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Full Length Research Paper

Differential responses of two rubber tree clones to chilling stress

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Chilling stress is one of the most important environmental factors that limit the growth, distribution and yield of rubber tree in China. The effects of chilling stress on the grated plants of two rubber trees clones, GT1 and Wenchang217, were studied by physiological methods in controlled light chamber in order to explore the physiological mechanism of cold tolerance in rubber tree. Our results show a significant change in the tested physiological parameters after chilling treatment between two rubber clones. In comparison with the case of rubber tree clone GT1, the level of malondialdehyde (MDA) increased while superoxide dismutase, especially peroxidase and catalase decreased significantly in the seedlings of rubber tree clone Wenchang217 in response to chilling stress. As the cold tolerance ability of rubber tree clone GT1 is stronger than that of rubber tree clone Wenchang217, activation of oxidative quenching enzyme system should be one of the important factors that determine the cold tolerance of rubber tree.

Key words: Chilling stress, cold tolerance, *Hevea brasiliensis* Muell. Arg., physiological parameter, seedling.

INTRODUCTION

Rubber tree (*Hevea brasiliensis* Muell. Arg.), a tropical rainforest plant, originates in South America Amazon River basin and thus has an instinctive taste for high temperature and humidity (Webster and Paardekooper, 1989). It frequently subjects to chilling injury in the area of South China, which is located in the tropical northern margin (latitude 18 to 24°N) (Huang and Pan, 1992). Chilling stress causes defoliation and dieback of chilling-tolerant rubber tree clones, whilst cause dead branches, damaged bark and latex leakage of middle tolerance clones, and even cause death of trunk and branches of the least tolerance clones. This provides a selection for the germplasm with chilling tolerance in the field.

It is widely accepted that chilling stress implies the formation of radicals and other reactive oxygen species (ROS) (Miller et al., 2004, 2008) and activation of antioxidant enzymes (Hodges et al., 1997), in plants such as maize (Prasad et al., 1994), cucumber (Lee and Lee, 2000) and rubber tree (Mai et al., 2010). The superoxide radical anion ($O_2^{\bullet -}$) is converted by superoxide dismutase (SOD; EC1.45.1.1) into hydrogen peroxide (H_2O_2), which can be turned into the highly reactive hydroxyl radical (•OH). ROS oxidize various cellular substances, including DNA, proteins and lipids (Halliwell and Foyer, 1978). The oxidatively damaged lipids, proteins and DNA have been suggested as indexes of oxidative stress in biological systems (Imlay, 2003; Venditti et al., 2010). The oxidative effects of ROS are minimized by a fairly complex system of antioxidant enzymes including catalase (CAT; EC1.11.1.6), ascorbate peroxidase (ASPX; EC1.11.1.11), etc. However, information concerning the effect of cold on rubber clones is relatively lacking (Mai et al., 2010).

GT1 was considered as chilling-tolerant clone, which planting in Hainan, Yunan and Guangdong province in China for 50 years. Wenchang217 was considered as

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cold-sensitive clone only planted in warm area in Hainan province. In this study, grated plants of these two rubber tree clones with different cold tolerance capacities were subjected to artificial chilling process so as to reveal some aspects of physiological processes associated with cold tolerance. This will provide fundamental knowledge for screening chilling-tolerant clone in China.

MATERIALS AND METHODS

Zhanjiang city is located at 109°41' to 110°58'E, 20°13' to 21°57'N. Two years old bud-graft plants of rubber tree clone GT1 (original clone breed in Indonesia) and Wenchang217 (crossed from Haiken1 and PR107, abbr. WC217 in figures) were used. Three years old epicormic shoots of GT1 were used as stocks for two clones. Grafted plants were put into a growth chamber CONVIRON E8 (Manitoba, Canada) with 28/25°C (day/night), photon flux density of 350 to 400 µmol m^{-2} s^{-1}, 16 h photoperiod, and relative humidity of 60 to 80%.

After 1 h of illumination by setting the temperature to 6°C, this was reached about 30 min later. At each sampling, leaves were collected from 4 to 6 separate branches of each plant as a single sample repeat.

