Cold-induced Changes of Proline, Malondialdehyde and Chlorophyll in Spring Canola Cultivars

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Abstract

Low temperature (LT) is an important environmental factor that limits the survival, productivity and geographical distribution of plants. Oil seeds are the second global food resources among which *Brassica napus* L. is the third annual oil seed in the world. In cold stress, some biochemical and physiological reactions occur in response to reactive oxygen species (ROS). Hence, the quantitative changes of proline, malondialdehyde (MDA) and chlorophyll (a, b, total) content were assessed in two spring canola (cv. Zarfam, cold tolerant and cv. Option 500, cold sensitive) seedlings exposed to early spring cold stress. They were first grown in a controlled growth room at 22/16 °C (day/night) and then at the 4th fully expanded leafy stage seedlings were transferred to a cold environment (10/3 °C) for 7 d, or they were maintained continuously at 22/16 °C (Control). Leaf samples were harvested at days 0 (transferred day), 2, 4 and 7 of cold exposure period. Analysis of variance showed significant differences between the temperature treatments and also cultivars for all physiological traits. The cold tolerant cultivar showed remarkable less LT-induced decrease in chlorophylls (a, b, total) and increase in MDA and accumulation of proline compared to the cold sensitive cultivar. This assay verified the superior response of cold tolerant Zarfam canola to cold temperature.

Keywords: *Brassica napus*; Canola; Chlorophyll; Cold stress; Lipid Peroxidation; Proline

Abbreviations Chl−Chlorophyll; LT−Low temperature; MDA−Malondialdehyde; ROS−Reactive Oxygen Species

Introduction

Abiotic stresses such as drought, high soil salinity and temperature extremes are found in many agricultural areas that lead to series of morphological, physiological and molecular changes that adversely affect plant growth and productivity. Among these stresses chilling (<10 °C) and/or freezing (<0°C) temperatures (Chinnusamy *et al.* 2007) are the major hazards to agriculture, limiting the survival, productivity and geographical distribution of plants in large areas of the world (Boyer 1982). Among crops, oil seeds the next to cereals, are the second global food resources. Canola (*Brassica napus* L.), the

next to soybean and cotton, is the third oil seed in the world. Spring cereals and oilseed rape are perceived as having a low frost resistance. This is consistent with field observations (Fowler and Carles 1979) and laboratory studies in which spring and winter forms showed distinct extents of metabolic changes during cold acclimation. Canola tolerates cold at 4-leaf rosette stage better than other developmental stages. Before this stage, efficient physiological processes such as the concentration of cell extract, enzymatic and protein changes induce cold-tolerance (Madani *et al.* 2005).

Various abiotic stresses lead to the overproduction of ROS in plants which are highly reactive and toxic and cause damage to proteins, lipids, carbohydrates and DNA which ultimately result in oxidative stress (Gill and Tuteja 2010). One approach that plants employ to protect themselves against these toxic oxygens, is the synthesis of cryoprotectant molecules such as soluble sugars, sugar alcohols and low-molecular weight nitrogenous compounds (proline, glycine betaine; Janska´ *et al.* 2010). Proline is a dominant organic molecule, (Naidu *et al.* 1991; Demiral and Turkan 2005) which contributes to the maintenance of enzymes from denaturation, interacts with membrane systems, regulates cytosolic pH, balances the ratio of $NADH/NAD^+$ functions as a source of energy and helps plants to detoxify ROS (Konstantinova *et al.* 2002). The latter can destroy membranes since their target includes phospholipids and glycolipids which produce free fatty acids, leading to more rigid and disassembled structures (Navari-Izzo *et al.* 1992). Furthermore, oxidation of polyunsaturated fatty acids leads to many different products, such as short-chain alcohols, aldehydes and alkanes (Kappus 1985; Chow 1991). Under most oxidative conditions, one of the most representative markers for membrane destruction after free-radical chain reactions is the formation and accumulation of MDA, an end product of peroxidation of the unsaturated membrane fatty acids (Halliwell and Gutteridge 2002). It is an important index for interpreting oxidative stress damages.

Rapid reduction of leaf temperature results in the accumulation of soluble carbohydrates (Azcón-Bieto 1983; Briiggemann *et al.* 1992; Paul *et al.* 1992). Data from transgenic tobacco (Stitt *et al.* 1990; Von Schaewen *et al.* 1990) have shown that soluble carbohydrate accumulation can also suppress photosynthesis by down regulating the levels of photosynthetic carbon reduction cycle enzymes and Chl a/b-binding proteins. In the present study, we examined whether two spring canola cultivars, cold-sensitive Option 500 (cv. 1) and cold tolerant Zarfam (cv. 2), respond differently to early spring cold stress in terms of the proline amount, MDA and changes in Chl (total, a and b) in leaves at temperature shifts from 22/16 °C to 10/3 °C.

