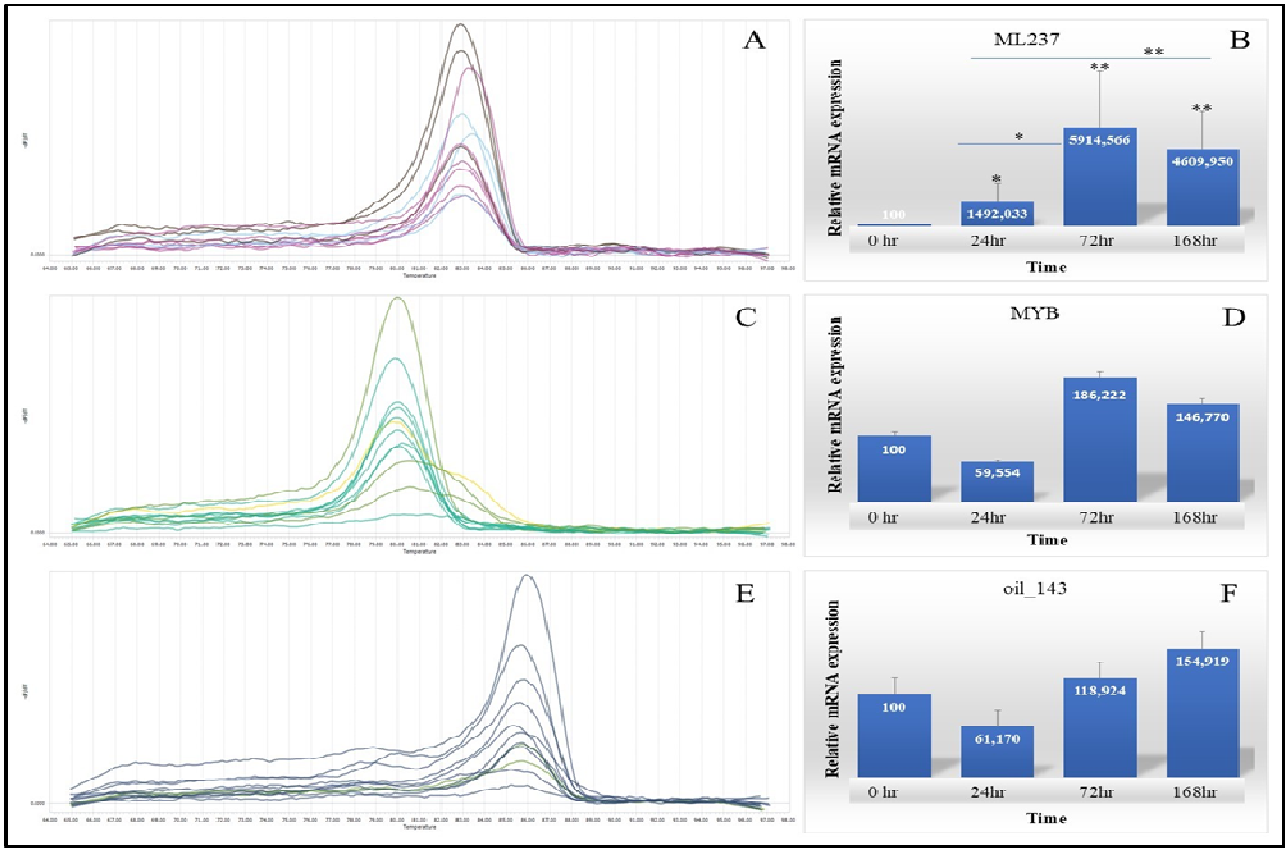


Figure 2. The melting curve graph of *ML237* (A); *MYB* (C); *oil_143* (E), and relative mRNA expression graphic of *ML237* (B); *MYB* (D); *oil_143* (F) genes (*, $p \leq 0,05$; **, $p \leq 0,01$; ***, $p \leq 0,001$).