Relative water contents

Determination of relative water content (RWC) was based on the formula according to Balsamo et al. (2006).

$$RWC = \frac{\text{Leaves fresh weight - Leaves dry weight}}{\text{Leaves turgid weight - Leaes dry weight}}$$

Analysis of soluble sugar and malondialdehyde (MDA)

Soluble sugar was measured by referring to the method of Creelman et al. (1990) and SCG4R (1956). 0.1 g of leaf samples was put it into centrifuging tubes with a volume of 10 and 5 ml of 80% alcohol added to the tube and heated in water for 30 min at 80°C. It was cooled down and the tube centrifuged at 1,000 *g* for 10 min. Sugar content was determined by the phenol-sulfuric acid method. MDA was assayed by the thiobarbituric acid method as described by Aust et al. (1985).

Starch measurements

Leaf segments were extracted with hot 80% ethanol until the tissue was pigment free. Starch grains were pelleted by centrifugation and then suspended in 1.0 ml of 0.2 N KOH and placed in boiling water for 30 min. After cooling, the pH of the mixture was adjusted to about pH 5.5 with 200 pl of 1.0 N acetic acid. To each sample, 1.0 ml of dialyzed amyloglucosidase (from *Aspergillus oryzae*, Sigma) solution (35 units/ml in 50.0 mm Na acetate buffer, pH 4.5) was added, and the tubes were incubated at 55°C for 30 min. After digestion, the tubes were placed in boiling water for 1 min and centrifuged, and the glucose in the supernatant was analyzed enzymically using hexokinase and glucose-6-P dehydrogenase (Jones et al., 1977). Quantitative hydrolysis of the starch to glucose under these conditions was confirmed by the complete hydrolysis of amylopectin added to the sample solution.

Enzyme activities

Peroxidase (POD; EC 1.11.1.7) activity was determined spectrophotometrically by the method of Van Loon and Geelen (1971). SOD (EC 1.15.1.1) activity was assayed by monitoring the inhibition of photochemical reduction of nitro blue tetrazolium (NBT), according to the method of Beyer and Fridovich (1987) with some modifications. For the total SOD assay, a 5 ml reaction mixture contained 50 mM HEPES (pH 7.6), 0.1 mM EDTA, 50 mM Na_2CO_3, 13 mM methionine, 0.025% (w/v) Triton X-100, 75 µm NBT, 2 µm riboflavin and an appropriate aliquot of enzyme extract. The reaction mixture was illuminated for 15 min at a light intensity of 350 µmol m^{-2} s^{-1}. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the reduction of NBT as monitored at 560 nm.

Catalase (CAT; EC1.11.1.6) is assayed spectrophotometrically by a kit supplied by OxisResearch (Percipio Biosciences, Inc. USA). The rate of dismutation of hydrogen peroxide (H_2O_2) to water and molecular oxygen is proportional to the concentration of catalase (reaction 1). The sample containing catalase is incubated in the presence of a known concentration of H_2O_2. After incubation for exactly 1 min, the reaction is quenched with sodium azide. The amount of H_2O_2 remaining in the reaction mixture is then determined by the oxidative coupling reaction of 4-aminophenazone (4-aminoantipyrene, AAP) and 3,5,-dichloro-2-hydroxylbenzenesulfonic acid (DHBS) in the presence of H_2O_2 and catalyzed by horseradish peroxidase (HRP) in reaction 2. The resulting quinone-imine dye (N-(4-antipyrl)-3-chloro-5-sulfonate-pbenzoquinonemonoimine) is measured at 520 nm according to the method of Aly and Shahin (2010).

Statistical analysis

All data were analyzed on statistical *package* for the social sciences (SPSS) analytical software package (version 19.0), One-way ANOVA and Duncan text were used to assess $P<0.05$ (probability level). Figures were drawn by Origin Data Analysis and Graphing Software, OriginPro8.0 (Version8E, OriginLab Corporation, Massachusetts, USA). All of the measurements were performed 6 times, and the means and calculated standard deviations (SD) are reported.

