Physiological and Cellular Ultrastructure Responses for Three Grass Species under Submergence

HANPING XIA, X. ZHANG, Z. LP, X. LU AND S. FU

ABSTRACT

To illustrate the tolerance mechanisms of different grass species to submergence, we examined the changes in biochemical and anatomical characteristics and leaf photosynthesis of three grass species. Vetivergrass (Vetiveria zizanioides), bahiagrass (Paspalum notatum), and carpetgrass (Axonopus compressus), the species selected for this study, are commonly used for erosion control under complete submergence in water-cultivation studies. After submergence, chlorophyll (a + b) and carotenoids of carpetgrass decreased by 10.4 and 32.2%, respectively, while those of vetivergrass increased by 39.8 and 39.5%, and bahiagrass increased by 35.9 and 27.8%, respectively, from 9 to 36 days. The PSII maximum photochemical efficiency and the quantum yield of carpetgrass decreased to a greater extent than that of vetivergrass and bahiagrass. The malondialdehyde content of carpetgrass increased more than that of vetivergrass and bahiagrass with increasing submergence time. The activity of superoxide dismutase and ascorbate peroxidase of carpetgrass decreased with submergence duration, whereas those of vetivergrass and bahiagrass remained stable or increased. When the stress duration was relatively short (<60 days), vetivergrass and bahiagrass had similar levels of tolerance with respect to photosynthetic features and biochemical response; however, the damage of submergence to the ultrastructure of some important organelles of bahiagrass was more severe than vetivergrass when the duration was longer than 60 days. The ultrastructure of vetivergrass was not damaged markedly unless it was exposed to 120 days of submergence, whereas that of carpetgrass was severely damaged by 32 days after submergence. Overall, vetivergrass was the most tolerant of the three species, while carpetgrass had the poorest tolerance.

Key words: enzymatic antioxidant, grass for erosion control, photosynthetic pigment, starch grains, stress tolerance.

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INTRODUCTION

Soil erosion and other ecological problems of riverbanks and reservoir slopes are receiving increasing attention (Hamed et al. 2002, Tian et al. 2004, Wang et al. 2006). Riverbanks and reservoir slopes can be protected through appropriate planting (Tabacchi and Tabacchi 2001, Fetzer et al. 2006), but few plants have been found to be effective for the planned ecological goals (Wilkinson 1999, Xia et al. 2002). A primary reason for failure is that riparian zones usually suffer from seasonal inundation, while almost all plants used for erosion control are xerophytes (Xia et al. 2000). As such most of these types of plants would not establish or grow and may perish after being submerged (Ewing 1996). Therefore, tolerance to flooding is a major consideration for selection of plant used for soil erosion control along riverbanks and reservoir slopes.

Tolerance to submergence varies greatly among plant species and is influenced by plant age, flooding time, condition of the floodwater, and site characteristics (Kozlowski 1997, Nabben et al. 1999). For example, flooding for 30 days is essentially lethal to Citrus aurantium with 90% of the trees killed or severely injured, while C. jambhiri trees show only 20% dieback from continuous flooding for 60 days (Vu and Yelenosky 1991). It was reported that submergence can result in physiological, biochemical, and morphological changes as well as affect plant anatomy, resulting in poor gas diffusion, and can limit light interception by plants (Bowes et al. 1979, Vervuren et al. 1999, Mommer et al. 2005a, 2005b, Voosenk et al. 2006, Wang et al. 2007). Flooding stress often inhibits the formation and expansion of leaves and internodes, decreases leaf chlorophyll content, and stomatal closure, inhibits photosynthesis, and results in an increase in starch and other nonstructural carbohydrate accumulation (Van et al. 1977, Gravatt and Kirby 1998, Mielke et al. 2003, Chen et al. 2005). Although the effects of submergence on eco-physiology and biochemistry of plants have been well documented, little information is available on the changes of leaf ultrastructure. Moreover, specific tolerance mechanisms of different plant species to complete submergence remain unclear.

