Full Length Research Paper

Effects of chilling stress on membrane lipid peroxidation and antioxidant system of *Nicotiana tabacum* L. Seedling

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Chilling stress is one of main constraint factors for tobacco production in many regions of the world. In order to study the lipid peroxidation and antioxidant system of flue-cured tobacco seedling after chilling stress, the experiment was conducted with different stressful period which was 2, 4 and 6 days under 5 to 7°C by the material named Yunyan87, Msk326 and Yunyan85, respectively. The results indicated that the rate of O$_2$- production in leaves of three cultivars significantly declined at short-term (2 to 4 days) chilling stress. The malondialdehyde (MDA) contents and electric conductivities presented increasing at 2 to 4 days chilling treatments for YY87 and YY85 while those were similar to that of control seedling for K326. Superoxide dismutase (SOD) activities of YY87 remained similar to that of control plants while SOD activities of K326 and YY85 took reverse change. Catalase (CAT) and peroxidase (POD) activities of YY87 and YY85 enhanced under chilling stress, and reached remarkable difference under 6 days chilling stress; As for K326, CAT activities were the maximum under 4 days chilling stress, and POD activities had a prominent decline under 2 days cold stress and increased significantly after that. Except glutathione (GSH) contents of YY87 declined at 6 days chilling, the contents of ascorbic acid (ASA) and glutathione (GSH) were significantly higher in leaves of others treatments comparing with that of controls.

**Key words:** Chilling stress, flue-cured tobacco, lipid peroxidation, antioxidant enzymes, ascorbic acid, glutathione.

INTRODUCTION

Temperature is one of important factors which affect yield and quality of plant finally. Low temperature is a major factor limiting the productivity and geographical distribution of chilling-sensitive plant species (Zhang et al., 2008). According to previous studies, stress can induce a great deal of physiological alterations to ameliorate oxidative stress, such as reducing accumulation of ROS and malondialdehyde (Chiang et al., 2006; Parvaiz and Prasad, 2012). Chilling stress affects growth of plant by accumulating reactive oxygen species (ROS). These cytotoxic active oxygen species (AOS), which are also generated during metabolic processes in the mitochondria and peroxisomes, can destroy normal metabolism through oxidative damage of lipids, proteins, and nucleic acids (McCord, 2000). Lipid peroxidation, induced by free radicals is also important in membrane deterioration (McCord, 2000). Chilling stress could impair membrane permeability by the transition of...
membrane lipids from a liquid–crystalline phase to a gel phase (Lukatkin 2003; Parvaiz; Prasad, 2012). To scavenge AOS, higher plants have developed several strategies to cope with oxidative stress (Hasan et al., 2009; Zhou et al., 2012). These defense tactics are involved of both enzymatic and non-enzymatic antioxidant mechanisms, which include superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) (Dazy et al., 2009), and non-enzyme antioxidant metabolites such as ascorbic acid (ASA) and glutathione (GSH) (Jebara et al., 2005). Activities of oxygen-scavenging enzymes under chilling stress are correlated with tolerance to the stress. Numerous experiments suggested that chilling tolerance was related to the composition and structure of plant membrane lipids (Guo et al., 2006; Morsy et al., 2007).

Tobacco is an economical leave crop and important multiplex mode organism which is cultured widely in the world. Chilling stress is one of primary constraints to tobacco production in many parts of the world (Xu et al., 2011). The growth of tobacco seedlings would be inhibited when exposed to nonfreezing temperature below approximately 5°C (Gechev et al., 2003). Chilling in its sowing of early spring is the most important hindrance which affects poor and erratic germination and culture of healthy and strong flue-cured seedling. These are not the only result in prolongs periods of seedling-raising, which does not help for regrowth after transplant. Previous reports have described fatty acid desaturation (Kodama et al., 1995), antioxidant enzymes (Gechev et al., 2003), chloroplastic NAD(P)H dehydrogenase (Li et al., 2004) of tobacco seedling under different chilling stress, respectively. In this paper, we simulated tobacco seedlings often suffer low temperature and its lasting period in practice to analyze lipid peroxidation and antioxidant system of tobacco seedling of three genotypes and increased their understanding of those reactive oxygen free radicals. And this would help researchers define how tobacco seedling adapts to chilling and make a right choice of varieties.

