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ORIGINAL PAPER

Physiological responses of bermudagrass (*Cynodon dactylon*) to submergence

Shuduan Tan · Mingyong Zhu · Quanfa Zhang

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Abstract A split-plot design was used to study the physiological responses of anti-oxidative enzymes and carbohydrate contents of bermudagrass to different durations (0, 3, 7, 15, 30, 60, 90, 120 and 150 days) and depths of submergence (0, 1, 5 and 15 m). The results suggest that submergence caused a higher production of malondialdehyde and more significant changes in the different submerged treatments. The activities of catalase (CAT), superoxide dismutase (SOD), peroxidase (POD), ascorbate peroxidase (APX) and glutathione reductase (GR) in roots increased with the increase of the durations and depths of submergence, implying an integrated pathway involving CAT, SOD, POD, GR and APX for protection against the detrimental effects of activated oxygen species under submergence. Total soluble carbohydrate and starch contents of shoots and roots decreased with the increase of the depth and duration of submergence, but remained at relatively high level at the end of the study, showing quiescence is one of the major strategies of bermudagrass under the stress of submergence. The results suggest that bermudagrass can endure long-term and deep submergence by balancing between the formation and detoxification of activated oxygen species, lowering metabolism and reserving high amounts of carbohydrate.

Keywords *Cynodon dactylon* · Deep submergence · Anti-oxidative enzymes · Carbohydrate

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S. Tan · M. Zhu · Q. Zhang (✉)
Key Laboratory of Aquatic Botany and Watershed Ecology, Wuhan Botanical Garden, Chinese Academy of Sciences, Wuhan 430074, People's Republic of China
e-mail: qzhang@wbgcas.cn

S. Tan
e-mail: tanshuduan@163.com

S. Tan
College of Bioscience and Biotechnology, Hunan Agricultural University, Changsha 410128, People's Republic of China

S. Tan · M. Zhu
Graduate School of the Chinese Academy of Sciences, Beijing 100049, People's Republic of China

Introduction

Submergence is one of the major abiotic stresses, primarily through restriction in the diffusion of oxygen and carbon dioxide in plant, and has a dramatic impact on biochemical activities, such as aerobic respiration and photosynthesis (Armstrong and Drew 2002). In anoxia plant cells, the oxidative stress reactions are associated with toxic-free radicals from the reduction of molecular oxygen to superoxide radicals, singlet oxygen, hydroxyl radicals and hydrogen peroxide (Lin et al. 2004). To counter the hazardous effects of reactive oxygen species (ROS), plants have evolved a complex anti-oxidative defensive system composed of both antioxidant enzymes and metabolites (Sarkar 1998; Ahmed et al. 2002; Sairam et al. 2002). High levels of antioxidant enzymes including catalase (CAT), superoxide dismutase (SOD), peroxidase (POD), glutathione reductase (GR) and ascorbate peroxidase (APX) are very important for the survival under oxidative stress of many plants such as rice (Ella et al. 2003), barley (Zhang et al. 2007), tomato, eggplant (Lin et al. 2004), mung bean (Ahmed et al. 2002) and wheat (Biemelt et al. 1998).

Periodic or prolonged deprivation of oxygen interferes with respiration at the level of electron transport (Lin et al.

2004). The lack of a suitable electron acceptor leads to saturated redox chains, accumulation of NAD(P), a decline in the generation of adenosine triphosphate (ATP), a center substance of energy metabolism (Kennedy et al. 1992; Crawford and Braendle 1996; Bor et al. 2003), and then energy starvation or even death. When plants are submerged, the energy metabolism is changed from aerobic respiration to alcoholic fermentation. Alcoholic fermentation is not as effective as aerobic respiration for energy production and consumes much more carbohydrate than aerobic respiration, thus causing depletion of carbohydrate reserves required for plant growth and maintenance. Carbohydrate contents, as the substrates for alcoholic fermentation, provide energy for maintenance and survival during anoxia and submergence. Several previous studies emphasized the importance of the level of non-structural carbohydrate content and its association with submergence tolerance and recovery ability in anoxic environments (Das et al. 2005; Sarkar et al. 1996, 1998; Sauter 2000; Singh et al. 2001; Vriezen et al. 2003).

