African Journal of Agricultural Research Vol. 7(22), pp. 3349-3359, 12 June, 2012
Available online at http://www.academicjournals.org/AJAR
DOI: 10.5897/AJAR11.1481
ISSN 1991-637X ©2012 Academic Journals

Full Length Research Paper

Chilling tolerance evaluation, and physiological and ultrastructural changes under chilling stress in tobacco

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Accepted 23 April, 2012

The physiological and ultrastructural changes of tobacco varieties with different chilling tolerances under chilling stress were investigated in this study. Twenty tobacco varieties were separated into three groups; chilling tolerant, intermediate chilling tolerant, and chilling sensitive varieties, with eight cluster analysis methods based on seven morphological parameters measured at low temperature. After a chilling stress for five days at 11°C, the activities of ascorbate peroxidase (APX), peroxidase (POD), superoxide dismutase (SOD), the concentrations of malondialdehyde (MDA), hydrogen peroxide (H_2O_2), superoxide anion radical (O_2^-), chlorophyll 'a' and 'b', total soluble sugar, and soluble protein were measured in the three varieties with different chilling tolerance. The cell ultrastructure of the three varieties was observed by transmission electron microscope. The results indicated that the values of APX, POD, SOD, MDA, H_2O_2, O_2^-, total soluble sugar, and soluble protein were increased under chilling stress, while the values of chlorophylls a and b were decreased in all the three varieties. The values of APX, POD, SOD, total soluble sugar, and protein in chilling tolerant variety were higher than chilling sensitive variety, the values of MDA, H_2O_2, O_2^-, and chlorophyll a and b in chilling tolerant variety were lower than those in chilling sensitive variety at 11°C. The separation of the cell membranes and chloroplasts from the cell wall along with an increase in the number of lipid droplets as well as the depletion of starch granules was observed in all tobacco varieties under the chilling stress, and these ultrastructural changes in chilling sensitive variety were more visible than chilling tolerant and intermediate chilling tolerant varieties.

Key words: Tobacco, chilling stress, physiological changes, ultrastructure.

INTRODUCTION

Tobacco (*Nicotiana tabacum* L.) is a warm season crop that grows best in warm days of 25 to 28°C, and stops growing when the temperature is lower than 10 to 13°C (Han, 2003). Chilling injury in tobacco is a common problem in China, especially in the southwest area. Long-term exposures of chilling-sensitive plants to low temperatures results in photo-oxidation, an oxygen-dependent bleaching of the carotenoid and chlorophyll pigments (Powels, 1984). Active oxygen species (AOS), such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2), were proven to play very important roles in photo-oxidation (Wise and Naylor, 1987). The enzymes involved in the scavenging of these AOS are ascorbate peroxidase (APX), peroxidase (POD), superoxide dismutase (SOD), etc. These enzymes could catalyze the disproportionation of superoxide to H_2O_2 and O_2^-, and play important roles for protection against superoxide derived (Fridovich, 1986). Gemel et al. (1986) reported that low temperature induced changes in chloroplast ultrastructure in relation to changes of hill reaction activity, manganese, and free fatty-acid levels in chloroplasts of tobacco. Tognetti et al. (2006) investigated the role of flavodoxin in chloroplasts under low temperature stress and the immunolocalization of flavodoxin in chloroplasts

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of mesophyll cells of tobacco. Additionally, low temperature induced changes in chloroplast of transgenic tobacco were studied by Liu et al. (2010). Although the ultrastructural changes in tobacco under low temperature have been described, the difference of these changes between tobacco varieties with different chilling tolerance is obscure. In addition, physiological changes of tobacco seedling under chilling stress had been reported in many papers (Han, 2003; Tognetti et al., 2006); however, little research is focused on the relation between physiological and ultrastructural changes under low temperature stress.

Cluster analysis is a useful method with objective classification. Many cluster analyses were conducted with a single cluster method (Hong and Hou, 2001). However, there are no unified standards for selecting a cluster method, and clustering results differ quite significantly in different cluster analysis methods, though it is not prudent to get the consequence by single cluster method. For this reason, in the present study, eight common cluster methods were used to compare the cold tolerance of 20 tobacco varieties. The objectives of this research are: to classify tobacco varieties according to their chilling tolerance using multiple cluster methods in order to ensure reliable clustering results; to view ultrastructural changes caused by low temperature in chloroplasts of tobacco varieties with different chilling tolerance; and to investigate the effect of low temperature on tobacco seedling, physiological traits, and the relation between physiological and ultrastructural changes.

MATERIALS AND METHODS

Plant materials

Seeds of 20 tobacco varieties: MD-609, MS Yunyan87 (MS Y-87), NC55, Yunyan 203 (Y-203), Yunyan 100 (Y-100), G-28, K346, TN90, MS K326, NC297, Yunyan 201 (Y-201), Yunyan 202 (Y-202), V2, RGH51, Basima 11 (BSM-11), MS Yunyan85 (MS Y-85), NC102, Yunyan 97 (Y-97), TN86-8, and Honghua dajinyuan (HHDJW) were supplied by Yunnan Provincial Academy of Tobacco Agricultural Sciences, China.

Seed germination test under low temperature stress

Tobacco seeds were sterilized in 0.5% sodium hypochlorite solution for 15 min, and then rinsed three times in distilled water. After that, three replicates of 50 seeds for each variety were placed in 9 cm Petri dishes containing two layers of moistened blotters with distilled water. The Petri dishes were then kept in a germination chamber under alternating cycle of 12 h of light with light intensity of 4000 lux and 12 h darkness for 16 days. In the first 7 days, the temperature of the chamber was 30°C (light)/20°C (darkness), then followed a low temperature stress at 11°C for 5 days. Finally, the temperature returned from 30 to 20°C for 4 days for a recovery period. The low temperature stress was set at 11°C, which can inhibit but not cease the germination and growth of tobacco seeds and seedlings (Li et al., 2009).

The germinated seeds were recorded every day, germination percentage (the germinated seeds relative to the total number of tested seeds), germination index (GI = ∑(Gt/Dt), where Gt is the number of the germinated seeds on day t, Dt is time corresponding to Gt in days) and vigor index (VI = S × GI, where GI is the germination index, S is average seedling length) were estimated on the 16th day (Hu et al., 2005). The root and seedling length, fresh and dry weight of seedlings were also measured on the 16th day. Three replicates of 30 seedlings for each parameter were used.