MATERIALS AND METHODS

Plant material and experiment design

Three varieties of flue-cured tobacco Msk326 (K326), Yunyan87 (YY87) and Yunyan85 (YY85), were used for experiment materials which was cultivated widely in Chinese tobacco region. After seeds of similar size germinated for 14 days in a Petri dish containing 2 layer of filter paper and distilled water at 25°C, 3 young seedlings were then planted in a plastic containers filled with commercial soil, and reared in a growth chamber, and supplementary lighting (12 h photoperiod), and irrigated water with 1/2MS solution. Tobacco seedling with 5 to 6 true leaves were divided in two groups for each cultivar, one group of flue-cured seedlings were put in a growth chamber with low temperature of 5 to 7°C for 2, 4 and 6 days for chilling stress treatments, and the other group remained at the initial temperature and illumination conditions, which was controlled and grew in temperature at 23 to 25°C with other similar conditions. 90 replicate plants (30 plastic containers) in each treatment. At the end of each treatment, 36 randomly selected replicate plants (12 containers) of each treatment were examined for antioxidant enzyme activities (SOD, CAT, POD) and antioxidant compounds (ASA, GSH). All treatments were done in four replicates.

Extraction and estimation of lipids peroxidation

The rate of O2− production was measured as described in Ke et al. (2002) by monitoring the nitrite formation from hydroxylamine in the presence of O2 with 6 ml of 65 mM sodium phosphate (pH 7.8), 0.5 g samples were grinded and centrifuged at 10,000 ×g for 15 min. Then, 0.5 ml of the supernatant was incubated with 0.5 ml of 50 mM phosphate buffer (pH 7.8) and 1 ml of 1 mM hydroxylamine hydrochloride at 25°C for 1 h. After incubation, 1 ml of 17 mM sultiamide and 1 ml of 7 mM a-naphthylamine were added to the incubation mixture. After reaction at 25°C for 20 min, the absorbance in the aqueous solution was read at 530 nm. A standard curve with NO2− was used to calculate the production rate of O2− from the chemical reaction of O2− and hydroxylamine.

Malondialdehyde (MDA) is a decomposition product of lipid peroxidation. The MDA content was determined by the reaction of thiobarbituric acid (TBA). Briefly, 0.5 g fresh leaves from each treatment were homogenized with 4 ml of 20% (w/v) trichloroacetic acid (TCA) and the homogenate was centrifuged at 3500 g for 20 min. Then 1 ml of the aliquot of the supernatant was mixed with 2 ml of 20% TCA containing 0.5% (w/v) TBA and 100 μl 4% (w/v) butylated hydroxytoluene in ethanol. The mixture was heated at 95°C for 30 min and then cooled down to room temperature and centrifuged at 10,000 g for 10 min. The optical density value of the supernatant was measured at 532 and 600 nm. The MDA content was calculated according to the extinction coefficient of the MDA (156 mM−1 cm−1).

Electric conductivity was measured according to Yao et al. (2009), 0.5 g of leaf discs with diameter of 0.35 cm were rinsed 3 times by deionized water, and then were dipped into deionized water in closed tube for 30 min vacuum pumping. The primary conductivity was measured using a DDS-11A (Shanghai, China) conductivity meter after 3 h oscillation, and CK conductivity was also measured. The tubes containing samples were treated with boiling water for 30 min and the final conductivity was measured when the samples were allowed to cool to room temperature. The results were expressed as: relative conductivity [%] = (primary conductivity − CK) × 100/(final conductivity − CK).

Antioxidant enzymes extraction and assay

1.0 g fresh leaves of flue-cured tobacco from each treatment were homogenized in a pestle and mortar with 0.05 M sodium phosphate buffer (pH 7.8) at the end of treated days. The homogenate was centrifuged at 10,000×g for 20 min, and the supernatant was used for analyzing SOD, POD and CAT. The above steps were carried out at 4°C.

