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Full Length Research Paper

Antioxidant enzyme and morphological characteristics of roots of three *Nicotiana Tabacum* L. genotype seedlings under chilling stress

C. Cui* and Q. Y. Zhou

College of Agronomy and Biotechnology, Southwest University, 400715 Chongqing, P. R. China.

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In order to study the morphological and physiological ecological response of root of Flue-cured tobacco seedling after low temperature stress, this experiment was conducted with different stressful period which was 2, 4 and 6 days under 5 to 7°C (day) by the material named Yunyan87, Msk326 and Yunyan85, respectively. The results showed that comparing with favorable temperature (23 to 25°Cday), activities of superoxide dismutase (SOD) and peroxidase (POD) and ascorbate peroxidase (CAT) had different change trends with difference variety, in which three parameters increased for YY87, and except activities of SOD rise for YY85, other parameters decreased after low temperature stress for short-term (2 days) as for K326 and YY85. When low-temperature stress period prolonged, these parameters come to rise gradually. As for morphological parameters, average diagram, surface area and volume of root declined significant after low-temperature stress for 2 days for YY87, while these morphological parameters are similar to normal level if prolonging stress to 6 days. However all morphological parameters are opposite for K326 comparing with YY87. Surface area, volume of root declined to some extent for YY85. These conclusion indicate different morphological and physiological ecological response mechanisms for roots of Flue-cured tobacco seedling of different variety after chilling stress.

Key words: Chilling stress, flue-cured tobacco, morphology, physiology, roots.

INTRODUCTION

Low temperature is a major factor limiting the productivity and geographical distribution of chilling-sensitive plant species (Thomashow, 1998; Sui et al., 2007; Zhang et al., 2008). Chilling can also result in weak growth, and further reduce crop production. Chilling stress could impair membrane permeability by the transition of membrane lipids from a liquid–crystalline phase to a gel phase (Murata and Los, 1997; Parvaiz and Prasad, 2012), numerous investigations experiments have suggested that chilling tolerance is related to the composition and structure of plant membrane lipids (Örvar et al., 2000). It was similarly observed in maize (Hodges et al., 1997; Takáč, 2004), coffee (Queiroz et al., 1998) and rice (Huang and Guo, 2005; Guo et al., 2006; Morsy et al., 2007). Chilling stress result in oxidative stress (Nayyar et al., 2005; Wang et al., 2009; Parvaiz and Prasad, 2012), higher plants have developed several strategies to cope with oxidative stress (Foyer and Noctor, 2005; Zhou et al., 2012). Antioxidant enzymes have been found to play a vital role in improving chilling tolerance (Bolkhina et al., 2003; Li and Zhang 2012), especially, superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) may protect the cellular membranes against the deleterious effects of reactive oxygen species (ROS) (Bowler et al., 1992). Activities of oxygen-scavenging enzymes under chilling stress have been correlated with tolerance to the stress.

Chilling stress can induce the overproduction of reactive oxygen species (ROS) in plants, which then in turn negatively affects cellular structures and metabolism by oxidative stress (Wang et al., 2009; Parvaiz and Prasad, 2012). Although much information is available

*Corresponding author. E-mail: cuigreeny@163.com. Tel: +86 23 68250617. Fax: +86 23 68251264

about the effects of sub- or supranormal temperatures on the physiological metabolism and functioning in the aerial parts (Hendrickson et al., 2004; Munro et al., 2004; Xu and Zhou, 2006; Dwyer et al., 2007), relatively little is known about the adaptation by plant roots to changes in substrate temperature (Xu and Huang, 2000; Rachmilevitch et al., 2006). Thus, metabolic damage caused by temperature stress could be alleviated to a great extent by keeping the root system at an optimal temperature since injury to the plants may be partly mediated by the disruption of root functions (Zhang et al., 2008). Roots are major organ of plants which is not only responsible for absorbing water, synthesizing, transmitting and storing, but also affect above-ground growth of plants. Roots are the primary site of perception and injury for several types of abiotic stress. In many circumstances, it is the stress-sensitivity of the root that limits the productivity of the entire plant (Steppuhn and Raney, 2005). In general, a highly structured root system is associated with vigorous plant development at the early stages (Richner et al., 1997). For example in cucumber, net photosynthetic rate (PN) and photochemical efficiency of photosystem 2 (PSII) were considerably decreased when the root temperature was lower than 15°C though the aerial temperature was kept optimal (Ahn et al., 1999). Therefore, roots are important organ for plants. Abiotic stresses, such as salinity (Cheng et al., 2009), P (Li et al., 2001), heavy metal ion (Chen et al., 2012) and so on, affect growth and development of roots. However, a few studies of root were found under abiotic stress.