Bermudagrass (*Cynodon dactylon*) is a perennial species adapted to tropical and subtropical climates. It has a fibrous, perennial developed root system with vigorous and deep rhizomes. Its optimal growth temperature is 20–35°C. Previous research has demonstrated that bermudagrass could endure 25-m deep submergence for 5 months with a survival rate of more than 90%, and is a promising species for revegetation in the water-level-fluctuation zone (unpublished data).

In this study, a split-plot design was used to study the physiological responses of bermudagrass to different durations and depths of submergence. The objectives are: (1) to determine oxidative damage in terms of malondialdehyde (MDA) concentration in roots; (2) to detect the anti-oxidative activities of roots in bermudagrass enduring long-term and deep submergence; and (3) to demonstrate the changes in carbohydrate contents of bermudagrass with the increase in the submerged duration and depth. The experiment was designed to investigate the physiological traits associated with the long-term and deep submergence tolerance of bermudagrass. The ultimate goal was to explore the possibility of using bermudagrass for revegetation in the water-level fluctuation area of China's Three Gorges Reservoir or other riparian areas.

Materials and methods

Plant materials

The current year's shoots of bermudagrass were collected from the natural water level fluctuation zone of the Three Gorges Reservoir, Hubei Province, China (E110°54′, N30°52′). Ten identical growth shoots were transplanted into 30 cm × 15 cm pot containing sterilized sandy loam of Lanling rivulet riverbank. Five drainage (0.5 cm in diameter) holes were drilled on the bottom of each pot. The transplanted plants were evenly spaced every 30 cm to ensure similar growth rate and size in an open field for about 2 months in the Lanling Creek, Zigui County, Hubei.

On 2 November 2008, the submergence treatment was carried out in a split-plot design with six replications. Four submerged depth (0, 1, 5 and 15 m) and nine submerged time (0, 3, 7, 15, 30, 60, 90, 120 and 150 days) were arranged as the sub-plot and main plot, respectively. Submergence treatments were administered by shifting the pots into the Lanling Creek, the tributary of Yangtze River.

At the time of 0, 3, 7, 15, 30, 60, 90, 120 and 150 days, six plots were taken out at each submerged depth for analysis. The plant samples were washed and clipped. Some roots were frozen in liquid nitrogen immediately. After being transported to the laboratory, the samples were stored in a −70°C freezer for MDA, CAT, SOD, POD, GR and APX activity analysis. The other shoots and roots were carried in an icebox to the laboratory immediately and used to determine the levels of soluble sugar and starch contents.

Malondialdehyde assays

Malondialdehyde was determined according to Li (2000). As much as 2 μl of enzyme extracts were incubated with 10% (w/v) trichloroacetic acid and 0.6% thiobarbituric acid solution in boiling water for 10 min. Then the mixture was centrifuged at room temperature and 10,000×*g* for 20 min. Absorbances in 450, 532 and 600 nm were recorded. The MDA content was calculated as described by (Li 2000):

$$C(\mu M) = 6.45(A_{532} - A_{600}) - 0.56A_{450}$$

Antioxidant enzyme activities assays

A total of 0.5 g of roots were collected and ground in a mortar with 5 ml of ice-cold 62.5 mM phosphate buffer (pH 7.8) with 1.0% (m/v) polyvinyl pyrrolidone. The homogenates were centrifuged at 4°C and 12,000×*g* for 20 min. The supernatants were stored at 4°C and then used for assays of enzymatic activities.

Catalase activity was assayed at 20°C in a 3-ml reaction volume containing 2.8 ml of 50 mM potassium phosphate buffer (pH 7, not containing EDTA), 120 μl of enzyme extract and 80 μl of 0.5 M H_2O_2. Activity was determined by UV spectrophotometer at 240 nm, which measures the decrease in absorbance for 1 min (Aebi and Bergmeyer 1983).

