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REGULAR ARTICLE

Exogenous catechin increases antioxidant enzyme activity and promotes flooding tolerance in tomato (*Solanum lycopersicum* L.)

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Abstract We studied the extent to which catechin applied as a soil drench modifies the effects of soil waterlogging on plant growth, the functioning of the free radical scavenging system and on oxidative stress levels. Forty-day-old tomato plants (*Solanum lycopersicum* L.) were treated with 0 and 2 *mM* catechin 48 h prior to 5 d waterlogging followed by a 4 d drainage period. Exogenous catechin increased total fresh and dry weight of flooded plants, reduced membrane damage, maintained chlorophyll concentrations, promoted photosynthesis and increased ATP concentration in the leaves, and raised sucrose synthase and alcohol dehydrogenase activities in the roots. Catechin pretreatment also reduced hydrogen peroxide and superoxide radical concentration and increased various components of the antioxidative system in leaves. Catechin treatment affected superoxide dismutase and catalase activities in close coordination with ascorbate peroxidases and glutathione reductase. Exogenous catechin can markedly reduce the waterlogging injury in leaves and roots of tomato by enhancing free radical scavenging system sufficiently to lower hydrogen peroxide and superoxide concentrations.

Keywords Alcohol dehydrogenase · Antioxidant enzyme · Pretreatment · Recovery · Sucrose synthase · Waterlogging

Abbreviations

ADH	alcohol dehydrogenase
APX	ascorbate peroxidase
C	control
Cat	catechin
CAT	catalase
Chl	chlorophyll
GR	glutathione reductase
MSI	membrane stability index
Pn	CO_2 assimilation rate
ROS	reactive oxygen species
RWC	relative water content
SOD	superoxide dismutase
SuSy	sucrose synthase
W	waterlogging

Introduction

Waterlogging is defined as the saturation of the soil with water around the roots (Anton et al. 2002). The negative impact of waterlogging on plant growth and

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development is largely the consequence of the slow diffusion rates of gases in water as compared with air and the relatively low solubility of oxygen in water (Vartapetian and Jackson 1997). Oxygen is vital for the central energy-providing pathway of cells; the presence or absence of oxygen determining metabolic activity and energy production. Reduction of root respiration is one of the earliest responses of plants under anoxia, regardless of whether the plants are flooding-tolerant or intolerant (Liao and Lin 2001). Kuo and Chen (1980) demonstrated that the relatively greater tolerance of the tomato genotype L-123 to soil flooding depends, in part, on its ability to transport oxygen internally from the aerial part to the roots. Oxygen deficiency due to waterlogging or flooding closes stomata, reduces internal CO_2 concentrations in leaves, and therefore slows photosynthesis severely (Olivella et al. 2000). As a consequence, the photosynthetic electron transport chain may become over-reduced, forming superoxide radicals (O_2^-) and singlet oxygen species in chloroplasts. Any reactive oxygen species (ROS) generated in excess due to flooding conditions could unbalance the cellular redox systems in favor of oxidized forms, resulting in oxidative damage to lipids, proteins and nucleic acids (Halliwell and Gutteridge 1989).

Waterlogging blocks the oxygen supply to the roots, thus inhibiting root respiration, resulting in a severe decline in the energy status of root cells that affects important metabolic processes of plants. Plants react to an absence of oxygen by switching from an oxidative to a solely substrate-level phosphorylation of ADP to ATP, the latter reactions predominantly involving glycolysis and fermentation. The overall yield of ATP produced during fermentation is only two molecules of ATP per glucose molecule, against 38 molecules of ATP produced during oxidative phosphorylation (Ricard et al. 2006). Sucrose synthase (SuSy), which catalyzes the reversible conversion of sucrose and UDP to UDP-glucose and fructose, plays a crucial role in providing an adequate sugar supply during anoxic stress (Ricard et al. 2006). Meristem death of maize seedlings was significantly alleviated in the presence of glucose in an anoxic incubation medium, indicating that an increase in glycolytic flux alone was sufficient to restore root tip viability (Ricard et al. 1998). The genotype T44 (tolerant) of the mung bean showed a greater increase in SuSy activity than the PB genotype (susceptible) under waterlogging conditions (Sairam et al. 2009). Continuous supply of fermentable sugars to roots is considered to be critical for long-term survival under anoxia or flooding. Therefore, the greater increase in SuSy activity under waterlogged conditions resulted in an increased availability of reducing sugars, which sustained their energy requirement.

Root anaerobic metabolism depends on alcoholic fermentation for ATP production. Alcohol dehydrogenase (ADH) is an essential fermentative enzyme which is supported by rapid increases in the activity of ADH in rice seedlings when oxygen supply was severely deprived (Rivoal et al. 1989). Maize and barley mutants that lack the *ADH1* gene are more sensitive to flooding injury than are wild-type plants (Roberts et al. 1989). Wang et al. (2009) found ADH activity of Kentucky bluegrass (*Poa pratensis* L.) to increase dramatically within 3 days of flooding but significantly decreased as root internal aeration increased. Higher ADH activity is often associated with anaerobic conditions in plants.

The susceptibility of plants to environmental stress, including waterlogging, has been considered to be associated with antioxidative ability (Zheng et al. 2009). To control the level of ROS and to protect cells under stress conditions, plant tissues contain several ROS scavenging enzymes, e.g., superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), ascorbate peroxidases (APX; EC 1.11.1.11), glutathione reductase (GR; EC 1.6.4.2), enzymes that detoxify lipid peroxidation products (glutathione S-transferases, phospholipid- hydroperoxide-glutathione peroxidase and ascorbate peroxidase), and a network of low-molecular-mass antioxidants (ascorbate, glutathione, phenolic compounds and α-tocopherol). Increases in the activities of antioxidant enzymes in response to various environmental stresses have also been reported in various studies. The comparatively greater antioxidant enzyme activities that result in less oxidative stress in pigeon pea genotype ICP 301 (tolerant) could be one of the determining factors of a greater tolerance to flooding as compared with Pusa 207 (susceptible) (Kumutha et al. 2009). Hossain et al. (2009) demonstrated that coordinated antioxidant activity, involving increased activities of SOD and CAT together with modulation of the ascorbate–glutathione cycle, allowed plants to cope with flooding-induced oxidative stress up to a certain point.

