J. BIOL. ENVIRON. SCI.,
2008, 2(6), 95-100

Peroxidase Activity and Lipid Peroxidation in Strawberry (*Fragaria* X *ananassa*) Plants Under Low Temperature

Hatice Gülen*, Cem Çetinkaya, Murat Kadıoğlu, Müge Kesici, Asuman Cansev and Atilla Eriş
Uludağ University, Faculty of Horticulture, Horticulture Department, Görükle 16059, Bursa

ABSTRACT

The activity of peroxidase (PRX) isozyme, lipid peroxidation (Malondialdehyde, MDA content) and cell membrane injury were studied during low temperature treatment for different periods in strawberry (*Fragaria x ananassa* cv. Camarosa) leaf tissues. Seedlings were grown for six weeks (plants had 4-5 leaves) in a greenhouse then the plants were transferred to a climate chamber with constant 5°C, 60% relative humidity, 14/10 h (light/dark) photoperiod regime and 4 LS light intensity for 1, 4, 7 or 10 days to impose a low temperature stress. In general, low temperature application during 10 days caused a linear increase in MDA content. Native polyacrylamide gel electrophoresis (PAGE) of both acidic and basic peroxidase (PRX) isozymes yielded a single sharp protein band with Rf=0.23 and Rf=0.17, respectively. In addition data indicated a strong relationship between band intensities and the duration of the low temperature treatment. However, the considerable increase of PRX activities could not stop the deleterious effects of low temperature, but reduced severity of stress, thus showing a reduction in the percentage of injury on the 7th day which is correlated with cold-acclimation of strawberry leaf tissues under low temperature.

Key Words: Cell membrane injury, lipid peroxidation, low temperature, peroxidase, strawberry (*Fragaria x ananassa*).

INTRODUCTION

Many temperate fruit crops are in the *Rosaceae* and their disturbution is restiricted by low temperature stress. However, commercially floral damage is often the main form of freeze damage, particularly in several of the early blooming members of the *Rosaceae* such as strawberry, cherry, peach (Rodrigo 2000, Owens et al 2003). One of the biochemical changes occurring when plants are subjected to low temperature stress is the production of reactive oxygen species (ROS). ROS are highly reactive and in the absence of any protective mechanism they can disrupt normal metabolism through oxidative damage to lipids, protein and nucleic acids (Allen 1995). Antioxidative enzymes like superoxide dismutase (SOD), catalase (CAT) and peroxidase (PRX) are the most important components in the scavenging system of ROS. Peroxidases are a family of isozymes found in all plants; they are heme-containing monomeric glycoproteins that utilize either H_2O_2 or O_2 to oxidaze a wide variety of molecules (Yoshida et al 2002). So peroxidases are known one of the important parts of the enzymatic defense of plant cells under stress conditions (Gaspar et al1982).

Plant species differ greatly in their ability to develop cold-tolerance through a process known as cold-acclimation. Biochemical changes that have been associated with cold-acclimation include alterations in lipid composition, increased sugar and soluble protein content, expression of spesific proteins, the appearence of new isozymes and so on (Thomashow 1999; Sarnighausen et al 2004; Cansev et al 2005; Eris et al 2007). In this respect, enhancement of cold-tolerance of species would be of considerable interest for preventing cold damage. Therefore figuring out the mechanisim of cold-acclimation of species is of a great importance even on the cultivar basis.

In this study, changes in the qualitative activities of PRX were analyzed in strawberry cv. Camarosa plants during low temperature treatments. The experiment was also conducted to examine the effect of low temperature stress on leaf injury and lipid peroxidation (malondialdehyde=MDA content) in cellular level. The main purpose of the study was to provide preliminary information for later, more detailed, studies on strawberry cold-acclimation and freezing tolerance.