Superoxide dismutase activity was measured as the amount of inhibition of photo-reduction of nitroblue tetrazolium (NBT) (Li 2000). The reaction mixture, with a

final volume of 3.0 ml, contained 1.5 ml of 62.5 mM phosphate buffer, 0.3 ml of 20 μM riboflavin, 0.3 ml of 130 mM methionine, 0.3 ml of 100 μM Na_2EDTA, 0.3 ml of 750 μM NBT, 0.05 ml of enzyme extract and 0.25 ml of deionized water. One unit of the enzyme activity was defined as the amount of enzyme required to result in a 50% inhibition of the rate of NBT reduction measured at 560 nm.

For the measurement of POD activity, 2.9 ml of 100 mM phosphate buffer + 20 mM guaiacol, pH 7.0, was mixed with 0.1 ml of enzyme extract and allowed to stand at room temperature for 3 min. To activate the reaction, 20 μl of 2% hydrogen peroxide was added. The absorbance at 470 nm was measured at 1-min intervals for 5 min, and an increase of 0.01 absorbance units per minute was equated to 1 U of POD activity (Cakmak and Marschner 1992; Li 2000).

Glutathione reductase activity was measured by oxidized GSH-dependent oxidation of NADPH. The reaction mixture contained 50 mM of Tris–HCl (pH 7.6), 5 mM of NADPH, 50 mM of GSSG and 1 ml of enzyme extract (Foyer and Halliwell 1976). The change in absorption at 340 nm ($E = 6.2\ mM^{-1}cm^{-1}$) was recorded over 3.5 min.

Activity of APX was measured by monitoring the rate of ascorbate oxidation at 290 nm ($E = 2.8\ mM\ cm^{-1}$) (Nakano and Asada 1981). The reaction mixture contained 50 mM (pH 7.0), 0.1 mM EDTA, 1.0 mM H_20_2, 0.25 mM AsA, and the enzyme aliquot.

Carbohydrate assays

Carbohydrate content was measured following the procedure described by Das et al. (2005) with slight modifications. Samples were oven dried at 60°C for 72 h and ground to a fine powder and extracted three times using 80% ethanol (v/v). The extract was then used for soluble sugar analysis after addition of anthrone reagent, followed by measurement of absorbance at 630 nm using a spectrophotometer. The residue after soluble sugars extraction was dried and extracted using 30% perchloric acid and then analyzed for starch using reagent of anthrone dissolved in acetic ether (2% p/v). The calibration curves were performed using commercial D-glucose or starch.

Statistical analysis

Statistical analysis was performed based on the split-plot design with six replications using SPSS 13.0. Multiple comparisons of means were performed by LSD test at the 0.05 significance level. Data were log_{10}-transformed when necessary to reduce data heterogeneity, and homogeneity was tested using Levene's test.

Results

Damage induced by submergence

Lipid peroxidation levels in roots of different treatments (determined as MDA content) are given in Fig. 1a. Plants without submergence treatment showed MDA levels ranging from 1.79 to 9.34 mmol $(g\ FW)^{-1}$. MDA production showed significant changes under different submergence stresses. Plants submerged with 1, 5 and 15 m depth and 150 days of stress reached levels of 18.61, 25.48 and 28.96 mmol $(g\ FW)^{-1}$, 2.6-, 3.5- and 4.1-fold higher than levels in control plants, respectively. From 90 to 150 days, submerged stress on plants increased more rapidly than that from 3 to 60 days.

Anti-oxidative enzyme activity

Catalase activities of submergence-treated plants showed similar changes compared to the control plants (Fig. 1b). The highest CAT activity was reached at 60 days after the onset of stress of submergence. Afterward, CAT activities in all plants decreased during the time of submergence, paralleling the pattern observed in control plants, although activities remained higher in all submergence plants than the control ones. At 150 days after the onset of stress of submergence, CAT activities were 1.25, 3.05 and 3.98 U $(g\ FW)^{-1}$ in roots of plants treated with 1, 5 and 15 m deep submergence stress, respectively, which means 1.5-, 3.7- and 4.8-fold increase with respect to control plants.

