Scientia Horticulturae 162 (2013) 333–340

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

Study of sponge gourd ascorbate peroxidase and winter squash superoxide dismutase under respective flooding and chilling stresses

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ARTICLE INFO

Article history:
Received 28 April 2013
Received in revised form 8 August 2013
Accepted 10 August 2013

Keywords:
Antioxidant enzyme
Flood stress
Chilling stress
Bitter melon
Sponge gourd
Winter squash

ABSTRACT

The objectives of this work were to study the responses of ascorbate peroxidase (APX), catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR), and physiological parameters of bitter melon (BM), sponge gourd (SG), and winter squash (WS) under waterlogged and low temperature conditions. The BM and SG plants were subjected to 0–72 h flooding treatments. Moreover, BM and WS plants were exposed to chilling at 12/7 °C (day/night) for 0–72 h. The results show that different genotypes responded differently to environmental stress according to their various antioxidant enzymes and physiological parameters. The activity of APX in roots and leaves of SG plants significantly higher than that of BM plants during continuous flooding. Significant increases in SOD activity in leaves of WS plants were also observed throughout the entire chilling duration compared to BM plants. On the basis of our observations, we conclude that increased APX and SOD activities provide SG and WS plants with increased waterlogging and chilling stress tolerance, respectively. Both APX and SOD activities can be used for selecting BM lines with the best tolerances to water logging and chilling stresses.

1. Introduction

The Cucurbitaceae (Cucurbit) family includes around 825 species derived from tropical and subtropical regions, including 26 species cultivated as vegetables (Henriques et al., 2012). Among the cultivated cucurbits, bitter melon (BM, *Momordica charantia* L.) is one of the most important vegetables. It is a white-to-green-colored immature fruit with a warty appearance and as the name suggests, it is valued for its unique bitter flavor. In addition, it is considered a prized vegetable because of its high nutritive value and medicinal properties. BM extract partitions are reported to show many pharmacological activities, including anti-inflammatory, antioxidant, and anti-radical activities (Lii et al., 2009).

Flooding conditions cause oxygen starvation, which arise from the slow diffusion of gases in water and from oxygen consumption by plant root. Problems caused by flooding may be solved by growing flood-tolerant crops or grafting intolerant plants onto tolerant ones. Sponge gourd (SG, *Luffa cylindrical*) is an annual upland crop vegetable originating in India and southern Asia, and is distributed mainly in tropical to warm-temperate areas. This species is flood tolerant in comparison to BM. In Taiwan, the yield of BM is increased by grafting with *Luffa* spp., which allows BM to survive in flooded soils (Shimamura et al., 2007). BM grows well in warm temperatures similar to those preferred for squash, but is chill-sensitive. Winter squash (WS, *Cucurbita moschata* L.) is a creeping, climbing, herbaceous, annual, monoecious plant. It has been used successfully in Taiwan as a cold-hardy rootstock that is resistant to chilling stress for winter production of BM.

Environmental stresses induce the production of reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), superoxide radicals ($^{\bullet}O_2^-$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($^{\bullet}OH^-$). Oxygen deprivation stress in plant cells is distinguished by three physiologically different states: transient hypoxia, anoxia and reoxygenation. Generation of ROS is characteristic for hypoxia and especially for reoxygenation (Blokhina et al., 2003). Toxic radicals can be removed by both enzymes and non-enzymatic compounds to protect plant cells against oxygen toxicity and counter the hazardous effects of ROS. The activities of antioxidant enzymes, which reflect the ROS pool, are often used as a measure of ROS-mediated oxidative stress. Antioxidant enzyme levels change differentially

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0304-4238/$ – see front matter.
http://dx.doi.org/10.1016/j.scienta.2013.08.016

in response to environmental constraints, depending on the magnitude of the stress and species-specific sensitivity to the stress. A coordinated increase in the activities of oxygen-detoxifying enzymes is necessary to protect plant leaves from the accumulation of oxygen radicals as a result of environmental stress. The complex antioxidant defense system that has evolved in plants is composed of antioxidant enzymes such as ascorbate peroxidase (APX), catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxidase (GPX), and peroxiredoxins (PRX). High levels of some antioxidant enzymes, either constitutive or induced, were found to be important in the pea (Moskova et al., 2009), mustard greens (Kumar et al., 2010), tobacco (Gechev et al., 2003), cucumber (Zhou et al., 2009), clover (Simova-Stoilova et al., 2012), corn (Chugh et al., 2011), sweet potato (Lin et al., 2006), and eggplant (Lin et al., 2004) in order to survive oxidative stress after being subjected to different flooding and chilling conditions. Nevertheless, there are few studies on the responses of antioxidant enzymes of BM, SG, and WS in terms of their ability to survive flooding and chilling stresses. The effects of flooding and chilling on the antioxidant activity of BM, SG, and WS, and the roles played by antioxidant enzymes in protecting plant cells from damage occurring due to flooding and chilling stresses were thus examined in this study.