Cluster analysis of tobacco varieties

The cluster analysis was conducted with the seven morphological parameters (GP, GI, VI, seedling length, root length, seedling fresh weight, and seedling dry weight). To minimize the genetic background difference of different tobacco varieties, "relative value" of these parameters was calculated based on the formula: relative value = x_L/x_N, where x_L is the value measured under low temperature and x_N is the value measured under normal temperature (Hodges et al., 1997). The tobacco varieties were then clustered according to the values measured under normal temperature and low temperature, as against the relative value. Cluster analyses were conducted with eight common methods (Centroid, Single, Average, Median, Flexible, McQuitty, Ward, and Complete) in SAS software. Euclidian distance was used in these eight procedures, and all variables were standardized to make mean 0 and standard deviation 1 by linear transformation.

Measurement of physiological changes in tobacco seedlings under low temperature stress

The shoots and roots of the tobacco seedlings, during germination, lasted for 16 days and gave five days chilling stress, used to determine the levels of antioxidant enzyme activities and malondialdehyde (MDA) concentration (Mai et al., 2009). Samples (0.3 g) were homogenized in 4 ml extraction buffer consisting of 50 mM L^{-1} phosphate (pH 7.8). The homogenate was centrifuged at 4°C for 20 min at 12000 ×g and the resulting supernatants were used for determination of enzyme activity and MDA level by using a spectrophotometer (UV-2450, SHIMADZU, Japan).

APX activity was measured according to Nakano and Asada (1981). 0.1 ml of extract was incubated with 2.9 ml of mix buffer (pH 7.0, 25 mM phosphate and pH 7.5, 7.5 mM ascorbate) at 25 ± 2°C. The reaction was started by adding 50 µl 10 mM H_2O_2. APX activity was determined as a decrease in absorbance at 290 nm for 30 s due to ascorbate oxidation. APX activity was calculated using an extinction coefficient of 2.8 $mM^{-1}cm^{-1}$. POD activity was assayed with guaiacol as the substrate in a total volume of 3 ml according to Zhang (1992). The reaction mixtures consist of 25 mM L^{-1} phosphate buffer (pH 7.0), 1.5% guaiacol, 0.4% H_2O_2, and 0.2 ml of enzyme extract. Increase in the absorbance due to oxidation of guaiacol (E = 25.5 mM L^{-1} cm^{-1}) was measured at 470 nm for 30 s. Enzyme activity was calculated in terms of µM of guaiacol oxidized g^{-1} FW min^{-1} at 25 ± 2°C. SOD activity was measured by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) according to Rao and Sresty (2000). The reaction mixture was 3 ml, which contained 50 mM L^{-1} phosphate buffer (pH 7.8), 13 mM L^{-1} methionine, 75 µM L^{-1} NBT, 2 µM L^{-1} riboflavin, 0.1 Mm L^{-1} EDTA, and 0.1 ml of enzyme extract. Reaction was started by adding 2 µM L^{-1} riboflavin and placing the reaction tubes under 15 W fluorescent lamps for 15 min. A complete reaction mixture without enzyme extract served as a control. Reaction was stopped by switching off the lamp. The photoreduction of NBT was measured at 560 nm and one unit of SOD was defined as being present in the volume of the extract that caused inhibition of the photoreduction of NBT by 50%.

MDA concentration was determined as 2-thiobarbituric acid

(TBA) reactive metabolites according to Hu et al. (2005). 1.5 ml extract were homogenized in 2.5 ml of 5% TBA made in 5% trichloroacetic acid (TCA). The mixture was heated at 95°C for 15 min, and then quickly cooled on ice. After centrifugation at 5000 ×g for 10 min, the absorbance of the supernatant was measured at 532 nm. Correction of non-specific turbidity was made by subtracting the absorbance value measured at 600 nm. The concentration of MDA was calculated in terms of nM of g^{-1} FW. The concentrations of H_2O_2 and O_2^- were measured according to Brennan and Frenkel (1977) and Wang and Luo (1990). Total soluble protein and sugar concentration were measured in accordance with Souza et al. (2004) and Bradford (1976). Chlorophyll a and b were measured in 80% acetone extracts by the method of Arnon (1949).

Ultrastructural changes in tobacco seedlings under low temperature stress

Ultrastructural changes of tobacco seedlings under chilling stress were observed using the seedlings cultured for 16 days, small pieces (1 × 2 mm) were cut from the middle region of the leaves, and immediately fixed in cold 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.6), vacuum-infiltrated for 10 min, and fixed in fresh fixative at 4°C for 4 h. Specimens were subsequently buffer-washed three times, post fixed in 1% OsO_4 (0.1 M phosphate, 4 h) and dehydrated in a graded ethanol series. They were then infiltrated via a propylene oxide series and embedded in Araldite. Sections were cut using a diamond knife, post-stained for 10 min in 1.5% uranyl acetate in methanol, 10 min in lead citrate, and examined with a Transmission Electron Microscope (JEM-1230 JEOL, Japan).

Statistical analysis

Statistical analysis of all data was done by means of analysis of variance (ANOVA) using SAS version 8.0 software (SAS Institute, Cary, NC). Fisher's least significant difference (LSD) tests ($P < 0.05$) were adopted for multiple comparisons. Percent data were transformed according to $y = arcsin[sqr(x/100)]$ before ANOVA.

RESULTS

Changes of seed germination and seedling characteristics under low temperature stress

The germination percentages of all varieties surpassed 75% at normal temperature (20 to 30°C), and their germination percentages decreased when they undergo chilling stress except the variety TN86-8 (Table 1). The values of relative germination percentage in HHDJW, TN86-8, Y-97, Y-201, BSM-11, and NC102 were higher than in Y-100. Meanwhile, the germination index and vigor index of all the varieties were reduced after chilling stress. The values of relative germination index in HHDJW and BSM-11 were higher than other varieties, and the value in K346 was the lowest. As for the relative vigor index, the values in HHDJW, BSM-11, and V2 were higher than in MS Y-87, Y-203, Y-100, and K346.

The seedling length, root length, seedling fresh weight, and seedling dry weight decreased in all varieties when they undergo chilling stress (Tables 2 and 3). The relative root length in TN90, RGH51, and BSM-11 were higher than in MD-609, MS Y-85, MS Y-87, and MS K326, the relative seedling length in V2, RGH51 and BSM-11 were higher than in MD-609, MS Y-85, MS Y-87, MS K326, NC55 and NC102 (Table 2). On the other hand, NC297 had the highest and MS K326 had the lowest value in relative seedling fresh weight, while HHDJW had the highest and NC102 had the lowest value in relative seedling dry weight (Table 3).