Superoxide dismutase activities of 5 and 15 m treatments gradually increased with the submergence time. SOD activities of control and 1-m treatments had the same increasing trends within 60 days after the onset of stress of submergence, and then decreased with the experiment time (Fig. 1c). Generally, the elevated SOD activities were in correlation with the degree of stress (durations and depths of submergence). There were significant changes among the different treatments.

A significant increase in POD activities was observed among all submerged plants in comparison with the control (Fig. 1d). POD activities increased with the submerged time and depth. The rate of increase in different treatments was different. The rate of increase of 15-m deep treatment was faster than that of 1 and 5 m. There was no significant difference between the treatments for 1 and 5 m depth.

Submergence induced a rapid increase of APX activity, only 7 days after the treatments. APX activities in the roots of treatments with 1, 5 and 15 m submerged depth were 1.2-, 1.5- and 1.9-fold higher than in controls, respectively (Fig. 1f). Afterward, APX activities followed an increasing

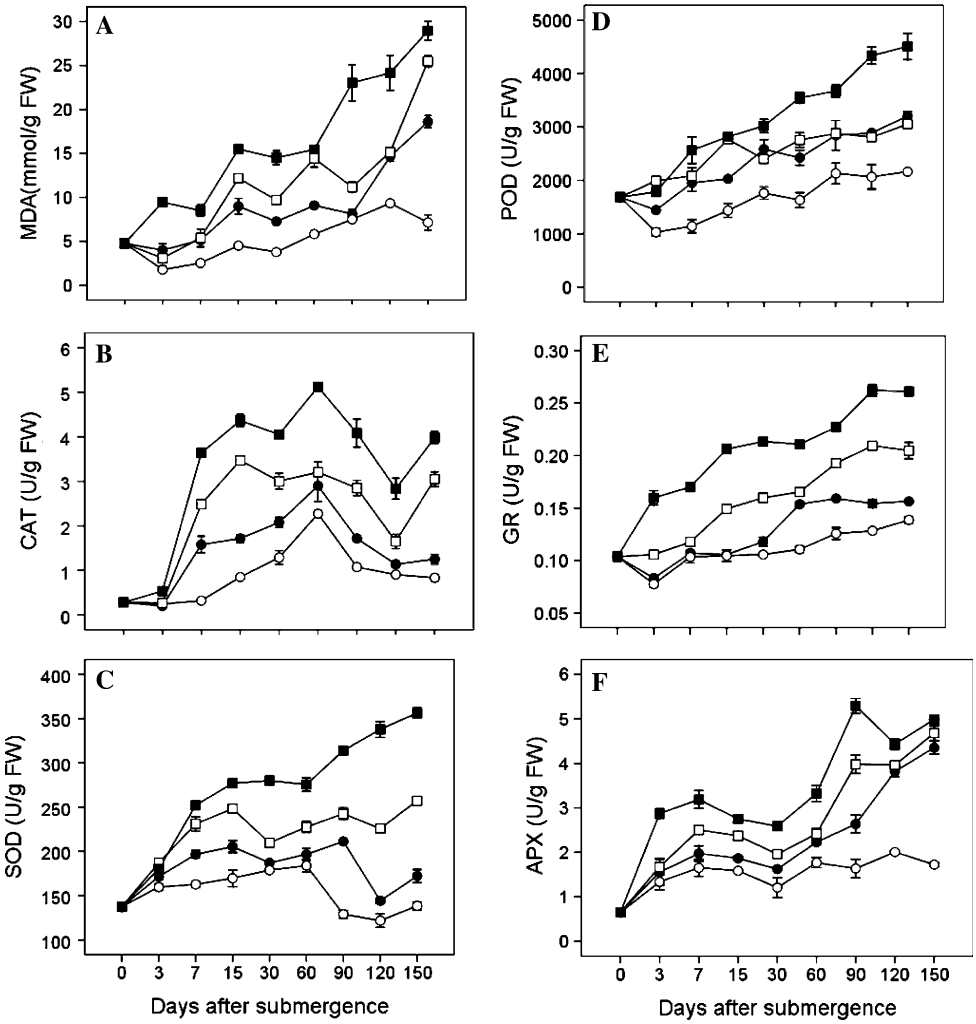

Fig. 1 The effect of different submergence stress on MDA (**a**), CAT (**b**), SOD (**c**), POD (**d**), GR (**e**), APX (**f**) of roots. Each point is the average value of six independent measurements ± SE. Control (*open circle*), non-submerged plants, and plants submerged at a depth of 1 m (*closed circle*), 5 m (*open square*) 15 m (*closed square*)

