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Paclobutrazol pre-treatment enhanced flooding tolerance of sweet potato

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KEYWORDS
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Summary

The objective of this experiment was to study changes of antioxidants and antioxidative enzymes in the flooding-stressed sweet potato leaf, as affected by paclobutrazol (PBZ) treatment at 24 h prior to flooding. Sweet potato 'Taoyuan 2' were treated with 0 and 0.5 mg/plant of PBZ, afterwards subjected to non-flooding and flooding-stress conditions for 0, 1, 3, and 5 d, followed by a 2 d drainage period. The study was conducted as a factorial experiment in completely randomized blocks with three replications maintained within a screen house. Plants with various antioxidative systems responded differently to flooding stress according to the duration of the flooding period and subsequent drainage period. The increased levels of antioxidants and antioxidative enzymes observed on different days of flooding afforded the sweet potato leaf with improved flooding tolerance. Glutathione reductase activity in the leaf was significantly enhanced over 5 d continuous flooding followed by a drainage period, in comparison with non-flooding conditions. Under non-flooding conditions, antioxidative system of leaf was regulated and elevated by PBZ pre-treatment. PBZ treatment may enable sweet potato 'Taoyuan 2' to maintain the balance between the formation and the detoxification of activated oxygen species. Our results also show that under flooding-stress conditions, the level of 'Taoyuan 2' antioxidative system is linked to PBZ treatment. Pre-treating with PBZ may increase levels of various components of antioxidative systems after exposure to different durations of flooding and drainage, thus inducing flooding tolerance. PBZ exhibited

Abbreviations: APX, ascorbate peroxidase; ASA, ascorbic acid or ascorbate; CAT, catalase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; MDA, malondialdehyde; PBZ, paclobutrazol; SOD, superoxide dismutase

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the important function of enhancing the restoration of leaf oxidative damage under flooding stress after the pre-application of 0.5 mg/plant. These findings may have greater significance for farming in frequently flooded areas.

Introduction

Plants are severely affected by flooding, because under such stress the production and the quenching of the reactive oxygen species (ROS) in plants will fail to be kept in a balanced state (Crawford and Brandle, 1996). One of the major consequences of soil flooding in plants is oxygen deficiency; consequently, the roots suffer from periodic or prolonged deprivation of oxygen and this interferes with respiration. The lack of suitable electron acceptors leads to the saturation of redox chains, the accumulation of NAD(P)H, and a decline in ATP generation (Kennedy et al., 1992; Perata and Alpi, 1993). In plant cells, oxidative stress reactions are associated with toxic free radicals yielded by the reduction of molecular oxygen leading to the production of superoxide radicals (O_2^-), singlet oxygen ($\bullet O_2$), hydroxyl radicals ($\bullet OH$) and hydrogen peroxide (H_2O_2). These radicals can deactivate various Calvin-cycle enzymes and are involved in oxidative systems, in which they mark proteins for degradation (Chaudiere and Ferrari-Ilious, 1999). The toxic radicals can be removed through the mobilization of antioxidant reserves, which react both enzymatically and chemically with the toxic molecular species and their products. Chemical constituents have been identified to scavenge free radicals and thus protect active plant cells against oxygen toxicity (Sairam et al., 1998; Ahmed et al., 2002). In order to counter the hazardous effects of ROS under stress, plants have evolved a complex antioxidative defense system composed of both antioxidant enzymes and metabolites, such as ascorbate peroxidase (APX), catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR), ascorbic acid or ascorbate (ASA), reduced glutathione (GSH), oxidized glutathione (GSSG), malondialdehyde (MDA) and vitamin E (Sairam et al., 2002). The ASA–glutathione cycle has been shown to be of great importance in multiple stress reactions (Drazkiewicz et al., 2003).

The interaction between the production and the scavenging of activated oxygen species maintains the plants in a relatively stable state. Plants are adapted to minimize radical damage using their natural defense mechanisms. Thus, maintaining the balance between the formation and detoxification of activated oxygen species is critical to cell survival during flooding stress. When roots are submerged, the anoxic conditions inhibit aerobic respiration and less energy is yielded. The roots transport less water to the leaves. The antioxidative enzymes, hormones, and other solutes entering the leaves via the transpiration stream may also undergo changes (Grichko and Glick, 2001; Voesenek et al., 2004). These changes may constitute physiologically active messages that modify leaf physiology and development; such modifications may include chlorophyll breakdown, protein degradation, decreased stem elongation coupled with increased stem thickness, a decrease in membrane permeability, peroxidation, slower leaf expansion, petiole epinasty and stomatal closure. Stomatal closure causes a decrease in internal CO_2 concentration. Subsequently, a concomitant decline in photosynthesis results from diminished availability of CO_2 for carbon fixation (Carvalho and Amancio, 2002). Reduction of CO_2 concentration increases the amount of harmful ROS within the leaf due to ongoing light reactions that can lead to senescence and even the death of the plant (Else et al., 1995; Kato et al., 2001). Flooding tolerance can be achieved using several adaptive mechanisms, and the modulation of antioxidative system levels may be part of the whole mechanism. High levels of some antioxidant enzymes and metabolites, such as SOD, CAT, APX, GR, GSH, ASA, MDA, AOX or GPX, are important in tobacco (Hurng and Kao, 1994a, b), corn (Yan and Dai, 1996), wheat (Biemelt et al., 1998), soybean (VanToai and Bolles, 1991), rice (Ushimaro et al., 1992), tomato and eggplant (Lin et al., 2004) and sweet potato (Hwang et al., 1999) survival under oxidative stress, after being subjected to different degrees of waterlogging. Some oxidative enzymes or oxidants have been useful in screening for flooding-tolerant plants (Lin et al., 2004).