The *ML237* gene in *M.* × *piperita*, a homolog of *Hsp70* in *A. thaliana*, defends cells and their metabolisms against oxidative stresses and heat shocks. This gene acts a very crucial role in the functions of supporting proteins to refold and preventing protein aggregation in inappropriate environmental conditions such as high and low temperatures, chemical agents (Usman *et al*., 2017). The *R2R3 MYB* gene, the *MYB 15* (*A. thaliana*) gene homologue in *Mentha* spp., is an transcription factor in upstream position that negatively controls the expression of C-rebinding factors (CBFs) (Agarwal *et al*., 2006). Cold stress negatively influences plant re-generation, development and growth. The most moderate species gain frost adaptation through a treatment called cold-hardening. Transcriptional control is interceded by the stimulater of the CBF transcriptional cascade, the C-rebinding factor (CBF) expression 1 (ICE1), and the CBF-independent regulation during cold acclimatization (Chinnusamy *et al*., 2007). In our study, the increase in these three genes in the first 72 hours of cold acclimation period before cryopreservation can be considered as an indicator of the response to applied cold stress conceivable.

In the current study, the *oil_143* gene corresponding to the *AtXH5* (*A. thaliana*) gene in *M.* × *piperita* plant increased after 168 hours of incubation in the cold adaptation pe-riod before liquid nitrogen application (Figure 2). Overexpression of *AtHAP5A* in *A. thaliana* was found to be highly effective in tolerance to freezing stress. *AtHAP5A* acts up-stream of *AtXTH21* in the freeze stress response in Arabidopsis and modulates freeze stress resistance through *AtHAP5A*'s interaction with the CCAAT motif of *AtXTH21* in *Arabidopsis* (Shi *et al*., 2014). In this context, when the control group compared to in the increasing periods of the cold-hardening period, in our study may suggest that the overexpression of the *oil_143* gene is effective in tolerance to freezing stress.

Consequently, cold stress effects almost all properties of cell metabolism, and therefore this is not surprising that the cold stress responses in plants are more related into cell metabolism at molecular levels (Galatali and Kaya, 2022). Using genetic analysis tools, newly technical devel-opments in gene expression, proteomics, transcriptomics, profiling of the small RNA and metabolomics have made it potential to study the complicated processes involved in cold stress responses. In plant species, cold stress controls the transcriptome via transcriptional, post-transcriptional, and post-translational mechanisms. Molecular studies on the cold stress tolerance in medicinal-aromatic plants such as *M.* × *piperita* will be crucial to de-termine key regulators of frost adaptaion in studies of long-term storage of similar plants by cryopreservation methods.

Conclusion: Within the scope of the current study, firstly, the genes expression levels considered to be induced by cold in plant tissues that cold-hardened before cryopreservation were examined by Real-Time PCR application. According to the T-Test result after the statistical evaluation of the results, a significant increases was observed the related genes. Since via-bility and successful regeneration of plant tissues after cryopreservation is thought to be associated with cold-hardening, plants were exposed to cold stress for 0 (control group) and 24, 72, 168 hours. The results obtained from the experiment were examined at the transcriptional level of mRNA expression levels. In the future, it is planned to examine the protein levels of these genes, which are expressed at the translational level.

Competing Interests: The authors declare that they have no competing interests.

Funding: Authors declare that they didn't receive any type of funds from any organization.

REFERENCES

Agarwal, M., Y. Hao, A. Kapoor, C.H. Dong, H. Fujii, X. Zheng and J.K Zhu (2006). A R2R3 type MYB transcription factor is ınvolved in the cold regulation of CBF genes and in acquired freezing tolerance. Int. J. Biol Chem. 281: 37636–37645. https://doi.org/10.1074/jbc.M605895200

Ahmadi, H., M.R. Morshedloo, R. Emrahi, A. Javanmard, F. Rasouli, F. Maggi, M. Kumafr and J.M. Lorenzo (2022). Introducing three new fruit-scented mints to farmlands: Insights on drug yield, essential-oil quality, and antioxidant proper-ties. Antioxidants. 11(5): 866. https://doi.org/10.3390/antiox11050866