Some polyphenols, especially those in seeds and green leaves inhibit oxidative damage through their

antioxidant activity (Rice-Evans et al. 1996). Catechins are the major polyphenolic compounds in green tea. These include (–)-epigallocatechin gallate (EGCG), (–)-epigallocatechin (EGC), (–)-epicatechin gallate (ECG), and (–)-epicatechin (EC). Collectively, they account for about 10% of tea leaf dry weight (Hara et al. 1995). Some phenolic compounds can establish cooperative redox interactions with the endogenous antioxidant substances, which reinforce synergistically the resistance of the system to suffer oxidative damage. The mechanisms of polyphenol antioxidant activity include their ability to scavenge radicals and interact with intracellular signal transduction pathways (Nijveldt et al. 2001; Williams et al. 2004). Tea catechins scavenge O_2^-, hydroxyl radicals, and oxidized copper-mediated low-density lipoproteins more effectively than alpha-tocopherol. Along with the protective effect against oxidative stress polyphenols might act as prooxidants (Chen and Chan 1996; Zhang et al. 1997; Lo et al. 2006).

In many Asian countries, heavy typhoon rains often disturb tomato production during the hot summer season (Lin et al. 2004). The physiological and biochemical responses of tomato plants to waterlogging stress have been extensively investigated (Jackson et al. 2003). Furthermore, exogenous antioxidant application is a convenient and effective approach for enhancing the stress tolerance of crops and eventually improving crop productivity. Treatment with 10*mM* L-ascorbic acid before re-exposure to air after varying periods of anoxia shows that this antioxidant can improve growth and survival of chickpea seedling during the post-anoxic recovery period (Crawford and Wollenweber-Ratzer 1992).

The present study is the first we know of that assesses the ability of catechin to ameliorate the effects of waterlogging on plant growth and link its effects to changes in ROS and components of the antioxidant system.

Materials and methods

Plant growth, waterlogging treatment and sample collection

Tomato seeds (*Solanum lycopersicum* L. cv. Koma) were sown in small pots (3 cm tall and 2 cm diameter) containing peat moss, vermiculite and perlite (1:2:2, v/v) and transferred to a growth chamber at 24°C under fluorescent light and tungsten light (350 μmol m^{-2} s^{-1}) with a 16-h photoperiod. Seven days after germination, seedlings were transplanted one plant per pot to 8 cm tall and 6 cm diameter plastic pots filled with similar compost. Plants were grown in a plastic greenhouse at National Ilan University, Taiwan, under natural sunlight and photoperiod for 4 weeks before being subjected to waterlogging stress. Plants were watered with half-strength Hoagland solution every other day to maintain optimal irrigation. Mid-day weather conditions during the experimental periods were characterized by relatively clear sky (i.e., photosynthetic photon flux density >900 μmol m^{-2} s^{-1}), maximum air temperature of 28–33°C and 72% relative humidity.

Each plant was treated with a single 50-mL dose of an aqueous solution of 2*mM* catechin (ca. 0.1*mmol*) (C1788, Sigma-Aldrich Laborchemikalien Gmbh, Seelze, Germany), applied as a soil drench. In preliminary experiments, when comparing the effect of Cat concentration, 2*mM* Cat proved to be the most effective dose. A lower concentration (1*mM*) was slightly effective, and 4*mM* Cat effects were little different to those of 2*mM* Cat. Forty eight hours after catechin treatment, the plants were waterlogged for 5 d by placing pots in 65 cm×35 cm×12 cm plastic containers filled with sufficient water to raise levels to 2 cm above the soil surface (Yiu et al. 2009). Treatments consisted of control (C), 1, 3, and 5 d of waterlogging, and recovery after 4 d drainage. Control (unwaterlogged) plants remained well-watered during the period of the experiment. More measurements, plants were removed from pots and plants (roots and leaves) were washed thoroughly with water and dried in paper towel, oven dried at 70°C to constant dry weight (DW).

Three replicates were allocated to each treatment, each replicate consisting of 30 plants. Physiological parameters and enzyme activities were measure on basal 3 full leaf of 2nd and 3rd node from the top and all parts of the root. Observations were recorded on relative water content (RWC), membrane stability index (MSI), chlorophyll (Chl) content, photosynthesis (*P*n), activity of SuSy and ADH, ATP, O_2^-, H_2O_2 concentration, and activity of antioxidant enzymes. After each treatment, leaves or roots were harvested, immediately frozen in liquid nitrogen and kept at −80°C until analysis.

Physiological measurements

Leaf RWC was estimated by recording the fresh weight of 0.5 g fresh leaf samples before and after floating on water for 4 h, followed by drying in hot air oven (75°C) until a constant dry weight was achieved (Weatherley 1950).

$$\text{RWC} = (\text{fresh weight} - \text{dry weight}) / (\text{fresh weight at full turgor} - \text{dry weight}) \times 100\%.$$

MSI was estimated by placing two sets of 200 mg of leaf or root material in test tubes containing 10 mL of double-distilled water (Sairam et al. 1997). One set was heated at 40°C for 30 min in a water bath, and the electrical conductivity of the solution recorded using a conductivity meter (C_1). The second set was boiled at 100°C on a boiling water bath for 10 min before conductivity (C_2) was measured as above. MSI was calculated as

$$\text{MSI} = [1 - (C_1/C_2)] \times 100$$

Chlorophyll concentration was estimated by extracting 0.5 g of the leaf material in 25 mL dimethyl sulfoxide (DMSO). Samples were heated at 60°C for 12 h, cooled to room temperature before measuring absorbance at 648.2 and 664.9 nm and calculating concentration according to Barnes et al. (1992).