Paclobutrazol (PBZ) ((2RS, 3RS)-1-4 (-chlorophenyl)-4, 4-dimethyl-2-1, 2, 4-triazol-1-yl-penten-3-ol) is a member of the triazole family. Triazoles have both fungitoxic and plant-growth regulatory effects. In addition, they can also protect plants against various stresses, including drought, low and high temperatures, UV-B radiation, air pollutants, and fungal pathogens. Therefore, the triazoles have been characterized as plant multi-protectants (Kraus and Fletcher, 1994; Pinhero and Fletcher,

1994; Kraus et al., 1995; Voesenek et al., 2003). Triazoles affect the isoprenoid pathway, and alter the levels of certain plant hormones by inhibiting gibberellin synthesis, reducing ethylene evolution, and increasing cytokinin levels (Kamountsis and Chronopoulou-Sereli, 1999). Some morphological changes observed in triazole-treated plants include the inhibition of plant growth, decreased internodal elongation, increased chlorophyll levels, enlarged chloroplasts, thicker leaf tissue, increased root to shoot ratio and elevated levels of epicuticular wax formation (Watson and Himelick, 2004). Triazole has also been used to promote massive adventitious bud development in in vitro propagation culture (Li and Zhang, 1999). The chloroplast is a major site of free-radical production and paclobutrazol in plants, protecting them by boosting antioxidant defense systems. PBZ-treated plants have a more efficient free-radical scavenging system that enables them to detoxify active oxygen (Kopyra and Gwozdz, 2003). Even though PBZ-induced stress tolerance and stress protection has been attributed to an increase in some antioxidant enzymes (Lurie et al., 1994; Paliyath and Fletcher, 1995; Pinhero et al., 1997), less is known about the extent to which the antioxidative response of PBZ applications to the sweet potato differs in the case of waterlogging tolerance.

The sweet potato is the world's fifth most important crop and is a major source of food and nutrition in developing countries (The International Potato Center Lima, Peru, 1998). It is a plant species that is, relatively speaking, more resistant to drought stress, because its deep-root system accesses more moisture. However, when subjected to waterlogging, leaf senescence such as chlorosis is observed in this plant species. Heavy rainstorms and overflowing waters can leave the soil saturated for days before drainage, making waterlogging a problem in many parts of the world. Air pockets in the soil become filled with water during saturation, creating hypoxic conditions that are followed by anoxia. Previously, we found that reduced susceptibility to waterlogging, together with high-light stress, are related to increases in SOD and CAT activities in the leaf of the sweet potato (Hwang et al., 1999). Usually, high light exerts this effect on the sweet potato following heavy rainfall during the summer in Taiwan. Flooding from heavy rainfall is a major risk to fresh market sweet potato production in Taiwan. Antioxidative enzymes responding to waterlogging in sweet potato thus take on greater importance. PBZ has been reported to confer protection to plants by reducing oxidative damage via the elevation of antioxidants or the reduction of oxidative enzyme activity. The hypothesis of this research is that pre-treating with PBZ can increase antioxidative enzyme activity and/or antioxidant levels under flooding stress, leading to higher flood tolerance in the sweet potato. The present research project studied the antioxidative system of the sweet potato leaf exposed to waterlogged conditions. The results provide information on PBZ pre-treatment for flooding tolerance to waterlogging stress.

Materials and methods

Plant materials, cultural practice, experimental design and treatments

Sweet potato (*Ipomoea batatas* (L.) Lam) 'Taoyuan 2', a popular variety grown in Taiwan for its leafy vegetable consumption, was used as the experimental material for this study. Cuttings about 30 cm in length were planted in plastic boxes 60 cm long, 22 cm wide and 15 cm deep, that contained a medium of sand, vermiculite and loamy soil in a volume ratio of 2:1:1. Specimens were planted during July 2000 in the screen house of Chinese Culture University. Plants were evenly spaced at intervals of 50 cm to encourage similar growth rate and size. Plants were watered with half-strength Hoagland solution (Hwang et al., 1999) every other day to maintain optimal irrigation, and allowed to grow for 45 d before flooding-stress imposition. The PBZ treatments were carried out in a factorial experiment of completely randomized design with three replications. Three factors with different levels were used. During the period of study, average day/night temperatures were 34/26 °C and the average day length was 14 h.

Two different quantities of PBZ (trade name, Bonsi; Zeneca Agrochemicals, Fermhursk Haslemere Survey GU 273JE, England), 0 and 0.5 mg/plant, were applied as a soil drench. The quantities of PBZ used in this study were determined in a preliminary experiment (data not shown). Twenty-four hours after PBZ treatment, the plants were subjected to two water conditions (non-flooding and flooding) for 0, 1, 3 and 5 d, followed by 2 d drainage. For each different flooding time treatment replication, samples were placed in a $140 \times 50 \times 35\,cm^3$ plastic bucket containing water at a level of 5 cm above the medium surface. Non-flooding conditions imposed on PBZ-untreated plants were considered to be a control to provide a basis to compare the effects of PBZ under flooding conditions. Young, fully expanded leaves from each

plant were clipped to measure for enzyme activity and antioxidant content.