Materials and Methods

a) Plant materials and growth conditions

Seeds of two spring canola (*Brassica napus* L., $2n = 4x = 38$) cvs., Option 500 as a cold sensitive (cv. 1) and Zarfam as a cold tolerant (cv. 2) (Valadiani and Tajbakhsh 2007), supplied from the Seed and Plant Improvement Institute (SPII), Karaj, Iran, were grown in plastic pots (150 mm diameter × 150 mm deep) filled with a mixture of five parts soft mold, two parts sand, two parts clay and two parts loamy soil (garden soil). Seedlings were grown in a controlled growth room at a constant air temperature of 22/16 °C (day/night) with illumination provided by white fluorescent tubes at a fluency rate of 300 μmol m^{-2} s^{-1} at soil level for 16 h d^{-1} until the 4-leaf developmental stage. At this time, half of the pots were maintained at these conditions continuously (control treatment) and other half were transferred to a cold growth room at 10/3 °C at the same fluency rate and photoperiod as above for 7 d (cold treatment).

b) Sampling times

Over the 7-d experimental protocol, the seedlings (shoot) were sampled randomly on days 0 (the day in which half of the pots were transferred to cold growth room), 2, 4 and 7 of cold exposure. At the same sampling times, samples were also taken from the control plants. The harvested leaves were frozen in liquid nitrogen, and then kept at -80 °C freezer for assaying of proline, MDA and Chl.

c) Measurement of proline content

Extraction and determination of free proline was carried out by the method described by Bates *et al.* (1973). A sample of 0.2 g plant tissue was homogenized in 10 ml of 3% sulphosalysilic acid solution. The homogenate was centrifuged at 1000 rpm for 15 min. Proline was analyzed by reacting 2 ml of the extract with 2 ml of glacial acetic acid and 2 ml of ninhydrin solution. The mixture was incubated in a boiling water bath for 1 h. After cooling, 4 ml toluene was added and vigorously shaken. The absorbance of the toluene phase was determined at 520 nm in a spectrophotometer (U-2800, Hitachi, Japan). The concentration of proline was reported using proline standard curve by mg g^{-1} fresh weight (Öncel *et al.* 2004).

d) Measurement of MDA content

The level of lipid peroxidation was measured in terms of MDA content following the method described by De Vos *et al.* (1991). MDA is a major cytotoxic product of lipid peroxidation and acts as an indicator of free radical production. Plant materials (0.2 g) was homogenized in 3 ml of 10% (v/v) trichloroacetic acid (TCA) following by filtering through Whatman filter paper. Aliquot of 1 ml supernatant was mixed with 1 ml of 0.5% thiobarbituric acid (TBA) and incubated in a boiling water bath with 95 °C for 30 min. After

cooling, the absorbance of supernatant at 532 nm was measured. After subtracting the non-specific absorbance at 600 nm, MDA concentration was determined, using the extinction coefficient of 155 mM^{-1} cm^{-1}.

e) Measurement of Chl (total, a and b) content

The amount of total Chl was measured, using a Chlorophyll meter (SPAD-502, Minolta, Japan). The content of Chls a and b was estimated by the method described by Helrich (1990). Chl extraction from leaf material was carried out with 80% (v/v) acetone. The absorbance of resulting supernatant was recorded at 664 and 647 nm. The concentrations of Chls a and b were calculated, using the following formulae:

Chl a = [12.7 (D 663) − 2.69 (D 645)] × V/1000 × W

Chl b = [22.9 (D 645) − 4.28 (D 663)] × V/1000 × W

f) Statistical analysis

Data were statistically analyzed, using 3-factor balanced analysis of variance (ANOVA) on the basis of completely randomized design (CRD) with three replications by Minitab Statistical Software (Minitab Inc., State College, PA, USA; Fry 1993; Ryan and Joiner 2001). The first factor included two cultivars (Option 500 & Zarfam), the second factor consisted of two temperatures (22/16 °C & 10/3 °C) and the third factor was sampling time at four different days (days 0, 2, 4 and 7; Table 1). For each cultivar/sampling time, mean comparisons between temperature treatments were performed by the LSD method. Furthermore, linear regressions of different characters under study with sampling times were fitted to the data. The significance of slopes were verified by the t-test.