Leaf CO_2 assimilation rate (Pn) was measured according to Yiu et al. (2009) on each of the selected leaves using infrared gas analyzers built into a leaf cuvette in an open-gas exchange system (Li-Cor-6400; Li-Cor Inc., Lincoln, NE, USA).

Enzyme assays on roots

Sucrose synthase assay of root tissue was based on the synthesis of sucrose from UDP-glucose and fructose and its estimation by anthrone reagent after the removal of untreated fructose by heating at 100°C (Zeng et al. 1999). Extraction buffer consisted of 200 *mM* HEPES buffer, pH 7.5, 1 *mM* DTT, 5 *mM* $MgCl_2$, 1 *mM* EDTA, 20 *mM* sodium ascorbate, 1 *mM* PMSF, and 10% (w/v) polyvinylpolypyrrolidone. The buffer-to-tissue ratio was 10:1. One gram root was ground to fine powder in liquid nitrogen in a chilled mortar and pestle followed by extraction with chilled extraction buffer. Extract was centrifuged at 14,000 × *g* at 4°C for 10 min, the supernatant dialyzed at 4°C for 24 h against extraction buffer diluted 1:40 and changed at least three times during dialysis. Dialyzed extract was used as an enzyme. The reaction mixture in 3 mL contained 50 *mM* HEPES–NaOH buffer (pH 7.5), 15 *mM* $MgCl_2$, 10 *mM* fructose, 5 *mM* UDP-glucose, 50 μL enzyme extract and water to make final volume 3.0 mL. Assays were at 30°C for 30 min in a shaking water bath and the reaction terminated by the addition of 1 mL of 30% KOH. Controls were terminated at 0 min. The un-reacted fructose is removed by subsequent heating at 100°C for 10 min. After cooling, each assay mixture was incubated with 1 mL of 0.14% anthrone in H_2SO_4 at 40°C for 20 min and absorbance recorded at 620 nm in UV–visible spectrophotometer. Activity was expressed as μmol per mg protein per min. Total protein was quantified according to Bradford (1976), with bovine serum albumin as the standard.

Alcohol dehydrogenase (ADH) activity in roots was assayed by the reverse reaction i.e., oxidation of ethanol by ADH with the help of NAD, resulting into the synthesis of acetaldehyde and NADH (Chung and Ferl 1999). The extraction buffer consisted of 50 *mM* Tris HCl and 15 *mM* DTT, pH 8.0. Root tissue (0.5 g) was first powdered with liquid nitrogen and then homogenized with 5.0 mL of extraction buffer. The extract was centrifuged at 12,000 g for 15 min at 4°C in refrigerated centrifuge and an aliquot of 50 μL taken for water-soluble protein concentration assay. The 3 mL reaction mixture contained 50 *mM* Tris buffer, 0.867 *mM* NAD, 20% ethanol, 50 μL enzyme, and water to make final volume of 3 mL. Reaction mixture except NAD was prepared in test tubes and each sample used as a blank to adjust to zero. NAD was then added to initiate the reaction. The resultant increase in absorbance at 340 nm was recorded for 1 min in UV–visible spectrophotometer. Amount of NADH formed was computed by drawing a standard curve of NADH at 340 nm, activity being expressed as nmol NADH formed per mg protein per min.

Measurement of ATP, O_2^- and H_2O_2 concentration in leaves

Leaf samples were prepared for ATP, O_2^- and H_2O_2 analyses by homogenizing 0.2 g of frozen leaf. Intracellular ATP was measured according to Yiu et al. (2009) using an ATP Bioluminescence Detection Kit (Promega Corporation, Madison, WI, USA). ATP

concentration was calculated from an ATP standard curve and expressed as nmol g^{-1} DW. Superoxide anion and H_2O_2 levels were determined according to Yiu et al. (2009). Determinations were performed on 5 replicates.

Protein extraction and antioxidant enzyme assay in leaves

Approximately 1.0 g fresh weight (ca. 10 leaves) were homogenized at 4°C in 1.5 mL of extraction buffer [100 *mM* potassium phosphate, pH 7.5, containing 1 *mM* EDTA and 1% PVP-40] with a mortar and pestle. The homogenate was then centrifuged at 25,000×*g* for 20 min and the supernatant used as the crude extract for SOD, CAT and GR assays. For the analysis of APX, the extraction buffer also contained 5 *mM* ascorbate as described by Rao et al. (1995). For the spectrophotometric assay of SOD, extracts were passed through Sephadex G-25 columns at 4°C using 1 mL of 0.1 M sodium phosphate, pH 7.8. All samples were made to 40% (v/v) with glycerol and then stored at −20°C until enzyme analysis. All spectrophotometric assays were performed in a Hitachi U-2001 UV/Vis spectrophotometer (Hitachi Ltd, Tokyo, Japan).

Total SOD activity was measured spectrophotometrically according to McCord and Fridovich (1969). The reaction was performed at 25°C in a 3-mL cuvette of 50 *mM* potassium phosphate, pH 7.8, containing 0.1 *mM* EDTA, 0.06 *mM* ferricytochrome *c*, 0.05 *mM* xanthine, and 10^{-8} M xanthine oxidase. One unit of SOD is defined as the amount of enzyme that inhibited the rate of ferricytochrome *c* reduction by 50% at A_{550}.

Total CAT activity was determined spectrophotometrically by following the decline in A_{240} as H_2O_2 (extinction coefficient 40 mM^{-1} cm^{-1}) was catabolized as described by Beers and Sizer (1952). The 2-mL reaction mixture contained 50 *mM* phosphate buffer (pH 7.0), 10 *mM* DTT, and 5 *mM* H_2O_2. One unit of CAT consumes 1 nmol of H_2O_2 min^{-1} under the assay conditions.

Total APX activity was measured at 25°C according to Nakano and Asada by the decrease in A_{290} due to ascorbate oxidation by H_2O_2 (extinction coefficient 2.8 mM^{-1} cm^{-1}) (Nakano and Asada 1981). The 2-mL reaction volume contained 100 *mM* potassium phosphate buffer (pH 7.0), 0.5 *mM* ascorbate, and 0.2 *mM* H_2O_2. One unit of APX forms 1 μmol of ascorbate oxidized min^{-1} under the assay conditions.