Enzyme extraction and activity determination

The cut leaves of each treatment were carried in an icebox to the laboratory and immediately frozen in liquid nitrogen. They were then stored in a −70 °C freezer for later analysis. Samples were prepared for SOD, CAT, APX, and GR activity analyses by homogenizing 0.2 g of frozen leaf in 990 μL of an ice-cold 100 mM HEPES buffer (pH 7.0) containing 1 mM PMSF (phenylmethysulfonyl fluoride) and 0.03 g PVP (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. The enzyme activities were determined by the spectrophotometer method.

CAT activity was assayed by measuring the initial rate of disappearance of H_2O_2 (Hwang and VanToai, 1991). Two milliliters CAT of assay reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 15 mM H_2O_2 and 20 μL enzyme extract. The decrease in H_2O_2 followed the decline in optical density at 240 nm, and activity was calculated using the extinction coefficient (40 $mM^{-1}\,cm^{-1}$ at 240 nm) for H_2O_2.

GR activity was measured by oxidized 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 enzyme extract (Foyer et al., 1997). The change in absorption at 340 nm ($E = 6.2\,mM^{-1}\,cm^{-1}$) 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 Na ASA, 1 mM H_2O_2 and 40 μL 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, Inc., Galthersburg, MD). The SOD Assay kit as a principle utilizes the WST working solution 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-tetrazolium that produces a water-soluble formazan dye upon reduction with a superoxide anion (O_2^-). The rate of the reduction with O_2^- is linearly related to the xanthine oxidase activity and is inhibited by SOD. Therefore, the 50% inhibition activity of SOD can be measured at 450 nm of absorbance. The specific activity of the SOD (inhibition rate) was calculated using the equation described in the protocol of the kit.

The total ASA content and total glutathione content were determined by dissolving 0.2 g homogenates in 1 mL of 5% *m*-phosphoric acid solution. The extract was then centrifuged at 13,000*g* for 10 min at 4 °C. The supernatant was used for total ASA and total glutathione assay. Both total ASA and the reduced ASA contents were determined spectrophotometrically at 525 nm using different reacting solutions; the former was a potassium phosphate buffer (pH 7.4), dithiothreitol and *N*-ethylmaleimide, the latter was trichloroacetic acid (TCA), *o*-phosphoric acid, α-dipyridyl in 70% alcohol and $FeCl_3$ (Cakmak and Marschner, 1992). Total ASA content and reduced ASA content were calculated using a standard curve plotted from a known concentration of ASA. The content of oxidized ASA (vitamin C) was calculated by subtracting the reduced ASA content from the total ASA content.

Total glutathione content was quantified spectrophotometrically at 412 nm using a reagent solution containing NADPH, Na_2HPO_4, EDTA, BSA, imidazole, yeast-GR and 5,5′-dithiobis-2-nitrobenzoic acid (Anderson, 1985). A standard curve was obtained as a reference to calculate the content.

MDA is one final decomposition product of lipid peroxidation and has been used as an index for the status of lipid peroxidation. Thiobarbituric acid reactive substances representing the lipid peroxidation product were extracted by homogenization of 0.2 g leaf in 5 mL of a solution containing 20% TCA. The mixture was heated at 95 °C for 30 min and the reaction was arrested by quickly transferring the mixture to 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 recorded. After subtracting the 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).

All spectrophotometer analyses were conducted on a 530 UV/VIS spectrophotometer (Pharmacia Biotech, Uppsala, Sweden). One unit of enzyme was defined as the amount of enzyme required to decompose 1 μmol substrate/min g fresh weight.

Statistical analysis

The enzyme measurements were analyzed by a three-factor, completely randomized ANOVA that compared the PBZ concentration, flooding conditions and days of treatment. With regard to the significant values, the means were separated by the least significant difference test at $P \leqslant 0.05$, 0.01 or 0.001, using PC SAS 8.2 (SAS Institute, Cary, NC). The results are presented in the following tables and figures.

Results

The enhancement of the production of both antioxidant enzymes and metabolites may play an important role in metabolic stress tolerance. The effects of PBZ on the sweet potato subjected to flooding stress were monitored by tracking changes in both antioxidative enzyme activities (CAT, GR, APX and SOD) and antioxidant content (ASA, total ASA, total glutathione and MDA) on various days of treatment. In this experiment, a factorial experiment design with a completely randomized arrangement was used.

ANOVA was used to uncover the main effects of the factors PBZ treatment, flooding and duration (P, F and D), and their interaction effects (P × D, P × F, F × D and P × F × D) for different antioxidative enzymes and antioxidants as summarized in Table 1. All the antioxidative enzymes and antioxidants displayed differences ($P \leqslant 0.001$, 0.01 and 0.05) of significance for the PBZ factor main effect, with the exception of CAT activity. APX and GR activity and total glutathione content constituted differences of significance for the flooding factor main effect. Moreover, duration of the treatment significantly affected the antioxidative system. When P × D interaction was calculated for significance, antioxidative enzyme and antioxidant values were all significantly different at 0.1%, 1% or 5% levels. Table 1 also illustrates that GR activity appears significantly different in both main effects (P, F and D) and interaction effects (P × D, P × F, F × D and P × F × D). Additionally, the quantities of SOD, GR, total ASA, ASA and MDA were significantly different in interaction effects.