MATERIALS AND METHODS

Plant material and low temperature treatments

Cold stored (frigo) seedlings of strawberry (*Fragaria* x *ananassa* cv. Camarosa) were planted in 12 cm pots using perlite:torf:soil (1:1:1) mixture. Plants were grown for six weeks (plants had 4-5 leaves) in a greenhouse with day/night mean temperature of 25/10°C day/night temperature, average relative humidity of 70%, average photoperiod of 14 h and watered on need basis to avoid any water stress by Actagro (7-7-7) (Actagro LLC, Biola, CA, USA) nutrient solution. The plants were transferred to a climate chamber

* Corresponding author: hsgulen@uludag.edu.tr

(SANYO MLR-351H, IL, USA) with constant 5^oC, 60% relative humidity, 14/10 h (light/dark) photoperiod regime and 4 LS light intensity for 1, 4, 7 or 10 days to impose a low temperature stress. Plants were kept watered by the nutrient solution during the low temperature treatments in the growth chamber. Also, control plants were kept in the greenhouse during the treatments.

Measurement of injury
Membrane thermostability was measured by using the procedure of Arora et al (1998), with some modifications of Gulen and Eris (2003) for strawberry leaf tissue. Leaf discs in 2 cm diameter were cut from fully expanded, uniform leaves from each of three plants (replicates) per treatment [unstressed- control, low temperature (5^oC) for 1, 4, 7 or 10 days]. Discs were lightly rinsed in distilled water, gently blotted with paper, and placed in test tubes (one disc per tube). Then, 20 ml of distilled water was added to each test tube. Samples were then vacuum infiltrated to allow uniform diffusion of electrolytes and incubated for 4 h at room temperature. After incubation, electrical conductivity of each solution was measured using a conductivity meter (WTW Cond 315i, Weilheim, Germany). Leaf discs were then killed in the same solution by autoclaving, and total conductivity was measured at room temperature. Percentage injury of each treatment was calculated from ion leakage data using the equation: $\%injury=[(\% L_{(t)}-\% L_{(c)}) / (100-\% L_{(c)}] \times 100$ (Arora et al 1992), where $\% L_{(t)}$ and $\% L_{(c)}$ are percentage ion leakage data for the treatments or control samples, respectively.

Lipid peroxidation (malondialdehyde=MDA content)
Lipid peroxidation was estimated by determining the malondialdehyde (MDA) contents in the leaves according to method of Rajinder et al (1981). A hundred milligram of leaf samples was homogenized in 5 ml of 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged at 10 000 × *g* for 5 min at 4 °C. Aliquot of 0.3 ml supernatant was mixed with 1.2 ml of 0.5% thiobarbituric acid (TBA) prepared in TCA 20%, and incubated at 95 °C for 30 min. After stopping the reaction in an ice bath for 5 min, samples were centrifuged at 10 000 × *g* for 10 min at 25°C. The supernatant absorbance at 532 nm was then measured using a Beckman UV-DU 520 spectrophotometer (Beckman Coulter, Inc., Fullerton, CA, USA). After subtracting the non-specific absorbance at 600 nm, MDA concentration was determined using the extinction coefficient 155 mM^{-1} cm^{-1}.

PRX extraction and Native polyacrylamide gel electrophoresis (PAGE) of acidic and basic isoperoxidases
Fully expanded leaf material was collected from each plant group of each treatment. Triplicate samples of leaf tissues were right away frozen and ground in liquid N_2 and stored at -80^oC until used. PRX was extracted from leaf tissues using the extraction methods described by Gulen et al (2002). Ground leaf tissues (0.1 g) were homogenized at 4^oC in 0.6 ml extraction buffer [0.1 M potassium phosphate pH 7.5, 30 mM boric acid, 50 mM L-ascorbic acid, 17 mM sodium metabisulfite, 16 mM dithiocarbamic acid, 1 mM EDTA and 4% (w/v) PVP-40 and final pH was readjusted to 7.5 with NaOH]. Homogenates were centrifuged at 15 000 rpm for 20 min and supernatant was used for electrophoresis.

Discontinuous PAGE was performed with a PROTEAN III vertical electrophoresis unit (Bio-Rad, Hercules, Calif.) for acidic and basic PRX, respectively, according to Davis (1964) and Reisfeld et al (1962). Five percent stacking gels and 10% separating gels were prepared for both systems. For each sample 20 µl of crude extract was loaded to the gel. Electrophoresis was performed at 20 mA for 30 min, followed by 40 mA for 3h. Gels were stained for PRX using the method of Wendel and Weeden (1989). The relative distance (Rf value) of the bands on the gel was calculated as described by Manganaris and Alston (1992) using Rf=1.0, distance to the fastest band and Rf=0.0, the starting point.