Cluster results

Cluster analyses were conducted and results were obtained for the seed germination and seedling growth (Tables 1 to 3). All varieties were clustered into three groups using each cluster method, and the results were summarized and displayed in a single table instead of eight cluster analysis trees (Table 4). As expected, the eight cluster analysis methods did not show a good correspondence in their cluster division. Results from the cluster methods of Flexible, McQuitty, Ward and Complete were the same, while results from the methods of Centroid, Single, Average, and Median differed markedly from each other. It was clear that the cluster results from methods of Centroid, Single, Average, and Median were unreasonable. The results from methods of Flexible, McQuitty, Ward and Complete were more reliable than the other four cluster methods. The tobacco varieties might be classified into three groups according to the methods of Flexible, McQuitty, Ward, and Complete: chilling sensitive varieties (MD-609, MS Y-87, NC55, Y-203, Y-100, G-28, K346, and TN90); intermediate chilling tolerant varieties (MS K326, NC297, Y-201, Y-202, V2, RGH51, and BSM-11); and chilling tolerant varieties (MS Y-85, NC102, Y-97, TN86-8, and HHDJW). Therefore, MS Y-87, V2, and HHDJW were used to investigate their physiological and ultrastructural changes, and they may represent the chilling tolerant, intermediate chilling tolerant, and chilling sensitive varieties, respectively.

Physiological changes in tobacco seedlings under low temperature stress

The activities of APX and POD were significantly higher in variety HHDJY than in MS Y-87 and V2 at normal temperature (Figure 1), while the concentration of MDA in HHDJY was significantly lower than in MS Y-87 and V2 (Figure 2). There was no significant difference in the activity of SOD between HHDJY and MS Y-87 at normal temperature. All the values of APX, POD, SOD and MDA were increased at low temperature, and the activities of APX and POD in HHDJY were still significantly higher than in MS Y-87 and V2, the concentration of MDA in HHDJY was still lower than in MS Y-87 and V2 at low temperature.

The concentrations of H_2O_2 and O_2^- were significantly

Table 1. Changes of germination percentage, germination index, and vigor index in tobacco under low temperature stress.

Variety	Germination percentage (%)		Relative Germination percentage	Germination index (GI)		Relative germination index	Vigor index (VI)		Relative vigor index
	Normal temperature	Low temperature		Normal temperature	Low temperature		Normal temperature	Low temperature	
MD-609	75.3_{i*}	74.0^{n}	0.982	10.67^{j}	9.51^{g}	0.891	9.85^{i}	6.55^{l}	0.666
MS Y-85	$94._{abc}$	88.0^{fgh}	0.93	16.86^{abcd}	14.91^{c}	0.884	23.93^{c}	15.57^{cd}	0.651
MS Y-87	85.3_{efgh}	81.3^{kl}	0.953	14.42^{ghi}	11.79^{de}	0.818	13.58^{gh}	8.29^{k}	0.612
MS K326	92.7_{abcde}	91.3^{def}	0.986	16.49^{bcde}	15.38^{bc}	0.933	17.58^{de}	12.1^{fg}	0.689
NC55	86.0_{fgh}	82.7^{jk}	0.961	14.55^{gh}	12.81^{d}	0.893	14.04^{fgh}	9.17^{jk}	0.669
NC102	95.3_{abcd}	94.7^{abc}	0.993	17.28^{ab}	15.57^{bc}	0.901	24.52^{c}	16.51^{c}	0.672
NC297	94.7_{abcd}	$92.0c^{de}$	0.972	16.72^{abcd}	14.41^{c}	0.862	17.39^{de}	11.73^{gh}	0.675
Y-97	96.0^{ab}	$95.^{3ab}$	0.993	17.58^{ab}	16.50^{ab}	0.939	25.87^{bc}	18.45^{b}	0.713
Y-201	88.3^{cdefgh}	88.0^{fgh}	0.996	15.61^{defg}	14.32^{c}	0.917	15.80^{ef}	11.67^{gh}	0.738
Y-202	94.0^{abcdef}	92.7^{cde}	0.986	16.47^{bcde}	14.63^{c}	0.888	17.60^{de}	12.30^{fg}	0.699
Y-203	80.7^{h}	79.3^{lm}	0.983	13.17^{i}	10.15^{fg}	0.771	12.82^{h}	8.11^{kl}	0.635
Y-100	84.0^{gh}	77.3^{mn}	0.921	14.06^{hi}	10.98^{ef}	0.781	14.63^{fg}	9.40^{ijk}	0.643
G-28	87.3^{defgh}	84.0^{jk}	0.962	14.65^{fgh}	$12.19d^{e}$	0.832	14.89^{fg}	10.26^{hij}	0.688
V2	96.0^{ab}	94.0^{abcd}	0.979	16.79^{abcd}	15.23^{bc}	0.907	17.67^{de}	13.59^{ef}	0.770
K346	89.3^{bcdefg}	83.3^{jk}	0.933	15.22^{efgh}	11.52^{def}	0.757	17.46^{de}	10.86^{gh}	0.624
TN90	90.7^{abcdef}	85.3^{hij}	0.941	15.00^{fgh}	11.68^{de}	0.779	15.88^{ef}	10.32^{hij}	0.650
TN86-8	97.3^{a}	97.3^{a}	1.00	17.86^{a}	15.67^{bc}	0.877	28.80^{a}	20.74^{a}	0.720
RGH51	94.0^{abcde}	89.3^{efg}	0.95	16.94^{abc}	14.63^{c}	0.864	19.14^{d}	14.00^{de}	0.732
HHDJW	96.7^{a}	96.0^{a}	0.993	17.72^{ab}	17.39^{a}	0.981	27.12^{ab}	21.55^{a}	0.797
BSM-11	88.0^{efgh}	87.3^{ghi}	0.992	15.86^{cdef}	15.51^{bc}	0.978	18.02^{d}	15.59^{cd}	0.868

lower in variety HHDJY than in MS Y-87 and V2 at normal or low temperature (Figure 3), and the concentration of H_2O_2 in MS Y-87 was approximately two times higher than in HHDJY. Both concentrations of H_2O_2 and O_2^- were increased after exposure to low temperature, and the concentrations of H_2O_2 and O_2^- in V2 was significantly lower than in MS Y-87. The concentrations of chlorophyll a and b were significantly lower in variety HHDJY than in MS Y-87 at normal or low temperature (Figure 4), and the concentrations of chlorophyll a in variety V2 were between the values of MS Y-87 and HHDJY at normal or low temperature.