Figure 1 presents the effects of PBZ pre-treatment on leaf SOD activity during flooding and drainage. In the case of non-PBZ and non-flooding conditions (−P/−F), SOD activity decreased after day 0 of flooding, increased on day 3 of flooding, and then dropped after day 5 of flooding. SOD activity under PBZ-addition and non-flooding conditions (+P/−F) remained low and stable during the first 3 d flooding, increasing thereafter, and then decreasing to a minimum level (0.41 μmol/g fw) on day 7 of flooding. The trend of change in SOD activity during non-PBZ treatment under flooding conditions (−P/+F) appeared to decline in the leaf from day 0 to day 5 of flooding, and then increased during drainage. Interestingly, PBZ pre-treatment and flooding stress together (+P/+F) resulted in a significant increase in SOD activity on both day 3 of flooding (0.95 μmol/g fw) and

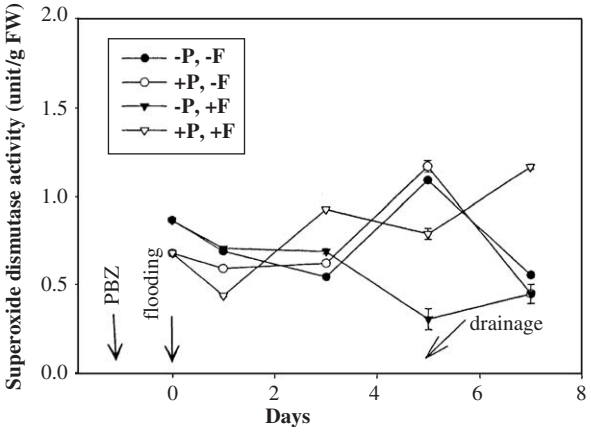

Figure 1. The effect of pre-treating paclobutrazol on superoxide dismutase activity in leaves of sweet potato 'Taoyuan 2' subjected to flooding stress and following drainage. +P: paclobutrazol pre-treated; −P: non-paclobutrazol; +F: flooding; −F: non-flooding. Vertical bar represents mean ± standard error.

Table 1. ANOVA of paclobutrazol concentration (P), flooding conditions (F), day of treatment (D) and their interactions (P × D, P × F, F × D, P × F × D) for SOD, CAT, APX, GR, total ASA, ASA, total glutathione and MDA content in the leaf of the sweet potato

Source of variance	Degree of freedom	*F*-value							
		SOD	CAT	APX	GR	Total ASA	Oxidized ASA	Total glutathione	MDA
P	1	12.08 **	3.74^{NS}	15.23 ***	17.78 ***	50.45***	25.11***	31.43***	5.89***
F	1	2.48^{NS}	0.15^{NS}	16.20***	57.25***	0.05^{NS}	1.18^{NS}	51.33***	0.13^{NS}
D	4	5.06**	6.78***	63.67***	58.28***	111.24***	40.63***	96.02***	86.07***
P × D	4	15.75***	5.72**	10.23***	41.50***	15.73***	14.65***	7.35*	20.95***
P × F	1	7.33*	2.70^{NS}	1.96^{NS}	61.89***	5.23*	5.14*	22.62***	37.54***
F × D	4	18.02***	3.09*	3.59**	13.64***	7.91***	8.48***	30.40***	6.87**
P × F × D	4	3.12*	3.91**	1.21^{NS}	17.13***	19.65***	26.04***	2.14^{NS}	8.52***

***$P \leqslant 0.001$, **$P \leqslant 0.01$, ***$P \leqslant 0.05$, NS: non-significant difference; P: paclobutrazol concentration, F: flooding condition, D: day of treatment; SOD: superoxide dismutase, CAT: catalase, APX: ascorbate peroxidase, GR: glutathione reductase, ASA: ascorbic acid or ascorbate, MDA: malondialdehyde.

drainage (1.24 μmol/g fw) in comparison to the other three interaction conditions (−P/−F, +P/−F and −P/+F). Thus we can conclude that pre-treating with PBZ under flooding stress may promote SOD activity in the plant. PBZ may lead to higher SOD activity under flooding stress. SOD may be a key enzyme for flooding tolerance in sweet potato leaves.

Figure 2 illustrates CAT activity in the sweet potato under flooding conditions, with or without PBZ pre-treatment, on days 0, 1-, 3-, 5 of flooding and drainage treatments. Compared to non-PBZ-treated plants under non-flooding conditions (−P/−F), the PBZ-treated plants grown under non-stressed conditions (+P/−F) had higher CAT activities on each day of the treatments. This observation exhibited traits characteristic of PBZ treatment. Additionally, significantly higher CAT activity was observed in the +P/+F condition on day 5 of treatment (0.47 μmol/g fw) as compared to other conditions (−P/−F, +P/−F and −P/+F). The addition of PBZ might lead to increased CAT activity in sweet potato leaves.