Vetivergrass (Vetiveria zizanioides [L.] Nash), bahiagrass (Paspalum notatum Flugge), and carpetgrass (Axonopus compressus Beauv.) have been widely used for erosion control, environmental protection, and ecological restoration in the tropics and subtropics, particularly in South China (Reynolds et al. 1999, Summerfelt et al. 1999, Xia et al. 2002, Pang et al. 2003, Xia 2004). In our previous study, these grass species were found to be quite different with regard to submergence tolerance: the most tolerant species was vetivergrass, enduring about 120 days of complete submergence; followed by bahiagrass for up to 60 to 70 days; the least tolerant was carpetgrass, lasting at most 40 days (Xia et al. 2003). We therefore hypothesized that the tolerance mechanisms to submergence among the three grass species must be different in several aspects, including photosynthesis, biochemical response, and cell ultrastructure. Clarification of these potential mechanisms will improve our knowledge of how to best utilize these grasses for protection of riverbanks and reservoir slopes.

In the present study, we conducted a water-cultivation experiment to investigate the effects of complete submergence on vetivergrass, bahiagrass, and carpetgrass. The objective was to identify mechanisms of tolerance to submergence in terms of the following three aspects: (1) photosynthetic characteristics such as chlorophyll (a + b) and carotenoid content, PSII maximum photochemical efficiency (FV/Fm) and quantum yield (Fv/Fm); (2) biochemical responses including changes in malondialdehyde (MDA) content and antioxidant activity; and (3) changes in leaf ultrastructure.

MATERIALS AND METHODS

Experimental Design

Mature and healthy plants were all collected from the grass nursery of the South China Botanical Garden. Vetivergrass is more erect in growth habit while the other two species are more procumbent.

Experiments were conducted using the method of water-cultivation described by Xia et al. (2003). Cement tanks with a volume of 2.0 m long by 2.0 m wide by 1.3 m high were constructed outdoors. Fully mixed local lateritic soil was filled into identical pots with a diameter of 25 cm and a height of 18 cm. We used 36 pots, with 12 pots for each species and 2 to 3 plants in each pot, and allowed 30 days growth before being transferred to the cement tank. To ensure adequate light for the two prostrate grasses, pots were placed on frames (80 cm high) previously installed inside the tank. Pots containing vetivergrass were placed directly on floor of the tank. After placement, tap water was used to fill the tank until all plants were completely submerged. Water was continuously added daily to the tank to ensure the complete submergence of the plants. On days 9, 18, 27, and 36 after initial submergence, three pots for each species were randomly selected for sample collection and chemical analysis. Plants grown under nonflooded conditions were used as controls.

To study the changes of cell ultrastructure under submergence, another experiment was conducted. The seedlings of the three grasses were planted, submerged, and managed as described in the previous experiment. Two mature leaves from each pot were collected for ultrastructure observation after carpetgrass plants were submerged for 16 and 32 days, bahiagrass for 30 and 60 days, and vetivergrass for 60 and 120 days. Plants grown under nonflooded conditions were used as control.

Biochemical Analysis and Observation

Determination of photosynthetic pigment contents. Leaf wafers with a diameter of 1 cm were sampled and then soaked with 80% (v/v) acetone for 3 days in dark. The contents of chlorophyll (a + b) and carotenoid in the extraction solutions were measured with a spectrophotometer (Lambdas 25, Perkin Elmer Inc., USA) and then calculated according to the method of Arnon (1949).

Measurement of chlorophyll fluorescence. Chlorophyll fluorescence kinetic emission was determined from the upper surface of mature leaves using a pulse amplitude-modulated chlorophyll fluorometer (PAM 101 Chlorophyll Fluorometer, Heinz Walz, Effeltrich, Germany). Initial fluorescence yield of leaves was recorded 30 min after dark adaptation. A

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single saturating pulse of white light (7000 µmol m⁻²s⁻¹) was then administered to obtain maximum fluorescence yield. The intensity of actinic light was 250 µmol m⁻²s⁻¹ with a flash of 2 s and an interval of 30 s. Chlorophyll fluorescence parameters, Fv/Fm and ΦPS₂, were calculated according to the formulas by Roháček and Barták (1999).

