膜脂过氧化作为扁桃品种抗寒性鉴定指标研究

潘晓云[1]，曹琴东[2]，王根轩[1]*

(1. 兰州大学干旱农业生态国家重点实验室，兰州 730000；2. 甘肃省绿色食品办公室，兰州 730030)

摘要：研究不同低温胁迫下(3℃，－2℃ 和 －10℃下分别处理 0.5、3、6、9、12h)扁桃离体叶片中 MDA 含量、CAT 活性和类胡罗卜素含量以及反映组织受伤害程度的膜透性变化和叶绿素降解间的关系，以探明膜脂过氧化在扁桃叶片低温伤害中的作用。3℃处理初期，CAT 活性、类胡罗卜素含量显著增加，叶片无明显伤害症状(水渍、黄化等)；－2℃和－10℃下随处理时间的延长，叶片膜透性增大、叶绿素含量下降、MDA 积累增多；同时 CAT 活性、类胡罗卜素含量显著下降。MDA 含量的增加与组织受伤害程度(膜透性增加及叶绿素降解)及抗氧化系统(CAT 和类胡罗卜素)水平下降之间具有极显著的相关性。以 7 个扁桃品种的田间越冬伤害指数及冬季所测枝条的 LT_{50}代表植株的冬季抗寒性，以－5℃下处理 4h 和 8h 后所测叶片 MDA 相对含量代表叶片的抗寒性，发现 2 者呈显著的相关性，表明 MDA 可以作为鉴定扁桃品种抗寒性的指标。

关键词：低温胁迫；膜脂过氧化；CAT；类胡罗卜素；选择指标；扁桃 (*Prunus dulcis*)

Evaluation of Lipid Peroxidation for Use in Selection of Cold Hardiness Cultivars of Almond

PAN Xiao-Yun[1], CAO Qin-Dong[2], WANG Gen-Xuan[1]* (1. *State Key Laboratory of Arid Agroecology, Lanzhou University, Lanzhou*, 730000, *China*; 2. *Green Food Office of Gansu, Lanzhou*, 730030, *China*)

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Abstract: Effects of low temperature stress on lipid peroxidation, membrane and chlorophyll stability, and catalase (CAT) activity and carotenoid content were evaluated under laboratory freezing tests (3℃, －2℃ and －10℃ for 0.5, 3, 6, 9, and 12h, respectively), for possible use as cold hardiness selection criteria, in detached leaves of two almond cultivars ('NePlus' and 'Nonpareil', differing in cold tolerance). The application of moderate chilling treatment (3℃ within 9h) to almond leaves had significantly elevated levels of CAT activity ('Nonpareil' ＋17%～94%, 'NePlus' ＋38%～107%) and carotenoid content ('Nonpareil' ＋6%～31%, 'NePlus'＋10%～32%). Severe freezing treatments (－2℃ and －10℃) caused considerable injury in almond leaves as indicated by reduction in chlorophyll content ('Nonpareil' －26%, －44% and 'NePlus' －42%, －67%, respectively) and increasing in electrolyte leakage ('Nonpareil' ＋76%, ＋275% and 'NePlus' ＋196%, ＋379%, respectively), and enhanced oxidative stress as indicated by increasing in malondiadehyde (MDA) content ('Nonpareil'＋43%, ＋81% and 'NePlus' ＋70%, ＋110%, respectively), and decreasing in levels of antioxidants (CAT activity and carotinoid content). The decrease in antioxidant system and the increase in oxidative damage are closely

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Biography: PAN Xiao-Yun (1969～), male, born in Lanzhou, Gansu, PhD. Majoring in both phant plysiology and ecology.

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* Author for correspondence E-mail: wanggx@lzu.edu.cn.

correlated. These results provide some evidence for the view that low-temperature responses in plants is due, at least in part, to oxidative stress, as a source of injury and as a signal to increase antioxidant defenses. We also found that the cold resistance of plant (measured by winter injury index of field plants and LT_{50} of current year shoots in winter) correlated to that of leaves (measured by MDA content in leaves under laboratory freezing test, −5 C for 4h and 8h) in 7 almond cultivars.