pattern until reaching the highest point at the end of the experimental period. GR activities showed a very similar pattern as APX activities and increased in all treated plants in response to submergence (Fig. 1e, f). There were significant differences in the increasing trends and rates of GR activities among different treatments.

Carbohydrate consumption

Total soluble carbohydrate (TSC) and starch content in all treatments remained at relatively high levels throughout the period of the study (Fig. 2). TSC and starch contents of shoots and roots decreased significantly with submergence depth and duration. Within 60 days after the treatments, TSC reserves were greatly depleted in all treatments. About 20, 40 and 50% of soluble carbohydrates were consumed within the time in 1, 5 and 15 m submerged shoots and roots, respectively. Shoot had higher starch content than root, but the starch decline in root was less dramatic than that in shoot.

Discussion

Damage after subjection to different submergence

The increased levels of submergence promoted different levels of damage in plants. Growth was impaired by submergence. During the experimental period, plants had green leaves, although the number decreased with the increase in duration. At about 60 days, submerged plants still had some leaves alive, green or yellow. After that, the leaves of all the submerged plants defoliated. Defoliation occurred earlier under longer duration and deeper submerged plants.

Submergence injury is a result of anti-oxidative chain reactions promoted by free radicals, which damage lipid membranes through peroxidation (Monk et al. 1989). The concentration of MDA reflects the extent of lipid peroxidation in the cell membrane under stress conditions (Zhang et al. 2005). In the current research, the injury of biological lipids by ROS, as indicated by MDA content, was clearly

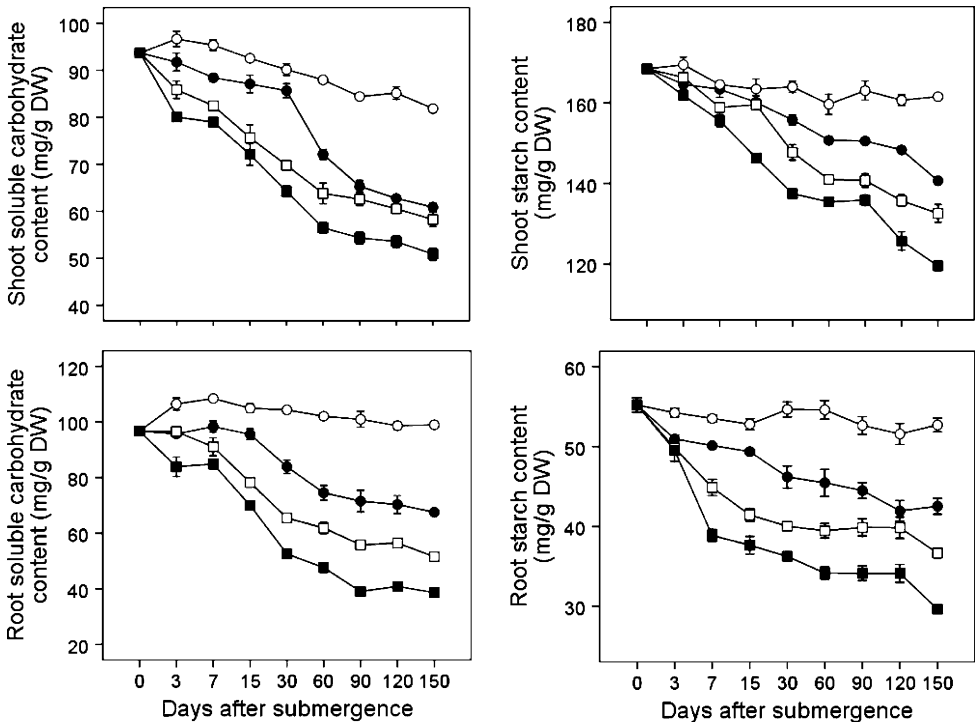

Fig. 2 The effect of different submergence stress on the total soluble sugar content and starch content of shoots and roots. Each point is the average value of six independent measurements ± SE. For *symbols* see Fig. 1