Tobacco (*Nicotiana tabacum L.*) is one of the most important economic crops in China. Poor and erratic germination at suboptimal temperature is the most important hindrance in its sowing of early spring (Xu et al., 2011). The growth of tobacco seedlings would be inhibited when exposed to nonfreezing temperature below approximate 5°C (Gechev et al., 2003). Thus, understanding the mechanisms of plants tolerance to chilling stress during early growth stage is a crucial environmental research topic, however, no systematic studies had, until now, been conducted to characterize the adaptation of tobacco roots to chilling in term of their physiological metabolism. In this present study, to better understand the adaptability of roots of tobacco seedlings chilling stress at early spring, we evaluated the stress tolerance of tobacco cultivars under 5 to 7°C treatment by assessing seedling growth and antioxidant enzyme activity in roots.

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 were cultivated widely in Chinese tobacco region. After seeds of similar size were germinated for 14 days in a Petri dish containing 2 layer of filter paper and distilled water at the 25°C, Per 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 1/2 MS solution.

Tobacco seedlings with 5 to 6 true leaves were divided in several groups. One group remained at the initial temperature and illumination conditions. Others plant flue-cured seedling were put in a growth chamber with in low temperature 5 to 7°C for 2, 4 and 6 days for chilling stress treatments, and control 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). The other seedlings were transferred to a growth chamber and grew at 23 to 25°C with other similar conditions for 10 days to measure roots characteristics. All treatments were done in four replicates.

Antioxidant enzymes extraction and assay

1.0 g fresh roots 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 aforementioned 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 mmol/L methionine, 0.3 ml 750 µmol/L nitroblue tetrazolium chloride (NBT), 0.3ml 100 µmol/L EDTA-Na_2, and 0.3 ml 20 µmol/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 enzyme 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 gram fresh weight (FW) of root.

The POD activity was examined according to the modified method of Zhang et al. (2005). The reaction mixture in a total volume of 6.9 ml 0.1 M of sodium phosphate buffer (pH5.5) containing 1 ml H_2O_2 (30 %), 2 ml deionized H_2O, 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 peroxidase activity unit defined as absorbance at 470 nm changes per minute) per gram of fresh weight of roots.

The CAT activity was assayed according to the method of Zhang et al. (2005). CAT activity was determined at 25°C in 2.7 ml reaction mixture containing 2.25 ml 0.05 M sodium phosphate buffer (pH07.8), 1.5 ml deionized water, and 0.45 ml 0.1 M H_2O_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_2O_2 consumption. Activity was expressed as units (one catalase activity unit defined as absorbance at 240 nm changes per minute) per gram of fresh weight of roots.

Roots characteristics

After flue-cured seedlings recovery grew for 10 days at 23 to 25°C, the roots were washed out of the soil; EPSON V750 was used for