Key words: low-temperature stress; lipid peroxidation; catalase (CAT); carotenoid; selection criteria; almond (*Prunus dulcis*)

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1 Introduction

In natural conditions crops are often exposed to various environmental stresses that decrease production. At the whole-plant level, the effect of stress is usually perceived as a decrease in photosynthesis and growth, and is associated with alterations in C and N metabolism[1]. At the molecular level, the negative effect of stress on leaves may be partially as a consequence of the oxidative damage to plant cells through their reaction with membrane lipids, protein, and DNA, as a result of the imbalance between production of activated oxygen and antioxidant defenses[2]. This hypothesis is very plausible because chloroplasts are major source of activated oxygen in plant[2], and because antioxidants, which may play a critical role in preventing oxidative damage, are greatly affected by environmental stress[3]. Other subcellular compartments of leaves, such as peroxisomes and mitochondria, are also potential generators of activated oxygen, mainly as a consequence of electron transport and enzymic reactions[4]. Fortunately, in optimal conditions leaves are rich in antioxidant enzymes and metabolites and can cope with activated oxygen, thus minimizing oxidative damage[2].

Low temperature injury is one of the main limiting factors to crop production and distribution of horticultural crops[5]. Despite the numerous research efforts devoted to reduce freezing injury, it still accounts for greater losses of fruits and vegetables than any other environmental or biological hazard[6]. Consequently, cold hardiness is often a main selection criterion in breeding programs[7] and much research has been focused on the mechanisms of freezing injury and acclimation to low temperatures. Low temperature responses of plant have been reported to involve biochemical and physiological changes[8~10] and alterations in gene expression[11,12]. More recently, the induction of oxidative stress by low temperature has been proposed as a major contributing factor to cold responses, as a source of injury and as a signal to increase antioxidant defenses[13~18].

Our early study suggested that low temperature was one of the major constrains for introducing California almond to southwestern China[19]. Therefore, in order to gain some underlying biochemical mechanisms which may bestow cold tolerance on almond cultivars, and to identify possible cold tolerance selection criteria, we made use of two almond cultivars differing in cold hardiness and examined: 1) the low temperature induced changes in the degree of membrane degradation through lipid peroxidation; 2) the activity of catalase and content of carotenoid, since they are both important antioxidants or antioxidant enzyme, and 3) changes in chlorophyll content and the permeability of membrane, both of which are known to be sensitive to low temperature. We also examined the cold hardiness of 7 almond cultivars by winter injure index of field plants and LT_{50}(the lowest temperature causing 50% damage) of current year shoots by electrolyte leakage test and variation of MDA content in leaves after laboratory freezing test under −5 C for 4h and 8h.

2 Materials and methods

2.1 Plant material

Two almond cultivars (Nonpareil and NePlus, all 3-year-old), which were introduced from Burchell Nursery Inc., California, USA, on March 1997, grew at the Fruit Tree Research Station of Gansu Academy of Agricultural Science (Gansu, China). Electrolyte leakage assays were conducted on the following tissues: 6 mm diameter discs collected from leaves of the current year shoots of each four plants of Nonpareil and NePlus on 12 to 17 June 2000.

2.2 Electrolyte leakage assays and cold hardiness determination

The samples were collected in the field, kept on ice, and brought to the laboratory for cold hardiness determination as described by Arora *et al*[20]. Leaves were cut into 6 mm diameter discs and placed into glass test tubes (20×2 cm^2) containing 1.5 mL cold (4℃) distilled deionized water. Leaf margins and midribs were excluded from the discs. To eliminate solute leakage from cut surfaces, all tissue samples were soaked for 1h in cold (4℃) distilled deionized water. The capped tubes containing samples to be frozen were randomly placed in an ethylene glycol freezing bath (model 2425 CH/P, Forma Scientific, Marietta, Ohio) set at 0℃. Additional tubes containing representative tissue samples were fitted with 30-gauge copper-constantan thermocouples and randomly placed in the freezing bath. The thermocouples were then attached to a programmable datalogger (CR7X, Campbell Scientific Inc., Logan Utah) for constant monitoring of temperature.