Table 1. Mean squares of the 3-factor balanced analysis of variance on the basis of completely randomized design for five physiological characteristics of canola cultivars

S.O.V.	df	MS				
		Proline	MDA	Total Chl	Chl a	Chl b
Temperature (T)	1	9.30^{***}	8.67^{***}	97.15^{***}	0.047^{***}	14.633^{***}
Cultivar (cv.)	1	9.30^{***}	6.04^{***}	80.35^{***}	0.108^{***}	4.258^{**}
Sampling time (S)	3	5.26^{***}	4.93^{***}	13.88^{*}	0.027^{***}	0.359^{ns}
cv. $\times$ T	1	0.62^{**}	2.59^{***}	50.43^{**}	0.000^{ns}	0.414^{ns}
T $\times$ S	3	1.44^{***}	1.72^{***}	11.06^{ns}	0.006^{ns}	2.509^{**}
cv. $\times$ S	3	0.68^{***}	0.94^{***}	2.30^{ns}	0.005^{*}	0.590^{ns}
cv. $\times$ T $\times$ S	3	0.23^{*}	0.40^{**}	6.61^{ns}	0.000^{ns}	0.691^{ns}
Error	32	0.08	0.091	4.35	0.001	0.389
CV%		9.0	10.0	8.1	5.4	20.8

Note: ^{ns} nonsignificant (P>0.05). $^{*,\,**,\,***}$Significant at P≤0.05, P≤0.01 and P≤0.001 levels, respectively.

Results

The result of ANOVA showed significant differences among temperature treatments, cultivars and sampling times for most of the measured physiological characteristics in the leaves of spring canola cultivars (Table 1). The three-way interactions were significant for the proline and MDA while only one two-way interaction was significant for total Chl (Cultivar $\times$ Temperature), Chl a (Cultivar $\times$ Sampling time) and Chl b (Temperature $\times$ Sampling time). In this study, we aimed to focus on the temperature treatments at each sampling time for each cultivar. Both cultivars showed similar incremental trend (Figure 1) for proline content in the cold-treated leaves compared to the controls: cv. 2 responded earlier from day 0 while cv. 1 delayed in response by two d (Figure 1). In both cultivars, remarkable increase in proline was obvious on days 4 and 7 of cold exposure (P<0.001) compared with their controls. On day seven, the LT-induced canola showed the highest amount of proline accumulated in their leaves in each cultivar compared to the controls (2.6-fold increase in cv. 1 and 1.4-fold increase in cv. 2, P<0.001). No proline changes were detected in the leaves of both control cultivars over the experimental period. In terms of MDA, both canola cultivars responded differently to LT treatment (Figure 2). MDA remained unchanged in the leaves of control plants of each cultivar during the experimental period while LT caused marked changes. The cold tolerant cv. 2 was affected less by LT treatment for accumulating MDA in their cold-treaded leaves compared to the controls. On day seven, the cold-induced leaves of cv. 2 showed 23% (P<0.001) increase in MDA compared to the control. In contrast, LT caused substantial effect on the cold sensitive cv. 1. In other words, as the LT treatment started on plants, MDA increased rapidly with time in the cold-treated leaves of this cultivar, reaching its maximum on day seven (3.4-fold increase, P<0.001, compared with the control, Figure 2). On the other hand, the positive cold-induced trend in MDA was significant (P<0.001) in each cultivar; however, a sharper 3.5-fold increased slope was observed in cv. 1 compared with that in

cv. 2. Similar to MDA, both canola cultivars responded differently to LT treatment in terms of Chl species (total, a and b; Figures 3-5). A dawn shift of temperature from 22/16 °C (day/night) to 10/3 °C caused decreasing effect of Chls in the leaves of both spring canola cultivars. The cold tolerant cv. 2 was influenced less than the cold sensitive cv. 1 against LT treatment. The slopes of cultivar 1 for Chl species were all significant while those of cv. 2 were not (P>0.05), indicating the superiority of the cold tolerant cv. 2 canola cultivar in response to the LT treatment.