Aksoy, H.M., S.F. Arslanoglu, M.F. Edbeib, Y. Kaya and S. Marakli (2021). Antibacterial activity of *Calendula officinalis* and *Echinacea purpurea* extracts against the causal agent of tomatoes' bacterial canker: *Clavibacter michiganensis* subsp. *michiganensis*. Bol. Latinoamericano Y Del Caribe De Plantas Med. Y Aromaticas 20(5): 496-502. 10.37360/blacpma.21.20.5.36

Anwar, F., A. Abbas, T. Mehmood, A.H. Gilani and N.U. Rehman (2019). *Mentha*: A genus rich in vital nutra-pharmaceuticals. Phytother Res. 33(10): 2548-2570. https://doi.org/10.1002/ptr.6423

Benson, E.E. (2008). Cryopreservation of phytodiversity: A critical appraisal of theory and practice. Crit Rev Plant Sci. 27(3): 141-219. https://doi.org/10.1080/07352680802202034

Chang, Y.J., R.E. Baker and B.M. Reed (2000). Cold acclimination improves recovery of

cryopreserved grass (*Zoysia* and *Lolium* sp.). Cryo-Lett. 11(5): 413-426.

Chew, Y.H. and K.J. Halliday (2011). A stress-free walk from arabidopsis to crops. Curr Opin. 22(2): 281-286. https://doi.org/10.1016/j.copbio.2010.11.011

Chinnusamy, V., J. Zhu and J.K. Zhu (2007). Cold stress regulation of gene expression in plants. Trends Plant Sci. 12(10): 0–451. https://doi.org/10.1016/j.tplants.2007.07.002

Crowe, J.H., J.F. Carpenter, L.M. Crowe and T.J. Anchordoguy (1990). Are freezing and dehydration similar stress vectors? A comparison of modes of interaction of stabilizing solutes with biomolecules. Cryobiology 27: 219–231. https://doi.org/10.1016/0011-2240(90)90023-W

Dai, X., Y. Xu, Q. Ma, W. Xu, T. Wang, Y. Xue and K. Chong. (2007). Overexpression of an *R1R2R3 MYB* gene, *OsMYB3R-2*, increases tolerance to freezing, drought, and salt stress in transgenic arabidopsis. Plant Physiol. 143(4): 1739-1751. https://doi.org/10.1104/pp.106.094532

Galatali, S. and E. Kaya (2022). Investigation of the cold stress effect on mac_4 (HSP80-Like) gene at transcriptional level for *Mentha* × *piperita* L. Mod Concep Dev Agrono. 10(5): 1057-1059. DOI: 10.31031/MCDA.2022.10.000746

Guldag, S., D.E. Ozkaya and E. Kaya (2023). Determination of genetic variations of post-micropropagated sweet orange (*Citrus Sinensis* (L.) Osbeck) micro-shoots by ISSR marker technique. CIACR. 10(3): 1393-1398. DOI: 10.32474/CIACR.2023.10.000336

Guy, C.L. (1990). Cold acclimation and freezing stress tolerance: Role of protein metabolism. Annu. Rev. Plant Biol. 41: 187–233. DOI: 10.1146/annurev.pp.41.060190.001155

Harding, K., J.W. Johnston and E. Benson (2009). Exploring the physiological basis of cryopreservation success and failure in clonally propagated *in vitro* crop plant germplasm. Agric Food Sci. 18(2): 103–116. DOI: https://doi.org/10.2137/145960609789267524

Hasan, H. and F. Abd Manan (2020). Total phenolic and flavonoid content of *Elaeis guineensis* leaves treated with different amount of nitrogen-potassium fertilizer. Int J. Life Sci. Biotechnol. 3(2): 156–163. https://doi.org/10.38001/ijlsb.699527

Jafari-Sales, A. and M. Pashazadeh (2020). Study of chemical composition and antimicrobial properties of Rosemary (*Rosmarinus officinalis*) essential oil on *Staphylococcus aureus* and *Escherichia coli in vitro*. Int J. Life Sci. Biotechnol. 3(1): 62–69. https://doi.org/10.38001/ijlsb.693371