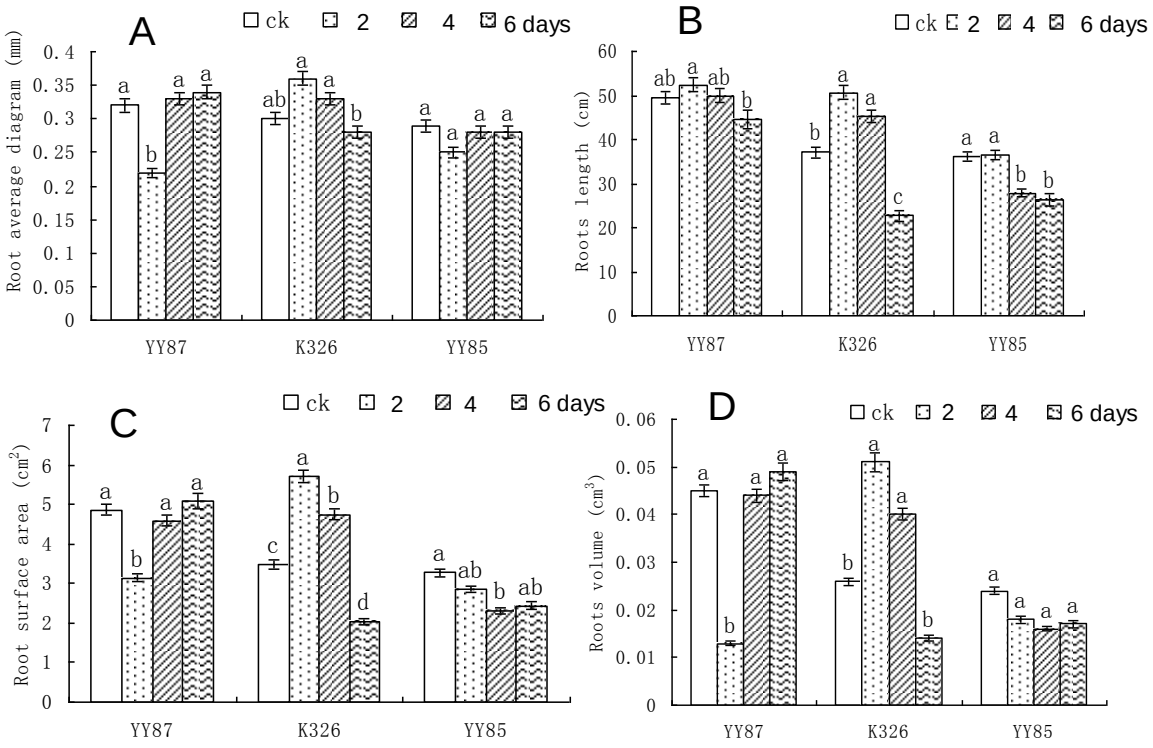

Figure 1. The effect of different cold periods on root morphological traits of tobacco seedling. A. Root average diagram; B. Root length; C. Root surface; D. Root volume.

image acquisition roots. Images were pre processed in Photoshop followed by digital images analysis in WinRHIZO (Regent Instrument Inc, Canada) to analyze roots characteristics, including root length, average diagram, surface area, volume and so on.

Statistical analysis

Analysis of variance (ANOVA) was used to detect the effects of chilling. Multiple comparisons were also performed to permit separation of effect means using the least significant difference test at significant level of P=0.05. All statistical analyses were done using the software statistical package (DPS) version 3.01.

RESULTS

Cold periods induced antioxidant enzymes systems of tobacco seedling

Antioxidant enzymes activities happened different changes according to varieties and cold stress periods (Figure 2). Superoxide dismutase (SOD) activities of YY87 and YY85 are beyond control seedling, furthermore, that is significant higher than control level for YY87 under 2 to 6 days chilling stress. SOD activities of K326 had a prominent decline under 2 to 4 days cold stress and increased significantly after that, which was the maximum under 6 day cold stress (Figure 2A). Change of CAT activities among three cultivars was inconsistent (Figure 2B). CAT activities of YY87 increased significantly less than 2 to 6 days chilling stress comparing with control seedlings. But for K326 and YY85, CAT activities were below the control level. The effect of chilling on POD activities were presented in Figure 2C. POD activity of YY87 was beyond the control level, but it was not remarkable at 4 to 6 days chilling stress. POD activity of K326 had a significant decline under 2 to 4 days cold stress and increased significantly by 27.31% under 6 day cold stress. POD activity of YY85 significantly declined comparing with control.

Response of root morphological traits of tobacco seedling on cold periods

The effect of chilling on root growth of flue-cured seedling varied for different genotype and treatment time (Figure 1). Low temperature treatment induced difference at the aspect of root average diagram. The decrease of average diagrams were more pronounced at short-term (2 days) chilling for YY87 and YY85 while it was on the contrast for K326 (Figure 1A).