Figure 1. Changes of proline content in the leaves of cold-sensitive Option 500 (cv. 1) and cold-tolerant Zarfam (cv. 2) spring canola cultivars in the control (solid line, 22/16 °C) and in the cold treatment (dotted line, 10/3 °C). Values are means (n = 3) ± SE, but where bars are absent, the variation about the mean was less than the diameter of the symbol.
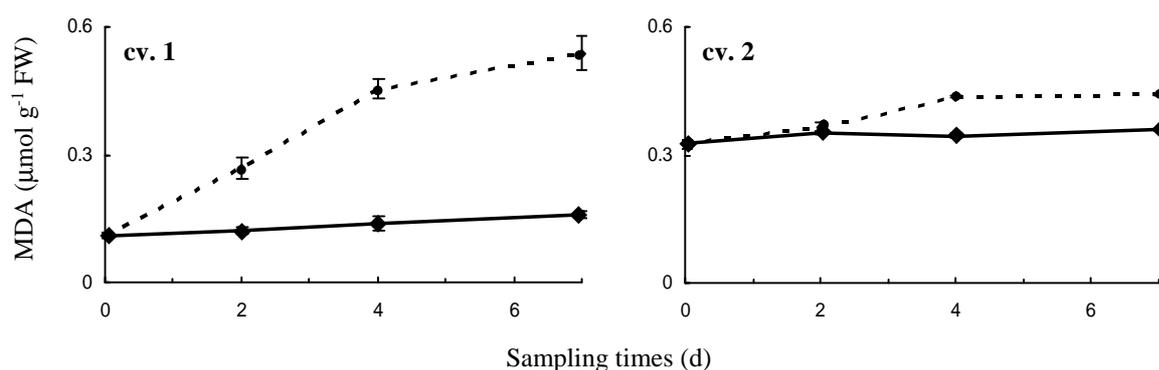

Figure 2. Changes of Malondialdehyde (MDA) amount in the leaves of cold-sensitive Option 500 (cv. 1) and cold-tolerant Zarfam (cv. 2) spring canola cultivars in the control (solid line, 22/16 °C) and in the cold treatment (dotted line, 10/3 °C). Values are means (n = 3) ± SE, but where bars are absent, the variation about the mean was less than the diameter of the symbol.

Discussion

Each plant species has its unique set of temperature requirements which are optimum for proper growth and development. LT is one of the abiotic stresses that is the principal cause of crop failure worldwide and reducing the yields for most major crops. It has been reported that cold stress induces the accumulation of proline, a well-known osmoprotectant. Proline content was increased in potato hybrids when plants subjected to cold treatment (Koç *et al.* 2010). In our study, during LT treatment, obvious accumulation of proline was detected in both spring canola cultivars over the experimental period time. Increase in the amount of proline was ascending during LT period such that it reached maximum level on the day 7, the final sampling time. Transgenic approaches revealed that proline accumulation leads to enhanced stress tolerance (Apse *et al.* 1999). Moreover, our results are in agreement to previous reports on different plants such as bread wheat (Jahanbakhsh-Godehkahriz *et al.* 2009; Javadian *et al.* 2010) and rice (Ghorbani *et al*. 2009), indicating that proline accumulation is an important mechanism of response to cold stress being capable to repair cell damages. In the present report, in the early stage of LT exposure, the cold tolerant cv. 2 showed more remarkable accumulation of proline in its leaves compared to cv. 1, suggesting that the cold tolerant spring canola has probably more quick adaptation capability to early spring temperature fluctuations. On the contrary, in some researches (Rouhaninia *et al*. 2006) on some apricot (*Prunus armeniaca*) cultivars and wheat (Petcu and Terbea 1995) proline content was increased in cold sensitive cultivars. In our work, linear LT-induced proline accumulation supported the findings of many researchers (Handa *et al*. 1986; Leyva *et al*. 1995; Konstantinova *et al*. 2002; Fahimirad *et al*. 2013).

MDA is a common product of lipid peroxidation and a sensitive diagnostic index of oxidative injury (Janero 1990). Increase in the lipid peroxidation was reported in many plants under various environmental stresses such as drought stress (Moran *et al*. 1994; Tatari *et al*. 2012), chilling-induced oxidative stress (Prassad 1996), high temperature (Ma *et al*. 2008), cadmium toxicity (Nouairi *et al*. 2009) and cold stress (Fahimirad *et al*. 2013). In the present study, in the case of cold stress, consistent to other researches on *Camptotheca acuminate* (Jiancan *et al*. 2004), *Fragaria xananassa* (Hatice *et al*. 2008) and *Oryza sativa* (Hassibi *et al*. 2008), cold stress led to increase in the level of MDA. In agreement to findings of Fahimirad *et al*. (2013), considerable differences were observed between the linear regression slopes of LT treatment and control conditions. During the experimental period, a rapid trend of MDA accumulation in the LT-treated leaves of cold sensitive cv. 1 compared to that in the cold tolerant cv. 2 was observed, perhaps signifying sever membrane phospholipids peroxidation in cv. 1. In other words, the less increased amount of MDA in the LT-treated leaves of cold tolerant cv. 2 compared to the cold sensitive cv. 1 may indicate its capability of tolerance to cold stress. In agreement to our data, Yadegary *et al*. (2008) reported that MDA content was decreased in the cold-treated leaves of soybean (*Glycine max*) during the cold exposure period, showing correlation with increased cold