The concentrations of total soluble sugar were significantly higher in variety HHDJY and V2 than in MS Y-87 at normal or low temperature (Figure 5). There was no significant difference in the concentration of soluble sugar among MS Y-87, V2, and HHDJY at normal temperature. The concentration of soluble sugar in V2 and HHDJY were significantly lower than in MS Y-87. Both concentrations of total soluble sugar and protein were increased at low temperature and the concentrations of total soluble sugar and protein in HHDJY were still the highest in the three varieties at low temperature. Additionally, the concentrations of total soluble sugar and protein in V2 were between the values of HHDJY and MS Y-87 at normal or low temperature.

Ultrastructural changes in tobacco seedling under chilling stress

The membranes were complete and clung to cell walls in the three varieties under normal condition (Figure 6). However, the membranes separated from the wall and formed a so-called “apoplastic

Table 2. Changes of root and seedling length in tobacco under low temperature stress.

Variety	Root length (mm)		Relative root length	Seedling length (mm)		Relative seedling length
	Normal temperature	Low temperature		Normal temperature	Low temperature	
MD-609	5.80^{i}	4.21^{j}	0.73	9.27^{g}	6.89^{m}	0.74
MS Y-85	8.93^{c}	6.41^{bc}	0.72	14.19^{b}	10.50^{bcd}	0.74
MS Y-87	5.86^{hi}	4.35^{ij}	0.74	9.42^{fg}	7.05^{lm}	0.75
MS K326	6.67^{defg}	4.92^{ghi}	0.74	10.65^{cde}	7.87^{jkl}	0.74
NC55	6.01^{ghi}	4.56^{hij}	0.76	9.62^{efg}	7.21^{klm}	0.75
NC102	8.87^{c}	6.62^{b}	0.75	14.20^{b}	10.6^{bc}	0.75
NC297	6.50^{efghi}	5.12f^{gh}	0.79	10.41^{cdef}	8.15^{hij}	0.78
Y-97	9.12^{bc}	6.93^{b}	0.76	14.74^{b}	11.2^{b}	0.76
Y-201	6.33^{fghi}	5.26^{efg}	0.83	10.12^{efg}	8.16h^{ij}	0.81
Y-202	6.68^{defg}	5.44^{defg}	0.81	10.69^{cde}	8.41^{hij}	0.79
Y-203	6.12^{ghi}	5.29d^{efg}	0.86	9.73^{efg}	7.99^{ijk}	0.82
Y-100	6.54^{defgh}	5.60^{def}	0.86	10.47^{cdef}	8.61^{ghij}	0.82
G-28	6.37^{efghi}	5.47d^{efg}	0.86	10.19^{defg}	8.41^{hij}	0.83
V2	6.60^{defg}	5.82^{cde}	0.88	10.53^{cdef}	8.92^{fgh}	0.85
K346	7.19^{d}	6.31^{bc}	0.88	11.48^{c}	9.46^{efg}	0.82
TN90	6.58^{defg}	5.92^{cd}	0.90	10.59^{cde}	8.83^{fghi}	0.83
TN86-8	10.10^{a}	8.64^{a}	0.86	16.11^{a}	13.20^{a}	0.82
RGH51	7.02^{def}	6.35b^{c}	0.90	11.30^{cd}	9.58^{def}	0.85
HHDJW	9.66^{ab}	8.16^{a}	0.84	15.30^{ab}	12.40^{a}	0.81
BSM-11	7.08^{de}	6.89^{b}	0.97	11.35^{c}	10.00^{cde}	0.88

*In each column, different letters behind data indicated significant treatment differences (P < 0.05) among 20 varieties at the same temperature for Fisher's LSD tests, data were the mean values of three replicates.

Table 3. Changes of seedling fresh and dry weight in tobacco under low temperature stress.

Variety	Seedling fresh weight (mg/plant)		Relative seedling fresh weight	Seedling dry weight (mg/plant)		Relative seedling dry weight
	Normal temperature	Low temperature		Normal temperature	Low temperature	
MD-609	12.75^{cd}	7.02^{de}	0.55	1.16^{bcde}	0.67^{cde}	0.58
MS Y-85	15.72^{ab}	9.98^{a}	0.63	1.43^{ab}	0.98^{ab}	0.69
MS Y-87	11.93^{d}	7.19^{de}	0.60	1.13^{cde}	0.59^{de}	0.52
MS K326	14.52^{abcd}	7.30^{cde}	0.50	1.29^{bcde}	0.66^{cde}	0.51
NC55	11.79^{d}	7.63^{cde}	0.65	1.08^{e}	0.6^{de}	0.56
NC102	15.76^{ab}	8.72a^{bcd}	0.50	1.44^{ab}	0.68^{cde}	0.47
NC297	12.77^{cd}	9.11^{abc}	0.71	1.18^{bcde}	0.66^{cde}	0.56
Y-97	15.15^{abc}	9.76^{ab}	0.64	1.38^{abcd}	0.70^{cde}	0.51
Y-201	12.91^{bcd}	7.35^{cde}	0.57	1.19^{bcde}	0.73^{cde}	0.61
Y-202	13.44^{bcd}	7.30^{cde}	0.54	1.17^{bcde}	0.59^{e}	0.5
Y-203	12.36^{cd}	6.66^{e}	0.54	1.10^{de}	0.57^{e}	0.52
Y-100	12.42^{cd}	7.53^{cde}	0.61	1.15^{bcde}	0.59^{de}	0.51
G-28	12.91^{bcd}	6.63^{e}	0.51	1.18^{bcde}	0.68^{cde}	0.58
V2	13.21^{bcd}	8.34^{abcde}	0.63	1.22^{bcde}	0.66^{cde}	0.54
K346	12.65^{cd}	7.03^{de}	0.56	1.12^{cde}	0.56^{e}	0.50
TN90	13.19^{bcd}	6.98^{de}	0.53	1.21^{bcde}	0.61^{de}	0.50
TN86-8	16.99^{a}	10.0^{9a}	0.59	1.59^{a}	0.7^{9bc}	0.50
RGH51	14.27^{abcd}	7.52^{cde}	0.53	1.25^{bcde}	0.68^{cde}	0.54
HHDJW	14.71^{abcd}	9.72^{ab}	0.66	1.39^{abc}	1.01^{a}	0.73
BSM-11	14.22^{abcd}	8.00^{bcde}	0.56	1.25^{bcde}	0.75^{cd}	0.60

*In each column, different letters behind data indicated significant treatment differences (P < 0.05) among 20 varieties at the same temperature for Fishers LSD tests, data were the mean values of three replicates.