Kami, D. (2012). Cryopreservation of plant genetic resources. In: Katgor, I. (Ed.), Current Frontiers in Cryobiology, 440-456. IntechOpen. DOI: 10.5772/34414

Kaya, E., M.A. Balci, O. Akguller, S. Galatali, S. Yeniocak, T. Mercan, S. Guldag, D.E. Ozkaya, B. Ozturk, O. Celik and I. Aktay. (2021). Development of an optimum proliferation medium via graph kernel statistical analysis method for genetically stable *in vitro* propagation of endemic *Thymus cilicicus* (Turkey). Acta Bot. Croat. 80(2): 199–207. https://doi.org/10.37427/botcro-2021-024

Kaya, E., F. Souza, E. Yilmaz-Gokdogan, M. Ceylan and M. Jenderek (2017). Cryopreservation of citrus seed via dehydration followedby immersion in liquid nitrogen. Turk. J. Biol. 41(1): 242-248. DOI: 10.3906/biy-1603-92

Kaya, E., F.V.D. Souza, J.A. dos Santos-Serejo and S. Galatali (2020). Influence of dehydration on cryopreservation of *Musa* spp. germplasm. Acta Bot. Croat. 79 (2): 99–104. https://doi.org/10.37427/botcro-2020-024

Khanam, D. and F. Mohammad (2016). Effect of structurally different plant growth regulators (PGRs) on the concentration, yield, and constituents of peppermint essential oil. J. Herbs Spices Med. Plants. 23(1): 1-10. https://doi.org/10.1080/10496475.2016.1254700

Kim, H.H., Y.G. Lee, D.J. Shin, H.C. Ko, J.G. Gwag, E.G. Cho and F. Engelmann (2009). Development of alternative plant vitrifcation solutions in droplet-vitrifcation procedures. Cryo-Lett. 30: 320–334.

Kizil, S., N. Hasimi, V. Tolan, E. Kilinc and U. Yuksel (2010). Mineral content, essential oil components and biological activity of two *Mentha* species (*M. piperita* L., *M. spicata* L.). Turkish J. Field Crop. 15(2): 148-153.

Lambardi, M. and A. de Carlo (2003). Application of tissue culture to the germplasm conservation of temperate broad-leaf trees. In Jain, S.M. and K. Ishii (Eds.), Micropropagation of Woody Trees and Fruits, Kluwer Academic Publishers, Springer, Dordrecht. 815-840. https://doi.org/10.1007/978-94-010-0125-0_28

Lang, V., E. Mantyla, B. Welin, B. Sundberg and E.T. Andpalva (1994). Alterations in water status, endogenous abscisic acid content, and expression of rab18 gene during the development of freezing tolerance in *Arabidopsis thaliana*. Plant Physiol. 104: 1341–1349. https://doi.org/10.1104/pp.104.4.1341

Li, Y., Y. Liu, A. Ma, Y. Bao, M. Wang and Z.L. Sun (2017). *In vitro* antiviral, anti-inflammatory, and antioxidant activities of the ethanol extract of *Mentha piperita* L. Food Sci. Biotechnol. 26: 1675–1683. https://doi.org/10.1007/s10068-017-0217-9

Lv, Y., M. Yang, D. Hu, Z. Yang, S. Ma, X. Li and L. Xiong (2017). The OsMYB30 transcription factor suppresses cold tolerance by interacting with a JAZ protein and suppressing β-amylase expression. Plant Physiol. 173 (2): 1475-1491. https://doi.org/10.1104/pp.16.01725

Ma, Q., X. Dai, Y. Xu, J. Guo, Y. Liu, N. Chen and X. Zhang. (2009). Enhanced tolerance to chilling stress in OsMYB3R-2 transgenic rice is mediated by alteration in cell cycle and ectopic expression of stress genes. Plant Physiol. 150 (1): 244-256. https://doi.org/10.1104/pp.108.133454

Maślanka, M. and A. Szewczyk (2021). Droplet-vitrification cryopreservation of *Tulipa tarda* Stapf. apical meristems. Plant Cell, Tissue Organ Cult. 144(1): 91-95.
https://doi.org/10.1007/s11240-020-01910-6