The SOD activity was detected according to the modified method of Zhang et al. (2005). The reaction mixture was made of 1.5 ml phosphate buffer (pH 7.8), 0.3 ml 130 mM/L methionine, 0.3 ml 750 μM/L nitroblue tetrazolium chloride (NBT), 0.3 ml 100 μM/L EDTA-NAs, and 0.3 ml 20 μM/L riboflavin. Appropriate quantity of enzyme extract was added to the reaction mixture. The reaction started by placing tubes below two 15 W fluorescent lamps for 15 min. Reaction stopped by keeping the tubes in dark for 10 min. Absorbance was recorded at 560 nm. One unit of SOD activity was defined as the quantity of SOD enzyme required to produce a 50% inhibition of reduction of NBT under the experimental conditions, and the specific enzyme activity was expressed as units per
The rate of O$_2$ production (nmol.mg$^{-1}$.min$^{-1}$.FW) in the reaction mixture in a total volume of 6.9 mL 0.1 M sodium phosphate buffer (pH 5.5) containing 1 mL H$_2$O$_2$ (30%), 2 mL deionized H$_2$O, and 1 mL 0.05 M guaiacol was prepared immediately before use. Then, 0.2 ml enzyme extract was added to reaction mixture. Increase in absorbance was measured at 470 nm at 1 min intervals up to 4 min using a UV-Vis spectrophotometer. Enzyme specific activity is defined as units (one CAT activity unit defined as absorbance at 470 nm changes per minute) per gram of fresh weight of leaves.

The POD activity was examined according to the modified method of Zhang et al. (2005). The reaction mixture in a total volume of 2.7 ml reaction mixture containing 2.25 ml 0.05 M sodium phosphate buffer (pH 7.8), 1.5 ml deionized water, and 0.45 mL 0.1 M H$_2$O$_2$ prepared immediately before use, and then 0.3 ml enzyme extract was added. The CAT activity was measured by monitoring the decrease in absorbance at 240 nm as a consequence of H$_2$O$_2$ consumption. Activity was expressed as units (one POD activity unit defined as absorbance at 240 nm changes per minute) per gram of fresh weight of leaves.

GSH contents were determined according to Israr et al. (2006). Over ice, 1 g of homogenized tissue was grounded with sterilized sand and 5 mL ice-cold 6% (v/v) phosphoric acid (pH 2.8) containing 0.5 mM EDTA in a mortar and pestle. The homogenate was centrifuged at 12000 g for 15 min and the supernatant was removed and used for estimation of glutathione. 0.5 mL of reaction buffer including 0.1 M phosphate buffer (pH 7) and 0.5 mM EDTA was added in 0.5 mL of above aliquot, and 0.05 mL of 3 mM 5-dithio-bis-(2-nitrobenzoic acid) was added. After 5 min, absorbance was taken at 412 nm.

**Figure 1.** Effect of chilling on oxidant of leaves of tobacco seedling A. the rate of O$_2$ production; B. malondialdehyde (MDA); C. electric conductivity. The bars with different letters are significant different (p < 0.05, t test).

**Measure of ascorbic acid and glutathione**

Ascorbic acid (ASA) was determined as described in Hodges et al. (1996). 0.5 g tobacco leaves were homogenized in 7 ml of cold 5% (w/v) m-phosphoric acid and centrifuged at 3000 g for 15 min. About 0.3 ml of supernatant was incubated for 5 min in a 0.7 ml total volume of 100 mM KH$_2$PO$_4$ and 3.6 mM EDTA. Color was developed with 0.4 ml of 44% α-phosphoric acid, 0.4 mL of 65 mM a,a′-dipyridyl in 70% ethanol, and 0.2 ml of 110 mM FeCl$_3$. The reaction mixtures were then incubated at 40°C for 1 h and quantified at 525 nm.