Table 4. Comparisons of eight cluster analysis methods to classify 20 tobacco varieties according to seven morphological parameters changes under low temperature stress.

Variety	Centroid*	Single	Average	Median	Flexible	McQuitty	Ward	Complete
*MD-609***	I***	I	I	I	I	I	I	I
MS Y-87	I	I	I	III	I	I	I	I
NC55	I	I	I	III	I	I	I	I
Y-203	I	I	I	III	I	I	I	I
Y-100	I	I	I	III	I	I	I	I
G-28	I	I	I	III	I	I	I	I
K346	I	I	I	III	I	I	I	I
TN90	I	I	I	III	I	I	I	I
MS K326	I	I	I	III	II	II	II	II
NC297	I	I	I	III	II	II	II	II
Y-201	I	I	I	III	II	II	II	II
Y-202	I	I	I	III	II	II	II	II
V2	I	I	I	III	II	II	II	II
RGH51	I	I	I	III	II	II	II	II
BSM-11	II	I	I	III	II	II	II	II
MS Y-85	III	III	II	II	III	III	III	III
NC102	III	I	III	III	III	III	III	III
Y-97	III	I	III	III	III	III	III	III
TN86-8	III	I	III	III	III	III	III	III
HHDJW	III	II	III	III	III	III	III	III

*Centroid = centroid hierarchical cluster analysis, Single = single linkage cluster analysis, Average = average linkage cluster analysis, Median = median hierarchical cluster analysis, Flexible = flexible-beta cluster analysis, McQuitty = McQuitty's similarity analysis, Ward = ward's minimum variance cluster analysis, Complete = complete linkage cluster analysis. The first three groups from the cluster analysis are displayed. **Italic variety names indicate group one (chilling sensitive), bold variety names indicate group two (intermediate chilling tolerant), and the normal letters are group three (chilling tolerant) according to the cluster methods of Flexible, McQuitty, Ward, and Complete. ***I = group one, I = group two, I = group three. Cluster-division was according to seven morphological parameters (Tables 1 to 3).

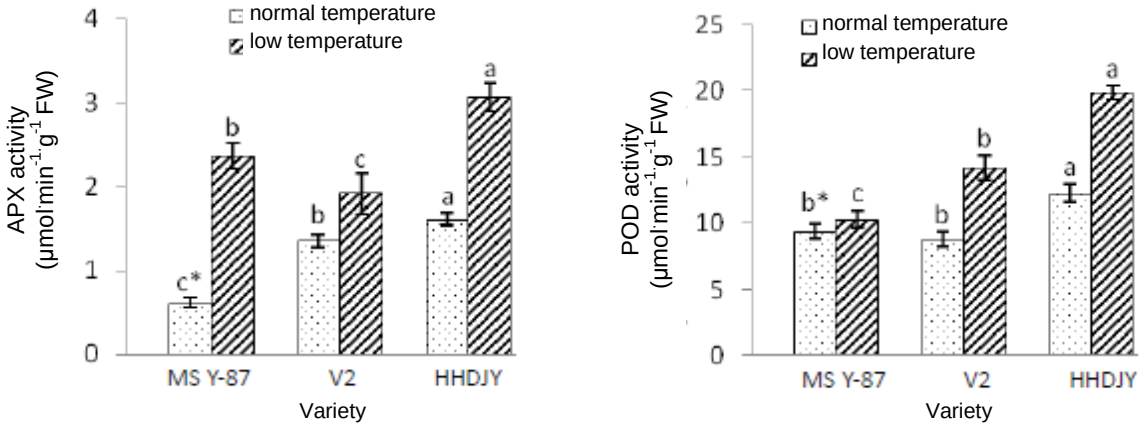

Figure 1. The activities of ascorbate peroxidase (APX) and peroxidase (POD) in seedlings of tobacco varieties with different chilling tolerance at normal (30/20°C) and low temperature (11°C). *In each graph, different letters indicated significant treatment differences ($P < 0.05$) among the three varieties at the same temperature for Fisher's LSD tests, mean values ± standard deviations of three replicates were shown.

space" when they undergo chilling stress. The apoplastic space in chilling-sensitive variety MS Y-87 was larger than other varieties. As for the variety MS Y-87, chloroplasts swelled and moved toward the center of cell

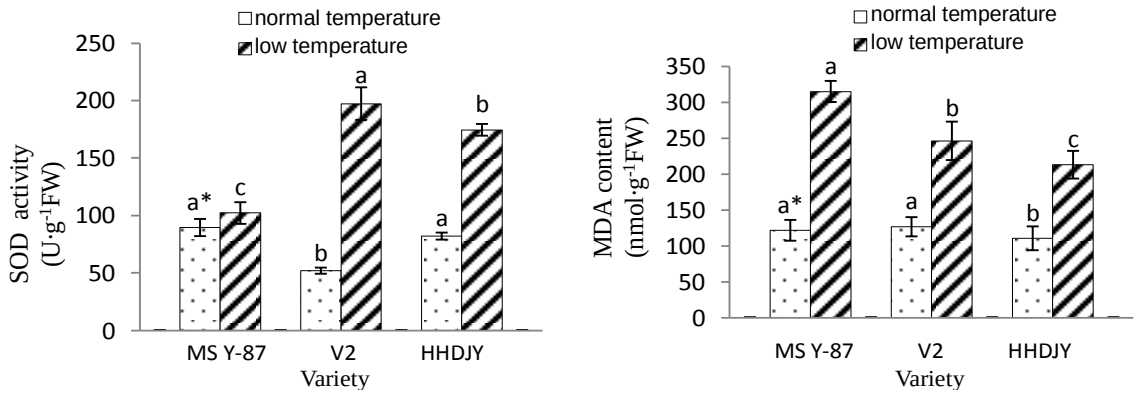

Figure 2. The activity of superoxide dismutase (SOD) and concentration of malondialdehyde (MDA) in seedlings of tobacco varieties with different chilling tolerance at normal and low temperature. *In each graph, different letters indicated significant treatment differences ($P < 0.05$) among the three varieties at the same temperature for Fisher's LSD tests, mean values ± standard deviations of three replicates were shown.