**Results and Discussion**

Effects of Submergence on Photosynthetic Characteristics

There were significant effects of plant species, submergence duration, and their interactions on chlorophyll (a + b) and carotenoid concentration (Table 1). The photosynthetic pigments showed strong interactions in their response to species and submergence duration treatments, and hence their variation was not independent. Submergence impact on chlorophyll (a + b) and carotenoid of grasses was positive for vetivergrass and bahiagrass but negative for carpetgrass. The contents of both chlorophyll (a + b; Figure 1A) and carotenoid (Figure 1B) in carpetgrass declined with increased submergence time, indicating that the photosynthetic pigments of this species were damaged by submergence. In contrast, the pigment contents of vetivergrass and bahiagrass increased gradually as submergence prolonged and was more pronounced for vetivergrass. After submergence, chlorophyll (a + b) and carotenoid of carpetgrass decreased by 10.4 and 32.2%, respectively, while those of vetivergrass increased by 39.8 and 39.5%, and bahiagrass increased by 35.9 and 27.8%, respectively, from 9 to 36 days. Chlorophyll is the main photosynthetic pigment for absorption light, and carotenoid is the assistant photosynthetic pigment for higher plants. Carotenoid plays an important role in quenching singlet molecular oxygen (¹O₂) produced in plants, thereby protecting the various membrane systems and especially the photosynthetic membrane of chloroplast from destruction due to induced peroxidation of unsaturated fatty acids (Telfer et al. 1994, Stahl and Sies 2003). Our results suggest that vetivergrass and bahiagrass may maintain high light-absorbing quantum by increasing photosynthetic pigments as a response to the decrease of light intensity under water. The increased tolerance of vetivergrass and bahiagrass to submergence is likely related to the maintenance of relatively high levels of photosynthesis, an important adaptation for some species in flooded environments (Chen et al. 2005).

There were significant interspecific differences for the Fv/Fm parameter, and significant differences between submergence durations with significant interaction between species and submergence duration (Table 1). Before submergence, the Fv/Fm values of the three grasses were similar, with 0.650, 0.676, and 0.683 for vetivergrass, bahiagrass, and carpetgrass, respectively (Figure 2A). After a 9-day submergence, all Fv/Fm values decreased significantly. As submergence prolonged, the Fv/Fm values of carpetgrass continued to decline while that of vetivergrass and bahiagrass remained somewhat stable. The Fv/Fm values of vetivergrass, bahiagrass, and carpetgrass decreased by 6.9, 9.9, and 30.3%, respectively, during the 36-day submergence period.

There were significant effects of plant species and submergence duration, and the interaction of species and submergence duration on ΦPS₂ of the three grasses (Table 1).
Strong interactions of species and submergence duration reflected that submergence impact on the Fv/Fm of grasses was slight for vetivergrass and bahiagrass but enormous for carpetgrass. The Fv/Fm values of the three grasses were similar before submergence. All declined after 9-days of submergence but recovered to the initial level after 18-days of submergence. Mateos-Naranjo et al. (2001) noticed that photosynthetic apparatus to tolerate environmental stress. Some chlorophyll fluorescence parameters such as Fv/Fm and ΦPSII are reported to be good indicators of submergence stress for plants (Bjorkman and Demmig 1987, Gordillo et al. 2001, Rohácek 2002), and the decrease in Fv/Fm ratio can be attributed both to the damage to the PSII reaction centre and the regulation capacity of PSII electron transport (Gordillo et al. 2001). Vervuren et al. (1999) noticed that photosynthetic rate and ΦPSII decreased in Arrhenatherum elatius (submergence-intolerant species), remained stable in Phalaris arundinacea (submergence-tolerant species), and increased in Rumex crispus (submergence-tolerant species) under submergence. Mateos-Naranjo et al. (2007) also found that continuous flooding conditions for a 2-month period reduced the efficiency of photosystem II, photochemistry of Spartina densiflora. In the present study, submergence resulted in a significant decrease of Fv/Fm and ΦPSII of carpetgrass, but showed limited impact on vetivergrass and bahiagrass (Figure 2).

Effects of Submergence on Peroxidation of Membrane Lipids and Activity of Enzymatic Antioxidants

Before submergence, the MDA contents of the three grasses in a nonflooded state were similar, with 52 to 58 nmol g⁻¹. In all three plants, MDA contents increased after the plants were submerged. On day 36 following submergence, the MDA content of carpetgrass had increased by 126.3%, whereas that of vetivergrass and bahiagrass increased only by 84.1 and 66.7%, respectively (Figure 3). The species and submergence duration and their interaction all significantly influenced the MDA content (Table 1). Reactive oxygen species (ROS) in plants can directly start the free-radical chain reaction of membrane lipid peroxidation, and MDA is the end product of membrane lipid peroxidation. Therefore MDA accumulation is an important indication that membrane structure and function are damaged and possibly destroyed (Halliwell 1981, Liu et al. 2006). Our results showed that membrane lipid peroxidation was significantly different among different species, with carpetgrass showing greater damage than vetivergrass and bahiagrass, possibly due to an increase or stabilization in carotenoid content in the latter two grasses during submergence (Figure 1B). Results suggest that vetivergrass and bahiagrass under submergence have a greater capacity to relieve the damage of membrane lipid peroxidation than carpetgrass.