Statistics
The experiment was arranged in a randomized block design with three replications. Data were tested by SPSS 13.0 for Windows program.

RESULTS

The percentage of injury (based on electrolyte leakage) in leaf discs as a function of low temperature for different duration was shown in Figure 1. The percentage injury increased until the 4th day of the low temperature treatment, followed by a decrease on the 7th day. Then the injury sharply increases over 50% level on the 10th day of the low temperature treatment.

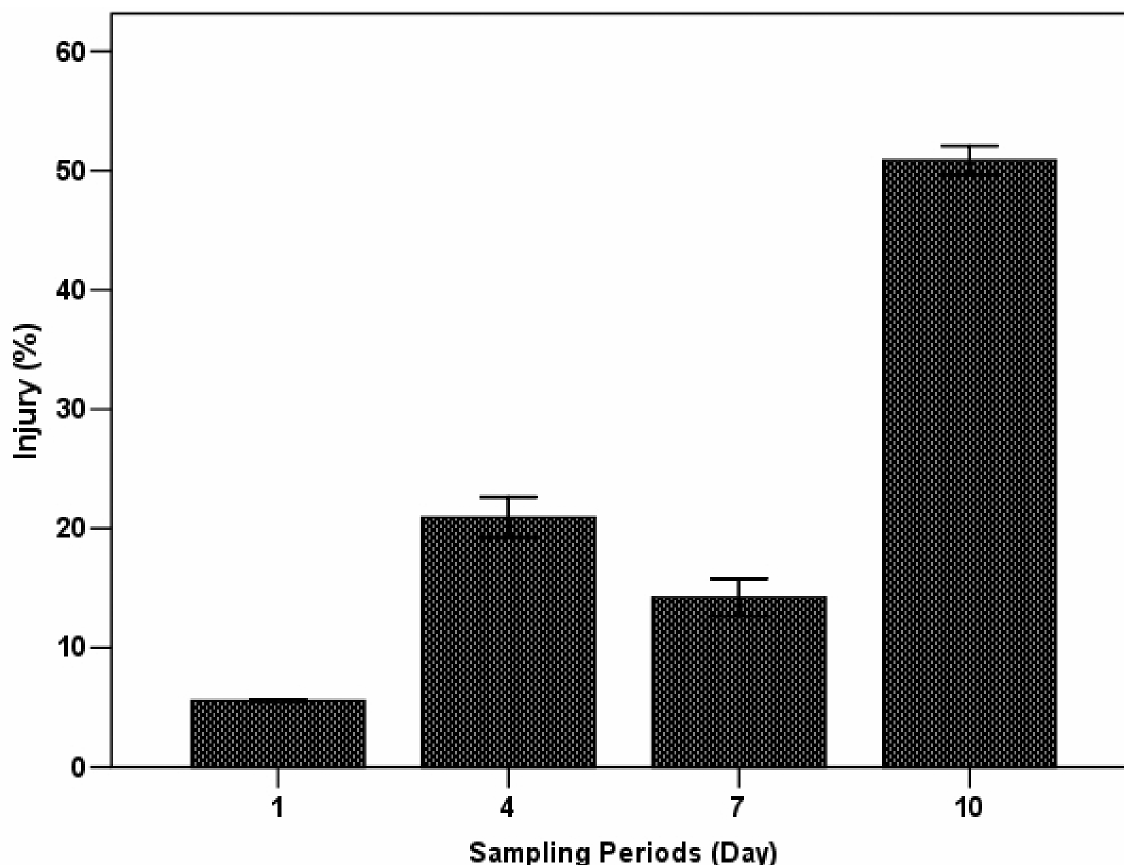

Figure 1. The percentage of injury (based on electrolyte leakage) in the leaf discs of strawberry cv. Camarosa plants under low temperature (5°C) treatment for the periods of 1, 4, 7 or 10 days, and unstressed (control) plants. Error bars represent ± SE of three replications.