The bath temperature was lowered to −1℃ at the rate of 1℃/h. When the tissue temperature reached −1℃, samples were nucleated with a small chip of ice (glass−distilled water) and held overnight at −1℃ to ensure complete tissue freezing. Next, the bath temperature was lowered at a rate of 1℃/h and the samples were held at each test temperature for 8h for leaf samples before 3 replicate tubes of each leaf category were removed. Leaf test temperatures of −2, −4, −6, −8, −10, and −12℃ were used because the results of preliminary freezing tests indicated that these temperatures produced tissue injury ranging from none to complete kill.

Tubes were removed at various treatment temperatures, fist placed in ice (2h minimum), and then transferred to 4℃ (overnight) to allow slow thawing. Discs were placed in 20 mL of distilled deionized water immediately after thaw, vacuum infiltrated at 0.17 MPa for a single 15-min cycle to ensure penetration of the bathing solution into the tissues. Measurements of electrical conductivity were made at 20 min after infiltration with an electrical conductivity meter (model CDM80, Radiometer, Copenhagen, Denmark) as described by Arora *et al*[21]., and percent injure (as described by Arora *et al*[20].) was then calculated using the percent ion leakage data.

Cold hardiness determinations of detached leaves indicated an LT_{50} of -8.75 ± 0.09℃ and -7.13 ± 0.05℃ for Nonpareil and NePlus, respectively . This cold resistance diffidence between Nonpareil and NePlus was consistent with the study of Buyukyilmaz and Kester[22].

2.3 Low-temperature treatments and parameters determination

Detached leaves were exposed to various low temperature (chilling temperature, 3℃; nonlethal freezing temperature −2℃; lethal freezing temperature −10℃) for 0.5, 3, 6, 9 and 12h, respectively. The procedure of freezing to various treatments and thawing as described by Arora (1996). And we made leaves from shoots which were kept in culture chamber (20℃, 45%～60% relative humidity (*RH*), the cut ends were soaked in distilled water) over the treatments time as controls. Cellular damage was estimated by the electrolyte leakage test[11, 21]. Malondiadehyde (MDA) content, a decomposition production from the peroxidation of polyunsaturated fatty acids[23], was measured as thiobarbituric acid-reactive material from centrifuged leaf extracts in 5% trichloroacetic acid[24]. The absorbance of the extract

was read at 532nm and the values were corrected for nonspecific turbidity by subtracting the absorbance at 600nm. The concentration of malondialdehyde was calculated using its extinction coefficient. Samples of 50mg fresh leaf tissue were extracted with 80% acetone, and their chlorophyll and carotinoid content were calculated with the extinction coefficients given by Lichtenthaler and Wellburn[25]. A 0.25mg of leaf sample was ground with cold (4℃) Tris-HCl buffer (pH 8.5) containing 5 mmol/L EDTA, 5 mmol/L DTT, 10% (w/v) insoluble polyvinylpolypyrrolidone (PVP) and 0.5 mmol/L phenylmethylsulfonyl fluoride. The homogenate was strained through one layer of Miracloth (Calbiochem) and centrifuged at 15,000g for 20 min. All operations were performed at 0 to 4℃. Catalase activity was determined by measuring the rate of decrease in absorbance at 240nm from 3 min in a solution of 12.5 mmol/L H_2O_2 in 50 mmol/L potassium phosphate (pH 7.0) at 25℃[26].

2.4 Data analyses

Analysis of variance (ANOVA) with temperature, treatment time and cultivars (fixed effects) and three independent samples (random effect) were performed on all five indices to test for responses of almond leaves to low temperatures. Differences between cultivar within times were tested by paired-samples t test at $P<0.05$. For all analyses, we used general linear models (procedure GLM, SPSS Inc., 1993). Correlationships between parameters expressed as % of control were also calculated. LT_{50}, the lowest temperature causing 50% damage, was determined by plotting conductivity data against temperature, using a logistic regression model by means of the Statistic program.