detected in the submerged and non-submerged plants (Fig. 1a). The MDA increases of the submerged plants were different for different submerged depth and duration, implying the capacity of their defensive system against ROS. The change of control plants showed that natural environmental stresses could induce oxidative stress damage because plants lived simultaneously under the effect of multiple stress factors in natural habitats or fields. In our study, the major stress factor of control plants was low temperature.

Levels of anti-oxidative enzymes

Accepting oxygen utilization under anoxia, anti-oxidative enzymes might be needed for scavenging active oxygen species. The increase in CAT is critical for plants under oxygen-deficient conditions because CAT can produce O_2 from H_2O_2. Since SOD is present in all aerobic organisms and most subcellular compartments that generate activated oxygen, it has been assumed that SOD has a central role in the defense against oxidative stress (Bowler et al. 1991; Scandalios 1993). Simultaneous increase in activity of H_2O_2-scanvenging enzymes, with accompanying increase in SOD activity, is crucial for effective defense against oxidative stress (Gossett et al. 1994). Zhang et al. (2007) reported that POD activity was higher in waterlogging stress-tolerant barley. The results of the present study showed that CAT, SOD and POD activities increased with submergence depth and duration. CAT, SOD and POD activities of all submerged treatments were higher than those of not submerged control plants [Fig. 1b–d; Ushimaru et al. (1999)].

APX 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 produces in cytosol or apoplast and diffuses from organelles (Asada 1992). Therefore, APX activity plays a major role in maintaining the balance between free radical production and elimination (Lin et al. 2004). GR is involved in maintaining a high ratio of GSH/GSSG, which is required for the regeneration of ascorbate (AsA), an important antioxidant in plant cells. The role of GR and glutathione in H_2O_2 scavenging in plant cells has been well documented (Peters et al. 1989; Broadbent et al. 1995). In the present study, GR and APX activities were increased by the submergence treatments, and there were significant differences among the different submergence depth and duration treatments (Fig. 1e, f), which ensures detoxification of reactive oxygen species (ROS) and regeneration of reduced AsA. At the same time, AsA could directly scavenge superoxide, hydroxyl radicals and singlet oxygen, and can serve as an enzyme cofactor (Fedoroff 2006).

From the enzymatic protective mechanism, our data are consistent with an integrated pathway involving CAT, SOD, POD, GR and APX for protection against detrimental effects of activated oxygen species under submerged stress. These data suggest that the anti-oxidative activities of roots play an important role in enduring long-term and deep submergence of bermudagrass. Generally, the elevated level of anti-oxidative enzyme activities was in correlation

with the degree of stress. The results suggest that the anti-oxidative system is regulated by various environmental factors in a complicated manner.

Total soluble carbohydrate and starch content

Within minutes of transfer to an O_2-depleted environment, cells reliant on external O_2 would limit processes that are highly energy consumptive and alter metabolism to increase anaerobic generation of ATP by cytosolic glycolysis (Drew 1997). Plant cells consume carbohydrates through energetically inefficient coupling of glycolysis and anaerobic fermentation when oxygen levels are restricted (Drew 1997). Therefore, energy depletion of submerged plants can be shown from the change of carbohydrate contents, because the metabolic response to O_2 deprivation is orchestrated by the availability and mobilization of carbohydrates and starch (Drew 1997; Frost-Christensen 2003). The amount of carbohydrate in plant parts was also found to be significantly and positively associated with submergence tolerance and regeneration growth (Panda et al. 2008).