Total GR activity was determined by following the oxidation of NADPH at 340 nm (extinction coefficient 6.2 mM^{-1} cm^{-1}) as modified by Foyer and Halliwell (1976). The 2-mL assay mixture contained 0.1 *M* Tris buffer (pH 7.8), 2 *mM* EDTA, 0.5 *mM* GSSG and 150 *mM* NADPH. One unit of GR oxidizes 1 μmol of NADPH min^{-1} under the assay conditions.

Statistical analysis

To calculate the significance of values, means were compared by Duncan's Multiple Range tests at $P<0.05$, using PC SAS 8.2 (SAS Institute, Cary, NC, USA). All data presented are means±SE.

Results

Physiological parameters

Chlorosis and leaf senescence were enhanced by soil flooding which also reduced leaf area and branching as flooding progressed. This injury was entirely eliminated by catechin treatment. Compared with the well-drained control plants, root length of flooded plants given catechin increased compared to that of plants waterlogged without catechin (W) (Fig. 1). Table 1 shows the time course of changes in whole plant biomass during waterlogging and recovery. After 9 d under non-waterlogging conditions, the FW and DW of the tomato roots and leaves increased when plants were treated with 2 *mM* catechin (Cat) but was little different to that of plants not receiving catechin. In waterlogged plants, FW and DW declined from day 1 to day 5 of flooding but increased again during 4 d recovery; However, waterlogged plants given catechin attained FW and DWs that exceeded those of untreated counterparts and grew larger that non-waterlogged plants. After 4 d of recovery, exogenous application of catechin under waterlogging conditions, gave whole plant FW and DW values that were, respectively, 46.8% and 61.6% greater than those of waterlogged plants not given catechin.

Waterlogging reduced leaf RWC over 5 d and was almost 45% below control values at the time plants were

Fig. 1 Photograph showing the effect of root-zone flooding on catechin and non-catechin tomato (*Solanum lycopersicum* L.) plants. The right plant was shown 40-day-old tomato plants were exposed to waterlogging conditions alone without catechin pre-treatment; the left plant was shown 40-day-old tomato plants were supplied with catechin exogenously for 48 h and then exposed to waterlogging stress for 5 days. Photograph was made after 4 d of recovery. Bars=5 cm

drained. However this decline was limited to 12% in plants given catechin (Table 2). Recovery of RWC during 4 d drainage was also larger. The Membrane Stability Index (MSI) of both leaves and roots decreased with the duration of waterlogging to a minimum on day 5. The decline in waterlogged plants was 60.2% and 59.1% in the leaves and roots, respectively. However, in flooded plants given catechin the decreases in MSI were only 26.8% and 25.4% in the leaves and roots, respectively (Table 2). Recovery was also faster in the catechin-treated plants.

Total chlorophyll concentration, decreased under waterlogging. Waterlogging alone for 5 d decreased chlorophyll by 47.6% although levels recovered slightly after 4 d recovery under well-drained conditions. Application of catechin to control plants was without effect but, in waterlogged plants, the loss of chlorophyll was substantially slowed with concentrations declining by only 24.92% after 5 d flooding and by only 11.85% after the 4 d recovery period (Table 3).

After 1 d, assimilation of CO_2 (*P*n) decreased significantly in waterlogged plants (by 12.5%) in comparison with non-waterlogged controls (Table 3) but catechin treatment prevented a statistically significant loss in *P*n. If waterlogging was prolonged for 5 d waterlogging, *P*n was halved in the absence of catechin but reduced by only a fifth when catechin was given. Thus, the presence of catechin, waterlogging inhibited *P*n to a much smaller extent. After 5 d recovery, *P*n levels remained at abt. 66% below control values in waterlogged plants while in catechin-treated plants *P*n had returned to control levels.

Enzyme activities

Sucrose synthase activity in the roots was inhibited by waterlogging for 1–5 d (Fig. 2a). In contrast, activity levels were stimulated strongly by catechin treatment to waterlogged plants with levels approximately double those of well-drained controls. Catechin given

Table 1 Effects of waterlogging and catechin treatment on the fresh and dry weight of the tomato leaves and roots

Days of waterlogging/ recovery	Fresh weight (mg)				Dry weight (mg)			
	C	Cat	W	Cat+W	C	Cat	W	Cat+W
0	631±35 d	628±31 d	625±34 a	619±29 c	31.6±1.8 d	31.9±1.6 d	31.3±1.7 a	30.9±1.5 c
1	658±40 cd	665±35 cd	610±39 a	636±33 c	33.5±2.1 cd	34.6±1.8 d	31.1±1.9 a	33.1±1.7 c
3	745±53 c	738±48 c	554±24 b	685±40 b	35.8±2.5 c	41.3±2.7 c	25.5±1.1 b	34.9±2.1 bc
5	874±57 b	866±46 b	493±30 c	727±52 ab	48.9±3.2 b	51.1±2.7 b	23.2±1.4 c	38.5±2.8 b
4 days after recovery	1219±71 a	1239±82 a	558±51 ab	819±57 a	73.1±4.3 a	74.3±4.7 a	27.9±2.6 ab	45.1±3.1 a

C: the group without catechin treatment or waterlogging; Cat: 40-day-old tomato plants were supplied with catechin alone; W: 40-day-old tomato plants were exposed to waterlogging conditions alone without catechin pre-treatment; Cat + W: 40-day-old tomato plants were supplied with catechin exogenously for 48 h and then exposed to waterlogging stress for 5 days, followed by a 4-day recovery period

Each value represents the mean ± SE (n=15) of 3 independent experiments. Different letters indicate significant differences between treatments (P<0.05).

