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Survival strategy in the wetland sedge *Carex brevicuspis* (Cyperaceae) in response to flood and drought: avoidance or tolerance?

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Survival strategies of plants to adapt to environmental stress include avoidance and tolerance. These strategies were investigated in the wetland sedge *Carex brevicuspis* (Cyperaceae) to determine the plasticity of its response to flood and drought. Individual plants were grown in PVC tubes and subjected to five water levels relative to soil surface: –40 cm, –20 cm, 0 cm, +20 cm, and +40 cm. After 60 days, plants were harvested and their biomass accumulation, number of leaves (a measure of growth), root and shoot elongation, aerenchyma formation (avoidance strategy), amount of malondialdehyde (MDA), amount of water-soluble carbohydrates (WSC), alcohol dehydrogenase (ADH) activity, amount of proline (tolerance strategy), and membrane permeability (a measure of degree of injury) were assessed. Relatively high amounts of MDA and proline and a high level of ADH activity, combined with relatively low aerenchyma area, short leaves, and low amount of WSC in the +20 cm and +40 cm treatments indicated that *C. brevicuspis* responded to floods with tolerance. A significant root elongation and WSC accumulation in the –20 cm treatment indicated that the species responded with high levels of avoidance to mild drought. High amounts of MDA and proline combined with relatively short roots and a low amount of WSC in the –40 cm treatment indicated a high tolerance strategy under severe drought. Overall, *C. brevicuspis* responded with tolerance to flood and adopted avoidance to mild drought, and it went back to tolerance under severe drought.

Introduction

Plants develop various strategies to cope with environmental stress such as freezing, flooding and drought. These strategies fall into two types: avoidance and tolerance (Levitt 1972). Avoidance means a plant's ability to escape from external stress by actively adjusting its morphology or physiology. Tolerance refers to the ability to alleviate or eliminate damage caused by environmental stress by adjusting physiology or biochemistry (McCue & Hanson 1990). Both strategies help plants resist environmental stress and maintain growth and survival under stressful conditions (Sugiyama & Nikara 2004, Manzur *et al*. 2009).

In floodplains, both floods and droughts are major stress factors. Floods reduce the soil oxygen availability and cause anoxic damage to plants (Blom & Voesenek 1996, Crawford 1996, 2003, Xie *et al*. 2008), while droughts lead to water deficit in the soil and cause physiological drought in plants (Baruch 1994a). Effective flood avoidance strategies that increase the oxygen supply to plant tissues include shoot or spacer elongation, increased shoot-to-root mass ratio (Laan & Blom 1990, Van der Sman *et al*. 1993, Vriezen *et al*. 2003, Sairam *et al*. 2008, Li & Xie 2009), formation of aerenchyma, and high porosity (Laan & Blom 1990, Xie *et al*. 2007). Accumulation of malondialdehyde (MDA), increase in alcohol dehydrogenase (ADH) activity, and depletion of water-soluble carbohydrates (WSC) are flood tolerance strategies that alleviate the damage to plant tissues caused by oxygen deficiency (Almeida *et al*. 2003, Chang *et al*. 2003). During droughts, a greater rooting depth and a higher root-to-shoot mass ratio are avoidance (Heschel & Riginos 2005) adjustments that can help plants gain more water. Drought tolerance strategies include increase in amounts of osmotically-active substances, such as MDA, proline, and WSC in leaves (Baruch 1994b, Proctor 2000, Vasellati *et al*. 2001, Bartels & Sunkar 2005, Luo *et al*. 2008).

Factors such as flooding depth and duration exert selection pressures on various traits of wetland plants (Colmer & Voesenek 2009). Plants appear to follow an avoidance strategy under mild stress and a tolerance strategy under severe stress (Parolin 2002, Colmer & Voesenek 2009, Manzur *et al*. 2009). For example, the shoots of *Lotus tenuis* (Fabaceae) elongated significantly (an avoidance strategy) during mild flood stress but remained quiescent, suffering a depletion of carbohydrates (a tolerance strategy), under severe flood stress (Manzur *et al*. 2009). Recent reviews provide detailed summaries of plant flood tolerance (Colmer & Voesenek 2009, Bailey-Serres & Colmer 2014).