The breeding of BM with broad abiotic stress resistance is hampered by the lack of practical selection tools like genetic markers because knowledge about genetic and physiology behind a successful rootstock is still very limited. It is necessary to identify physiological characteristics that reflect the complex underlying genetic make-up. These physiological biomarkers, ideally easy to measure, could be used as generic tools to develop a reliable method to support the selection of a BM having flooding and chilling-stress resistances. With this approach, BM and its specific stress-tolerant rootstocks, SG and WS, were studied with regard to efficient use of time, labor, and space. Because SG and WS have been used as rootstocks for anoxic and chilling stresses, respectively, to avoid reduced BM production, the hypothesis of this research was that increases in the activities of APX and SOD were part of the reason for the higher flooding and chilling tolerance in these rootstocks. The long-term goal of our work is to help breed a flood- and chill-tolerant BM to be grown in summer and winter seasons. The present research project studied the antioxidant enzymes of BM, SG, and WS under waterlogged and low temperature conditions.

2. Materials and methods

2.1. Plant materials, cultural practices, and stress treatments

Seeds of BM (*Momordia charanthia* L. cv. Yu-Hwa), SG (*Luffa cyllindria* Roem cv. Son-Yi), and WS (*C. moschata* cv. Gon-Zon) were purchased from Known-You seed company, the largest seed company in Taiwan. Bitter melon is a flood- and chill-sensitive variety, and requires optimum growing temperatures for satisfactory production. However, the SG and WS varieties are more flood- and chill-tolerant than BM, respectively, and are used as rootstocks during summer and winter in Taiwan. Seeds were surface-sterilized with 0.1% mercuric chloride for 5 min and washed with double-distilled water before use. Seeds were then sown in a commercial potting soil mixture, and seedlings were transplanted into 15.4-cm diameter plastic pots and placed in a growth chamber under 400 $\mu mol\ m^{-2}\ s^{-1}$ light with a 14-h photoperiod provided by fluorescent and incandescent light. The temperature of the BM plants was maintained at 30 °C and 25 °C (day and night), with the SG and WS plants kept at 25 °C and 20 °C (day and night), both at a relative humidity (RH) of 70%. Plants were watered with a half-strength Hoagland solution (Hoagland and Arnon, 1950) every other day to maintain optimal irrigation and growth for 30 days before imposition of flooding and chilling stresses.

Pots of BM and SG plants were divided into control groups receiving no flooding treatment and flooding treatment groups wherein plants were subjected to five flooding treatments for periods of 6, 12, 24, 48, and 72 h. For each treatment, three replications were used. Pots were randomly placed in 28 cm × 14 cm × 14 cm plastic buckets and subjected to flooding by filling the buckets with tap water to 5 cm above the soil surface. Pots were removed from the buckets at different times following flooding, and plants were removed and their roots rinsed with tap water. Roots and leaves from each plant were clipped, frozen in liquid nitrogen, and stored at −80 °C in an ultrafreezer until used. Three plants from each flooding period were harvested at the same time of the day and used for the enzyme measurements. Moreover, pots of BM and WS plants were randomly placed in a growth chamber under a 14 h photoperiod with an irradiance of 400 $\mu mol\ m^{-2}\ s^{-1}$ and an RH of 70%. All of the plants were exposed to chilling at 12/7 °C (day/night) for 0, 6, 12, 24, 48, and 72 h. Three replicates of each treatment were randomly placed in a growth chamber. The experiment was performed twice independently for a randomized design of growth environment, sampling day, and biochemical analysis. Following each treatment, young, fully expanded leaves from each plant were clipped to measure enzyme activities.