On the other hand, 5 to 7°C low temperature for 2 days helped for elongation of roots length of three varieties seedling, which were above that of control, especially for K326, roots length significantly increased by 36.82% ($P<0.05$) above that of control (Figure 1B). However,

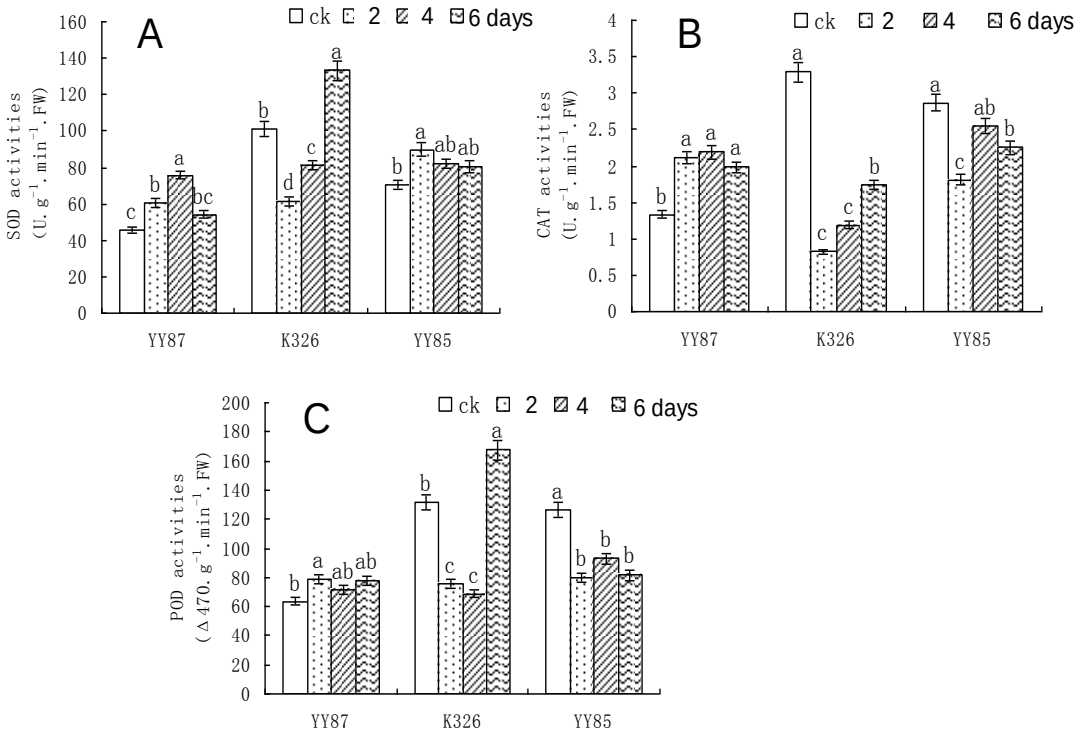

Figure 2. Effect of chilling on antioxidant enzyme activities of roots of tobacco seedling. A. SOD activities; B. CAT activities; C. POD activities.

when roots length for each of treatments decreased under chilling for 6 days, prominent influence were observed for K326 and YY85 ($P<0.05$). There were different results of roots surface area among varieties after chilling (Figure 1C). Short-term (2 to 4 days) chilling led to decline of roots surface area for YY87 and YY85, however, helped for increasing of surface area of roots for K326, which ascended by 64.08 and 36.49% comparing with control, respectively.

Roots volume at first (2 days) decreased significantly, and were similar to control seedlings at 4 and 6 days treatment for YY87; Progressive increased in roots volume due to 2 and 4 days chilling were apparent with for K326, which was enhanced by 96.15 and 53.84% of control samples, respectively, while roots volume declined the minimum after 6 days chilling; It was descendant but not significant for YY85 after 2, 4 and 6 days due to chilling (Figure 1D).

DISCUSSION

Antioxidant enzymes systems

Antioxidant enzymes are endogenous factors that protect cells from oxidative damage caused by ROS (Chiang et al., 2006). SOD catalyzes the dismutation of the O_2^- to molecular oxygen and H_2O_2, which in turn is metabolized to harmless water and oxygen by CAT (Chiang et al., 2006). Although, roots that were rapidly increase in SOD activities for YY87 and YY85 significantly decreased for K326. Previous studies showed that the level of CAT decreased during chilling (EL-SAHT, 1998; Lee et al., 2004). Our result was not in completely accordance because CAT activities of YY87 significant enhancement under chilling stress. Peroxidase activity can be induced under a variety of stress conditions, for example, drought, chilling, salinity, γ-radiation, and toxic contamination (Qadir et al., 2004; Kim et al., 2005; D'Arcy-Lameta et al., 2006). POD is generally involved not only in scavenging of H_2O_2 but also in diverse plant physiological processes, such as plant growth, development, lignification, and suberization (Passardi et al., 2005). In a previous study, Wang et al. (2009) reported that POD activity in roots of alfalfa decreased when subjected to chilling stress, and both cultivars showed similar levels of POD activity. In this present study, POD activities showed different change trend among three tobacco cultivars. Abiotic stresses are limiting in crops production. Much of the injury to plants caused by stress is associated with oxidative damage at cellular level (Bowler et al., 1992). Activities of antioxidant enzymes under chilling stress have been correlated with tolerance to the stress. Tolerant plant species generally have a better capacity to protect themselves from chilling-induced oxidative stress, *via* the enhancement of antioxidant enzyme activity. Higher contents of defense enzymes were correlated with Higher chilling tolerance. Chilling-tolerant cultivars had