RESULTS

The effects of chilling stress on water and osmosis matter contents

The water contents in both leaves of Wenchang217 and GT1 rubber clone seedlings showed little changes during 5-day chilling treatment. Relative water contents in GT1 leaves were lower than those in Wenchang217.

Low temperature affected starch contents more severe. The starch contents of Wenchang217 were lower than those in GT1 especially from 1 to 3 days. However, during 4 to 5 days, the starch contents increase at 1.27 and 0.74 mg/g. On the contrary, the starch contents in leaves of GT1 decreased sharply from 2.78 mg/g at 3-day to 0.39 and 0.54 at 4 and 5-day, respectively. Chilling stress reduced the soluble sugar contents at 2-day periods in 2 rubber clones. Both clones increased at highest levels at 3-day. However, a decrease from 0.068% at 3-day to 0.044% at 4-day were found in GT1.

Also, on the contrary, an increase from 0.056% at 3-day to 0.060% at 4-day were observed in Wenchang217.

The effects of chilling stress on oxidation and antioxidative enzyme activities

The SOD enzyme activities did not show significant difference between 2 rubber clones. However, low temperature increase SOD enzyme activities at 1 day then decreased significantly afterwards. An increase from 123.0 U/min/g protein at 3-day to 173.6 U/min/g protein at 4-day were found in GT1. An increase from 140.4 U/min/g protein at 3-day to 158.0 U/min/g protein at 4-day were observed in Wenchang217. The SOD enzyme activities in GT1 were significantly higher than those in wenchang217 ($P < 0.01$). The POD enzyme activities in 2 rubber clone seedling under different chilling stress were showed different patterns. When treated at 6℃, chilling stress increased the POD activities at 4-5d periods in GT1 (113.1 and 72.4 enzyme activities). While Wenchang217 showed continuous reduction pattern during all 5 days. Chilling stress significantly increase the CAT activities at 3 and 4 days, and were significantly higher than those in Wenchang217 ($P<0.01$).

The effects of chilling stress on lipid membrane stabilities

The MDA contents in 2 rubber clone seedling under chilling stress were showed similar patterns. Chilling stress increased the MDA contents at 4 to 5 days periods significantly. An increase from 3.45 mmol/g FW at 3-day to 9.28 mmol/g at 4-day were found in GT1. An increase from 4.98 mmol/g at 3-day to 11.24 mmol/g at 4-day were observed in Wenchang217.

DISCUSSION

Low temperature is one of the important environmental factors that limits the growth and distribution of tropical rubber tree in China (Huang and Pan, 1992). Under chilling stress conditions, the membrane system is the most sensitive parts of cell feeling of low temperature stress, but also the key structures of the plant and resistance subject to chilling injury (Kaniuga, 2008; Kratsch and Wise, 2000). Using the conductance method to measure the plant cell electrolyte leakage change, is a classic method to reflect the membrane systems of varying degrees of damage and cold hardiness (Mai et al., 2010). The level of MDA content, a lipid peroxidation product, reveals the lipid peroxidation level (Kaniuga, 2008). GT1 was introduced from Indonesia, which considered as chilling-tolerant rubber clone in China. Wenchang217 was considered as cold sensitive clone. MDA contents of GT1 were significantly lower than those of Wenchang217 at 6℃ which suggested the lipid oxidation of the GT1 was lower than that of Wenchang217 (Figure 4). Thus, the physiological changes between GT1 and wenchang217 would help in the selection of chilling tolerance germplasm in rubber tree breeding program.

Accumulation of soluble carbohydrates is one of the best-known responses of plants to cold. It begins early during the response to cold (Tabaei-Aghdaei et al., 2003). Closely relationship was found between photosynthesis carbohydrate accumulation and plant cold resistance (Öquist and Huner, 1993), plants with cold hardiness generally accumulated more soluble sugar (Tabaei-Aghdaei et al., 2003). Because sugar accumulation can increase the osmotic pressure and played frozen protective agent role. It also provided energy and substrate induction of cold resistance physiological and biochemical processes such as protein synthesis, help to improve cold hardiness. In this study, the relative water contents did not show significantly difference (Figure 1). Chilling treatments led to loss of leaf turgor were found in other crop. Low temperature affected starch contents more severe in leaves of GT1. However, the starch degradation did not increase the soluble sugar content suggest starch convert to triose phosphate or maltose rather than glucose (Figure 2) (Smith et al., 2005).