When PBZ treatment/flooding conditions were compared across different days of treatment, −P/−F conditions displayed lower APX activity than other conditions, with the exception of elevated values (13.9 μmol/g fw) during drainage (Fig. 3). On the contrary, higher levels of APX activity were detected under +P/+F conditions on each day of flooding, save the value for drainage (8.2 μmol/g fw). These observations suggest that the addition of PBZ may raise APX activity under flooding stress, and this may mitigate flooding-stress effect. As a consequence, the pre-application of PBZ at 0.5 mg/

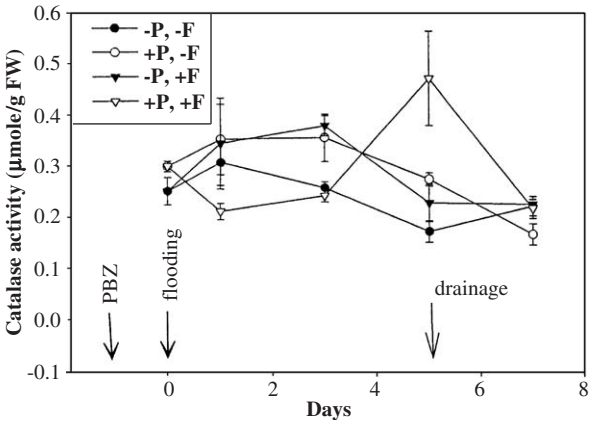

Figure 2. The effect of pre-treating paclobutrazol on catalase activity in leaves of sweet potato 'Taoyuan 2' subjected to flooding stress and following drainage. +P: paclobutrazol pre-treated; −P: non-paclobutrazol; +F: flooding; −F: non-flooding. Vertical bar represents mean ± standard error.

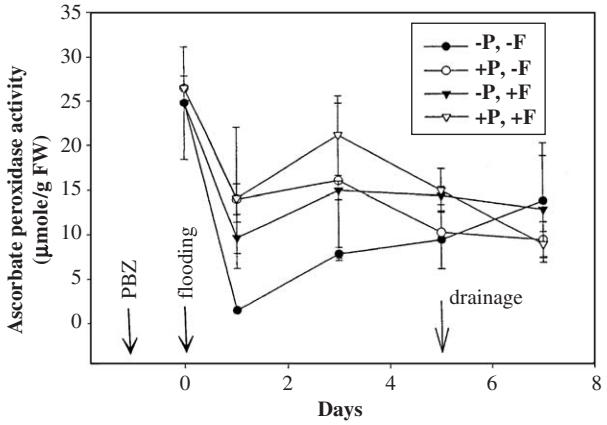

Figure 3. The effect of pre-treating paclobutrazol on ascorbate peroxidase activity in leaves of sweet potato 'Taoyuan 2' subjected to flooding stress and following drainage. +P: paclobutrazol pre-treated; −P: non-paclobutrazol; +F: flooding; −F: non-flooding. Vertical bar represents mean ± standard error.

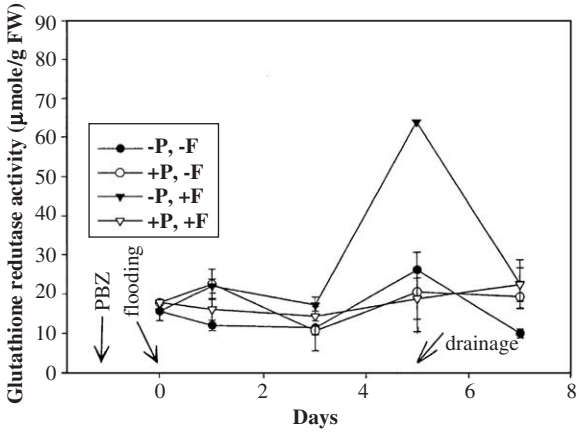

Figure 4. The effect of pre-treating paclobutrazol on glutathione reductase activity in leaves of sweet potato 'Taoyuan 2' subjected to flooding stress and following drainage. +P: paclobutrazol pre-treated; −P: non-paclobutrazol; +F: flooding; −F: non-flooding. Vertical bar represents mean ± standard error.

plant proved to be important for countering flooding stress.

GR activity was significantly higher for −P/+F condition compared to other conditions on day 5 of flooding (63.3 μmol/g fw), as seen in Fig. 4. In addition, −P/−F conditions also resulted in significantly lower GR activity than other conditions after 2 d drainage (9.3 μmol/g fw). GR activity in the 'Taoyuan 2' leaf induced by flooding stress may be involved in imparting flooding tolerance.

