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The influence of low temperature on photosynthesis and antioxidant enzymes in sensitive banana and tolerant plantain (*Musa* sp.) cultivars

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Abstract

Low temperature (LT) is one of the major factors that limit crop production and reduce yield. To better understand the cold-tolerance mechanism in the plantains, a sensitive cultivar Williams (*Musa acuminata* AAA cv. Williams) and a tolerant cultivar Cachaco (*Musa paradisiaca* ABB cv. Dajiao) were used. LT resulted in increased malondialdehyde (MDA) content, elevated contents of hydrogen peroxide (H_2O_2) and superoxide radical ($O_2^{\bullet -}$), and decreased photochemical efficiency (F_v/F_m) and net photosynthetic rate (P_N), but *cv*. Cachaco showed better LT tolerance than *cv*. Williams. After LT treatment for 120 h, total scavenging capability (DPPH$^\bullet$ scavenging capability) in Williams showed a significant decrease but no significant alternations was found in Cachaco. Ascorbate peroxidase (APX) and peroxidase (POD) displayed a significant increase but superoxide dismutase (SOD) showed no significant alternations and catalase (CAT) showed a significant decrease in Cachaco after 120 h of LT treatment. All the four antioxidant enzymes above showed a significant decrease in Williams after 120 h of LT treatment. Our results suggest that higher activities of APX, POD, SOD, and DPPH$^\bullet$ scavenging capability to a certain extent can be used to explain the higher cold tolerance in the plantain, which would provide a theoretical guidance for bananas production and screening cold-resistant variety.

Additional key words: antioxidant enzyme; banana; low temperature; photosynthesis; plantain.

Introduction

Banana and plantain (*Musa* sp.), belonging to the family Musaceae, are crops of tremendous economic and social importance in the humid and subhumid tropical regions of the world (Robinson 1996). Its perennial nature and ability to produce fruit throughout the year provides staple food for millions of people across the globe (Rowe 1981, Kulkarni and Ganapathi 2009). Bananas and plantains (except the Fe'i bananas) are derived from *Musa acuminata* Colla. or hybrids of these species with *M. balbisiana* Colla. (Gawel and Jarret 1991). *Musa* species grow in the tropical and subtropical world regions and have varied human uses, ranging from the edible bananas and plantains of the tropics to cold-hardy fiber and ornamental plants. Bananas originate from tropical regions and several abiotic stresses affect their growth. Low temperature (LT) is perhaps the most important environmental constraint for plant distribution on land (Ishitani *et al.* 1997) and an important factor that limits the geographical area suitable for growing a particular plant species (Ishitani *et al.* 1998, Lee *et al.* 2004).

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Abbreviations: APX – ascorbate peroxidase; ASC – ascorbate; CAT – catalase; DAB – diaminobenzidine; DPPH$^\bullet$ – 1,1-diphenyl-2-picrylhydrazyl; F_v/F_m – maximum photochemical efficiency of photosystem II; g_s – stomatal conductance; H_2O_2 – hydrogen peroxide; LT – low temperature; MDA – malondialdehyde; NBT – nitroblue tetrazolium; $O_2^{\bullet -}$ – superoxide radical; $^1O_2$ – singlet oxygen; $^\bullet$OH – hydroxyl radical; P_N – net photosynthetic rate; POD – peroxidase; SOD – superoxide dismutase; TCA – trichloroacetic acid.
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Temperature, humidity and rainfall need to be high (22–31°C, 2,000 to 2,500 mm year^{-1}, Robinson 1996) and evenly distributed for optimal growth and high yield of bananas. The major banana-producing areas in China are mainly in the subtropics, and since temperature in the subtropical regions is marginal in winter (Turner and Lahav 1983), bananas production is often threatened by low temperature, causing reduced growth and slower development of plants. Therefore, low temperature in winter has become one of the most limiting environmental factors for growth of bananas in southern China.