Table 2 Effects of waterlogging and catechin treatment on the relative water content (RWC) in leaves (%) and the membrane stability index (MSI) in the leaves and roots of the tomato

Days of waterlogging/ recovery	RWC (%)				MSI (%) leaves				MSI (%) roots			
	C	Cat	W	Cat+W	C	Cat	W	Cat+W	C	Cat	W	Cat+W
0	81.35±2.8 a	81.33±2.9 a	81.25±2.8 a	81.08±2.6 a	82.41±3.5	82.65±2.2 a	82.11±3.1 a	82.35±2.7 a	81.22±2.8 a	81.42±3.1 a	81.33±2.4 a	80.75±2.8 a
1	81.31±2.8 a	81.25±2.7 a	71.44±3.1 b	78.26±2.7 a	82.51±2.8 a	82.73±2.3 a	70.36±2.8 b	76.17±2.9 b	81.15±3.3 a	81.12±2.8 a	56.72±2.7 b	73.15±3.1 b
3	81.31±3.1 a	81.31±2.4 a	59.34±2.5 c	75.71±3.2 ab	82.45±2.9 a	82.51±2.7 a	55.72±2.2 c	70.79±3.3 c	80.99±3.5 a	80.98±2.7 a	44.18±2.6 c	68.62±2.7 b
5	81.35±2.7 a	81.29±2.6 a	45.62±2.3 e	71.32±2.4 b	82.39±3.4 a	82.43±2.4 a	32.65±2.1 e	60.86±3.1 d	81.08±2.7 a	80.83±2.9 a	33.26±2.4 d	60.21±2.9 c
4 days after recovery	81.29±2.5 a	81.27±2.6 a	52.61±2.9 d	79.27±2.8 a	82.56±3.5 a	82.67±2.7 a	49.54±3.1 d	71.98±2.5 c	81.18±2.4 a	81.22±2.4 a	43.28±2.9 c	70.56±3.5 b

C: the group without catechin treatment or waterlogging; Cat: 40-day-old tomato plants were supplied with catechin alone; W: 40-day-old tomato plants were exposed to waterlogging conditions alone without catechin pre-treatment; Cat+W: 40-day-old tomato plants were supplied with catechin exogenously for 48 h and then exposed to waterlogging stress for 5 days, followed by a 4-day recovery period

Note: Each value represents the mean±SE (n=15) of 3 independent experiments. Different letters indicate significant differences between treatments (P<0.05).

to well-drained plants had no effect. During recovery, waterlogged plants showed a slight increase in SuSy activity, while no further increase was observed in waterlogged plants given catechin.

Alcohol dehydrogenase activity in the roots was increased by 1–5 d waterlogging (Fig. 2b). This effect was very much larger in the presence of catechin; activity increasing to approximately 14.7 times pre-stress levels after 5 d Activity of ADH declined after the termination of treatment in both the presence and absence of catechin.

ATP concentration and free radical production

In the leaves of flooded plants, a rapid decrease in ATP concentration was observed after 3 d or 5 d compared to well-drained controls (Fig. 3a). Although exogenous catechin did not significantly alter the ATP concentration of control plants, it prevented a statistically significant loss of ATP in flooded plants. After 4 d recovery, the benefit of catechin to ATP concentrations in flooded plants was still very marked.

Flooding increased oxidative stress levels of the leaves. A 1.9-fold increase in O_2^- (Fig. 2b) and a 1.4-fold increase in H_2O_2 (Fig. 3c) was observed on day 5 for non-catechin treated plants. However, exogenous catechin reduced the accumulation of O_2^- and H_2O_2 by 44.5% and 36.7% under flooding treatment, respectively. After 4d recovery both O_2^- and H_2O_2 concentrations decreased although levels in waterlogged plants not given catechin remained substantially above those of controls and of flooded plants treated with catechin.

Antioxidant enzyme activities

Soil flooding modified both enzymatic activities and metabolites of the ascorbate–glutathione cycle in the leaves. Among the different antioxidant enzymes, SOD and CAT exhibited early responses, whereas APX and GR exhibited late responses (Fig. 4a). SOD activity significantly was increased by 1 d of waterlogging but levels fell to below control values thereafter, including after 4 drainage. Catechin changed this flooding response by raising SOD activity to levels that exceeded those of control plants. This effect persisted after 4 d recovery.

In waterlogged plants, CAT followed a similar trend to SOD. Although exogenous catechin had no

Table 3 Effects of waterlogging and catechin treatment on *Chl* a+b and *P*n in the tomato leaves

Days of waterlogging/ recovery	*Chl* a+b (μg g^{-1} FW)				*P*n (μmol CO_2 m^{-2} s^{-1})			
	C	Cat	W	Cat+W	C	Cat	W	Cat+W
0	2881±66 a	2897±60 a	2879±78 a	2845±72 a	13.98±0.7 a	13.95±0.6 a	14.05±0.8 a	13.92±0.7 a
1	2892±69 a	2901±69 a	2512±67 b	2796±70 a	13.94±0.6 a	13.99±0.7 a	12.21±0.7 b	13.11±0.6 a
3	2894±59 a	2888±72 a	2094±89 c	2488±63 b	13.97±0.7 a	14.01±0.5 a	10.55±0.6 c	12.25±0.4 b
5	2886±64 a	2891±55 a	1509±92 e	2136±49 c	13.92±0.5 a	13.91±0.6 a	7.33±0.3 e	11.05±0.5 b
4 days after recovery	2882±61 a	2899±59 a	1855±64 d	2508±57 b	13.97±0.6 a	13.94±0.5 a	9.26±0.5 d	12.85±0.4 a

C: the group without catechin treatment or waterlogging; Cat: 40-day-old tomato plants were supplied with catechin alone; W: 40-day-old tomato plants were exposed to waterlogging conditions alone without catechin pre-treatment; Cat+W: 40-day-old tomato plants were supplied with catechin exogenously for 48 h and then exposed to waterlogging stress for 5 days, followed by a 4-day recovery period

Note: Each value represents the mean±SE (n=15) of 3 independent experiments. Different letters indicate significant differences between treatments (P<0.05).