3 Results

3.1 Low-temperature injuries on almond leaves

The effects of low temperatures on detached leaves of almond are highly variable and depend on the characteristics of both the intensity and duration exposed to low temperatures and the plant genotypes (Table 1). At 3℃, there were no any visual symptoms of chilling damage in detached leaves of almond until the duration of treatment reached to 9h. Damage symptoms in the more severe treatments (−2℃ over 3h and −10℃) appeared as waterlogged appearance and a chlorosis of the tissue. Irrespective of the cultivars, while the chlorophyll content decreased significantly, the electrolyte leakage increased sharply with the intensity and duration of the low temperatures (Fig. 1). At 3℃for 12h, the content of chlorophyll in NePlus and Nonpareil decreased 31.5% and 18.2%, and electrolyte leakage increased 85.6% and 43.6%, respectively. At −2℃ for 12h, chlorophyll content decreased 67.4% and 45.9%, and electrolyte increased 368.7% and 195.6%, respectively. The rate and extent of variation were

Table 1 Results of analyses of variance for physiological and biochemical traits of two almonds, cv 'Nonpareil' and 'NePlus', after three types of low-temperature treatments (3℃, −2℃, −10 ℃ and untreated control) **for five treatments time** (0.5, 3, 6, 9, and 12h) **in the darkness**

Source of variation	Chlorophyll content		Electrolyte leakage		MDA content		CAT activity		Carotinoid content	
	df	MS	df	MS	df	MS	df	MS	df	MS
Replication	2	0.005*	2	1.184^{NS}	2	0.012^{NS}	2	0.016^{NS}	2	0.002*
Cultivar (C)	1	2.537***	1	129.958***	1	4.983***	1	5.075***	1	0.201***
Temperature (T)	3	3.335***	3	5448.093***	3	121.042***	3	94.840***	3	0.612***
Time (t)	4	0.863***	4	746.440***	4	25.931***	4	6.181***	4	0.133***
$C\times T$	3	0.079***	3	376.301***	3	2.030***	3	0.575***	3	0.001*
$C\times t$	4	0.006**	4	11.033***	4	0.613**	4	1.383***	4	0.003***
$T\times t$	12	0.078***	12	158.041***	12	5.721***	12	2.801***	12	0.029***
$C\times T\times t$	12	0.004***	12	8.296***	12	0.178***	12	2.401***	12	0.002***
Error	78	0.001	78	1.351	78	0.025	78	0.021	78	<0.001

Note: NS, not significantly different at $P<0.05$ *, * *, * * *, significantly different at $P<0.05$, $P<0.01$ and $P<0.001$, respectively.

significantly higher in 'NePlus' than in 'Nonpareil' (Fig. 1). Based on the observation above it was obvious that 'NePlus' was more sensitive to freezing than 'Nonpareil'. This result was consistent with the study of Buyukyilmaz and Kester[22].