2.2. Enzyme extraction and activity determination

Samples were prepared for SOD (EC 1.15.11), CAT (EC 1.11.1.6), APX (EC 1.11.1.11), and GR (EC 1.6.4.2) activity analyses by homogenizing 0.2 g of each frozen leaf in 990 μl of ice-cold 100 mM HEPES buffer (pH 7.0) containing 1 mM phenylmethysulfonyl fluoride and 0.03 g polyvinylpyrrolidone. The extracts were centrifuged at 13,000 × *g* at 4 °C for 15 min. The supernatants were then collected in a fresh tube for enzyme assays. Enzyme activities were determined using a spectrophotometer. CAT activity was assayed by measuring the initial rate of disappearance of H_2O_2 (Hwang and VanToai, 1991). Two milliliters of the CAT assay reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 15 mM H_2O_2, and 20 μl of the enzyme extract. The decrease in H_2O_2 followed the decline in optical density at 240 nm, and activity was calculated with the extinction coefficient (40 $mM^{-1}\ cm^{-1}$ at 240 nm) for H_2O_2. Meanwhile, GR activity was measured by the GSH-dependent oxidation of NADPH. The reaction mixture contained 25 mM Tris–$MgCl_2$ (pH 7.6), 5 mM NADPH, 50 mM GSSG, and 1 ml of the enzyme extract (Foyer et al., 1997). The change in absorption at 340 nm (NADPHε = 6220 $M^{-1}\ cm^{-1}$) was recorded over 2.5 min. The assay for APX activity was carried out in a reaction mixture containing 166 mM HEPES (pH 7), 1.5 mM sodium ascorbate, 1 mM H_2O_2, and 40 μl of the enzyme extract. The change in absorption at 290 nm was recorded 80 s after the addition of H_2O_2 (Nakano and Asada, 1981). SOD activity was determined using the SOD Assay Kit – WST (Dojindo Molecular Technology, Gaithersburg, MD, USA). The SOD assay kit utilizes the WST working solution 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-tetrazolium, which produces a water-soluble formazan dye upon reduction with superoxide anions ($^{\bullet}O_2^-$). The rate of the reduction with O_2^- is linearly related to the xanthine oxidase activity and is inhibited by SOD. Therefore, 50% of the SOD inhibitory activity can be measured at an absorbance of 450 nm. The specific activity of SOD (inhibition rate) was calculated using the equation described in the protocol of the kit. One unit of enzyme was defined as the amount of enzyme required to decompose 1 μmol of substrate [$min^{-1}\ g^{-1}$ fresh weight (FW)].

2.3. Determination of lipid peroxides, electrolyte leakage (EL), chlorophyll fluorescence (CF), and chlorophyll content (CC)

Thiobarbituric acid (TBA)-reactive substances representing lipid peroxidation products were extracted by homogenization of 0.2 g of leaf in 5 ml of 0.6% (v/v) TBA solution in 10% (v/v) trichloroacetic acid (TCA). The mixture was heated to 95 °C for 30 min, and the reaction was stopped by quick placement in an ice-bath. The cooled mixture was centrifuged at 13,000 × *g* for 10 min at 25 °C, and the absorbance of the supernatant at 532 and 600 nm was determined. After subtracting the value for non-specific turbidity at 600 nm, the MDA concentration was determined by its molar extinction coefficient 155 mM^{-1} cm^{-1} (Kosugi and Kikugawa, 1985).

Cell membrane stability was estimated by measuring the ion leakage of leaves according to the method of Huang and Guo (2005). Leaves were excised and immersed in 15 ml of distilled water in test tubes overnight at room temperature. Initial conductivity of the water was determined using a conductivity meter (model CDM 210, Radiometer, Cedex, France). Tubes were placed in boiling water for 15 min and then cooled to room temperature. The relative EL (%) was calculated as the ratio of conductivity before boiling to that after boiling.

Components of CF were quantified with a portable modulated fluorometer (Mini-Pam Photosynthesis Yield Analyzer, Waltz, Effeltrich, Germany). Measurement of the yield to the ratio of variable fluorescence (Fv) to maximum fluorescence in light-adapted leaves (Fm) was previously described (Lin et al., 2007). Relative CC unit leaf area was determined using a SPAD (Soil Plant Analysis Development) analyzer (SPAD-502 Chlorophyll Meter, Konica Minolta, Tokyo, Japan).

2.4. Statistical analysis

All data are presented as the mean value of two independent sets of experiments. Measurements of enzymes and physiological parameters were analyzed by a completely randomized analysis of variance (ANOVA) that compared the varieties and time periods. For significant values, means were separated by the least significant difference (LSD) test at $p \leq 0.05$, using PC SAS 8.2 (SAS Institute, Cary, NC, USA).

3. Results

3.1. Physiological characteristics of bitter melon (BM) and sponge gourd (SG)

Table 1 demonstrates the effects of water logging on leaves and roots in BM and SG monitored by measuring the changes in antioxidant enzyme activities. The trends of change in CAT activity of BM plants and SG plants did not differ significantly in any tissue from 0 to 48 h duration. GR activities also showed no significant differences under flooding stress treatment between tissues over time for either BM or SG plants, with the exceptions of leaves at 24 h (1.39 > 0.81 μmol g^{-1} FW) and roots at 48 h (0.52 > 0.24 μmol g^{-1} FW). APX activity in leaves of SG plants increased from 0 h (10.25 μmol g^{-1} FW) to 6 h (72.02 μmol g^{-1} FW), but dropped to 66.04 μmol g^{-1} FW after 12 h, and then sharply increased up to a peak of 138.50 μmol g^{-1} FW at 72 h duration. A similar trend and rate of increase in APX activity over time was observed in roots of SG plants. In general, APX activities were more expressed in the roots than in the leaves of SG plants. Although APX activities of all plants were detected at all times, both leaves and roots of BM plants showed rather low activity in all of the flooding-stressed times. When genotypes were compared across time, APX activities in SG plants were exhibited more strongly and

Table 1
Effect of flooding treatment on catalase (CAT), glutathione reductase (GR), ascorbate peroxidase (APX), and superoxide dismutase (SOD) activities (μmol g^{-1} fresh weight) of bitter melon and sponge gourd.