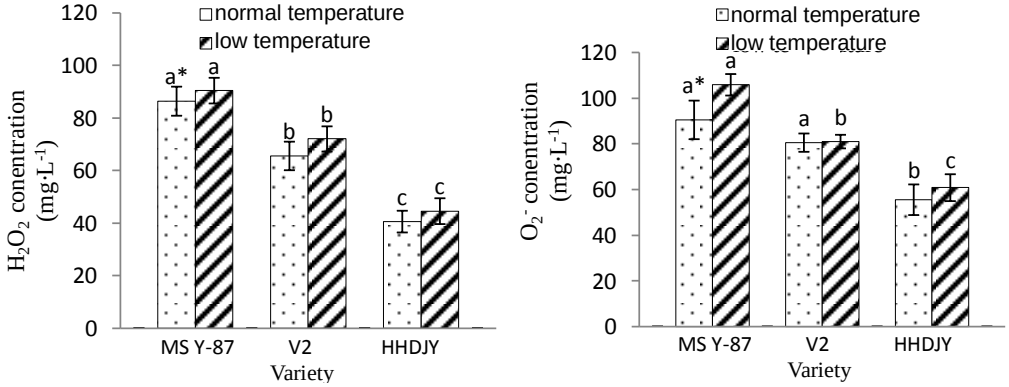

Figure 3. The concentrations of hydrogen peroxide (H_2O_2) and superoxide anion radical (O_2^-) in seedlings of tobacco varieties with different chilling tolerance at normal and low temperature. *In each graph, different letters indicated significant treatment differences ($P < 0.05$) among the three varieties at the same temperature for Fisher's LSD tests, mean values ± standard deviations of three replicates were shown.

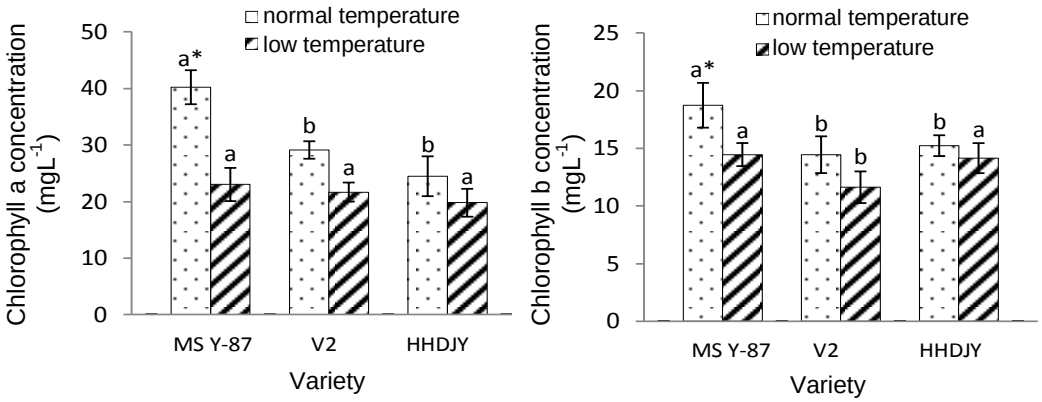

Figure 4. The concentrations of chlorophyll a and b in seedlings of tobacco varieties with different chilling tolerance at normal and low temperature. *In each graph, different letters indicated significant treatment differences ($P < 0.05$) among three varieties at the same temperature for Fisher's LSD tests, mean values ± standard deviations of three replicates were shown.

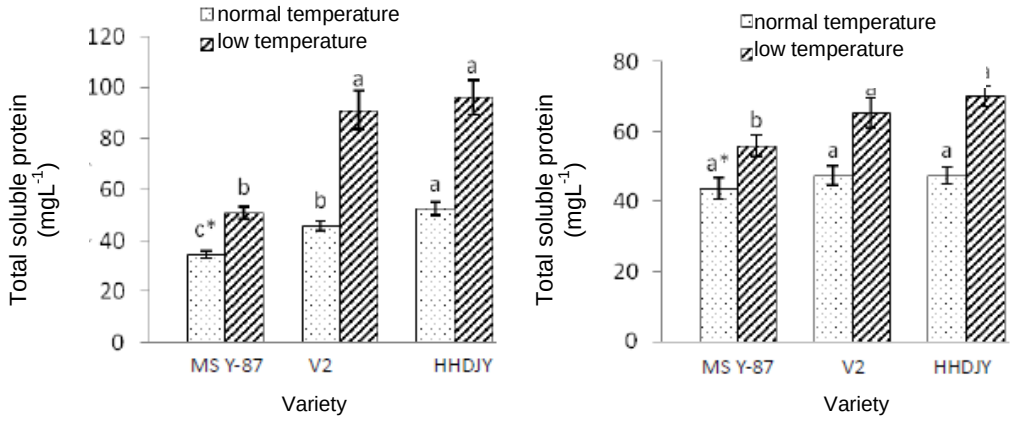

Figure 5. The concentrations of total soluble sugar and protein in seedlings of tobacco varieties with different chilling tolerance at normal and low temperature. *In each graph, different letters indicated significant treatment differences ($P < 0.05$) among the three varieties.