Plant growth and productivity are severely limited by temperature (Noctor *et al.* 2002). Photoinhibition increased with decreasing temperature and with increasing photon irradiance in plants (Smillie *et al.* 1988, Damasco *et al.* 1997). The stress caused by such low temperature can lead to extensive damage to the plants, *e.g.* D1-protein degradation, damage to construction of thylakoid membrane and inhibition of the photochemical activity of chloroplasts *etc.* (Havaux *et al.* 1984, Pinedo *et al.* 2000, Holá *et al.* 2008). Reactive oxygen species (ROS) are known to be generated in plant cells even during normal metabolic process, such as photosynthesis and respiration (Apel and Hirt 2004). Plants will suffer oxidative stress once the balance between the production of ROS and the scavenging activity of antioxidant is upset, resulting in oxidative damage (Elstner *et al.* 1988, Smirnoff 1993, Moller 2001). ROS such as superoxide ($O_2^{\bullet -}$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\bullet}OH$) are highly toxic to plant cell and by reaction with proteins, lipids, carbohydrates, and DNA, and they can cause cell death (Singla-Pareek *et al.* 2006, Shao *et al.* 2008, Kumar and Yadav 2009). To protect themselves from the toxic oxygen intermediates, plants employ defense systems that include enzymes such as superoxide dismutase (SOD, EC1.12.1.1), ascorbate peroxidase (APX, EC1.11.1.11), catalase (CAT, EC1.11.1.6), and peroxidase (POD, EC 1.11.1.7) that scavenge the ROS (Sundar *et al.* 2004).

In the production practice in the field, plantains showed a relative higher cold-tolerance as compared to bananas; however, it is still not clear about the physiological mechanisms of cold-tolerance in the plantains. In the present study, the effects of cold-stress on the leaves of saplings between a sensitive banana cv. Williams (AAA genome) and a tolerant plantain cv. Cachaco (ABB genome) were compared in order to explore the physiological mechanisms of cold-tolerance in the plantain, which would provide a theoretical guidance for bananas production and screening of cold-resistant variety.

Materials and methods

Plants and low-temperature treatment: Saplings of the cold-sensitive banana cv. Williams (*M. acuminata* AAA cv. Williams) and the cold-tolerant plantain cv. Cachaco (*M. paradisiaca* ABB cv. Dajiao) with a uniform growth stage were obtained from Biotechnology Institute, Guangxi Academy of Agricultural Sciences, Nanning. They were grown in plastic plots with soil and sand mixture (10:1, v/v) for this study. The saplings were placed in a growth cabinet under normal growth conditions [30°C/22°C day/night, 75% relative humidity (RH), 12-h photoperiod with a photosynthetic photon flux density (PPFD) of 250 μmol(photon) m^{-2} s^{-1}] and supplied with the same levels of water and MS nutrient. Two months after the transfer from *in vitro* to a soil, uniform and healthy saplings (leaf number: 5–6; height: about 20 cm) were selected for the present experiment. For low temperature treatment, these saplings were treated with 7°C/7°C day/night with the same light/dark regime and RH mentioned above and the saplings which were placed in the growth cabinet under normal growth conditions were used as controls. All five replicate measurements of the physiological index were performed on the leaves of the saplings of the banana and the plantain studied which were selected at random from each treatment.

Lipid peroxidation determination and electrolyte leakage test: Lipid peroxidation was determined by estimating the malondialdehyde (MDA) content according to Karabal *et al.* (2003) with some modifications (Sun *et al.* 2006). After 120 h of low-temperature treatment, approximately 0.5 g of fresh leaves were collected from each treatment and cut into small pieces and homogenized by the addition of 5 mL of 5% trichloroacetic acid (TCA) in ice bath. The homogenates were centrifuged at 10,000 × *g* for 10 min; 1 mL of the supernatant was added with 4 mL of 20% TCA containing 0.5% thiobarbituric acid. This resulting mixture was incubated at 98°C for 40 min, then cooled to room temperature and centrifuged at 10,000 × *g* for 5 min. The supernatant was subjected to analysis with a spectrophotometer. The MDA content was calculated from $A_{535} - A_{600}$ using coefficient of absorbance of 155 mM^{-1} cm^{-1} (Lei *et al*. 2010).