Entry	CAT					GR					APX					SOD				
	Duration (h)					Duration (h)					Duration (h)					Duration (h)				
	0	6	12	24	48	72	0	6	12	24	48	72	0	6	12	24	48	72	0	6	12	24	48	72
Bitter melon leaf	3.40bB	6.78bA	2.84bB	2.09bBC	3.18bB	1.07dC	0.68aB	0.55aC	0.61aBC	0.81bB	0.77aB	1.24aA	13.14aD	19.25cC	23.76bBC	26.57cB	28.91cB	33.02cA	25.10bA	24.0bA	23.2cAB	22.89cB	25.65cA	24.81cA
Sponge gourd leaf	3.02bB	7.96bA	4.02bB	3.85bB	3.21bB	4.18cB	0.90aB	0.70aC	0.91aB	1.39aA	0.65aC	1.35aA	10.25aF	72.02bD	66.04aE	97.85aC	116.09bB	138.50bA	27.89abC	26.30bC	30.79bBC	32.90bB	33.75bB	36.95bA
Bitter melon root	19.84aA	11.71aC	14.07aB	20.68aA	18.91aA	19.00bA	0.14bC	0.08bC	0.29bB	0.32cB	0.24cB	0.68bA	11.92aC	15.88cBC	19.98cB	19.72dB	24.99cA	27.94cA	30.41aC	32.24aB	33.50bB	35.78bB	34.28bB	38.08bA
Sponge gourd root	16.11aC	10.25aD	17.60aC	23.54aB	22.89aB	29.94aA	0.18bD	0.15bD	0.17bD	0.36cC	0.52bB	0.82bA	12.38aF	94.47aC	61.55aE	80.04bD	167.22aB	190.11aA	28.18aD	33.70aC	42.62aB	44.36aB	45.04aB	49.66aA

Among four entries (column), means with the same *lowercase letter* do not significantly differ by the least significant difference (LSD) test at $p \leq 0.05$ with a completely randomized design. Among six flooding times (row), means with the same *capital letter* do not significantly differ by the least significant difference (LSD) test at $p \leq 0.05$ with a completely randomized design. Each value is the mean of three replicates of each entry or for each time period.

significantly higher than BM plants in any tissue. Slight increases in SOD levels were noted in leaves of SG plants and roots of BM plants as flooding times were extended. SOD activities appeared significantly different among genotypes in any tissue after 12 h of flooding.

Table 2 illustrates comparisons of MDA, EL, Fv/Fm, and CC values under waterlog treatment at six different times in the leaves of bitter melon and sponge gourd. MDA levels in SG plants (21.20–23.33 μmol g^{-1} FW) remained low and stable, and were significantly lower than in BM plants (24.68–28.64 μmol g^{-1} FW) treated with 12–72 h of flooding. As MDA is a product of lipid peroxidation, the elevation in MDA content clearly reflected cell wall damage. Lipid peroxidation measured in terms of MDA increased in all flood-stressed plants from 6 to 72 h comparing with non-flooded control plants. The patterns and trends in leaf EL (%) were similar to those of the MDA content in that all SG plants (18.74–26.37%) had significantly lower EL values compared to all BM plants (37.45–50.34%) after the 12 h treatment. Overall, the study showed differential responses of both varieties toward differential MDA and EL for different treatment durations. As shown in Table 2, levels of Fv/Fm in all plants progressively decreased as flood-stress durations were extended. Fv/Fm in the BM plants showed significantly lower values after 24 h with flooding treatment compared to the SG plants, and the lowest value (0.60) was found at 72 h. When different flood-stress treatments across time were compared, all of the BM plants exhibited significantly lower CC levels (8.84–27.12) than SG plants (15.23–34.65) at all times; thus, the CC of different genotypes responded totally different to flooding stress.