As shown in Fig. 5, PBZ-treated plants subjected to flooding stress (+P/+F) exhibited significantly higher total glutathione content than

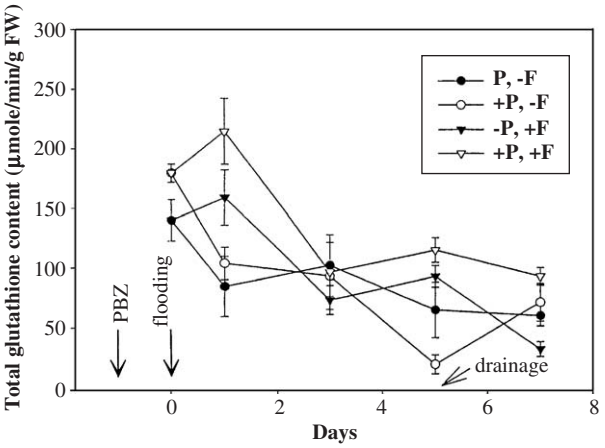

Figure 5. The effect of pre-treating paclobutrazol on total glutathione content in leaves of sweet potato 'Taoyuan 2' subjected to flooding stress and following drainage. +P: paclobutrazol pre-treated; −P: non-paclobutrazol; +F: flooding; −F: non-flooding. Vertical bar represents mean ± standard error.

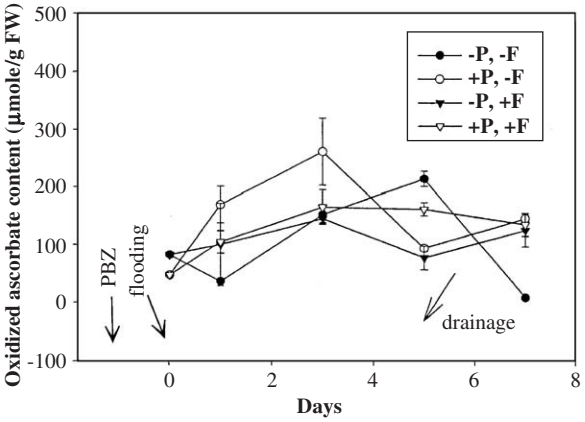

Figure 6. The effect of pre-treating paclobutrazol on oxidized ascorbate content in leaves of sweet potato 'Taoyuan 2' subjected to flooding stress and following drainage. +P: paclobutrazol pre-treated; −P: non-paclobutrazol; +F: flooding; −F: non-flooding. Vertical bar represents mean ± standard error.

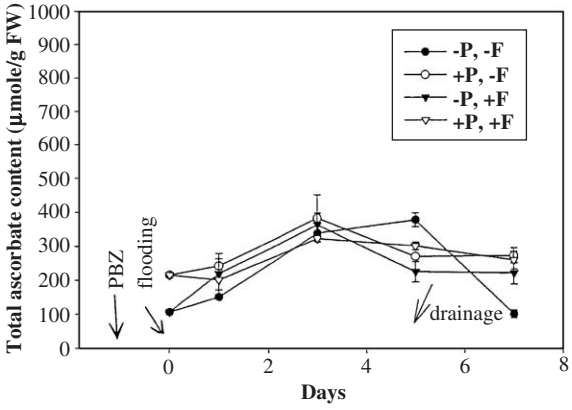

Figure 7. The effect of pre-treating paclobutrazol on total ascorbate content in leaves of sweet potato 'Taoyuan 2' subjected to flooding stress and following drainage. +P: paclobutrazol pre-treated; -P: non-paclobutrazol; +F: flooding; −F: non-flooding. Vertical bar represents mean ± standard error.

non-PBZ-treated plants under flooding stress (−P/+F) on days 1 and 5 of flooding and drainage. This result indicates higher total glutathione content in the 'Taoyuan 2' leaf under flooding stress can be achieved by pre-treating with 0.5 mg PBZ. A rise in total glutathione stimulated by pre-treating with PBZ plays an important role against flooding stress. PBZ treatment may enable the leaf to withstand better flood stress conditions.

Figure 6 compares the oxidized ASA content under PBZ/flooding conditions throughout the period of treatment. −P/−F conditions resulted in significantly higher oxidized ASA amount on day 5 of flooding than −P/+F conditions; this implies that flooding might result in a decrease in the level of oxidized ASA and the occurrence of oxidized damage in the plant. On the other hand, the level of oxidized ASA under −P/−F conditions was also higher than under +P/−F conditions, which suggests that pre-treating with PBZ can alter the oxidative system and limit the production of oxidized ASA under non-stressed conditions.

All four conditions showed an accumulation of total ASA content from day 0 to day 3 (or day 5), of flooding, which fell thereafter (Fig. 7). The level of total ASA under the −P/+F conditions accumulated at different rate from day 0 (102.8 μmol/g fw) to day 3 of flooding (370.1 μmol/g fw), and was followed by a decrease to 241.6 μmol/g fw after drainage.

Figure 8 shows that the MDA content responded differently to four treatments. −P/+F conditions yielded a higher amount of MDA compared to +P/+F conditions on day 0 and day 1 of flooding. These results suggest that the addition of PBZ may reduce MDA content under flooding stress, in other words decreased lipid peroxidation in the leaves. Pre-treating with PBZ could mitigate the adverse impact of flooding stress and induce flooding tolerance in the sweet potato.