tolerance. Therefore, this trait can be suggested as an assay character of LT-tolerance to be used in the breeding programs. LT can potentially influence photosynthetic activity in plants. It was reported that Chls a and b content decreased in plants subjected to cold treatment (Wise and Naylor 1987b). In our study, the Chl species (total, a and b) were less influenced by a dawn shift of temperature from 22/16 °C (day/night) to 10/3 °C in the canola leaves of cold tolerant cv. 2 compared to the cold sensitive cv. 1. Such decline of the amount of Chls induced by LT was supported by the finding of Koç *et al.* (2010) on pepper (*Capsicum annuum* L.) under cold stress. Studies of photosynthetic acclimation of overwintering cereals have shown that cold tolerance is strongly correlated with the capacity to increase photosynthesis and with the capacity to increase soluble carbohydrate pools during cold hardening (Tognetti *et al.* 1990; Oquist *et al.*
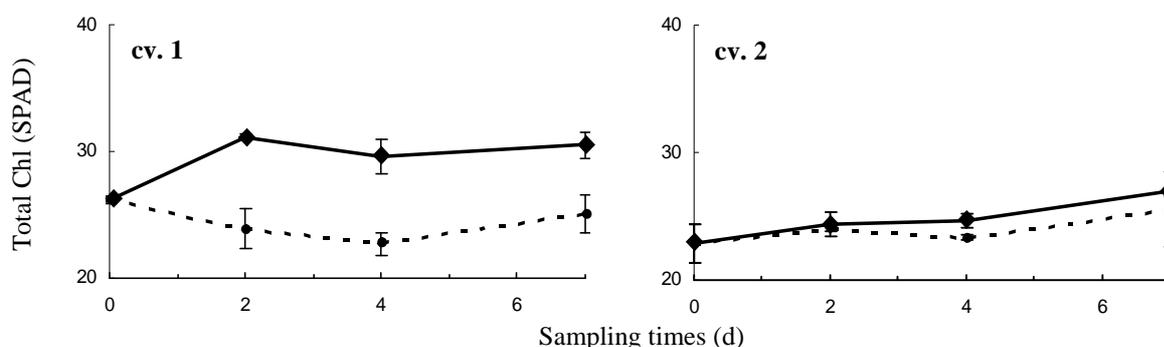

Figure 3. Changes of total Chlorophyll content in the leaves of cold-sensitive Option 500 (cv. 1) and cold-tolerant Zarfam (cv. 2) spring canola cultivars in the control (solid line, 22/16 °C) and in the cold treatment (dotted line, 10/3 °C). Values are means (n = 3) ± SE, but where bars are absent, the variation about the mean was less than the diameter of the symbol.
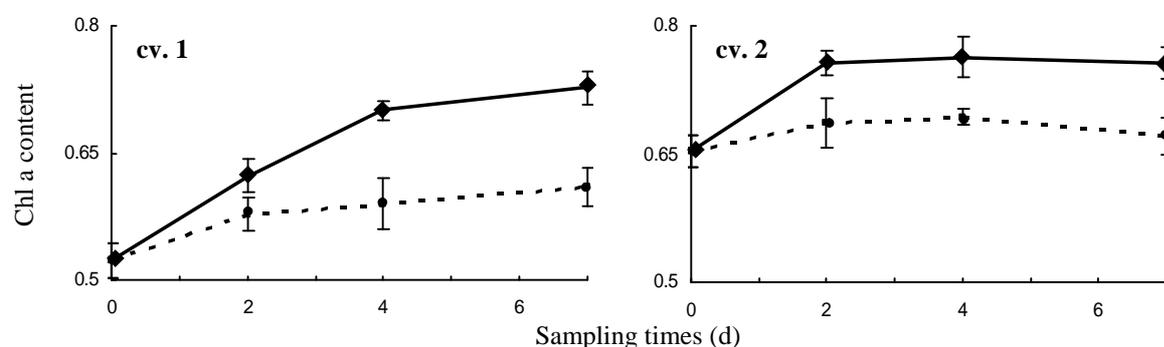