The percentage of electrolyte leakage was measured to evaluate the degree of low-temperature injury in the leaves of banana and plantain saplings. After low-temperature treatment for 120 h, five leaf discs (diameter: 1.3 cm) were collected from each treatment and were immersed in deionized water for 1.5 h at room temperature, followed by a 30-min boiling treatment. The conductivity as a solution of leaked electrolytes before and after boiling was determined using a *DDS-11 A conductmeter* (*Shanghai Dapu Instruments*, Shanghai, China).

H_2O_2 and $O_2^{\bullet -}$ localization *in situ*: The method of ROS localization was conducted according to Romero-Puertas

et al. (2004) and Zeng *et al.* (2010) with some modifications. After 120-h treatment, samples were collected for analysis of H_2O_2 and $O_2^{\bullet -}$ localization *in situ*. For H_2O_2 *in situ* localization, H_2O_2 was visualized by diaminobenzidine (DAB) staining. Leaves from each treatment were excised and immersed in a solution of DAB 1 mg ml^{-1} in 50 mM phosphate buffer (pH 7.0), vacuum-infiltrated for 10 min and then incubated at room temperature for 8 h in the absence of light. Then the leaves were illuminated with white light (80 μmol m^{-2} s^{-1}) until the appearance of brown spots, characteristic of the reaction product of DAB with H_2O_2. Chlorophyll was then removed by immersion in boiling ethanol (75%, v/v) to visualize the brown spots and leaves were photographed by a digital camera.

For $O_2^{\bullet -}$ localization *in situ*, leaves from each treatment were excised and immersed in a 0.1 mg ml^{-1} solution of nitroblue tetrazolium (NBT) in 50 mM K_2HPO_4-KH_2PO_4 buffer (pH = 6.4), containing 10 mM NaN_3, and then were vacuum-infiltrated for 5–10 min and illuminated until the appearance of dark spots, characteristic of blue formazan precipitates. The subsequent steps were the same as for localization of H_2O_2.

Photosynthetic capacities and chlorophyll fluorescence: After the low-temperature treatment of 0, 48, and 96 h, photon-saturated gas exchange was measured on five fully developed leaves from five plants per species with a portable infrared gas analyzer (*LI-6400, LI-COR*, Lincoln, NE, USA). The PPFD of 800 μmol m^{-2} s^{-1} on the cuvette surface was provided by a LED source, which was photosynthetically saturating for the two species. CO_2 concentration inside the leaf chamber was maintained at 380 cm^3 m^{-3} through the CO_2 controlling system of the *LI-6400* attached to a tiny CO_2 cylinder. During measurements, the relative air humidity was about 75% and leaf temperature was maintained at 25°C for each species and treatment. Chlorophyll fluorescence on the same leaf was measured with a portable pulse-modulated fluorometer (*PAM 2100, Walz*, Effeltrich, Germany) with the *PamWin* software. Fluorescence was measured with relatively weak measuring light pulses (<1 μmol m^{-2} s^{-1}) at a low frequency (600 Hz) for measurement of F_o (minimum fluorescence yield of a dark-adapted leaf). Maximal fluorescence yield of a dark-adapted leaf (F_m) was measured during an 800-ms exposure to a PPFD of approximately 2,700 μmol m^{-2} s^{-1}. All fluorescence measurements were started after an additional 15-min dark adaptation. The maximum photochemistry efficiency (F_v/F_m) of photosystem II (PSII) was measured after the low-temperature treatment of 0, 24, 48, 72, and 96 h. For the measurement of photosynthetic capacities and chlorophyll fluorescence, the saplings were used for the measurement after exposed at room temperature for 10 min.