3.2. Physiological characteristic of bitter melon (BM) and winter squash (WS)

Average enzyme activities in the leaves of BM plants and WS plants under chilling treatments are summarized in Table 3. CAT activity showed no significant difference for all plants under chill-stressed conditions, with the exception of significantly higher values (46.38 and 70.21 μmol g^{-1} FW) in WS plants than (32.94 and 33.55 μmol g^{-1} FW) in BM plants at 24 and 72 h, respectively. GR activities of WS plants (0.30 and 0.25 μmol g^{-1} FW) were significantly lower than in BM plants (0.90 and 1.11 μmol g^{-1} FW) from 48 to 72 h under chilling stress. No significant differences of APX activity were detected in any treatment duration pair in any of the chill-stress treatments. SOD activities in WS plants (128.34–203.08 μmol g^{-1} FW) over time with chilling treatments were significantly higher than those of BM plants (87.67–99.35 μmol g^{-1} FW). It is noteworthy that WS (203.08 μmol g^{-1} FW) displayed a twofold greater increase over BM (99.35 μmol g^{-1} FW) at 72 h. This increase in SOD activity was clear with chilling treatments, which suggests that it was a result of the oxidative stress induced by the low temperature.

Table 4 presents the effect of chilling time on the MDA, EL, Fv/Fm, and CC in BM and WS plants. MDA levels responded differently under chill-stressed conditions for the two plant genotypes. A significantly lower MDA content over time was observed in the WS plants (1.16–1.98 μmol g^{-1} FW) compared to BM plants (3.01–4.23 μmol g^{-1} FW). Only the 72 h duration exhibited a significant difference in EL%. In addition, BM and WS plants exhibited similar patterns of increased EL values as chilling durations increased. As shown in Table 4, the Fv/Fm values for 6–72 h tests in BM plants (0.68–0.31) were significantly lower than in WS plants (0.79–0.70). In general, low temperatures reduced Fv/Fm values, and gradual decreases in Fv/Fm values were observed with both varieties over time. Chlorophyll content also gradually decreased in all plants during all time intervals over the course of the experiment. Furthermore, BM plants (30.02–17.38)

Table 2
Effect of flooding treatment on malondialdehyde (MDA, μmol g^{-1} fresh weight), electrolyte leakage (EL, %), Fv/Fm value, and chlorophyll content (CC) in leaves of bitter melon and sponge gourd.

Entry	MDA					EL					Fv/Fm					CC				
	Duration (h)					Duration (h)					Duration (h)					Duration (h)				
	0	6	12	24	48	72	0	6	12	24	48	72	0	6	12	24	48	72	0	6	12	24	48	72
Bitter melon	20.12aC	22.95bB	24.68aB	26.05aAB	27.80aA	28.64aA	15.51aE	35.88aD	37.45aD	42.60aC	50.34aA	46.60aB	0.82aA	0.81aA	0.73aB	0.69bC	0.62bD	0.60bD	27.12bA	20.62bB	13.87bC	11.05bC	10.92bCD	8.84bD
Sponge gourd	18.47aC	30.69aA	21.20bB	22.17bB	24.76bB	23.33bB	14.26aD	37.02aA	26.37bB	19.18bC	18.74bC	21.56bC	0.81aA	0.79aA	0.76aB	0.75aB	0.72aC	0.68aD	34.65aA	27.34aB	20.56aC	18.59aC	16.25aD	15.23aD

Among two entries (column), means with the same *lowercase letter* do not significantly differ by the least significant difference (LSD) test at $p \leq 0.05$ with a completely randomized design. Among six flooding times (row), means with the same *capital letter* do not significantly differ by the least significant difference (LSD) test at $p \leq 0.05$ with a completely randomized design. Each value is the mean of three replicates of each entry or for each time period.

Table 3
Effect of chilling treatment on leaf catalase (CAT), glutathione reductase (GR), ascorbate peroxidase (APX), and superoxide dismutase (SOD) activities (μmol g^{-1} fresh weight) in leaves of bitter melon and winter squash.

Entry	CAT						GR						APX						SOD					
	Duration (h)						Duration (h)						Duration (h)						Duration (h)					
	0	6	12	24	48	72	0	6	12	24	48	72	0	6	12	24	48	72	0	6	12	24	48	72
Bitter melon	52.46aA	34.75aC	36.14aC	32.94bC	47.02aB	33.55bC	0.27aD	0.24aD	0.22aD	0.63aC	0.90aB	1.11aA	1.32bB	0.51aC	1.19aB	1.00aB	0.87aC	2.15aA	84.26aD	87.67bC	90.53bB	92.19bB	93.86bB	99.35bA
Winter squash	35.27bC	32.11aC	33.91aC	46.38aB	44.83aB	70.21aA	0.19aC	0.17aC	0.14aC	0.54aA	0.30bB	0.25bB	2.85aA	0.43aE	1.01aC	0.83aCD	0.76aD	1.88aB	86.81aE	128.34aC	116.03aD	120.48aC	142.90aB	203.08aA

Among two entries (column), means with the same *lowercase letter* do not significantly differ by the least significant difference (LSD) test at $p \leq 0.05$ with a completely randomized design. Among six chilling times (row), means with the same *capital letter* do not significantly differ by the least significant difference (LSD) test at $p \leq 0.05$ with a completely randomized design. Each value is the mean of three replicates of each entry or for each time period.