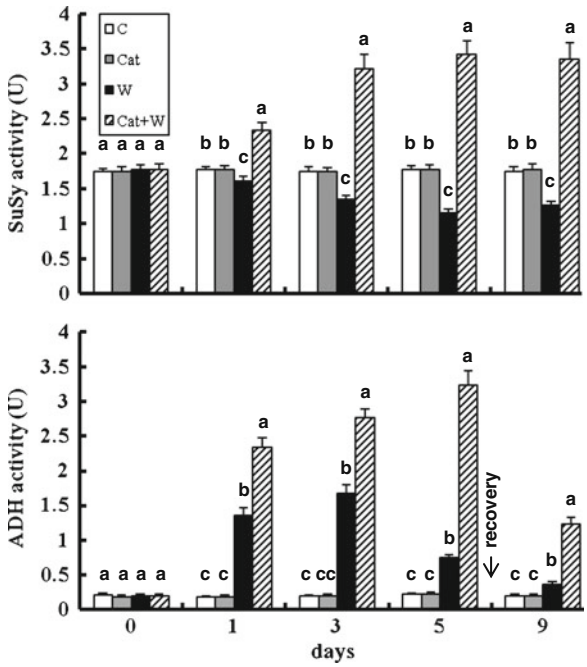

Fig. 2 Effect of waterlogging and catechin treatment on the activity of SuSy (**a**) and ADH (**b**) in root tissues. Letters above bars indicate a statistically-significant difference (P≤0.05) according to Duncan's multiple range test. Vertical bars show ± SE of mean. C: the group without catechin treatment or waterlogging; Cat: 40-day-old tomato plants were supplied with catechin alone; W: 40-day-old tomato plants were exposed to waterlogging conditions alone without catechin pre-treatment; Cat+W: 40-day-old tomato plants were supplied with catechin exogenously for 48 h and then exposed to waterlogging stress for 5 days, followed by a 4-day recovery period

effect on non-waterlogged controls this treatment increased CAT activity by 53.7% in flooded plants, and up to twice the activity found in waterlogged plants without catechin (Fig. 4b). These effects were slightly diminished after 4 d recovery.

APX activity was decreased by 3 and 5 d waterlogging (Fig. 4c), to 27.2% of control plants. The depression was still evident after 4 d recovery after draining. Catechin treatment to non-waterlogged plants raised APX activity by up to 20.1% on the 9th day of the experiment. In flooded plants, catechin boosted APX activity after 3 d and 5 d waterlogging and after 4 days recovery. The effect was sufficient to give APX activities closely similar to those of non-waterlogged control plants.

Waterlogging for 5 d reduced GR activity by 22.6% compared to controls and the effect extended to the end of the 4 d recovery period (Fig. 4d). Catechin treatment increased GR activity by 22.7% in non-waterlogged plants with respect to controls. Catechin treatment to flooded plants completely restored the GR activity to control levels and, at the end of the experiment, GR activity was 24.9% more than in flooded plants not treated with catechin.

Discussion

The commercial production of tomatoes is mostly limited to the hot and wet summer season in Taiwan. Excess water produces hypoxic soil conditions within a few hours, and prolonged waterlogging results in anoxia. As a result of 5 d of waterlogging, the tomato

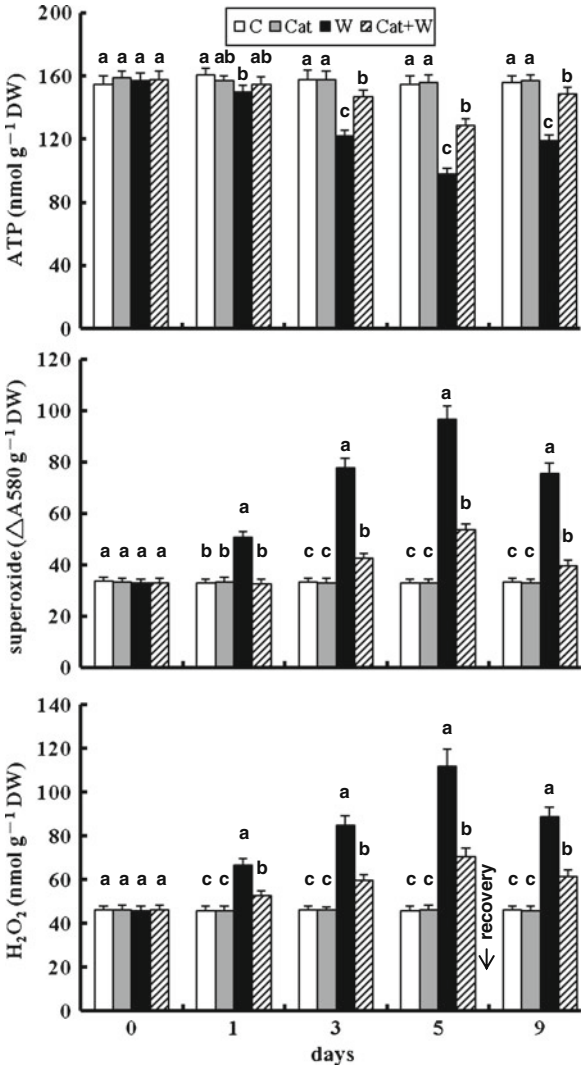

Fig. 3 Physiological and biochemical responses of waterlogging stress-treated tomato leaves. The ATP, O_2^- and H_2O_2 concentrations of control (drained) and waterlogging-treated plant leaves are shown in (**a**), (**b**) and (**c**), respectively. Each point represents the average of three individual identical experiments (±SE). Letters above bars indicate a statistically-significant difference ($P \leq 0.05$). C: the group without catechin treatment or waterlogging; Cat: 40-day-old tomato plants were supplied with catechin alone; W: 40-day-old tomato plants were exposed to waterlogging conditions alone without catechin pre-treatment; Cat + W: 40-day-old tomato plants were supplied with catechin exogenously for 48 h and then exposed to waterlogging stress for 5 days, followed by a 4-day recovery period