Total antioxidant capacity (DPPH$^{\bullet}$ scavenging capability): After treatment for 120 h, samples were collected for analysis of DPPH$^{\bullet}$ scavenging capability. The 50% (v/v) ethanol extracts were prepared from the fresh leaves (0.1 g of fresh mass, FM) of control and low-temperature-treated plants, followed by centrifugation at 5,000 × *g* for 15 min. The resulting supernatant was mixed with colored DPPH$^{\bullet}$ solution. The decrease of colored DPPH solution absorbance was measured at 525 nm using the spectrophotometer (*Lambda 25*, *Perkin-Elmer,* Waltham, MA, USA) as described by Peng *et al.* (2000).

Measurement of antioxidant enzymes: After treatment for 120 h, samples were collected from the fresh leaves (0.2 g FM) of each treatment for analysis of antioxidant enzymes activities. All enzyme activities were assayed using a visible-UV spectrophotometer (*Lambda 24, Perkin-Elmer,* Waltham, MA, USA). The content of protein in various extracts was estimated following the Bradford method (Bradford 1976). Standard was prepared with BSA and used for protein estimation.

Total SOD activity was assayed by monitoring the inhibition of nitroblue tetrazolium photochemical reduction (NBT; Giannopolitis and Ries 1977). One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the reduction rate of NBT as monitored at 560 nm (Aebi 1984). APX activity was measured following the oxidation of ASC at 290 nm for 1 min (Nakano and Asada 1981). POD activity was determined specifically with guaiacol at 436 nm following the method of Putter (1974), and the activities of CATwere measured by the determination of the rate constant of hydrogen peroxide decomposition according to the method of Aebi (1974).

Statistical analysis: All of the data were from five measurements. Differences of all the parameters for both the species between the treatment and the control were tested by a Student *t*-test. Data were checked for normality and homogeneity of variances. Statistical analyses were performed with *SPSS12.0* (*SPSS*, Chicago, IL, USA).

Results and discussion

Low temperature is one of the major abiotic factors limiting the agricultural productivity world over. The exposure of plants to low temperature induces many changes in physiological and biochemical parameters.

The low-temperature treatment increased the MDA content in the two cultivars, especially in cold-sensitive Williams (Fig. 1*A*), did not significantly increase the cell-membrane permeability rates in Cachaco, but significantly increased the cell-membrane permeability rates in Williams (Fig. 1*B*), which suggested that the plasma

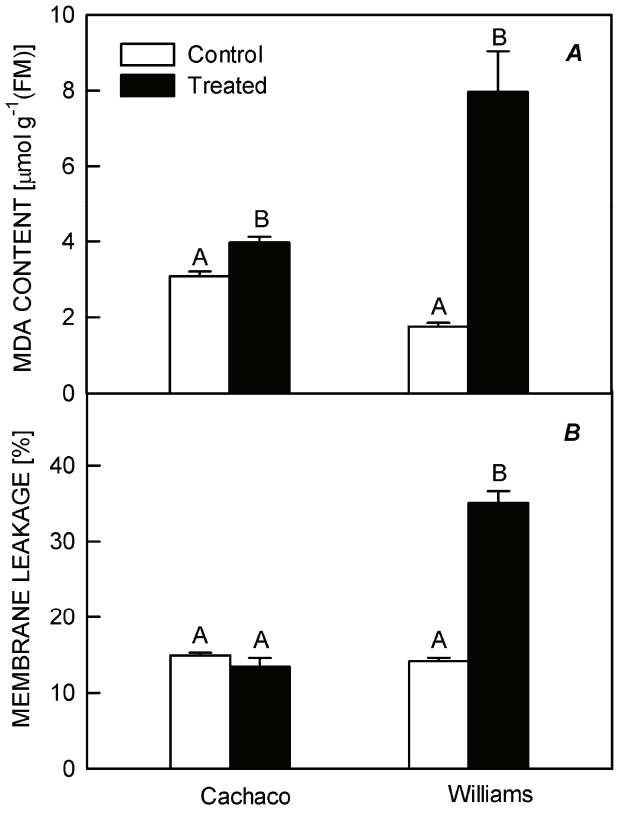

Fig. 1. Effects of low-temperature treatment for 120 h on the malondialdehyde (MDA) content and membrane leakage in the leaves of two *Musa* cultivars. Data are the mean ± SE (n = 5). *Different letters* indicate significant differences at P<0.05 between control and treatment.