The role of dry periods in floodplains has been mostly ignored, despite growing evidence that drought affects species distribution patterns in floodplains (Parolin *et al*. 2010). Drought may limit the survival of local vegetation more severely than flooding, as many wetland plants complete their life cycles during dry periods (Baruch 1994a, Lenssen & de Kroon 2004). Therefore, identifying plant strategies under different levels of drought and flood stress could provide important information on plant development in floodplains.

In the present study, adaptive strategies of the wetland sedge *Carex brevicuspis* (Cyperaceae) were investigated. *Carex* spp. occur in a wide range of habitats throughout the world, especially in north-temperate and arctic regions (Bernard 1990). *Carex brevicuspis* is a dominant species in the Dongting Lake wetlands in Hunan, China. The pseudo-culm of the plant, which is composed of a series of overlapping leaf sheaths, is usually 20–55 cm high. The plants form mono-dominant communities or co-dominate with other *Carex* spp. or with *Miscanthus sacchariflorus* (Chen *et al*. 2011). At Dongting Lake, *C. brevicuspis* flowers and fruits from April to May. The plants are distributed in habitats that are subject to flooding and drought.

We tested the hypothesis that *C. brevicuspis* would follow an avoidance strategy under mild stress levels and a tolerance strategy under severe stress levels. Specifically, we expected that at mild levels of flooding (water level increased by 20 cm), avoidance through an increase in the relative aerenchyma area and leaf length to counteract submergence would be more common, whereas under severe flooding (water level increased by 40 cm) tolerance through an increase in ADH activity, an increase in the amounts of MDA and proline, and the depletion of WSC would prevail. In addition, we expected that when subjected to mild drought (water level decreased by 20 cm),

plants would respond with avoidance by increasing root depth to gain more water, while at severe levels of drought (water level decreased by 40 cm), they would respond with tolerance to alleviate drought injury by increasing the amounts of MDA, proline, and WSC. Plant biomass accumulation and leaf number represent plant growth responses. The leaf membrane permeability represents plant injury degree at different stress levels.

Material and methods

Experimental design

Plants (ca. 100 individuals, ca. 15 meters apart) with roots and rhizomes were randomly collected from a mature population of *C. brevicuspis* in the village of Chunfeng (29°13´49.72´´N, 113°02´32.79´´E) on East Dongting Lake (Hunan Province, China). They were transplanted into plastic containers containing 10 cm of sand and placed in a greenhouse, where the temperature remained 25 ± 2 °C during the day and 17 ± 2 °C at night, and the illumination was provided by 400 W SON-T ARGO sodium lamps (Philip Company, Guildford, UK) at a photon flux density of 600 μmol m^{-2} s^{-1}, the photoperiod being 14 h/10 h light/dark. After shoot emergence, 25 similarly sized ramets (about 16.5 cm in height, with five leaves) were planted in PVC tubes (45 cm in height and 25 cm in diameter) that were filled with soil (depth 40 cm) collected from the location where *C. brevicuspis* was collected.

Twenty-five PVC tubes with plants (one tube for each treatment per basin) were randomly placed in five outdoor cement basins (130 × 110 × 100 cm) filled with tap water (depth 85 cm, pH 7.21, 4.3 μM NH_4-N, 16.8 μM NO_3-N, 1.9 μM PO_4-P). For the five water-level treatments, the PVC tubes were placed in the basins so that the water surface relative to the soil surface was at –40 cm (severe drought), –20 cm (mild drought), 0 cm, +20 cm (mild flood), or +40 cm (severe flood). A completely randomized design was adopted in our experiment: there were five levels of treatments with five replicates of each treatment. During the experiment (14 August–14 October 2009) the water in the basins was completely replaced every week.

Harvest and measurement

Plants were harvested on 14 October, 60 days after planting, by removing the soil core with the intact root system. The plants were then cleaned with tap water and transported to the laboratory, where the lengths of leaves and roots were measured. Next, leaves and roots were separated. The dry weights of plants were measured after drying at 70 °C for 48 h in an oven. Biomass accumulation was the sum of leaf and root mass.