The time courses of MDA content in the leaf tissues subjected to low temperature were given in Figure 2. In general, low temperature application for 10 days caused a linear increase in MDA content. Even though there was no statistical difference between the 1st and the 4th day of the low temperature treatment, MDA content increased linearly and reached to the highest level (~115 nmol g FW^{-1}) on the 10th day of low temperature in comparison with control plants (~70 nmol g FW^{-1}).

Native PAGE analysis of the samples was repeated at least three times with similar results. Data from a single, representative analysis are presented herein. Native PAGE of PRX was performed to obtain acidic and basic isozyme profiles of leaf tissues subjected to the low temperature for various durations. Native PAGE of both acidic and basic isozymes yielded a single sharp protein band with Rf=0.23 and Rf=0.17, respectively (Figure 3). Although both acidic and basic bands were faint and barely observed in control (unstressed) sample, the bands were observed commonly with different band intensities in all over the low temperature treatment. The intensities of the bands significantly increased depending on the low temperature durations and reached to highest levels on the 10th day in both acidic and basic PRX.

DISCUSSION

Cell membrane stability has been widely used to express stress tolerance, and higher membrane stability could be correlated with abiotic stress tolerance (Premachandra et al 1992). Uemura et al (2006) recently indicated the necessity of an increase in membrane stability during cold-acclimation both under natural and artificial conditions. Moreover, it was stated that there are compositional, structural and functional changes occurring in the plasma membrane, which are increased stability of the plasma membrane under cold conditions. It is known that many of the changes during acclimation to temperature stress are reversible, but if the stress is too great, irreversible changes can occur and these can lead to death (Lester 1985). Indeed,

Shilpi and Narendra (2005) suggested that the symptoms of stress induced injury in plants appear from 48 to 72 h later; however, this duration varies from plant to plant and also depends upon the sensitivity of individual plant to cold-stress. In this respect, percentage injury increased depending on the duration of low temperature treatment with an exception in present study (Figure 1). The decrease in injury on the 7th day of the low temperature treatment was correlated to cold-acclimation of the leaf tissues. Longer period of low temperature treatment which is 10 days had more deleterious effect on the cell membrane, since the injury was over 50% on the 10th day.