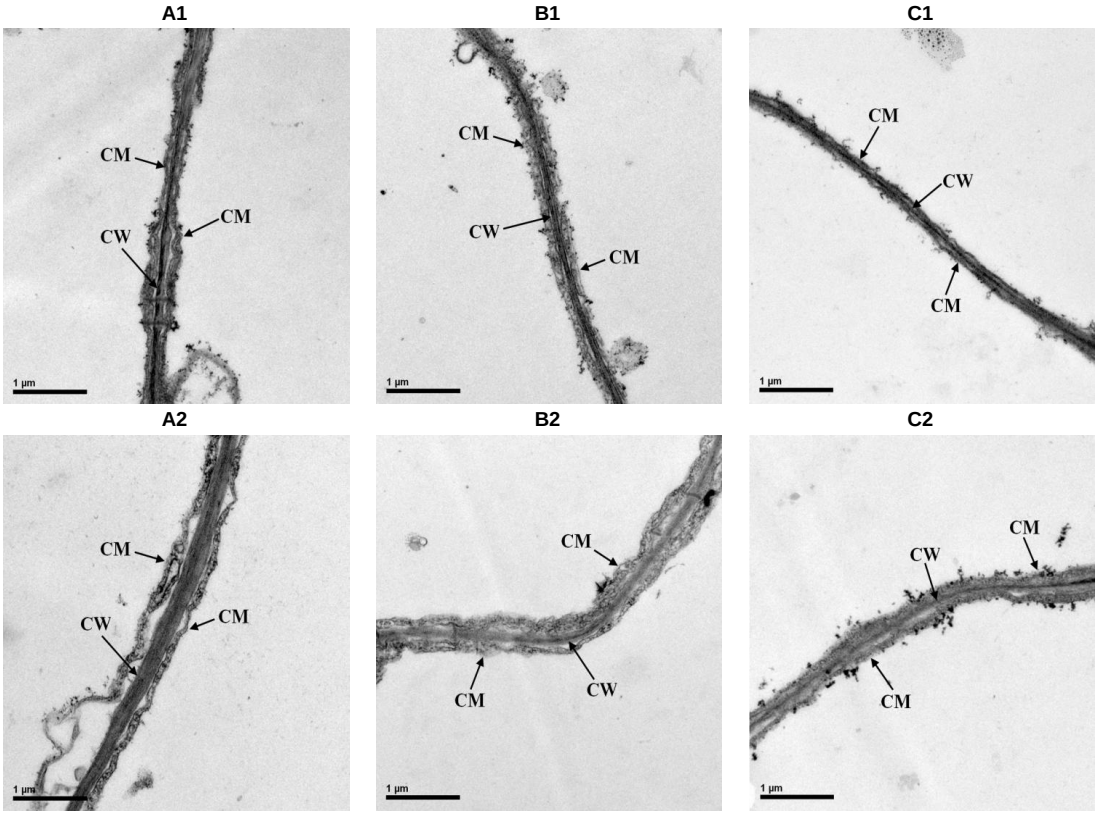

Figure 6. Ultrastructural changes of cell membranes in tobacco varieties A1: MS Y-87 at normal temperature and A2: MS Y-87 at low temperature, B1: V2 at normal temperature and B2: V2 at low temperature, and C1: HHDJY at normal temperature and C2: HHDJY at low temperature. CW: cell wall, CM: cell membrane.

at low temperature while the chloroplasts clung to the cell membranes at normal temperature, but there were no obvious differences of the chloroplasts between normal and low temperature in varieties of V2 and HHDJY (Figure 7). In addition, the structures of nucleus were intact in the three varieties and there were no significant differences between normal and low temperature in the three varieties.

The lipid droplets were increased in number in all chilled plants; however, the lipid droplets changes in size were not obvious (Figure 8). Starch granules in chloroplasts were depleted in the chilled plants,

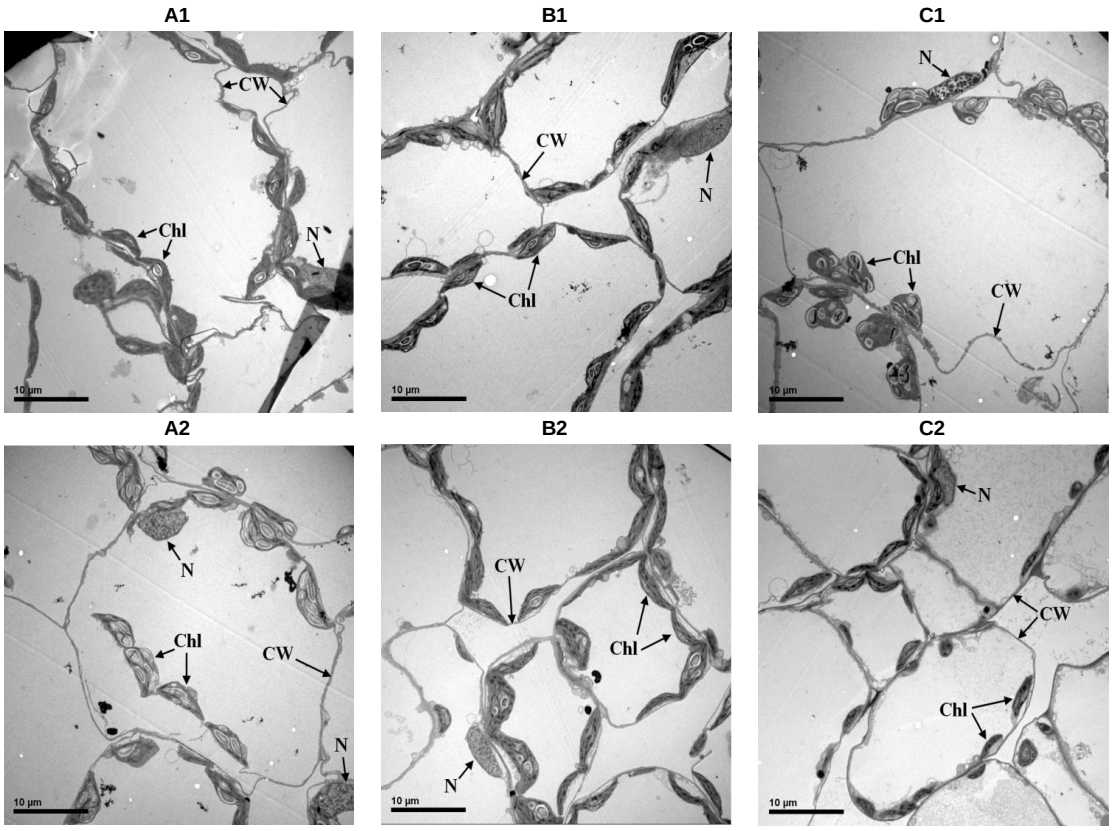

Figure 7. Ultrastructural changes of cells in tobacco varieties. A1: MS Y-87 at normal temperature and A2: MS Y-87 at low temperature, B1: V2 at normal temperature and B2: V2 at low temperature, and C1: HHDJY at normal temperature and C2: HHDJY at low temperature. N: nucleus, CW: cell wall, Chl: chloroplast.

especially in the varieties of MS Y-87 and V2. There were no obvious differences of the structures of grana lamella between normal and low temperature in the three varieties.

DISCUSSION

In some papers, varieties had been clustered according to their characteristics with a single cluster method (Hong and Hou, 2001). However, the cluster result from a single cluster method may not be reliable. Our studies showed that results from the methods of Flexible, McQuitty, Ward, and Complete were the same and in agreement with the germination and growth results. But, the cluster results of Centroid, Single, Average, and Median differed markedly from each other. It was necessary to use multiple cluster methods and compare these methods for getting a reliable cluster result.