higher antioxidation activities than the susceptible cultivar (Takáč, 2004; Huang and Guo, 2005). Activities of SOD, POD and CAT were inconsistent among the three tobacco varieties. After chilling stress, three antioxidant enzyme activities of YY87 were higher than control level to certain extent. And all of them decreased except raise of SOD and POD activities under 6 day's cold stress for K326. As for YY85, CAT and POD activities declined despite SOD activities increased under cold stress. In this study, these results suggest that YY87 is a variety of Chilling-tolerant. Root growth of YY87 suggests that it is functional to attenuate oxidative stress by increasing antioxidant enzyme activities. Antioxidant enzymes activities affected by genotypes of flue-cured and chilling periods, which depend on comprehensive ecophysiological adaptation because of difference of seedlings physiological situations, triggering of gene of antioxidant enzyme expression and cold resistance of varieties. In other words, it might be possible that triggering of different antioxidant enzyme protection mechanism is different among three flue-cured tobacco cultivars.

Root characteristics analysis

Soil temperature is below optimal temperature in which reduce water and nutrient uptake and limits growth of roots. As a consequence, growth rate of plant come to slow. Queiroz et al. (1998) reported that root tissue exposed to 10°C evolved significantly lower rates of metabolic heat compared with controls grown at 25°C, and the values were closely associated with the observed root growth inhibition. In cucumber, net photosynthesis and the photochemical efficiency of PSII are considerably decreased at root temperatures below 15°C, even if the aerial temperature remains optimal (Ahn et al., 1999). Plants possess a series of detoxification systems that break down highly toxic ROS *via* antioxidant enzymes, for example, superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT), as well as by non-enzyme antioxidants, such as glutathione (GSH), ascorbate (AsA), á-tocopherol, and carotenoids, thereby limiting oxidative damage under stress conditions (Zhang et al., 2007). One of main aims of raise seedling is to cultivate healthy and strong seedling with developed roots, so that converted to heat and light energy for re-growth after transplant as soon as possible. In this present study, there were big differences at the aspect of roots growth among the three cultivars. Short-term (2 days) chilling prominent decreased average diagram, surface area and volume of root for YY87, inversely; it helped for enhancement to root average diagram, length, surface area and volume of K326. On the other hand, long-term chilling (6 days) increased root average diagram, surface area and volume of YY87, but it is on the converse for K326. This might be because root of K326 have stronger tolerance of short-term chilling than that for YY87 and YY85, which result in great accumulation of root comparing with the control. The result also showed that long-term chilling (6 days) limited root growth for K326, which could not absorb enough water and nutrient at long-term chilling stress and maintain normal the process of metabolism. As for YY87, root was sensitive to the short-term (2 days) chilling, but it can gradually well-adjust at molecule and physiological level and adapt the unfavorable situations.

Our results support the fact that there are big differences at the aspects of antioxidant defense systems and roots growth among the three cultivars. YY87 present strong cold resistance, and had well-adaptation by increasing activities of three enzymes to keep roots prolonging for long-term chilling (6 days). The roots of K326 grew well for short-term chilling though decreasing activities of three enzymes. This might partly explain that the result from genetic composition and expression is difference in space-time to adapt chilling stress among cultivars. Therefore, improvement of chilling tolerance of crops requires a detailed knowledge about tolerance mechanisms in plants, which comprise a wide range of responses on molecular, cellular, whole plant levels, and include others the synthesis of compatible osmolytes and radical scavenging mechanisms, all of them need further research.

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