Discussion

Sweet potato production is limited to the hot and wet summer season in Taiwan. Flooding is an important factor affecting sweet potato production

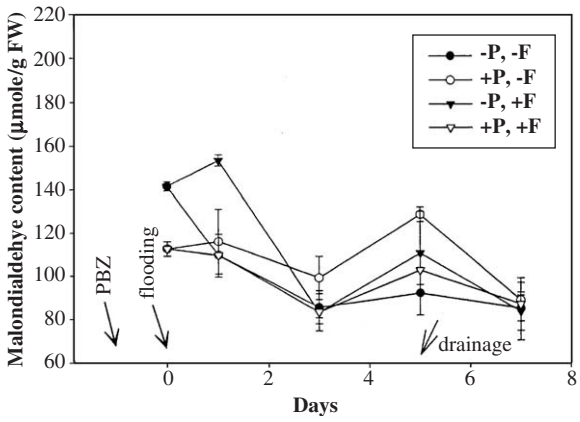

Figure 8. The effect of pre-treating paclobutrazol on malondialdehye content in leaves of sweet potato 'Taoyuan 2' subjected to flooding stress and following drainage. +P: paclobutrazol pre-treated; −P: non-paclobutrazol; +F: flooding; −F: non-flooding. Vertical bar represents mean ± standard error.

during the summer. Oxygen limitation is the primary stress to plants growing in flooded soils. Determination of the role of any observed response is one of the most complex issues in plant stress physiology; in the attempt to understand responses to flooding stress, many enzymes induced by flooding have been identified and characterized (Pinhero et al., 1997). Nonetheless, scarcely any studies have been conducted on the response of the antioxidative system in the sweet potato with regard to the ability to survive under flooding stress. Plants receiving PBZ that are under environmental stress have significantly higher antioxidative system levels compared to plants under non-stressed conditions (Lurie et al., 1994; Paliyath and Fletcher, 1995). This study examined the effects of PBZ pre-treatment on antioxidative enzymes and antioxidants in the sweet potato leaf under flooding stress.

A comparison of non-PBZ-treated plants under non-stressed conditions (−P/−F, control) and non-PBZ-treated plants subjected to flooding stress (−P/+F)

The involvement of the antioxidative system in the regulation of free-radical metabolism was followed by measuring changes in enzyme activity and antioxidant content under flooding conditions. Without PBZ pre-application, SOD, CAT, APX and GR activity on day 3 of flooding stress was significantly higher than under non-flooding conditions (Figs. 1–4). Plants under flooding stress also exhibited significantly higher levels of APX, GR, total glutathione, oxidized ASA, total ASA and MDA on day 1 of flooding compared to non-flooding conditions (Figs. 3–8). Figures 3 and 4 demonstrate that APX and GR activity markedly increased under flooding stress on days 1, 3 and 5 of flooding in comparison to non-stressed conditions. Hence, SOD, CAT, especially APX and GR might act as flooding-tolerant enzymes to overcome flooding stress. Plants may prepare for oxidative damage by up-regulating their antioxidative enzyme activity under 5 d flooding stress. The degree of flooding injury seems to result from an enhancement of APX and GR in sweet potato 'Taoyuan 2'. Anoxic conditions are a major problem in plants under flooding stress. As the plant encounters anoxic stress, higher oxidative system levels illustrate their superior tolerant mechanism in terms of H_2O_2, O_2^-, $\bullet O_2$ or $\bullet OH$ scavenging over the plant.

The lower leaves of the sweet potato under flooding conditions showed epinasty and senescence after 3 d flooding. However, under non-flooding conditions, most leaves looked green and healthy in the boxes (data not shown). Flooding stress had a harmful effect on sweet potato leaves. When significant flooding injury occurred, oxy-radical production increased. The entire antioxidative system (Figs. 1–8) in sweet potato leaves was affected by flooding stress (−P/+F) on different days of the treatment. The leaves of flooded plants maintained certain levels in the antioxidative system, and this consumed at least a part of the ROS, including H_2O_2, $\bullet O_2$, $\bullet OH$, and O_2^-. Antioxidative enzyme activity and antioxidant content play major roles in maintaining the balance between free-radical production and elimination. Enhancement of SOD activity under waterlogged environments may be an indicator of superoxide production (Fig. 1). High levels of SOD should be followed by the scavenging of H_2O_2 catalyzed by APX and CAT. Asada (1992) reported that the APX found in organelles is believed to scavenge H_2O_2 produced from 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 which has diffused from organelles. In the chloroplast, H_2O_2 can be detoxified by the ASA–GSH–NAPDH system that has been catalyzed by APX (Mehlhorn et al., 1995). Herein, the GSH is a composition of two stoichiometric forms, and is oxidized to GSSG. High levels of glutathione are the result of increased GSH biosynthesis. The level of MDA is a measure of whether plant cells were confronted with oxidative stress damage. Lower levels of MDA indicate better flooding tolerance. ROS scavenging is important in imparting tolerance

against flooding stress. Certain enzyme activities and levels of antioxidant content may limit the defense mechanisms of susceptible plants under waterlogged conditions. It may be presumed that enhancement of the antioxidative system favors flooding resistance. Antioxidative enzymes may augment antioxidants in the removal of ROS from plant cells.