Low temperature stress caused a large number of free radicals has toxic effects on plant cells (Michaeli et al., 1999a, b, 2001). Antioxidative enzymes, such as POD, SOD and catalase were the most important intracellular free radical scavenging system (Apel and Hirt, 2004). Under low temperature stress, these enzymatic activity, expressed initially as an upward trend, in order to effectively protect cells, enzyme activity gradually decreased when the temperature is below a certain limit or effect more than a time limit. So their activities can generally be used as the physiology of plant cold hardiness indicators (Figure 3).

Superoxide dismutase is a metal-containing antioxidant enzyme. The active oxygen scavenging reaction is at the core where superoxide anion radical is rapidly oxidized to hydrogen peroxide and molecular oxygen. SOD can be induced by reactive oxygen species generated under stress conditions, thereby reducing damage to the cell membrane. The optimal substrate of POD is hydrogen peroxide, but others are more active with organic hydroperoxides such as lipid peroxides. The function of catalase is to convert hydrogen peroxide to water. In this way, two toxic species, superoxide radical and hydrogen peroxide are converted to the harmless product water. In this study, the SOD enzyme activities of two rubber clones showed significantly difference at 4-day. These were in accordance with the changes in POD and CAT. These changes indicated that chilling stress induced radical molecular as well as the antioxidative enzyme activities. High POD activities were helpful for the oxidation of lipid peroxides resulted in the little damage of

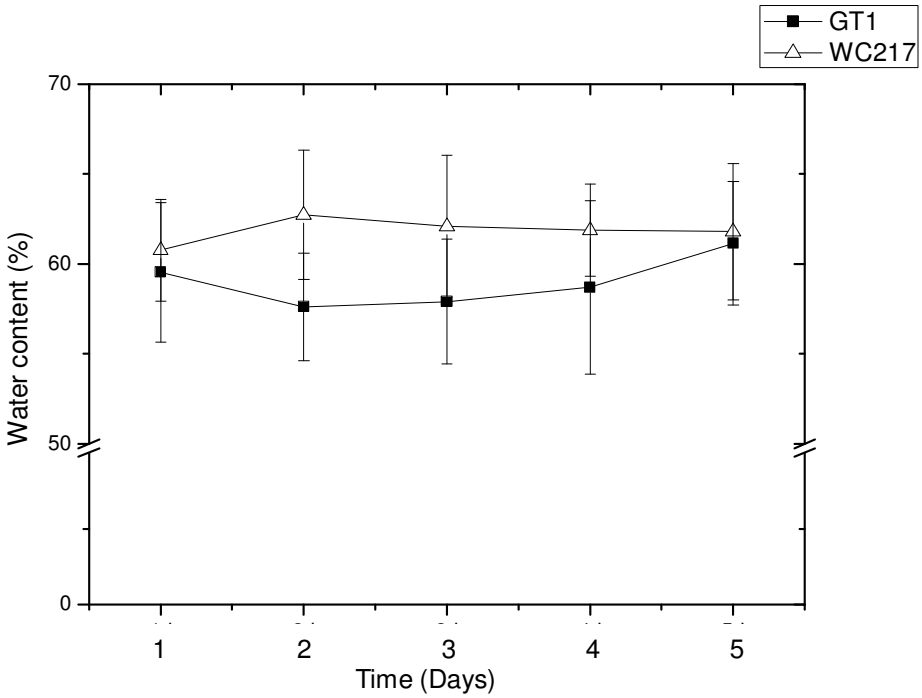

Figure 1. Water contents in the leaves of the seedlings of two rubber tree clones under different chilling stress treatments at 6℃.