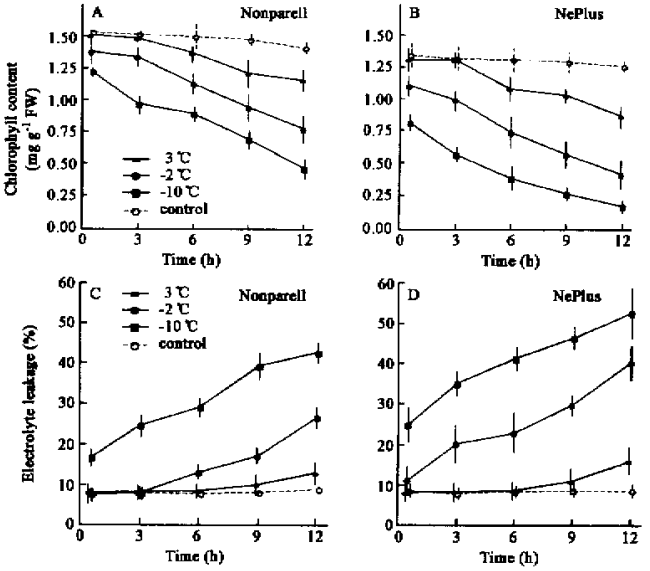

Fig. 1 Changes in chlorophyll content (A, B) and electrolyte leakage (C, D) of 'Nonpareil' and 'NePlus' leaves after low-temperature treatments. Values represent the means of three replications. Error bars represent 95% confidence.

3.2 Lipid peroxidation

In the leaves of two almonds the content of MDA on a fresh weight basis, increased significantly with temperature ($P<0.001$) and duration of treatment ($P<0.001$) (Table 1). At 3℃, −2℃ and −10℃ for 12h, MDA content increased 19.2% (Nonpareil) and 36.7% (NePlus), 86.7% (Nonpareil) and 134.4% (NePlus), 111.8% (Nonpareil)and 163.5% (NePlus), respectively (Fig. 2). And the extent of increasing in MDA content was significantly higher in 'NePlus' than in 'Nonpareil' (Fig. 2). Furthermore, there were close correlations between low temperature injures (as measured by decreasing in content of chlorophyll (Fig. 4 D) and enhanced electrolyte leakage) and increase in content of MDA (Fig. 4 C), indicating that freezing increased obvious lipid peroxidation, which was related to low-temperature injures in almond leaves.

3.3 CAT activity and carotenoid content

To determine if the low temperature responses in almond leaves were associated with differences in antioxidants that protect against oxidative stress, CAT activity and carotenoid content were measured. At 3℃, CAT activity and carotinoid content firstly increased rapidly but later decreased with duration of the treatment (Fig. 3). In 'Nonpareil', the peak of CAT activity (194% of control) and carotinoid content (131% of control) occurred at 9h and 6h, respectively (Fig. 3). In 'NePlus', the time reached the peak of CAT activity (207% of control) and carotinoid content (132% of control) were both 3h (Fig. 3). This suggested that the application of moderate chilling treatment (3℃ for 3～9h) on almond leaves led to increase in concentrations of antioxidant. There were no changes or only small changes in the content of

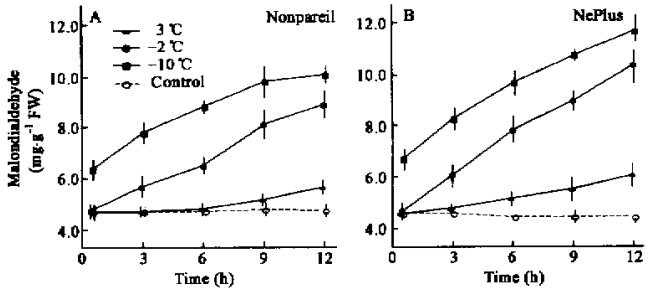

Fig. 2　Changes in malondialdehyde (MDA) content of ‘ Nonpareil ’ and ‘ NePlus ’ leaves after low-temperature treatments. Values represent the means of three replications. Error bars represent 95% confidence