**RESULTS**

Cold periods induced lipid peroxidation of tobacco seedling

The rate of O$_2$ production of YY87 and YY85 decreased significantly below those of controls under 2 and 4 days chilling stress, but it jumped quickly and prominently higher than that of control for YY87 and was similar level to the control for YY85 with 6 days chilling stress (Figure 1A). The rate of O$_2$ production for K326 declined gradually with the development of chilling periods.
The MDA contents in YY85 and YY87 increased quickly and significantly under 2 to 4 days chilling stress, and that in K326 remained at control’s level after treatment by chilling, while that of YY87 increased gradually with treatment (Figure 1B). K326 showed little change of electric conductivity compared with its control plants. And electric conductivity of YY87 was insignificant different under 2 days chilling stress, but obviously increased after 4 days chilling (Figure 1C). While electric conductivity of YY85 rise up firstly and dropped later, which ascend by 94.59% than control seedling under 4 days chilling stress. Taken together, it showed that YY85 is sensitive and K326 is tolerance to 5 to 7°C low temperature, and YY87 between them.

Cold periods induced antioxidant enzymes systems of tobacco seedling

To determine the role of ROS scavenging systems in combating the oxidative stress, antioxidant enzymes were characterized in leaves of three genotype tobacco cultivars (Figure 2). Under 2 to 6 days chilling conditions, SOD activities of YY87 remained similar to that of control plants while SOD activities of K326 and YY85 were the maximum and the minimum for 4 days chilling stress, respectively (Figure 2A), which took reverse change. Catalase (CAT) activities of YY87 and YY85 enhanced with the development of the chilling stress, which went beyond 49.51 and 30.66%, respectively, with remarkable difference under 6 days chilling stress (Figure 2B). CAT activities of K326 were the maximum under 4 days chilling stress, which ascend by 86.31% comparing with that of control. POD activities of YY87 and YY85 were significant higher than those of their control under chilling stress for 2 to 6 days (Figure 2C). POD activities of K326 had a prominent decline under 2 days cold stress and increased significantly after that, which was the maximum under 6 days cold stress. Change of POD activities under chilling stress for 2 to 6 days showed that it was one of very important enzyme of oxygen-scavenging and defense system.

Response of ascorbic acid and glutathione of tobacco seedling on cold periods

After chilling stress for 2 to 6 days concentrations of ASA and GSH of three genotype flue-cured tobacco seedling significantly increased in comparison with control seedlings (Figure 3A and B). Under 4 days chilling stress, ASA contents of YY87 and YY85 were the maximum and increased by 95.77 and 105.36% and GSH contents were times of 1.97 and 2.19 above those of control, respectively.

Figure 2. Effect of chilling on antioxidant enzyme activities of leaves of tobacco seedling. A. SOD activities; B. CAT activities; C. POD activities. The bars with different letters are significant different (p < 0.05, t test); d, days.
While ASA contents of K326 elevated by 244.99% and GSH contents were times of 3.77 of controls under 2 days chilling stress. Therefore, ASA and GSH might play critical role in oxygen-scavenging system and protection of cell membrane for tobacco seedling.

**DISCUSSION**

ROS can cause lipid peroxidation resulted in breakdown of functional and structural integrity of biological membranes, in turn damage cell membrane increase the permeability of plasma membrane, leakage of K⁺ ions, and eventually cause cell death (Tewari et al., 2008). MDA formation is used as the general indicator of the extent of lipid peroxidation resulting from oxidative stress (Yong et al., 2013). Electric conductivity reflected the damage of stresses to the plasmalemma. In our study, enhancement of MDA and electric conductivity for YY87 and YY85 suggested that membrane stability had been damage and happened ion leakage, which might mean the protective mechanisms against oxidative stress was not enough to keep balance and control of ROS. This showed that YY87 and YY85 were sensitive to 5 to 7°C chilling, and K326 could resist 5 to 7°C chilling stress during periods of seedling judged from small changes of MDA contents and electric conductivity. Antioxidant enzymes, such as SOD, CAT, and POD are endogenous factors that protect cells from oxidative damage caused by ROS. SOD catalyzes the dismutation of the O₂⁻ to molecular oxygen and H₂O₂.