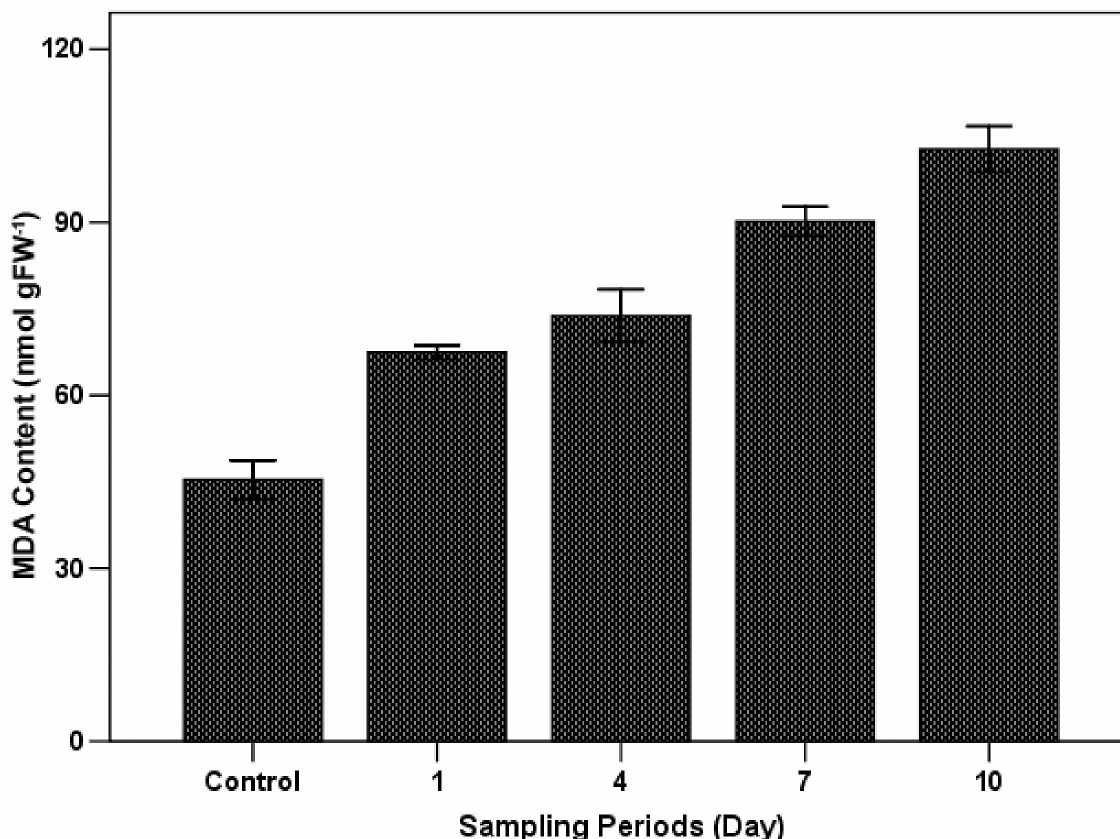

Figure 2. Lipid peroxidation (malondialdehyde=MDA content) in the leaf tissues of strawberry cv. Camarosa plants under low temperature (5°C) treatment for the periods of 1, 4, 7 or 10 days, and unstressed (control) plants. Error bars represent ± SE of three replications. FW indicates fresh weight.

The peroxidation reactions differ among the fatty acids depending on the number and position of the double bounds on the acyl chain. Oxidation of unsaturated fatty acids by singlet oxygen produces distinctly different products such as MDA (Bradley and Min 1992). MDA is a common product of lipid peroxidation and a sensitive diagnostic index of oxidative injury (Janero 1990). In this respect increase in lipid peroxidation was reported in many plants under various environmental stresses (Moran et al 1994; Prassad 1996). In the present study, time course of MDA content as an expression of lipid peroxidation was increased by low temperature treatment (Figure 2). These data also indicated to severity of cell membrane injury.

Peroxidases are a large group of isoenzymes with an extreme range of isoelectric points, serving a multitude of functions (van Huystee 1987). Each group is thought to have a different function in the cell. Acidic peroxidases are the isoenzymes most likely involved in lignin formation and wall-associated, whereas function of basic isoenzymes has been suggested that they might provide H_2O_2 for other peroxidases (Walter 1992). Regarding the temperature stress, one basic isoperoxidase band (Rf=0.22) was correlated with lignification and recovery of cell membrane damage under heat stress in strawberry leaf (Gulen and Eris 2004). Recently, Cansev et al (2005) reported expression of acidic peroxidase bands with different band intensities which are responsible for tolerance to freezing stress of 15 olive cultivars. In the present study, data from native PAGE indicated one acidic and one basic isoperoxidases with different intensities (Figure

3). Since data indicated a linear relationship between band intensities and the duration of the low temperature treatment, it is correlated to cold-acclimation of strawberry leaf tissues.