Figure 4. Changes of Chlorophyll a content in the leaves of cold-sensitive Option 500 (cv. 1) and cold-tolerant Zarfam (cv. 2) spring canola cultivars in the control (solid line, 22/16 °C) and in the cold treatment (dotted line, 10/3 °C). Values are means (n = 3) ± SE, but where bars are absent, the variation about the mean was less than the diameter of the symbol.
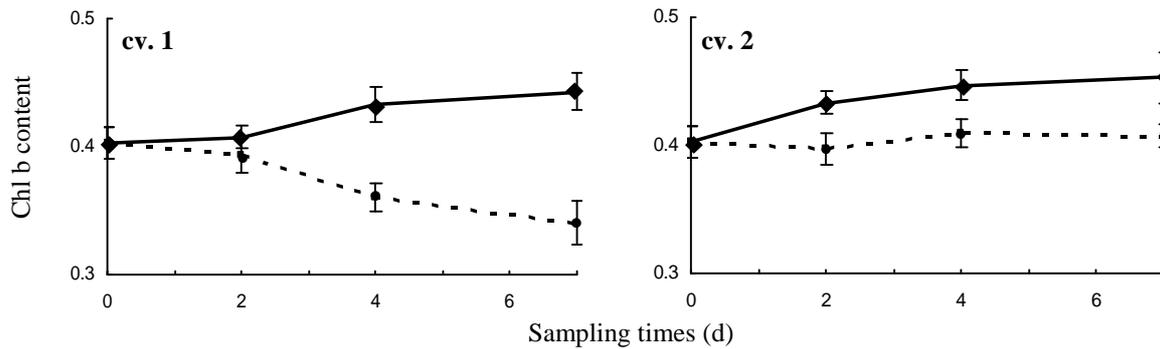
Figure 5. Changes of Chlorophyll b content in the leaves of cold-sensitive Option 500 (cv. 1) and cold-tolerant Zarfam (cv. 2) spring canola cultivars in the control (solid line, 22/16 °C) and in the cold treatment (dotted line, 10/3 °C). Values are means (n = 3) ± SE, but where bars are absent, the variation about the mean was less than the diameter of the symbol.

1993). Therefore, an important factor in successful cold adaptation is the regulation of photosynthetic approaches to obtain favorable yield under LT (Ensminger *et al*. 2006). In the present work, it was considered that cold tolerant spring canola cv. 2 was more successful and efficient in the regulation of photosynthetic approaches in the early spring LT circumstances. This is in agreement with the findings reported by previous researches on canola (Fahimirad *et al*. 2013) and other crops, e.g. rice (Wise and Naylor 1987a) and pea (Yong *et al*. 2003). Furthermore, in agreement to Jahanbakhsh-Godehkahriz *et al*. (2009) on wheat, the photosynthetic systems undergo fewer injuries under LT stress in cold tolerant cultivars. Javadian *et al*. (2010) represented that cold tolerance mechanisms were activated more efficiently in winter wheat cultivar than in the spring one, so that the leaves were protected better from photooxidative damage to photosynthesis in the cold tolerant winter cultivars. Majdi *et al*. (2008) in the assay of vernalization temperature on winter and spring cultivars of bread wheat confirmed a rising trend of the Chl content with the development of cold period which was more intense in the winter and cold tolerant cultivar. These damages in the cold

sensitive cultivar may be due to the disorders in light reactions and electron transport chain in photosystems that cause to produce ROS and consequently photo-oxidative reactions, protein and carotenoid oxidation and finally reduction of quantum efficiency of photosystems (Kuk *et al*. 2003). Yuanyuan *et al*. (2009) hypothesized that photosynthetic activity is regulated in a feedback manner by soluble sugars under cold stress. The photosynthetic machinery might be down-regulated by sugars due to the cold-girdling petioles to prevent sugar export out of the leaf. Also, in these cases, genes related to photosynthesis were repressed, including those for Chl binding protein and Rubisco (Smeekens, 2000). As a result, decrease in the amount of Chls content could be a typical symptom of oxidative stress. In this direction, Chen *et al*. (2011) considered the difference in Chl contents of leaves between the plants transferred by *BnCOR25*, playing an important role in plant tolerance, and wild type in response to cold treatment.

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