Table 4
Effect of chilling treatment on malondialdehyde (MDA, μmol g^{-1} fresh weight), electrolyte leakage (EL, %), Fv/Fm value, and chlorophyll content (CC) in leaves of bitter melon and winter squash.

Entry	MDA						EL						Fv/Fm						CC					
	Duration (h)						Duration (h)						Duration (h)						Duration (h)					
	0	6	12	24	48	72	0	6	12	24	48	72	0	6	12	24	48	72	0	6	12	24	48	72
Bitter melon	2.38aC	3.01aB	3.45aB	3.89aAB	4.08aA	4.23aA	20.50aE	25.16aD	42.47aC	44.02aB	47.38aB	57.92aA	0.80aA	0.68bB	0.61bBC	0.52bC	0.37bD	0.31bD	30.02bA	26.57bB	25.66bB	22.18bB	20.46bC	17.38bC
Sponge gourd	1.99aA	1.16bC	1.60bB	1.74bB	1.80bAB	1.98bA	18.61aE	23.87aD	38.53aC	41.39aBC	43.45aB	47.76bA	0.81aA	0.79aA	0.78aA	0.76aA	0.70aB	0.70aB	43.94aA	42.18aA	40.75aA	36.84aB	32.64aB	28.77aC

Among two entries (column), means with the same *lowercase letter* do not significantly differ by the least significant difference (LSD) test at $p \leq 0.05$ with a completely randomized design. Among six chilling times (row), means with the same *capital letter* do not significantly differ by the least significant difference (LSD) test at $p \leq 0.05$ with a completely randomized design. Each value is the mean of three replicates of each entry or for each time period.

exhibited significantly lower levels of CC compared to WS plants (43.94–28.77) at all time intervals.

4. Discussion

4.1. Flooding and chilling stresses effects on the activities of antioxidant enzyme

Tables 1 and 3 demonstrate that various enzymes acted differently under flooding and chilling stresses; however, each enzyme is not necessarily equally significant in protecting plants against stress. Higher APX activity in the SG genotype at all times of water logging signified its relative tolerance to flood stress, while the flood-sensitive BM genotype was inferior in this regard (Table 1). Most importantly, both genotypes can be identified by APX activity with as little as 6 h of flooding. Exposure to various flooding durations for at least 12 h caused significant increases in SOD activity in SG plants compared to BM plants. However, in general, CAT and GR activities did not seem to be affected by waterlogging stress throughout the entire duration of the study in all plants (Table 1). Water logging changes plant metabolic activity. One of the metabolic features in roots affected by flooding is the antioxidant system. When antioxidant enzyme activities between leaf and root were compared, roots had higher CAT, APX, GR, and SOD activities than leaves at most flooding durations. It is possible to attribute a higher ROS accumulation in roots under flooding treatments because roots also generate ROS (Zhou et al., 2009). The differential responses of antioxidant enzymes may be one of the possible mechanisms of the differences in flooding sensitivities of the two species, and APX has a direct relation to the sensitivity of the two varieties to flooding. When SG plants underwent waterlog stress for a longer time (72 h), they required CAT, APX, GR, and SOD to detoxify ROS, suggesting that the interactions of CAT, APX, and SOD, and their involvement in scavenging ROS, are complex. The response to environmental oxygen deprivation has been studied in species that range from flood-sensitive to flood-tolerant (Fukao and Bailey-Serres, 2004). The flood-tolerant ability of SG plants to different durations might be considered compensatory, because they buffer the effects of flooding shifts on their metabolic systems. Moreover, the measurement of changes in APX activity in SG plants exposed to flooding overtime confirmed that APX was upregulated by flooding stress. Taken together, these data suggest that different plant genotypes may respond to oxidative stress by upregulating antioxidant activities during flooding conditions, and that APX is deeply involved in this process.

SOD activity levels in WS plants under 6–72 h chilling treatments were significantly higher than in BM plants (Table 3). In other words, genotypes can be identified after 6 h of chilling by measuring SOD activity in leaves. On the contrary, activities of CAT, GR, and APX in WS plants under chilling stress overtime were either similar to or lower than in BM plants. This implies that ROS (i.e., H_2O_2) homeostasis can be maintained in plant cells by various antioxidant enzymes, and SOD activity appeared to be more sensitive to chilling stress in plants compared to GR, CAT, and APX. Enhanced SOD activity in WS plants favors chilling tolerance. This was caused by induction of low-temperature stress, and it explains why WS plants are more resistant to chilling than BM plants. Enhancement of SOD activity in chilled WS plants may be an indicator of superoxide production. Dismutation of the superoxide anion by SOD might be the primary step in a defense mechanism against low temperature conditions. The chilling-induced decrease in the proportion of electron flux for photosynthetic carbon reduction might be accompanied by an increase in O_2-dependent alternative electron flux and a significant increase in the activity of SOD. As this enzyme is important for chilling tolerance, its elevated levels may partially compensate the low activities of catalase and enzymes of the ascorbate–glutathione cycle. It might be due to removal of H_2O_2 if carried out by SOD, so an upgrade in CAT activity is not required. In WS plants, either SOD is not very active under non-stressed conditions, and therefore excess H_2O_2 is not being produced, or SOD is functional all the time and is capable of reducing the H_2O_2 produced. These findings strongly suggest that SOD is involved in increasing chilling tolerance, and might have a function related to stress signaling. Winter squash plant response to chilling stress might then be correlated with resistance to oxidative stress.