A comparison of non-PBZ-treated plants under non-flooded conditions (−P/−F, control) and pre-treatment with PBZ followed by non-stressed conditions (+P/−F)

As shown in Figs. 2 and 5–8, under non-stressed conditions, pre-treating with PBZ induced a significantly higher level of CAT, total glutathione, total ASA, lower oxidized ASA and MDA contents on day 0 of treatment. One day after the plants received PBZ, the chemical immediately caused increased levels of CAT, total glutathione and total ASA and decreased levels of oxidized ASA and MDA. Figures 3 and 4 demonstrate that under non-stressed conditions, PBZ pre-addition significantly enhanced APX and GR levels in 'Taoyuan 2' on day 1 of treatment. Thus it is clear that plants are highly regulated by PBZ, which can drastically enhance components of the antioxidative system. Pre-treating with PBZ may influence the ability to maintain a balance between the formation and detoxification of activated oxygen species, leading to leaf vulnerability against oxidative stress. From our observations, in comparison to control plants (−P/−F), the PBZ-treated plants under non-stressed conditions (+P/−F) appeared healthy and had greener leaves throughout the duration of the experiment.

A comparison of non-PBZ-treated plants subjected to flooding conditions (−P/+F) and pre-treating with PBZ followed by the imposition of flooded conditions (+P/+F)

Significantly higher total glutathione (Fig. 5) and lower MDA (Fig. 8) content were found under +P/+F conditions compared to −P/+F conditions early on day 1 of flooding. Significantly greater SOD activity (Fig. 1) was shown under +P/+F than under −P/+F on day 3 of flooding. Significantly enhanced levels of CAT (Fig. 2), oxidized ASA (Fig. 6) and total ASA (Fig. 7) were exhibited under +P/+F in comparison to −P/+F up to day 5 of flooding. Interestingly, +P/+F conditions showed a significantly higher total glutathione than −P/+F on each day of flooding and drainage (Fig. 5). +P/+F conditions simply induced the same level of GR activity as under −P/+F conditions (Fig. 4). These results suggest that enhancement of the level of most components in the antioxidative system was attributable to PBZ pre-treatment, and the sweet potato 'Taoyuan 2' exhibited flooding tolerance as a result. PBZ application prior to flooding protects 'Taoyuan 2' from the adverse effects of flooding. It is possible that PBZ is one modulator of the cellular regulation factors following flooding-stress damage.

Pinhero et al. (1997) have shown that PBZ treatment of maize induced several changes in the antioxidative system profiles and enhanced antioxidative enzyme activity, especially that of SOD, GR and APX, in addition to inducing chilling tolerance. Kraus and Fletcher (1994) proposed that PBZ-induced protection in wheat from damage caused by heat stress was mediated by increased SOD, APX and GR activity. Our results support the findings presented in Fletcher et al. (2000), namely that PBZ protected wheat seedlings from injury due to flooding, and that PBZ treatment reduced all symptoms of damage at various stages of growth. From our study, we may determine that pre-dripping PBZ could be an important strategy to alter the behavior and survival of the sweet potato 'Taoyuan 2' under flooding stress. Flooding-stress protection conferred by PBZ was mediated to some extent by the enhancement of the antioxidative system, in particular of total glutathione. Our results suggest that flooding stress effects on sweet potato 'Taoyuan 2' can be mitigated by pre-treating with PBZ at 0.5 mg/plant.

A comparison of non-PBZ-treated plants under non-flooding conditions (−P/−F) and pre-treating with PBZ followed by the imposition of flooding conditions (+P/+F)

GR activity and the total MDA content in 'Taoyuan 2' that had been pre-treated with PBZ, in the end showed comparable levels after flooding stress as those of non-PBZ-treated plants under non-flooding conditions (Figs. 4 and 8). Moreover, PBZ pre-treatment enhanced SOD, CAT and APX activity, and total glutathione content under flooding stress compared to non-PBZ-treated plants under non-flooding conditions (Figs. 1–3 and 5). PBZ pre-treatment could only be seen to lower oxidized ASA and total ASA content under flooding in relation to non-PBZ-treated plants under non-flooding conditions (Figs. 6 and 7). In sum, pre-treating with PBZ enhanced the antioxidative system in 'Taoyuan 2' under flooding stress to a level higher than that of the control.

In conclusion, on separate days of treatment the plants responded differently to oxidative injury under flooding stress, according to the state of their antioxidative system. Some of the enzymes and antioxidants were characterized by a tendency to increase after flooding on different days of treatment, especially GR in the plant leaf, which was significantly greater than under non-flooding conditions on days 1, 3, 5 and drainage of treatments. Sweet potato leaves under waterlogged stress generate ROS that may then be removed by GR or other flooding-tolerant antioxidative enzymes and antioxidants. The antioxidative system level in the sweet potatoes was highly regulated by the PBZ treatment under non-flooding conditions. Our results indicate that different oxidative systems in the leaf were largely induced by PBZ pre-treatment at 0.5 mg/plant on various days of treatment. PBZ adding under non-stress increased the levels of antioxidative enzymes and antioxidants to combat against the adverse effects of ROS. Furthermore, SOD, CAT and APX activity, and total glutathione, oxidized ASA and total ASA content were enhanced by the addition of PBZ prior to flooding stress. PBZ can protect the sweet potato from flooding stress through its influence on the plant's oxygen detoxifying system; thus, pre-applying 0.5 mg/plant of PBZ 24 h prior to flooding could mitigate flooding stress. According to these findings, this paper proposes that paclobutrazol would be a beneficial pre-treatment for plants that are grown in wetlands, lowlands or areas that are prone to flooding, such as where there is natural gleying of the soil, or in areas subject to short and intense rainfall.

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