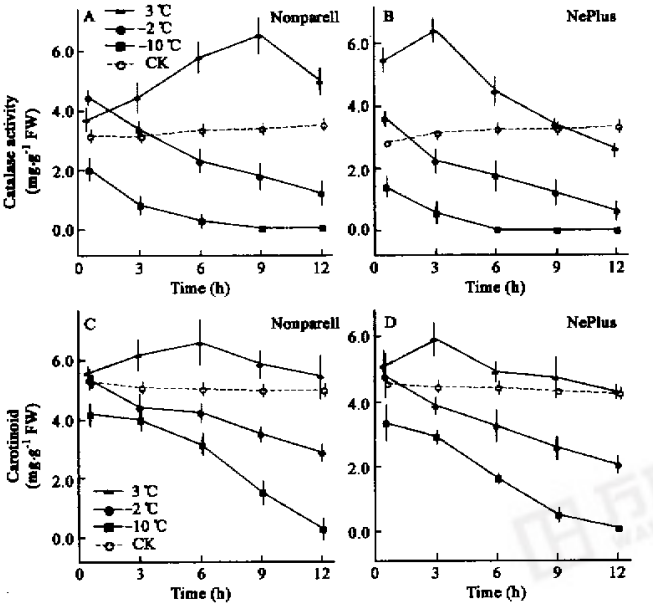

Fig. 3　Changes in catalase (CAT) activity (A, B) and caroteniod content (C, D) of ‘Nonpareil’ and ‘NePlus’ leaves after low-temperature treatments. Values represent the means of three replications. Error bars represent 95% confidence

chlorophyll or MDA and the electrolyte leakage, when the CAT activity and carotinoid content increased (Fig. 1 and Fig. 3). But over the peak of CAT and carotinoid, chlorophyll content decreased while MDA content increased obviously (Fig. 3, Fig. 2 and Fig. 1). At −2 C for 0.5h there was still a significant increase in CAT activity (‘Nonpareil’ +41% and ‘NePlus ’ +28%) (Fig. 3), but after that they decreased sharply. At −10 C, CAT activity and carotenoid content showed significantly the same decline tendency with treatment time (Fig. 3). There were close correlations between increase in MDA content and decrease in carotinoid content (Fig. 4 B) or CAT activity (Fig. 4 A). This suggested that induction of oxidative stress by low-temperature was related to a weakening in antioxidant defenses.

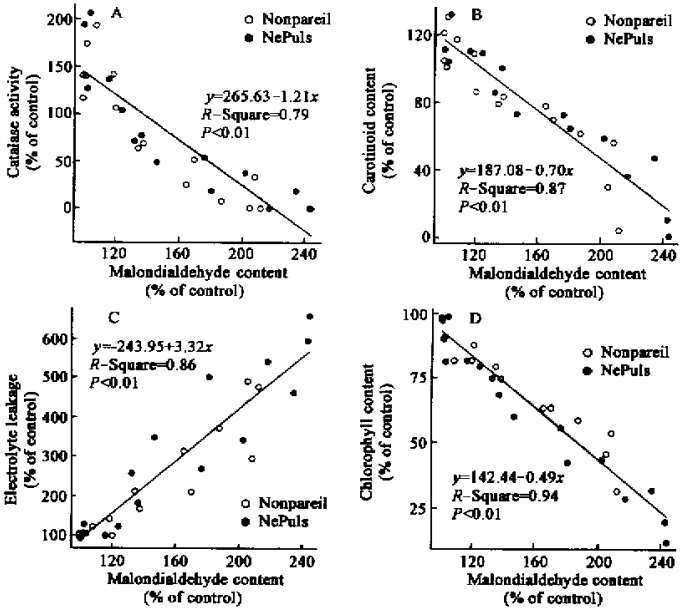

Fig. 4 Correlations between increase in MDA content and reductions of antioxidants levels [CAT activity (A) or caroteniod content (B)] in almond leaves; and correlations between increase in MDA content and low-temperature injuries [indicated by elevated electrolyte leakage (C) or reduction in chlorophyll content (D)] in almond leaves ($n=30$)

3.4 Correlation between cold resistance of leaves and shoots in almond

A preliminary study revealed that the cold resistance difference (as measured by electrolyte leakage) in leaves of 7 almond cultivars was more definite at nonlethal freezing temperature than at chilling or lethal freezing temperature (data not shown). We then determined the cold resistance of 7 almond cultivars by the variation of MDA content and electrolyte leakage in leaves after laboratory freezing test under -5℃ for 4h and 8h (Table 2). As shown in Table 2, the tolerance to cold stress in terms of oxidative damage (relative increment of MDA content) in almond leaves largely depends on cultivars: the higher resistance of 'Butte' and 'Padre' and the lower tolerance of 'NePlus' and 'Sonora'. Statistical correlation was found between MDA content and electrolyte leakage (for 4h, $R^2=0.897$, $p<0.01$; for 8h, $R^2=0.830$, $p<0.01$). Winter injure index of field plants and LT_{50} of current year shoots of 7 almond cultivars in winter in 1999～2001[27] again confirmed the higher resistance of 'Butte' and 'Padre' and the lower tolerance of 'NePlus' and 'Sonora' (Table 3). There were significant correlations between the cold resistance of leaves and that of plants (Fig. 5) in 7 almond cultivars.