membranes in Williams suffered more damage than in Cachaco. LT in Cachaco had zero effect on membrane leakage although increased ROS production and inhibited strongly PSII (Figs. 1*B*, 2, 3), which indicated that other non-ROS-related processes were still of importance in *Mus*a species. The basis for life is the ability of the cell to maintain ion gradients across biological membranes (Ronquist and Waldenström 2003), thus it is necessary to maintain membrane protein stability and function for plants in order to survive in low-temperature conditions. MDA is a harmful lipid peroxidation product and H_2O_2 represents a highly toxic active oxygen species that can damage many important cellular components (Kuzniak and Urbanek 2000). The amount of increase of MDA content in Cachaco was significantly lower than that in Williams (Fig. 1*A*), thus the membrane of the former suffer less damage as compared to the latter.

High levels of ROS accumulate in the cell when plants are continuously exposed to low temperature. H_2O_2 and superoxide radical were detected in the leaves of Williams and Cachaco cultivars with DAB and NBT staining, respectively (Fig. 2). In H_2O_2 detection, the brown deposits are the result of the reaction of DAB with H_2O_2. The control leaves of the two *Musa* cultivars were stained by some pale brown deposits, without significant differences between them. By contrast, with comparing to the Cachaco leaf, more brown deposits could be observed in Williams leaves when both were exposed to low-temperature stress for 120 h. In superoxide radical detection, where the blue formazan deposits were characteristic of reaction of NBT with superoxide radical, the result was very similar with the H_2O_2 detection. When

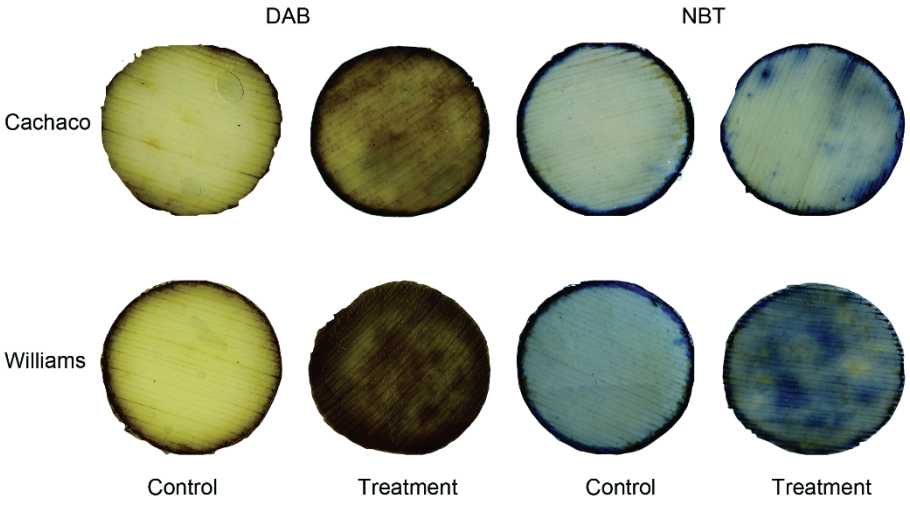

Fig. 2. H_2O_2 (*left panel*) and superoxide radical (*right panel*) localization *in situ* in the leaves of two *Musa* cultivars. The control and treated leaves of two *Musa* cultivars with low temperature (7°C) for time indicated, followed by infiltrating with diaminobenzidine (DAB) or nitroblue tetrazolium (NBT) for visualizing H_2O_2 and superoxide radical, respectively. The figure was obtained from the three repeated measurements, the disks are of diameter 1.3 cm.