Table 1. Analysis of variance of photosynthetic pigments, fluorescence parameters, MDA, SOD, and APX activity.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Source</th>
<th>d.f.</th>
<th>Mean square</th>
<th>F-value</th>
<th>P-value</th>
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<tr>
<td>Chlorophyll (a + b)</td>
<td>Duration (D)</td>
<td>3</td>
<td>0.050</td>
<td>6.51</td>
<td>0.001</td>
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<td></td>
<td>Species (S)</td>
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<td>0.034</td>
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<td></td>
<td>D × S</td>
<td>6</td>
<td>0.043</td>
<td>5.60</td>
<td>0.001</td>
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<tr>
<td>Carotenoid</td>
<td>Duration (D)</td>
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<td>0.005</td>
<td>10.2</td>
<td>0.001</td>
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<tr>
<td></td>
<td>Species (S)</td>
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<td>206</td>
<td>0.001</td>
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<tr>
<td></td>
<td>D × S</td>
<td>6</td>
<td>0.010</td>
<td>18.3</td>
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<tr>
<td>Fv/Fm</td>
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<td></td>
<td>D × S</td>
<td>8</td>
<td>0.004</td>
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<td>ΦPSII</td>
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<td>0.018</td>
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<td>MDA</td>
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<td>4541</td>
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<td>APX</td>
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<tr>
<td></td>
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<td>2.76</td>
<td>5.64</td>
<td>0.001</td>
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</table>

Furthermore, the SOD activity of the latter two species was significantly higher than that of the former (Figure 4A). The activity of APX was influenced by plant species, submergence duration, and the interaction of plant species and submergence duration (Table 1), reflecting that submergence impact on the APX of grasses was positive to vetivergrass and bahiagrass but negative to carpetgrass. The APX activity of all three species increased from day 9 to 18 of submergence. Subsequently, the APX activity of carpetgrass decreased whereas that of vetivergrass and bahiagrass continued to increase (Figure 4B).

Active oxygen scavenging capacity and antioxidation ability of plants are marked by the activity of enzymatic antioxidants such as SOD and APX, and non-enzymatic antioxidants like carotenoids (Orendi et al. 2001, Yordanova et al. 2004, Ghanati et al. 2005, Xu et al. 2006). The content of these two substances represent the ability of plants to resist peroxidation and ROS damage (Candan and Tarhan 2003, Song et al. 2005, Almeselmani et al. 2006). The activity of SOD and PAX for vetivergrass and bahiagrass showed a rising pattern over time during the submergence while carpetgrass showed the opposite (Figure 4), implying that the former two species had a stronger ability to scavenge ROS than the latter (Panchuk et al. 2002).

**Effects of Submergence on Leaf Ultrastructure**

The ultrastructure of plant leaves is a sensitive index reflecting environmental stress. Under the transmission electron microscope, a circuit of closed vascular bundle sheath cells forming a garland was seen in the control leaf samples of carpetgrass (Figure 5.1). The sheath cells had many large chloroplasts whose shape was like a spindle due to the presence of starch grains (Figures 5.4 and 5.7). There were well-developed granum thylakoids and stroma lamellas in the chloroplasts (Figure 5.10). After submergence for 16-days, the garland in the vascular bundle began to deform (Figure 5.2), the chloroplasts swelled into an elliptical shape, the starch grains in chloroplast abated markedly (Figures 5.5 and 5.8), and the granum and stroma thylakoids began to
break into sparse strips (Figure 5.11). At 32 days after submergence, chloroplasts further swelled into a round shape (Figures 5.3 and 5-6), stroma thylakoids and stroma lamellas became disorganized, and the number decreased markedly (Figures 5.9 and 5.12). In addition, vacuolar lipids increased with submergence duration (Figures 5.9 and 5.12).

For the control samples, the leaf ultrastructure of bahiagrass showed a similar feature to that of carpetgrass (Figures 6.1, 6.4 and 6.10). However, almost no starch grains could be seen in chloroplasts of bahiagrass (Figure 6.7). At 30 days after submergence, the starch grains in chloroplasts increased distinctly (Figure 6.8), stroma thylakoids and stroma lamellas were partly or completely ruptured, and the number decreased (Figure 6.11). At 60 days after submergence, the number of starch grains continued to increase (Figure 6.9), and stroma thylakoids were further destroyed (Figure 6.12).