4 Discussion

More recently, the induction of oxidative stress by low temperature has been proposed as a major contributing factor to cold responses, as a source of injury and as a signal to increase antioxidant defenses[13～18]. Our results tend to support this interpretation. In our study, an application of moderate chilling treatment (3～9h) to almond leaves had significantly elevated levels of CAT activity and carotinoid content, while there were no changes or only small changes in content of chlorophyll or MDA

and degree of electrolyte leakage.

Table 2　Effects of low-temperature treatments (−5 C for 4 and 8h) on MDA content and electrolyte leakage in leaves of seven almond cultivars

Cultivar	MDA content (nmol • g^{-1} FW)			Electrolyte leakage (%)		
	Control	−5 C, 4h	−5 C, 8h	Control	−5 C, 4h	−5 C, 8h
Butte	5.06±0.14+	5.94±0.12 (17.4)++	6.89±0.10 (36.1)	9.62±0.16	14.12±0.27 (46.8)	27.52±0.36 (186.1)
Padre	5.12±0.10	6.28±0.08 (22.6)	7.30±0.19 (42.6)	9.31±0.18	14.64±0.19 (57.3)	28.96±0.23 (211.1)
Price	4.83±0.11	6.36±0.11 (31.7)	7.54±0.22 (56.3)	9.95±0.13	17.60±0.28 (76.9)	30.53±0.35 (206.8)
Nonpareil	4.48±0.20	6.21±0.22 (38.6)	7.24±0.12 (61.5)	9.51±0.20	16.48±0.26 (73.3)	30.39±0.32 (219.6)
Monterey	4.58±0.12	6.13±0.14 (33.8)	7.73±0.12 (68.8)	9.60±0.17	16.80±0.34 (75.0)	31.23±0.41 (225.3)
Sonora	4.05±0.19	5.88±0.09 (45.3)	7.09±0.15 (75.1)	9.24±0.19	19.95±0.35 (115.9)	33.85±0.51 (266.3)
NePlus	4.35±0.07	6.58±0.20 (51.3)	7.93±0.13 (82.2)	8.96±0.22	19.30±0.49 (115.4)	32.67±0.55 (264.6)

Note: + mean±SD, $n=4$　　++ Relative increment (+% of control)

Severe freezing treatments (−2 C and −10 C) caused considerable injuries in almond leaves as measured by decrease in chlorophyll content and increase in electrolyte leakage, and enhanced oxidative stress as indicated by increase in MDA content and decrease in levels of antioxidants (CAT activity and carotenoid content). These results may serve as another evidence for postulation that chilling tolerance was associated with treatments that increased the levels of antioxidants.

Table 3　Survey of LT_{50} and winder injure index in seven almond cultivars

Cultivar	LT_{50}(C)	Winter injure index (%)
Butte	−24.4	29.4±1.99
Padre	−24.5	30.9±1.70
Price	−21.2	41.4±2.78
Nonpareil	−20.7	41.6±1.59
Monterey	−20.9	37.1±1.67
Sonora	−18.0	67.5±3.61
NePlus	−18.5	68.1±3.02