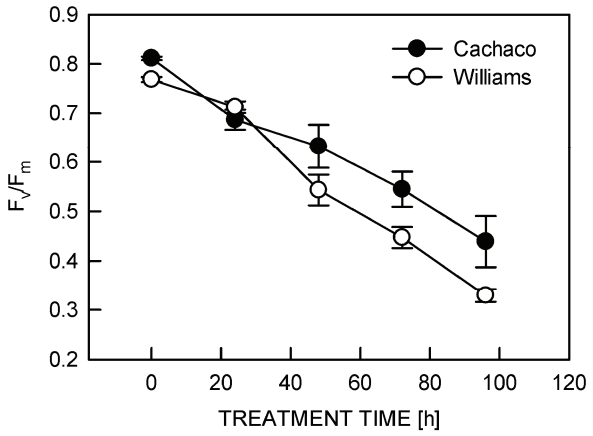

Fig. 3. Effects of low-temperature treatment on maximum photosystem II efficiency (F_v/F_m) in the leaves of two *Musa* cultivars. Data are the mean ± SE (n = 5).

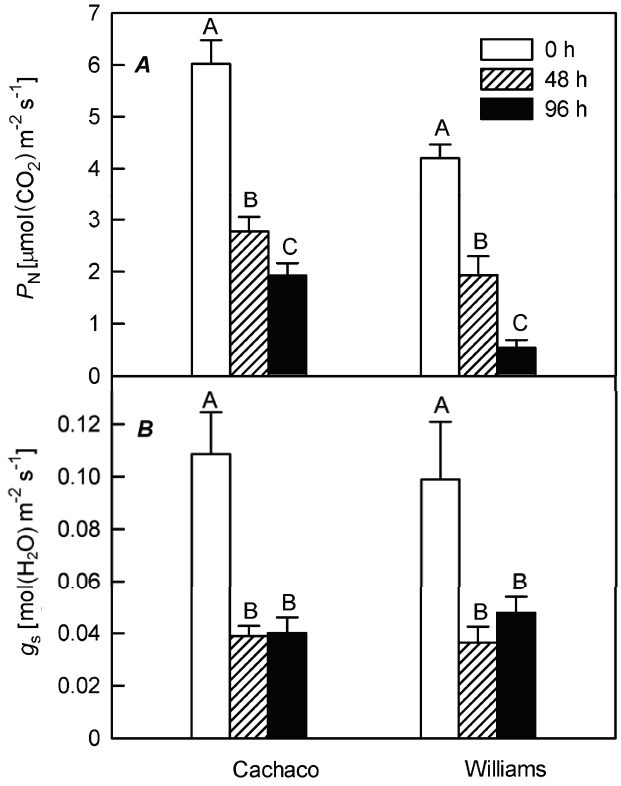

Fig. 4. Effects of low-temperature treatment on maximum net photosynthetic rate (P_N) and stomatal conductance (g_s) in the leaves of two *Musa* cultivars. Data are the mean ± SE (n = 5). *Different letters* indicate significant differences at P<0.05.

exposed to the low temperature, a leaf of Williams was stained more intensely than Cachaco leaf. The results indicated that H_2O_2 and superoxide radicals were produced when the leaves of *Musa* cultivars exposed to low temperature and Cachaco leaves may be more sensitive to low temperature stress.

Low-temperature-induced photoinhibition of photo-

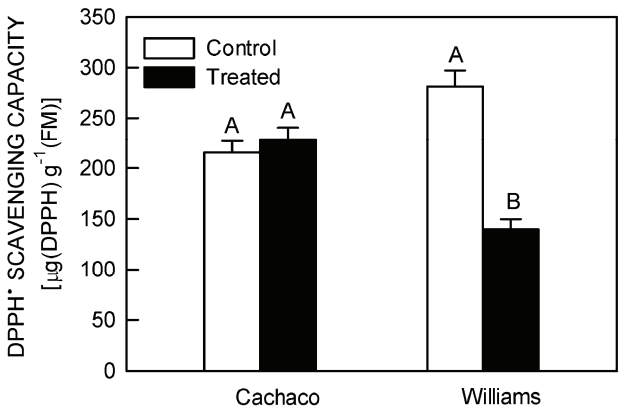

Fig. 5. Changes in the total antioxidant capacity (DPPH• scavenging capability) in the leaves of two *Musa* cultivars after 120 h of low-temperature treatment. Data are the mean ± SE (n = 5). *Different letters* indicate significant differences at P<0.05 between control and treatment.