The control samples of vetivergrass showed a very similar structure to the other two grass species (Figures 7.1, 7.4, 7.7, and 7.10). On day 60, its vascular bundle structure was not deformed (Figure 7.2), neither granum thylakoids nor stroma thylakoids showed clear disorganization (Figure 7.8), the outer mitochondrial membranes also kept intact, and the mitochondrion cristae were ruptured only slightly (Figure 7.11). However, the chloroplasts became round and the starch grains vanished, compared with the control (Figure 7.5). On day 60 and 120, the vascular structure and chloroplast shape remained similar (Figure 7.3 and 7.6), but granum and stroma thylakoids were broken, and their number decreased greatly (Figure 7.9). In addition, the outer mitochondrial membranes burst, and its mitochondrion cristae was further disorganized (Figure 7.12).

Cell ultrastructure of plants has been increasingly used in recent years to evaluate the response of plants to environmental stresses such as drought (Popova 1998), air pollution (Oksanen et al. 2001), heavy metals (Yuan et al. 2005), and heat (Xu et al. 2006), but little information is available on how flooding stress affects the changes of plant cell ultrastructure. This study illustrated that the structure disaggregation of the vascular and chloroplasts in carpetgrass resulted in an accumulation of membrane lipid, which was likely why the lipid bodies increased (Xu et al. 2003). Moreover, submergence increased the number of mitochondria of carpetgrass and intensified its respiration (data not shown); as a result, its food stores and energy were successively consumed, and its leaves were accelerated to wither and fall as observed during the process of the experiment. The damage of submergence stress to the cell ultrastructure of both vetivergrass and bahiagrass, especially to the former, was much slighter than to carpetgrass. Chloroplasts are the center of photosynthetic response and photosynthetic systems (PSI and PSII), which are distributed on the membranes of granum and stroma thylakoids. Photosynthesis is severely influenced if these thylakoids are destroyed (Xu et al. 2003, 2006), which is perhaps the main reason why vetivergrass and bahiagrass were slightly influenced whereas carpetgrass was severely inhibited with special reference to photosynthesis (Figures 1 and 2). In addition, a change in starch, an important energy source, might reflect the photosynthetic activity of plants and the dynamic process of glucide metabolism (Dong et al. 2002). Voesenek et al. (2006) surmised that leaves of submergence-tolerant plant species can acclimate to an underwater environment, resulting in enhanced CO₂ uptake.
uptake for photosynthesis and therefore providing a supply of sugars to the plant. Increased starch contents in response to flooding have been found in *Helianthus annuus* (Wample and Davis 1983), *Fraxinus pennsylvanica* (Gravatt and Kirby...
1998), and Lepidium latifolium (Chen et al. 2005). Different from vetivergrass and carpetgrass, the number of starch grains in chloroplasts of bahiagrass increased with increasing submergence time (Figure 6.9), a likely adaptive mechanism that suggests bahiagrass is relatively tolerant to submergence.

In conclusion, vetivergrass and bahiagrass minimized the deleterious effects of submergence on plants through a series of adaptations and mechanisms as follows: (1) the two species may maintain high light-absorbing quantum by increasing photosynthetic pigments as a response to the decrease of light intensity.
under submergence; (2) submergence might have caused little damage to the PSII reaction center and to the regulation capacity of PSII electron transport of the two grasses; (3) the two species had a stronger ability to scavenge ROS; and (4) the damage of submergence stress to the cell ultrastructure of the two species was limited.

Figure 7. Leaf ultrastructure of vetivergrass. 1-3: Vascular bundles in control, 60-day, and 120-day submergence, respectively (Bar = 5 µm for 3 plates); 4-6: Chloroplasts in control, 60-day, and 120-day submergence, respectively (Bar = 2 µm for Plate 4, and 1 µm for Plates 5 and 6); 7-9: Thylakoids of chloroplast in control, 60-day, and 120-day submergence, respectively (Bar = 100 nm for 3 plates); 10-12: A mitochondrion in control, 60-day, and 120-day submergence, respectively (Bar = 100 nm for Plates 10 and 12, and 200 nm for Plate 11).
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LITERATURE CITED