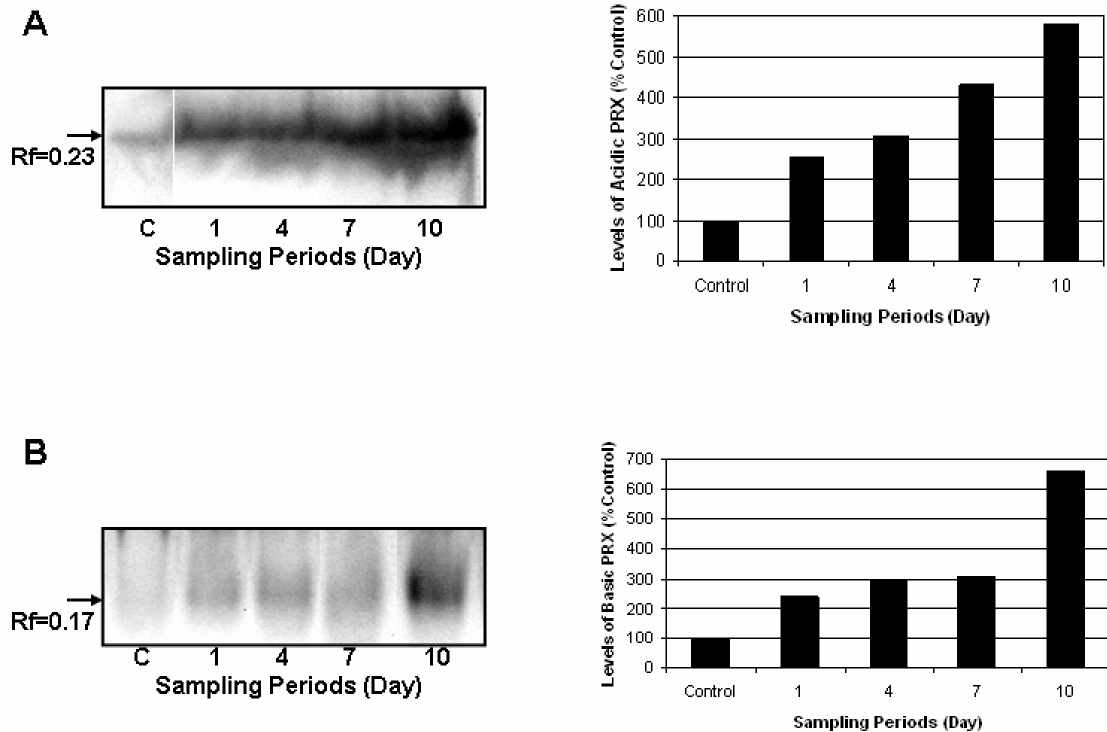

Figure 3. Native PAGE of acidic (A) and basic (B) PRX activities of the leaf tissues of strawberry cv. Camarosa plants under low temperature (5°C) treatment for the periods of 1, 4, 7 or 10 days, and unstressed (control) plants and their band intensities based on the quantitative measurements. Equal volumes of the crude extracts, 20 µl, were loaded in each lane. Arrows to the left mark indicate the Rf=23 (A) and Rf=17 (B) isoperoxidases.

In conclusion, our data indicated a correlation between PRX activities and cellular damage provoked by low temperature treatment. The considerable increase of PRX activities could not stop the deleterious effects of low temperature, but reduced stress severity thus showing a reduction in the percentage of injury on the 7th day followed by a sharp increase in the 10th day. This fluctuation in the percentage of injury was correlated with cold-acclimation of the plants. In addition MDA content significantly increased under low temperature, which is another indication of cellular damage. One acidic (Rf=0.23) and one basic (Rf=0.17) PRX bands was observed commonly in all samples with different band intensities, and, therefore, might be associated with cell membrane recovery and cold-acclimation in strawberry cv. Camarosa plants under low temperature stress.

ACKNOWLEDGEMENTS

The authors thank to Yaltır A.Ş. (Adana, Turkey) for supplying the strawberry seedlings and to Hekimoğlu Sözmen Ltd. Şti. (Mersin) for providing Actagro nutrient solution used in the study.

REFERENCES

Allen R (1995). Dissection of oxidative stress tolerance using transgenic plants. Plant Physiol 107:1049-1054.

Arora R, Wisniewski M, and Scorza R (1992). Cold acclimation in genetically related (sibling) deciduous and evergreen peach (*Prunus persica* [l.] Batsch) I: seasonal changes in cold hardiness and polypeptides of bark and xylem tissues. Plant Physiol 99: 1562-1568.

Arora R, Pitchay DS, and Bearce BC (1998). Water-stress induced heat tolerance in geranium leaf tissues: A possible linkage through stress proteins? Physiol Plant 103: 24-34.

Bradley DE, and Min DB (1992). Singlet oxygen oxidation of foods. Cat Rev Food Sci Nutr 31: 211-236.

Cansev A, Köksal N, Gülen H, İpek A, and Eriş A (2005). Düşük Sıcaklık Stresi Altındaki Bazı Zeytin Çeşitlerinin Peroksidaz Aktivitesine Göre Gruplanması. XIV. Biyoteknoloji Kongresi, Bildiriler Kitabı, 313-317.

Davis BJ (1964). Disc electrophoresis. Method and application to human serum proteins. Ann NY Acad Sci 121: 404-427.

Eris A, Gulen H, Barut E, and Cansev A (2007). Annual patterns of total soluble sugars and proteins related to cold-hardiness in olive (*Olea europaea* L. 'Gemlik'). J Hort Sci Biotech 82: 597-604.