synthesis is a major factor, limiting yield in many plants (Ebrahim *et al.* 1998, Hurry *et al.* 1998, Bertamini *et al.* 2005). An obvious decreasing trend in F_v/F_m in the leaves of the saplings of Williams and Cachaco cultivar was observed during the low-temperature treatment (Fig. 3). A more rapid decrease in the photochemical efficiency of PSII in the leaves of Williams revealed that the Williams cultivar was more sensitive to low temperature than Cachaco cultivar. In addition, Fig. 4 shows the effects of low temperature treatment on the photosynthetic capacities and stomatal conductance in the leaves of the saplings of Williams and Cachaco cultivars. After treatment for 48 h, the values of P_N in the saplings of Williams and Cachaco cultivars decreased by 54.0% and 53.9%, respectively. After treatment for 96 h, the values of P_N in the saplings of Williams and Cachaco cultivars decreased by 87.2% and 68.1%, respectively. There was a significant difference in the values of g_s after 48 h and 96 h of low-temperature treatment. After 48-h and 96-h treatment, the values of g_s in the saplings of Williams and Cachaco cultivars showed a significant decrease, however, no significant difference was found in the values of g_s after 48 h and 96 h of low-temperature treatment (P>0.05).

Tolerance to low temperature in plants should be accompanied by an increase in the activities of antioxidant enzymes (Sundar *et al.* 2004) and also in the total antioxidant capacity.

In recent studies, the capacity of a biological reagent to scavenge DPPH• has been used as an index of its antioxidant capacity and reflects approximately the resistance of the plant antioxidant system to extrinsic free radicals (Larrauri *et al.* 1998, Peng *et al.* 2000), which is a rapid, simple, sensitive, and practical assay for the evaluation of the antioxidant capacity of plants (Peng *et al.* 2000). The low-temperature treatment improved the DPPH•-scavenging capacity in cold-tolerant Cachaco, but