Increased activity of SOD also appeared in chilling-exposed cucumber leaves under low light and subsequent recovery (Zhou et al., 2004). The importance of SOD for chilling tolerance was demonstrated by over-expression of the enzyme in tobacco (Roxas et al., 2000). Kumar et al. (2010) reported that SOD was increased after chilling treatment in comparison to normal seedlings of *Brassica juncea*. Previously, we found that the reason for eggplant being more flood tolerant than other cultivars and thus often used as a rootstock for propagating tomatoes was that the increased APX activity contributed to increased waterlogging stress tolerance in its roots (Lin et al., 2004). In the current study, the three genotypes exhibited unique abilities and specificities through antioxidant enzymes in response to specific stress. Hence, different stress conditions might generate different antioxidant mechanisms for tolerance. Enzyme activities responded differently to flooding and chilling stress treatments, indicating that different enzyme components in the ascorbate–glutathione cycle play different roles in the detoxification of ROS. The degree of stress-induced injury seemed to be a result of APX and SOD enhancement in SG and WS plants, respectively. Sponge gourd's APX activity (Table 1) and WS's SOD activity (Table 3) were maintained at significantly higher levels under specific stress treatments than in BM leaves; therefore, they could be used to develop physiological markers to select flood- and chill-tolerant germplasm or to create a tolerant bitter melon through genetic manipulation.

4.2. Effects of flooding and chilling on the MDA, EL, Fv/Fm and CC

The oxidative status of leaves in the three genotypes under flooding and chilling stresses is shown in Tables 2 and 4. The flood-tolerant SG genotype showed significantly lower MDA and EL content than the sensitive BM genotype when flooded for 12–72 h (Table 2), which reflects its tolerant nature. High electrical conductivity due to a high concentration of electrolytes in the leaf sap of BM plants should lead to alterations in membrane permeability and a reduced ability to retain solutes during flooding. However, the ability of SG plants to tolerate flooding stress strongly depends on adjustments in ionic leakage (Table 2). At the physiological level, the many effects of flooding stress indicate the importance of protecting plants from oxidative damage caused by the overproduction of ROS that is elicited by increased ion leakage (Kangasjarvi et al., 2008). Membrane damage under flooding stress conditions is mainly caused by ROS generation. The extent of oxidative stress causing membrane and cellular damage possibly differs depending on the flooding stress imposed. In BM plants, the results are primarily ionic imbalance and hyperosmotic stress, and the effect of this imbalance in homeostasis occurs in the leaf (Jithesh et al., 2006). However, SG plants osmotically adjust additionally to compensate for flooding stress. It is possible that the superior flood tolerance of SG was associated with its ability to maintain higher APX activity (Table 1), resulting in lower lipid peroxidation (indicative of higher membrane stability).

Significantly higher MDA was observed in chill-stressed BM plants compared to WS plants (Table 4), which may cause peroxidation of unsaturated membrane lipids, increase membrane rigidity, and result in later leakage of electrolytes and soluble materials out of the cell into intercellular spaces of the leaves. By measuring MDA

and EL content with BM and WS plants in this study, it became clear that WS plants having low temperature tolerance may decrease the degree of lipid peroxidation and electrolyte leakage induced by chilling stress (Table 4). Increases in the steady state level of membrane lipid peroxidation products are considered to reflect oxidative stress. Chilling in plants may result from the formation of ROS, leading to the oxidation of macromolecules and oxidative stress, which can induce lipid peroxidation. A direct relationship can be assumed between efficient antioxidant machinery and a delay in the onset of MDA accumulation (Yiu et al., 2009). Comparing the BM and WS plants indicates that this difference is related to differences in ROS accumulation. When ROS is increased with chilling stress, a profound up-regulation in the capacity to detoxify these compounds via ROS-scavenging enzymes can be expected. Higher activity of SOD in WS plants was in agreement with this phenomenon (Table 3). Our results are also in agreement with the results of Meloni et al. (2003), who found that the lesser degree of membrane damage and higher activity of SOD in NaCl-treated cotton were correlated with higher salinity tolerance.