As submergence proceeds, a progressive reduction occurs in the TSC of the shoots and roots of bermudagrass (Fig. 2). Apparently, carbohydrate contents decreased with the submergence depth and duration, and the decrease was progressive and proportional to the magnitude of the submergence stress, since carbohydrate status is susceptible to genotype, plant age, nature of submergence (durations and depths of submergence) and submergence environment (turbidity, O_2 and CO_2 concentrations, temperature and pH) (Ram et al. 2002). According to our observations, the leaves of all submerged plants were shed as a consequence of submersion. This action in tolerant genotypes during submergence can be considered as a means of preserving carbohydrate for sustained metabolism and prolonged energy supply (Setter and Ella 1994), which ultimately leads to higher carbohydrate status after submergence and better survival and recovery growth (Singh et al. 2001; Ram et al. 2002).

Since the assimilation of carbohydrate slows down, the submerged plants have to rely on the reserves (starch) for their respiratory substrate. Consequently, starch content decreased during submergence, the ratio of reduction being higher in the longer-and-deeper submerged treatments (Fig. 2). It has been reported that α-amylase activity plays a pivotal role in inducing the degradation of starch, particularly in cereal grains (Perata et al. 1998) and lowland or deepwater rice (Septiningsih et al. 2009). Shoot starch content was more than that in root, but the starch decline in root was less dramatic than that in shoot (Fig. 2). These results reveal that submerged shoots experience a more rapid starch starvation during submergence.

In spite of a general reduction, all submerged plants had relatively higher starch content at the end of submergence (Fig. 2). This slow consumption of starch allows the rhizomes to sustain a low level of metabolism that affords survival energy for long periods of submergence. These kinds of responses have also been emphasized by Greenway and Setter (1996) as being among the mechanisms of tolerance to submergence in plants. Furthermore, bermudagrass is dormant from late November to late March of each year according to the life history. It seems that the life cycle of bermudagrass was correlated with 'quiescence strategy' (Perata and Voesenek 2007; Kawano et al. 2008). At the same time, the life cycle is basically in accordance with the hydrological regime in the Three Gorges Reservoir, i.e., land in summer (May to October) and submergence in winter (November to April). This plays an important role for bermudagrass survival after long-term and deep submerged stress.

Given the above traits, the mode of carbohydrate metabolism of bermudagrass during submergence seems to be an important factor in submergence tolerance, and this 'quiescence strategy' is characterized by slow expansion growth that is presumed to conserve energy and carbohydrates (Singh et al. 2001). Higher POD activities were also closely associated with reduced expansion growth in plants such as mung bean (Goldberg et al. 1987), peanut (Zheng and Van Huystee 1992) and rice (Ismail et al. 2009). The POD activities of bermudagrass have been maintaining at higher levels during submergence (Fig. 1d), which may contribute to economize carbohydrate and warrant the supply of needed energy of bermudagrass under the anoxia stress.

Conclusions

From the enzymatic protective mechanism, our data are consistent with an integrated pathway involving CAT, SOD, POD, GR and APX for protection against detrimental effects of activated oxygen species under submerged stress. These data suggest that the anti-oxidative activities of roots play an important role in enduring long-term and deep submergence of bermudagrass. Generally, the elevated level of anti-oxidative enzyme activities was in correlation with the degree of stress.

The carbohydrate contents decreased with the submergence depth and duration, and the decrease was progressive and proportional to the magnitude of the submergence stress. All submerged plants had relatively higher total soluble carbohydrate and starch contents at the end of submergence. The mode of carbohydrate metabolism of bermudagrass during submergence seems to be an important factor in submergence tolerance, and this 'quiescence strategy' is characterized by slow expansion growth that is

presumed to conserve energy and carbohydrates (Singh et al. 2001).

In summary, bermudagrass can endure long duration and deep submerged stress because they can balance between the formation and detoxification of activated oxygen species, lower metabolism and reserve high amounts of carbohydrate. The results suggest that bermudagrass is a promising species for revegetation in water-level fluctuation areas of the Three Gorges Reservoir. However, future experiments are required to understand the morphological and molecular mechanisms of the endurance.

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