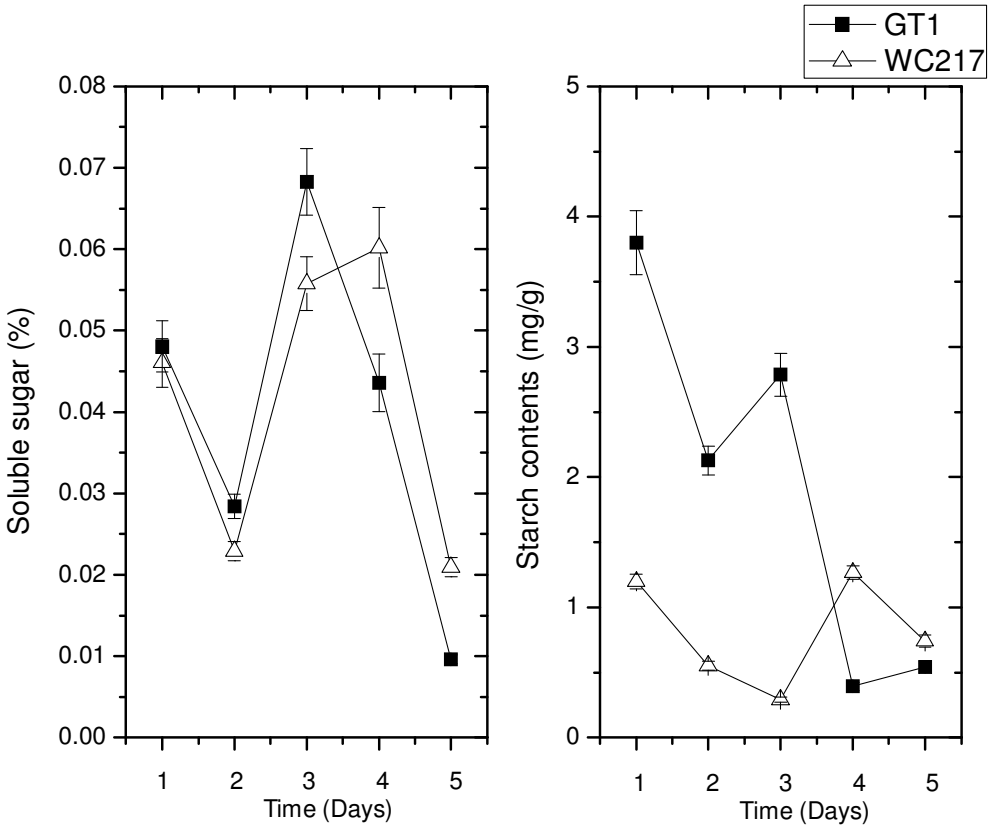

Figure 2. Soluble sugar and starch contents in the leaves of the seedlings of two rubber tree clones under different chilling stress at 6℃.