The exposure to flooding for at least 24 h provoked greater reductions in Fv/Fm values in BM plants than SG plants, with values being <0.8 (Table 2). In addition, low temperatures also significantly decreased the leaf Fv/Fm in BM plants than in WS plants in 6–72 h durations (Table 4). The chlorophyll fluorescence emission parameter, Fv/Fm that is widely used as a proxy for the maximum quantum efficiency of PS II photochemistry, was correlated with flooding and low temperature tolerance. In healthy leaves, the Fv/Fm value is close to 0.8, which is a typical value for uninhibited plants. A lower value indicates that some proportion of the PSII reaction centers are damaged, which is often observed in plants under conditions of stress (Camejo et al., 2006). Flooding and low temperatures inhibit photosynthetic CO_2 fixation, and damages photosynthetic electron transport at the site of PSII where exists a very sensitive photosynthesis apparatus. This reduction in the CO_2 assimilation rate observed in BM plants was generated by effects on the Calvin cycle and also on PSII functioning. Exposure of BM plants to flooding and low temperature may lead to a reduction in the net photosynthetic rate, stomatal closure, and cell activities that damage photosynthetic membranes. Jiang and Huang (2001) also reported that heat stress caused declines in Fv/Fm and increases in EL in tall fescue and perennial ryegrass. These results suggest that the chlorophyll fluorescence parameters were stress specific and were not expressed solely in response to increasing excess of photon energy. Chloroplast development in BM plants may be particularly sensitive to low temperature and flooding. Alternatively, the pigments of the sensitive variety might have been destroyed because of a high sensitivity to oxidative stress.

Similar parallel losses of chlorophyll were observed in BM, SG, and WS leaves under flooding and chilling stresses, but responded at different levels (Tables 2 and 4). BM plants suffered greater CC losses than SG and WS plants over durations of 6–72 h. Flooding and chilling may induce stomatal closure and consequently reduce CC levels. Typically, CC is reduced in amount by stressful conditions. This is consistent with our observations that chlorophyll loss was more pronounced in waterlog- and chill-sensitive BM plants in accordance with the more pronounced and increased visible symptoms of leaf injury. Leaf curling or folding were the initial and most obvious changes observed. Most of the leaves in BM plants progressively looked necrotic, epinastic, or wilted over the course of time; however, most leaves of SG and WS plants visually appeared green and healthy after 72 h of flooding and chilling (photos not shown). Along with visual symptoms, reduced CC could be used to monitor flooding and chilling damage in green or senescent leaves. The flooding and chilling stresses applied in this study (0–72 h) influenced plant growth, but these effects were not lethal. Oxygen is required for stress-induced inactivation of photosynthesis to take place, which suggests that the formation of ROS seemed to be the cause of the damage to chlorophyll during exposure to stress. A decrease in the use of radiation absorbed by pigments can lead to the production of potentially dangerous ROS (Ahmed et al., 2002), which are then enhanced in the leaves of BM plants. To keep the electron transport chain oxidized and prevent ROS formation, electrons must be efficiently consumed in the leaves of BM plants by the Calvin cycle or other electron sinks. Results from Tables 2 and 4 demonstrate that the chlorophyll losses in BM plants were in concert with increasing MDA and EL accumulations in this genotype after 6 h of flooding and chilling treatments as an index of oxidative damage of cell constituents in general. The results suggest that Fv/Fm and CC can effectively be used as reporter signals for screening of flooding and low temperature tolerances. Among the three genotypes, SG and WS plants had higher APX and SOD activities than BM plants under flooding and chilling stresses, respectively (Tables 1 and 3). These results agree with SG and WS plants having higher Fv/Fm and CC, and lower MDA and EL with different stress treatments overtime compared to BM plant (Tables 2 and 4). These findings are important for farming in low temperature areas and wetlands or other areas subject to short and intense rainfall events.

5. Conclusions

Different varieties displayed variations in their antioxidant enzymes, and the differential activities of each genotype were associated with stress responses. Higher activities of APX and SOD in flood- and chill-treated plants can reduce the accumulation of H_2O_2 and alleviate damage to cell membranes. Therefore, APX and SOD are essential components of an adaptive defense mechanism against flooding and chilling stresses, and are thus considered to be flood- and chill-tolerant enzymes in the sponge gourd and winter squash, respectively. They may be the rate-limiting factors of antioxidant enzymes for use as genetic tools to develop an effective method for the selection of bitter melon to improve adaptability to flooding and chilling. The systems identified herein could thus be used for rapid monitoring and early detection of flooding and chilling injuries, i.e., during the seedling stage. In this way, hundreds of individual plants can be cost-effectively screened per day, providing the scope for discovery of individuals exhibiting tolerance to flooding and chilling.

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