plants in our tests suffered severe losses in growth, FW, DW, and MSI in both the roots and leaves, and RWC, chlorophyll and *P*n in the leaves (Fig. 1, Tables 1, 2 and 3) leading to stunted plants (Zhang et al. 2007). However, the growth in FW and DW of the waterlogged plants (roots and leaves) benefited markedly from pre-treatment with catechin (Table 1). Sairam et al. (2009) reported a decrease in RWC during flooding, which further declined with the duration of flooding stress. Electrolyte leakage is an indicator of cell membrane stability and is widely used to screen to evaluate plant species or cultivars in terms of their tolerance to temperature and water stresses (Sapra and Anaele 1991). The higher MSI in flooded plants given catechin indicate an enhanced capability to maintain root cell membrane stability. This probably contributed significantly to the better waterlogging tolerance (Table 2). Sairam et al. (2009) reported that 4 and 8 d of waterlogging in the mung bean lowered chlorophyll concentrations but also noted a rise following drainage, indicating partial restoration of the photosynthetic machinery once oxygen re-entered the wet soil. The data in Table 3 demonstrate that catechin benefitted the flooded plants by maintaining higher chlorophyll concentrations and a resultant higher rate of CO_2 assimilation (*P*n).

Carbohydrate starvation has been shown to be one of the numerous reasons for hypoxia/anoxia-induced injury (Schluter and Crawford 2001). SuSy is a key enzyme responsible for the hydrolysis of sucrose to fructose and glucose (UDP-glucose) under oxygen deprivation (Noguchi 2004). Both glucose and fructose (reducing sugars) are substrates for the glycolytic pathway, which is the major source of energy under hypoxia in the absence of oxidative phosphorylation (Greenway and Gibbs 2003). In this context, the greater and increasing activity of SuSy in flooded plants given catechin plants as compared with those without catechin and with non-waterlogged plants under catechin suggest a carbohydrate-based tolerance mechanism in tomato roots (Fig. 2a). The essentiality of SuSy in anoxic tolerance has been demonstrated in maize using a double-mutant of the enzyme (Ricard et al. 1998). Furthermore, Sairam et al. (2009) reported that the greater increase in the SuSy activity under waterlogged conditions in the tolerant T44 genotype of the mung bean resulted in an increased availability of reducing sugars, thus helping to fulfill their energy requirement. ADH activity is critical for the recycling of NADH and thus for the continuation of the glycolytic pathway (Johnson et al. 1994). Increases in ADH activity may reduce the detrimental effects of the accumulation of toxic acetaldehyde or lactate

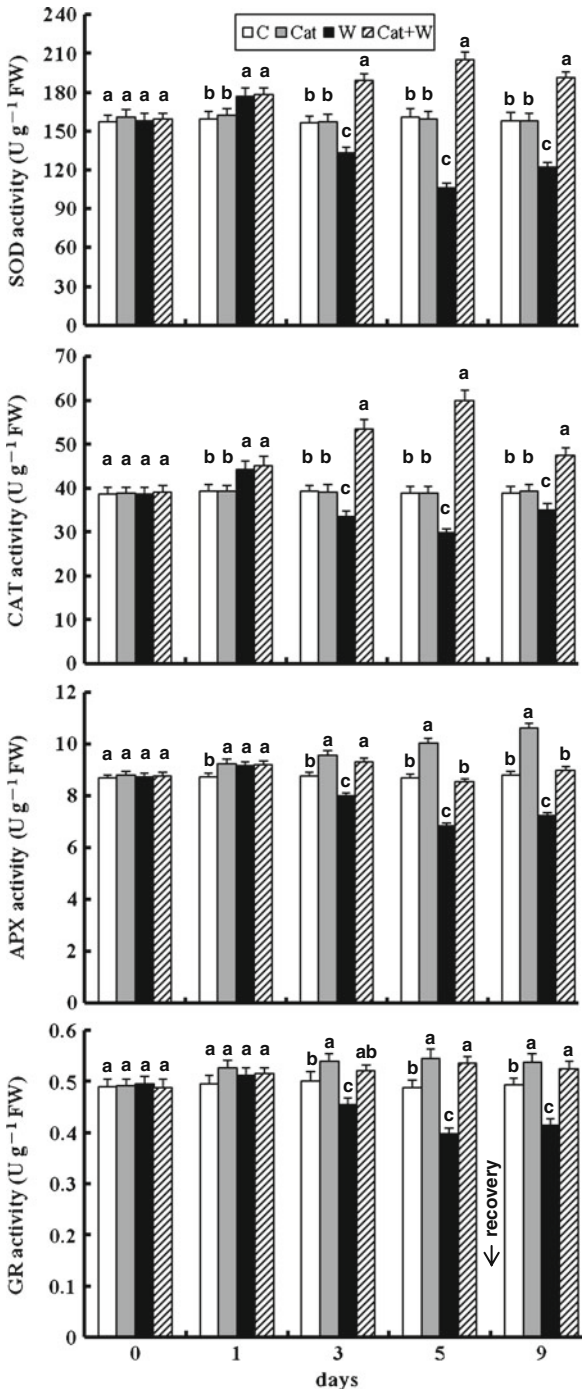

Fig. 4 Effects of waterlogging and catechin treatment on the activities of SOD, CAT, APX and GR in tomato leaves. Each value represents the mean of three replicates with SE. Letters above bars indicate a statistically-significant difference ($P \leq 0.05$). C: the group without catechin treatment or waterlogging; Cat: 40-day-old tomato plants were supplied with catechin alone; W: 40-day-old tomato plants were exposed to waterlogging conditions alone without catechin pre-treatment; Cat + W: 40-day-old tomato plants were supplied with catechin exogenously for 48 h and then exposed to waterlogging stress for 5 days, followed by a 4-day recovery period

under flooding conditions (Perata and Alpi 1993) and help maintain ATP production in the absence of O_2 (Dennis et al. 2000). The importance of ADH in flooding tolerance has been investigated in a study of a maize mutant deficient in one of its *ADH* genes, therefore unable to produce a functional ADH enzyme. As there was no counterbalance to lactic dehydrogenase, and the pH continued to decline to very low levels, this mutant was more sensitive to flooding injury than the wild-type plant, and died after 3 d of submergence (Roberts et al. 1984). The activity of ADH in the roots was tomatoes was significantly increased by catechin plants and especially so under waterlogging conditions and during recovery after draining the soil (Fig. 2b). Recently, gene expression studies during 24 h waterlogging on *SuSy* and *ADH* gene expression suggested that the flooding susceptible PB genotype of mung bean failed to express *SuSy* and *ADH* mRNA under waterlogged conditions (Sairam et al. 2009).