Mehrotra, S., S. Verma, S. Kumar, S. Kumari and B.N.T. Mishra (2020). Transcriptional regulation and signalling of cold stress response in plants: An overview of current understanding. Environ Exp Bot. 104243. https://doi.org/10.1016/j.envexpbot.2020.104243

Murashige, T. and F. Skoog (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant 15: 473- 497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

Nor Kamal, N.H. and F. Abd Manan (2020). Photosynthetic-related properties of oil palm leaves treated with different amount of fertilizer. Int J. Life Sci. Biotechnol. 3(1): 70–80. https://doi.org/10.38001/ijlsb.697738

Ozkaya, D.E., F.V.D. Souza and E. Kaya (2022). Evaluation of critical points for effective cryopreservation of four different *Citrus* spp. germplasm. Hortic. 8(11): 995. https://doi.org/10.38001/ijlsb.697738

Ozudogru, A., D.P.C. da Silva, E. Kaya, G. Dradi, R. Paiva and M. Lambardi (2013). *In vitro* conservation and cryopreservation of *Nandina domestica* an outdoor ornamental shrub. Not. Bot. Horti Agrobot. Cluj-Napoca 41(2): 638-645. DOI: https://doi.org/10.15835/nbha4129335

Ozudogru, E.A., E. Kaya, E. Kirdok and S. Issever-Ozturk (2011). *In vitro* propagation from young and mature explants of thyme (*Thymus vulgaris* and *T. longicaulis*) resulting in genetically stable shoots. In Vitro Cell. Dev. Biol. – Plant, 47(2): 309-320. https://doi.org/10.1007/s11627-011-9347-6

Ozudogru, E.A., E. Kirdok, E. Kaya, M. Capuana and C. Benelli (2012). *In vitro* conservation of redwood (*Sequoia sempervirens*) by slow growth storage and cryopreservation. Acta Hortic. 961: 291-296. DOI: 10.17660/ActaHortic.2012.961.38

Ozudogru, E.A., E. Kirdok, E. Kaya, M. Capuana, C. Benelli and F. Engelmann (2011). Cryopreservation of redwood (*Sequoia sempervirens* (D. Don.) Endl.) *in vitro* buds using vitrification-based techniques. CryoLett. 32(2): 99- 110.

Rasooli, I., S. Shayegh, M. Taghizadeh and S.D.A. Astaneh (2008). Phytotherapeutic prevention of dental biofilm formation. Phytother Res. 22(9): 1162-1167. https://doi.org/10.1002/ptr.2387

Reed, B.M. (1988). Cold acclimation as a method to improve survival of cryopreserved rubus meristems. CryoLett. 9: 166–171.

Reed, B.M. (1999). *In vitro* storage conditions for mint germplasm. HortScience 34(2): 350-352.

Rihan, H.Z., M. Al-Issawi and M.P. Fuller (2017). Advances in physiological and molecular aspects of plant cold tolerance. J. Plant Interact. 12(1): 143-157. https://doi.org/10.1080/17429145.2017.1308568

Rosa, M., C. Prado, G. Podazza, R. Interdonato, J.A. Gonzalez, M. Hilal and F.E. Prado (2009). Soluble sugars–metabolism, sensing and abiotic stress. Plant Signal Behav. 4(5): 388–393. https://doi.org/10.4161/psb.4.5.8294

Saharkhiz, M.J., M. Motamedi, K. Zomorodian, K. Pakshir, R. Miri and K. Hemyari (2012). Chemical composition, antifungal and antibiofilm activities of the essential oil of *Mentha piperita* L. Int Sch Res Notices 2012: 1-6. https://doi.org/10.5402/2012/718645

Sakai, A. and Y. Nishiyama (1978). Cryopreservation of winter vegetative buds of hardy fruit trees in liquid nitrogen. HortScience 13: 225–227.