Gaspar T, Penel CL, Thorpe T, and Greppin H (1982). Peroxidases. A survey of their biochemical and physiological roles in higher plants. Universite de Geneve Press, Geneve.

Gulen H, and Eris A (2003). Some physiological changes in strawberry (*Fragaria x ananassa* cv. Camarosa) plants under heat stress. J Hort Sci Biotech 78: 894-898.

Gulen H, and Eris A (2004). Effect of heat stress on peroxidase activity and protein content in strawberry plants. Plant Sci 166: 739-744.

Gulen H, Arora R, Kuden A, Krebs SL, and Postman J (2002). Peroxidase isozyme profiles in compatible and incompatible pear-quince graft combinations. J Amer Soc Hort Sci 127: 152-157.

Janero DR (1990). Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. Free Radic Biol Med 9: 515-540.

Lester GE (1985). Leaf cell membrane thermostabilities of *Cucumis melo*. J Amer Soc Hortic Sci 110: 506-509.

Manganaris AG, and Alston FH (1992). Inheritance and linkage relationships of peroxidase isozymes in apple. Theor Appl Genet 83: 392-399.

Moran JF, Becana M, Iturbe-Ormaetxe I, Frechilla S, Klucas RV, and Aparicio-Trejo P (1994). Drought induces oxidative stress in pea plants. Planta 194: 346-352.

Owens CL, Iezzoni AF, and Hancock JF (2003). Enhancement of freezing tolerance of strawberry by Heterologous expression of *CBF1*. Acta Hort 626: 93-100.

Prassad TK (1996). Mechanisms of chilling-induced oxidative stress injury and tolerance in developing maize seedlings: changes in antioxidant system, oxidation of proteins and lipids, and protease activities. Plant J 10:1017-1026.

Premachandra GS, Saneoka H, Fujita K, and Ogata S (1992). Leaf water relations, osmotic adjustment, cell membrane stability, epi-cuticular wax load and growth as affected by increasing water deficits in Sorghum. J Exp Bot 43: 1569-1576.

Rajinder SD, Dhindsa PP, and Thorpe TA (1981). Leaf Senescence: Correlated with increased levels of membrane permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase. J Exp Botany 32: 93-101.

Reisfeld RA, Lewis UJ, and Williams DE (1962). Disk electrophoresis of basic proteins and peptides on polyacrylamide gels. Nature, 195: 281-283.

Rodrigo J (2000). Spring frosts in deciduous fruit trees – morphological damage and flower hardiness. Scientia Hort 85: 155-173.

Sarnighausen E, Karlson DT, Zeng Y, Goldsbrough PB, Raghothama KG, and Ashworth EN (2004). Characterization of a novel Y_nSK_n class of dehydrin-like cDNAs from cold acclimated red-oiser dogwood (*Cornus sericea* L.) xylem. In: Adaptations and Responses of Woody Plants to Environmental Stresses, (Ed.: R. Arora). The Haworth Press, New York, pp. 17-36.

Shilpi M, and Narendra T (2005). Cold, Salinity and drought stresses: An overview. Archives Biochem Biophys 444: 139-158.

Thomashow MF (1999). Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. Annu Rev Plant Physiol Plant Mol Biol 50: 571-599.

Uemura M, Tominaga Y, Nakagawara C, Shigematsu S, Minami A, and Kawamura Y (2006). Responses of the plasma membrane to low temperatures. Physiol Plant 126: 81-89.

van Huystee RB (1987). Some molecular aspects of plant peroxidases biosynthetic studies. Ann Rev Plant Physiol 38: 205-219.

Walter MH (1992). Regulation of lignification in defense. In: Plant Gene Research – Genes Involved in Plant Defense, (Eds.: T. Boller and F. Meins). Springer- Verlag, pp. 329-352.

Wendel JF, and Weeden NF (1989). Visualization and interpretation of plant isozymes. In: Isozymes in Plant Biology, (Eds.: D.E. Soltis and P.S. Soltis). Dioscorides Press, Portland, Oregon, pp. 5-44.

Yoshida K, Kaothien P, Matsui T, Kawaoka A, and Shinmyo A (2002). Molecular biology and application of plant peroxidase genes. App Microb Biotech 60: 665-670.