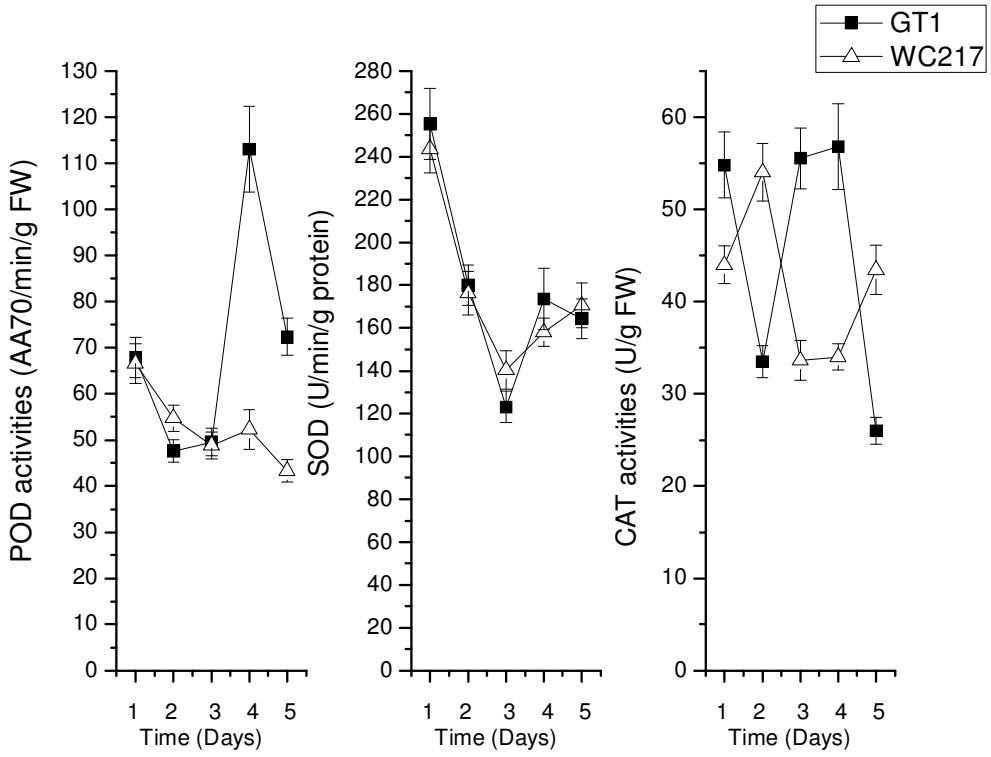

Figure 3. Antioxidative enzyme activities in the leaves of the seedlings of two rubber tree clones under chilling stress.

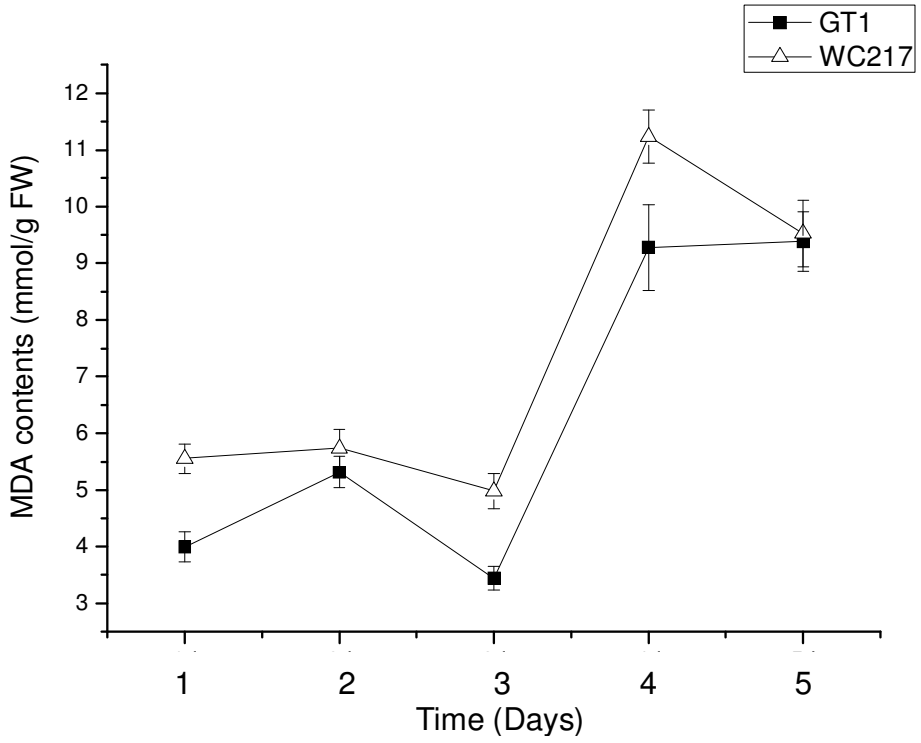

Figure 4. MDA contents in the leaves of the seedlings of two rubber tree clones under chilling stress.

membrane lipid in GT1 leaves. These results were same with MDA results. As mean time, SOD and CAT worked together converting superoxide radical and hydrogen peroxide to the harmless product water. These enzymes also help in the protection of membrane under chilling stress. It was antioxidative enzyme activities rather than soluble sugar contents played key role in the chilling tolerance of GT1.

Conclusion

The level of MDA decreased while SOD, especially POD and CAT increased significantly in the seedlings of rubber tree clone GT1 (chilling tolerant) compared to Wenchang217 in response to chilling stress. It was safely assumed that the activation of oxidative quenching enzyme system (SOD, CAT) should be one of the important factors that determine the cold tolerance of rubber tree.

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