Sandasi, M., C. Leonard and A. Viljoen (2010). The *in vitro* antibiofilm activity of selected culinary herbs and medicinal plants against *Listeria monocytogenes*. Lett Appl Microbiol. 50(1): 30-35. https://doi.org/10.1111/j.1472-765X.2009.02747.x

Senula, A., E.R. Keller, T. Sanduijav and T. Yohannes. (2007). Cryopreservation of cold-acclimated mint (*Mentha* spp.) shoot tips using a simple vitrification protocol. Cryo-Lett. 28(1): 1-12.

Shi, H., T. Ye, B. Zhong, X. Liu, R. Jin and Z. Chan (2014). AtHAP5A modulates freezing stress resistance in arabidopsis through binding to

CCAAT motif of AtXTH21. New Phytol. 203: 554–567. https://doi.org/10.1111/nph.12812

Sokovic, M., P.D. Marin, D. Brkic and L.J. van Griensven (2008). Chemical composition and antibacterial activity of essential oils against human pathogenic bacteria. Food 1(2): 220-226. https://doi.org/10.1111/nph.12812

Souza, F.V.D., E. Kaya, L. de Jesus Vieira, A. da Silva Souza, M.D.J. da Silva Carvalho, E.B. Santos, A.A.C. Alves, and D. El-lis (2017). Cryopreservation of Hamilin sweet orange [(*Citrus sinensis* (L.) Osbeck)] embryogenic calli using a modified aluminum cryo-plate technique. Sci. Hortic. 224: 302–305. https://doi.org/10.1016/j.scienta.2017.06.042

Souza, F.V.D., E.H. de Souza, E. Kaya, L. de Jesus Vieira and R.L. da Silva (2018). Cryopreservation of pineapple shoot tips by the droplet vitrification technique. Methods Mol. Biol. 815: 269-277. https://doi.org/10.1007/978-1-4939-8594-4_18

Su, L.T., J.V. Li, D.Q. Liu, Y. Zhai, H.J. Zhang, X.W. Li, Q.L. Zhang, Y. Wang and Q.Y. Wang (2014). A novel MYB transcription factor, GmMYBJ1, from soybean confers drought and cold tolerance in *Arabidopsis thaliana*. Gene 538(1): 46-55. https://doi.org/10.1016/j.gene.2014.01.024

Telci, I., O. Kacar, E. Bayram, O. Arabaci, I. Demirtas, G. Yilmaz and E. Goksu (2011). The effect of ecological conditions on yield and quality traits of selected peppermint (*Mentha piperita* L.) clones. Ind Crop Prod 34(1): 1193-1197. https://doi.org/10.1016/j.indcrop.2011.04.010

Thomashow, M.F. (1998). Role of cold-responsive genes in plant freezing tolerance. Plant Physiol. 118: 1–7. https://doi.org/10.1104/pp.118.1.1

Towill, L.E. (2002). Cryopreservation of *Mentha* (mint). In: Towill, L.E. and Y.P.S. Bajaj (eds.), Cryopreservation of Plant Germplasm II. Biotechnology in Agricul-ture and Forestry, Springer, Heidelberg, Berlin. 151–163. https://doi.org/10.1007/978-3-662-04674-6_11

Tyagi, A.K. and A. Malik (2010). Liquid and vapour-phase antifungal activities of selected essential oils against *Candida albicans*: Microscopic observations and chemical characterization of cymbopogon citratus. BMC Complement Altern Med. 10(1): 1-11. https://doi.org/10.1186/1472-6882-10-65

Usman, M.G., M.Y. Rafii, M.Y. Martini, O.A. Yusuff, M.R. Ismail and G. Miah (2017). Molecular analysis of Hsp70 mechanisms in plants and their function in response to stress. Biotechnol. Genet. Eng. Rev. 33(1): 26-39. https://doi.org/10.1080/02648725.2017.1340546

Vazquez-Hernandez, M., I. Romero, M.I. Escribano, C. Merodio and M.T. Sanchez-Ballesta. (2017). Deciphering the role of CBF/DREB transcription factors and dehydrins in maintaining the quality of table grapes cv. autumn royal treated with high CO_2 levels and stored at 0 ˚C. Front Plant Sci. 8: 1591. https://doi.org/10.3389/fpls.2017.01591