However, H₂O₂ is still toxic to plants, and it is metabolized to harmless water and oxygen by CAT (Chiang et al., 2006). Under short-term (2 days) chilling stress, the rates of O₂⁻ production of three flue-cured varieties evidently decreased while activities of SOD and CAT remained similar to controls. This might be explained that SOD and CAT were efficient O₂⁻ scavenger in that SOD catalyzed the conversion of O₂⁻ into H₂O₂ and CAT converted it into harmless water and oxygen. With the increasing of natural scavengers such as CAT and SOD, MDA in tissues reduced to low levels.

A positive relationship between peroxidase activity and resistance has been reported (Edreva et al., 1989). Cell wall-bound peroxidases were probably involved not only in the oxidative polymerization of hydroxylated cinnamyl alcohols but also in the generation of hydrogen peroxide necessary for lignification (Coffey and Cassidy, 1984; Edreva et al., 1989; Goldberg et al., 1985). Strong localization and high intensity of the peroxidase response around the sites of infection were characteristic of systemic resistance induced by Mg²⁺ in tobacco seedlings (Edreva et al., 1989). In our experiments, the activities of POD were a few higher in seedling treated with chilling in most treatments for three varieties, this showed long-term (4 to 6 days) chilling might catalysed aged and lignification of tobacco seedling, also exerted influences on some pathogens induced, for peroxidase could not only participate in the biosynthesis of antimicrobial compounds and lignin but also serve as a regulator for the entire metabolic process (Peng and Kuc, 1992). On the other hand, K326 could resist in infection under short-term (2 days) chilling stress because of low POD contents to some extent. The result disclosed that K326 is tolerant to chilling and easy to raise strong seedling while YY85 and YY87 is much susceptible to chilling.

Ascorbic acid and glutathione are important ROS scavenging metabolites. ASA was a non-enzymatic antioxidant acting as a substrate for extracellular enzymes for attenuation ROS levels (Burkey et al., 2006). Glutathione is crucial for biotic and abiotic stress management. Oxidative stress enhances ascorbate and glutathione levels. In the present study, enhancement of ASA and GSH contents showed that the chilling-treated flue-cured seedling accumulated greater amount of toxic oxygen compounds than the control, and long-term chilling stress resulted in attenuating of physiological metabolism and enhancement of antioxidant compound contents. ASA and GSH had the positive effects on
protecting tobacco seedling from chilling injury.

Antioxidant system took complicated changes and had been affected by chilling periods and genotypes of flue-cured. Chilling periods influenced physiological difference of seedling and triggering of gene of antioxidant enzyme expression. In this study, with the development of chilling stress, the rate of \( O_2^- \) production, MDA contents, electric conductivity and non-enzymatic antioxidant had different changes among three varieties, and three types antioxidant enzymes activities were the maximum for YY87 and YY85 under 6 days chilling stress while SOD and CAT activities reached to be the maximum for K326 under 4 days chilling stress. It is possible that triggering of different antioxidant enzyme protection mechanism is different among three genotype flue-cured tobacco, this need to be further study.

**Conclusion**

The rate of \( O_2^- \) production in tobacco leaves of three cultivars significantly declined at short-term (2 to 4 days) chilling, but happened different change at 6 days chilling. The MDA contents and electric conductivities presented increasing at 2 to 4 days chilling treatments for YY87 and YY85 while that remain similar to level of control for K326. SOD activities of YY87 were similar to that of control plants while SOD activities of K326 and YY85 took reverse change. CAT and POD activities of YY87 and YY85 enhanced under the chilling stress, and reached to remarkable difference under 6 days chilling stress; CAT activities of K326 were the maximum with 5 to 7°C for 4 days and POD activities of K326 had a prominent decline under 2 days cold stress and increased significantly after that. Except GSH of YY87 at 6 days chilling, the contents of ASA and GSH were significantly higher in leaves of others treatments comparing with that of controls. On the whole, K326 is tolerant to chilling and easy to raise strong seedling while YY85 and YY87 is much susceptible to chilling.

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