Changes in membrane structure were considered as the primary lesion of chilling injury and lead to a loss of membrane permeability and metabolic dysfunction (Lyons, 1973; Marangoni et al., 1996). Active oxygen species such as O_2^- and H_2O_2 accumulate under chilling stress. However, antioxidant enzymes such as APX, POD, and SOD could protect plants from oxidative damages, and these enzymes played a key role in cellular defense against active oxygen (Elster, 1982; Lee et al., 1987; Hernández et al., 2000). The results of the present study also showed the increase of the activities of antioxidant enzymes and the concentrations of active oxygen species under chilling stress. However, the increases of active oxygen species concentrations were slight, which may be the result of the protection from antioxidant enzymes.

The accumulation of soluble sugar and protein were considered as significant physiological changes of plants during chilling stress (Souza et al., 2004). The concentrations of total soluble sugar and soluble protein of these three tobacco varieties were increased obviously under low temperature. These results supported the positive roles of total soluble sugar and soluble protein as osmoregulators to maintain osmotic potential, and thus enhanced the ability of dehydration tolerance of plants, which seemed to act as a survival mechanism for plants under chilling stress (Irigoyen et al., 1992). There were significantly higher increasing rate in total soluble sugar and soluble protein of tobacco varieties of HHDJW and

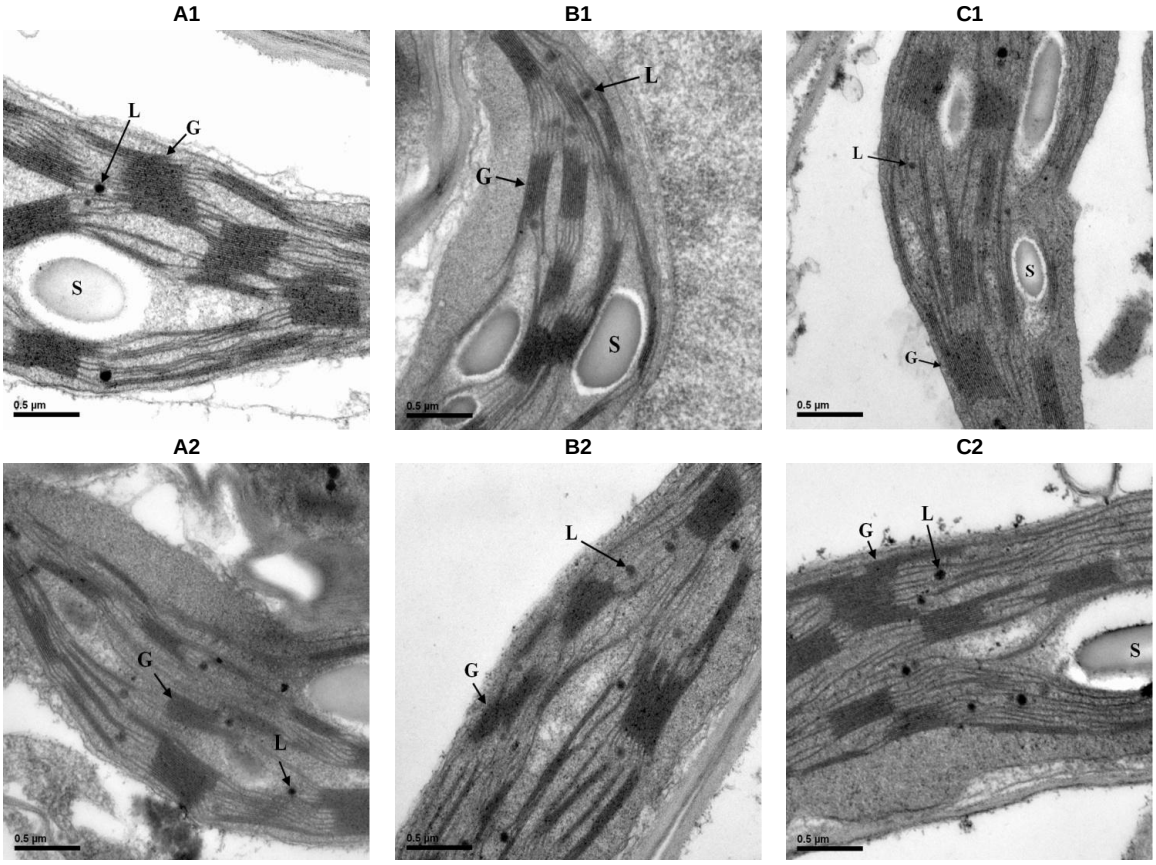

Figure 8. Ultrastructural changes of chloroplasts in tobacco varieties. A1: MS Y-87 at normal temperature and A2: MS Y-87 at low temperature, B1: V2 at normal temperature and B2: V2at low temperature, and C1: HHDJY at normal temperature and at low temperature. G: grana lamella, L: lipid droplet, S: starch granule.

V2; it may be one of the reasons why the chilling tolerance of HHDJW and V2 were higher than MS Y-87. The concentration of chlorophyll is closely related to the intensity of photosynthesis. The concentrations of chlorophyll a and chlorophyll b in three tobacco varieties were decreased under low temperature stress, this was because the enzymes in chlorophyll synthesis were affected and the chlorophyll itself can be degraded by low temperature (Zhou et al., 2008).

Ultrastructural changes in tobacco variety MS Y-87 were more visible than another two varieties under low temperature stress, this result was in agreement with previous observations that the more sensitive a plant is to chilling the more extensive the ultrastructural changes are (Nessler and Wernsman, 1980; Wise and Naylor, 1987). The most important organelle in cells is the nucleus because it is the "brain" of cells (Wu, 2003). An intact nucleus structure may be the key to helping plants survive low temperature stress. Chloroplast was considered the earliest visible site of ultrastructural injury in plant cells at low temperature (Kimball and Salisbury, 1973); it also acted as reservoir for starch, which provided a carbon skeleton such as malate when it was required (Kratsch and Wise, 2000). The location and extent of chloroplast swelling were dependent upon the cold tolerance of varieties. Thylakoid disintegration was a common symptom under chilling stress, it would result in the accumulation of lipid droplet (Lee et al., 2002), and our research showed the similar results that lipid droplet accumulated in the three kinds of tobacco varieties under chilling stress. Besides the inherent sensitivity of species to chilling, the duration of chilling is another important factor that may exacerbate injury to plants (Kratsch and Wise, 2000). Future research may focus on the factor of the duration of chilling and the synergistic effect of these two factors.

ACKNOWLEDGEMENTS

The research was supported by key project of Natural Science Foundation of Zhejiang Province (No. Z3100150) and Science and Technology Department of Yunnan Province (No. 2009EB073), P. R. China. The authors are grateful to the editor and anonymous reviewers for valuable comments.

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