Light-dependent electron transported by chlorophyll can be converted into stable chemical energy and prompt ATP formation via ATPase in the chloroplasts. In wheat, the suppression of the activities of Mg^{2+}-ATPase and Ca^{2+}-ATPase in the chloroplasts may have depressed ATP synthesis under waterlogging stresses (Zheng et al. 2009). Thus, the suppression in ATP synthesis might have damaged PSII, resulting in an excess of absorbed energy by chlorophyll, and the formation of ROS. Figure 3a shows that the ATP concentration markedly decreased under flooding stress on days 1, 3 and 5 of flooding in comparison with non-stressed conditions. Remarkably, catechin pre-treatment significantly enhanced the ATP concentration in the leaves under waterlogging stress. The role of catechin (Cat) in plant defense to environmental stress has not been widely discussed, so the accurate mode of their action still remains to be explored. Based on the above results, we propose that Cat may function as a stress-signaling regulator.

A very striking observation under waterlogging is the production of various ROS, especially O_2^- and H_2O_2, leading to an increase in lipid peroxidation. This increase in ROS during waterlogging is primarily

due to an increase in DPI-sensitive NADPH oxidase-dependent O_2^- production (Kumutha et al. 2009). The waterlogging-induced increase in O_2^- (Fig. 3b) and H_2O_2 (Fig. 3c) levels in leaf tissue was reduced in catechin-treated plants. This suggests that catechin is also involved in the direct scavenging of free radicals, thereby reducing oxidative stress. ROS in the leaves induced by oxygen shortage at the roots could also serve as a signal for the induction of antioxidant enzymes (Agarwal et al. 2005).

The results observed for the antioxidative enzymes SOD, CAT, APX and GR in the leaves in waterlogged plants given catechin reveal a continuous increase in all four enzymes up to 1 d of waterlogging. In plants waterlogged without catechin, an increase in antioxidative enzymes (SOD and CAT) was noticed only in 1-d waterlogged plants, and at subsequent stages there was a decline in the activity of all antioxidant enzymes as compared with well-drained plants (Fig. 4). Enhancement of SOD activity under waterlogged environments may be an indicator of O_2^- production (Fig. 4a). The increase in SOD activity in response to flooding plants might be related to the overproduction of O_2^- and associated with oxygen deprivation at the roots because of inbalances in energy input and usage as photosynthesis is inhibited through wilting (low RWC— see Table 2) and stomatal closure (Else et al. 2009). Hence, the gradual increase in the H_2O_2 concentration in continuously-flooded plants may be partially responsible for the decline in SOD activity (Figs. 3c and 4a). High levels of SOD should be followed by the scavenging of H_2O_2 catalyzed by APX and CAT. The early activation of SOD and CAT enzymes observed in continuously-flooded plants appeared to be an active and efficient response of flooded plants enriched with catechin (Fig. 4a, b). Tseng et al. (2007) demonstrated that transgenic Chinese cabbage plants over-expressing SOD and CAT isoforms were able to tolerate salt stress better than the non-transgenic line. These results allow us to suggest the co-ordinated involvement of these two enzymes in mitigating oxidative stress damage.

The excess O_2^- generated under waterlogging stress could dismutate into H_2O_2 through the action of SOD, which is then metabolized by the components of the ascorbate— glutathione cycle. Asada (1992) reported that the APX found in organelles scavenges H_2O_2 produced by the organelles, whereas the function of cytosolic APX is probably to eliminate H_2O_2 that is produced in the cytosol or apoplast and that diffused from organelles. APX and CAT activities increased in the leaves of flooded plant treated with catechin but were much depressed by waterlogging without catechin (Fig. 4b, c). This response indicates a strong H_2O_2 scavenging ability by leaves of flooded plants given catechin. The observed correlation between the two enzyme activities strongly supports the coordinated action of APX and CAT. Previous work by Zhang et al. (2007) showed that in the early stages of waterlogging, both peroxidase and CAT activities significantly increased in tolerant barley cultivar Xuimai 3, while they decreased in the flooding sensitive cultivar Gerdner. It can be concluded that rapid development of higher peroxidase and CAT activities under stress are a trait of tolerant plant species or genotypes, enabling plants to protect themselves against oxidative stress (Zhang et al. 2007). Therefore, an increase in the activity of H_2O_2-scanvenging enzymes with an accompanying increase in SOD activity is crucial for an effective defense against oxidative stress (Gossett et al. 1994).

GR helps maintain high ratios of GSH/GSSG, which is required for the regeneration of ascorbate, an important antioxidant in plant cells. GR activity can effectively recycle GSH at the expense of NADPH. GR activity in flooded tomatoes pre-treated with catechin was comparable with non-flooded plants (Fig. 4d). In flood-sensitive genotypes of citrus, an impairment of the collaborative action of GR and APX has been shown to a lower the ability for AOS detoxification and to raise the sensitivity to flooding (Arbona et al. 2008). Overall, these results suggest that the enhancement in the level of most components in the antioxidative system by catechin pre-treatment resulted in much greater resilience of tomato plants to 5 d flooding stress.

To the best of our knowledge, this study is the first to demonstrate catechin as an effective antioxidant in tomato plants subjected to soil flooding. High levels of ROS could be reduced in the leaves after catechin treatment to the soil and roots. Increased flooding tolerance from catechin treatment was associated with increased antioxidant enzyme activity and, a reduction in lipid peroxidation, indicating that the antioxidant property of catechin involves protection against membrane oxidation. Therefore, we conclude that exogenous catechin treatment has the potential to reduce injury

from stress caused by waterlogging of the soil by affording protection against oxidative stress.

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