Oxidative free radicals can be highly reactive toward cell components, and therefore, the ability of the cell to remove these undesirable species might be view as an important feature in improved resistance to low temperature stress. CAT (and peroxidase, POD) appeared to be an important H_2O_2-scavenging enzyme in plant cells. Cold acclimation of maize seedlings at 14 C for 3d led to an increase in the expression of Cat3mRNA and catalase enzyme activity and induced low temperature tolerance[15~17]. Additionally, the plant growth regulator paclobutrazol has been reported to increase antioxidant levels in tomato plant, which was correlated with a reduction in chilling induced degradation of membrane lipids[28]. Increased activities of oxidative stress defense enzymes following treatment with the growth regulator uniconazol were associated with enhanced chilling tolerance in cucumber seedlings[29]. Kuroda and Sagisaka[13] also reported that H_2O_2 produced during freezing and thawing rendered the peroxide-scavenging systems dysfunctional in apple flower buds, indicating that freezing injury is associated with the accumulation of H_2O_2. Carotenoid is another important noenzymes antioxidants. Gao *et al*[30]. showed that under low concentration of Na_2SO_4 stress for a short time the content of carotinoid in *Festuca arundinacea* and *Seriphidium kokanianum* tended to increase; while high concentration of Na_2SO_4 or a long time treatment caused a decrease in carotinoid content. Another study[31] showed that the content of chlorophyll

in conifer was degraded to various extents in winter while the carotenoid was still synthesized during the soil-frozen period. Taken together, these results suggest that, during adaptation to low temperatures, moderate increases in oxidative stress increase oxidative stress defense mechanisms and the tolerance of plant to low temperatures. Thus, oxidative stress plays a dual role in low-temperature responses, as a source of injury and as a signal to increase antioxidant defenses.

Additionally, it is clear that the response of antioxidants to an environmental stress depends on the severity of stress and on the species and age of plants. But there is also a differential sensitivity of cultivars to low-temperature stress with respect to the induction of oxidative stress. Our study indicated, at the biochemical level, 'Nonpareil' is more tolerant to low temperature than 'Ne Plus Ultra'; the decrease in antioxidant protection and the increase in oxidative damage are closely related. The study of isoenzymes for almond cultivar identification also showed that catalase was one of the enzymatic systems with the highest discriminating possibilities, especially for 'Nonpareil' and 'NePlus'[32].

In summary, moderate chilling treatments (3℃ within 9h) did not cause obvious damages in almond leaves and this was accompanied by enhanced levels or activities of several antioxidants including catalase and carotenoid. More severe freezing treatments (−2℃ and −10℃) led to elevated lipid peroxidation significantly, which was due to , at least in part, a weakening in antioxidants defenses, and correlated to low-temperature injuries as indicated by reduction in chlorophyll and enhanced electrolyte leakage. These results provide some evidence for the view of earlier studies[13~18] that the induction of oxidative stress by low temperature has been proposed as a major contributing factor to cold responses, as a source of injury and as a signal to increase antioxidant defenses[13~18]. Our results also suggested that there is a differential sensitivity of almond cultivars to low-temperature stress with respect to the induction of oxidative stress (Table 2). These differences can be used as criteria for determination or selection of cold handiness almond cultivars.

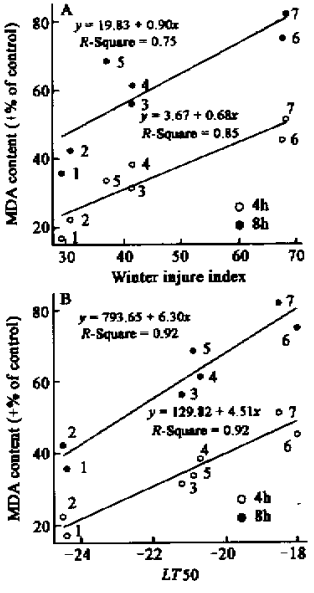

Fig. 5 Correlations between cold resistance of leaves (measured by relative content of MDA in leaves under −5℃ treatment for 4h and 8h) and that of plants (measured by winter injure index of field plants[A] and LT_{50} of current year shoots[B] in winter in 1999～2001[27]) in seven almond cultivars. ($n=7$) 1-Butter, 2-Padre, 3-Price, 4-Nonpareil, 5-Monterey, 6-NePus and 7-Sonora

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