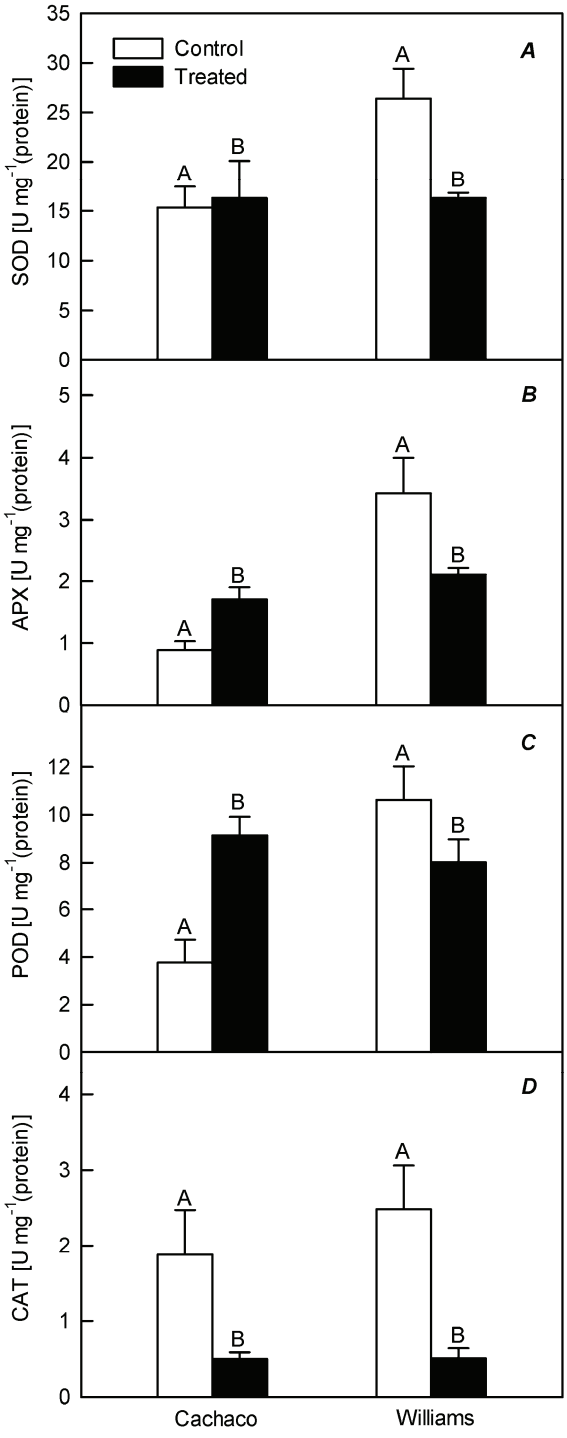

Fig. 6. Changes in the activities of superoxide dismutase (SOD; *A*), ascorbate peroxidase (APX; *B*), peroxidase (POD; *C*) and catalase (CAT; *D*) in leaves of two *Musa* cultivars after 120 h of low-temperature treatment. Data are the mean ± SE (n = 5). *Different letters* indicate significant differences at $P<0.05$ between control and treatment.

decreased that in cold-sensitive Williams (Fig. 5). After 120-h treatment, the DPPH$^{\bullet}$-scavenging capability in Cachaco showed a little increase as compared to the control, but the difference was not significant ($P>0.05$). On the contrary, the DPPH$^{\bullet}$-scavenging capability in Williams showed a significant decrease compared to the control after 120 h of low-temperature treatment. The high stable DPPH$^{\bullet}$-scavenging capability in cold-tolerant Cachaco to some degree increased its cold-tolerance.

The activity of SOD in cold-tolerant Cachaco kept stable under low temperature stress (Fig. 6*A*), which acts as a first line of defense against ROS, dismutating superoxide to H_2O_2 and hence decreasing the risk of hydroxyl radical formation from superoxide *via* the metal-catalyzed Haber-Weiss-type reaction (Apel and Hirt 2004). Moreover, low temperature enhanced the activity of APX and POD in Cachaco (Fig. 6*B,C*). PODs are the primary H_2O_2-scavenging enzymes that detoxify H_2O_2 in the chloroplasts and cytosol of the plant cells. The greater increase in the POD activity that was observed in Cachaco plays a crucial role in the detoxification of H_2O_2 in the leaves. However, low temperature resulted in decrease of CAT in Cachaco (Fig. 6*D*). The responses of SOD, APX, POD, and CAT to low-temperature stress in Williams showed a different pattern compared to Cachaco (Fig. 6*A–D*).

High activities of oxygen-free radical-scavenging enzymes have been associated with tolerance to low-temperature stress (Apel and Hirt 2004). Exposure to low temperature may increase the amount of ROS not only in cold-sensitive plants like Williams cultivar, but also in cold-tolerant plants like Cachaco cultivar. Low temperature not only resulted in a significant decrease in the photosynthetic rate in *Musa* species, but also clearly imposed an enhanced oxidative stress with important consequences. It is obvious that any ROS, promoters of cold-enhanced photooxidation, must be scavenged rapidly generally *in situ* to avoid a damaging radical chain reaction affecting DNA, lipids, and proteins (Sundar *et al.* 2004). Compared to the cold-sensitive cultivar Williams, the cold- tolerant Cachaco cultivar has an effective antioxidant mechanism to neutralize ROS. The present study describes the effect of low temperature on some key enzymes of the antioxidant system in the banana and the plantain cultivars. Results indicate that the plantain Cachaco cultivar exhibits higher activity of free radical scavenging enzymes at low temperature as compared to the banana Williams cultivar, which to a certain extent can be used to explain the higher cold tolerance in the plantain. Our results would provide a theoretical guidance for bananas production and screening of the cold-resistant variety.

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