Root anatomy

Typical roots, starting at 2 cm from the root tip in each treatment, were chosen for anatomical analysis. Fresh roots were fixed and preserved in FAA (70% ethanol:10% formalin:5% acetic acid, 90:5:5 by volume) for at least 48 hours and the air in the tissue was evacuated using an oil rotary vacuum pump. The samples were dehydrated in an ethanol series (50%, 70%, 85%, 95%, and 100%) and embedded in paraffin wax in 62 °C. Sections were cut into slices 10–12 μm thick with a rotary microtome (Leica RM 2145, Germany), stained using safranin/fast green, and permanently mounted on a glass slide. Observations were made using a light microscope (100×, Nikon ECLIPSE E200, Japan) and the microscopic images were taken using a microscopy camera (Nikon DXM1200F, Japan). The relative aerenchyma area (%) was calculated as the ratio of total aerenchyma area to root area (Purnobasuki & Suzuki 2004) and measured using Adobe Photoshop CS3 (Adobe Labs, USA).

MDA in leaves

MDA in leaves was determined by two-component spectrophotometry (Hodges *et al.* 1999). Leaf samples were homogenized in 2 ml of 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged at 15 000 rpm for 10 min, after which 0.5 ml of the supernatant was added to 1.5 ml thiobarbituric acid (TBA) in 20% TCA. The mixture was incubated at 90 °C in a shaking water bath for 20 min, and the reaction was stopped by cooling the reaction tubes in an

ice water bath. Samples were then centrifuged at 10 000 rpm for 5 min, and absorbances at 532 nm (D_{532}), 600 nm (D_{600}), and 450 nm (D_{450}) were determined (Hernandez *et al*. 2001). MDA was calculated using the equation:

$$\text{MDA} = 6.45(D_{532} - D_{600}) - 0.56D_{450}.$$

Membrane permeability

Membrane permeability was determined as described by Masood *et al*. (2006). Fresh leaves (200 mg) were cut into 5-mm-long pieces and placed in test tubes containing 10 ml of distilled deionized water. The tubes were incubated in a water bath at 32 °C for 2 hours and the initial electrical conductivity of the medium (EC_1) was measured. The samples were autoclaved at 121 °C for 20 min to release all electrolytes, then cooled to 25 °C, after which final electrical conductivity (EC_2) was measured. The electrolyte leakage (EL) was calculated by the following formula:

$$\text{EL} = (EC_1/EC_2) \times 100\%.$$

ADH activity

Fresh roots were cut into sections about 0.5 cm long, from which fresh samples weighing 0.01–0.05 g were assayed (4 °C) in 5-ml assay mixtures (50 mM phosphate buffer pH 7.5, 5 mM $MgCl_2$, 1 mmol l^{-1} phenylmethyl sulfonyl fluoride). Plant extracts were then centrifuged at 15 000 rpm for 20 min at 4 °C, and 0.1 ml of extract was added to a reaction cuvette containing 2.85 ml assay buffer (15% 1.0 M Tris pH 8.0, 3% 0.01 M NAD+, 82% distilled water), after which 0.03 ml of 95% ethanol was added to start the reaction. ADH activity was detected spectrophotometrically by monitoring the change in absorbance of the reaction mix at 340 nm for 10 min (Tang 1999).

Amount of WSC in leaves

To measure the amount of WSC, samples of leaf tissue weighing approximately 0.1 g dry weight (dw) were milled and heated to 80 °C in 25 ml of 80% ethanol for 1 h. The supernatant was filtered twice, diluted with 25 ml of distilled water, and heated to 60 °C for 1 h. Next, 80% ethanol was added to all supernatants up to 100 ml and aliquots were mixed with anthrone reagent [200 mg of anthrone in 75% (v/v) H_2SO_4], left to stand for 30 min, and shaken and heated for 10 min in an 80 °C water bath (Yemm & Willis 1954). Absorbance was recorded at 620 nm, and the total soluble carbohydrate concentration was calculated according to a glucose calibration curve. The amount of WSC was expressed in mg g^{-1} dw.

Amount of proline in leaves

Free proline was extracted and determined as described by Bates *et al*. (1973). Approximately 0.5 g of leaves was homogenized in a mortar after the addition of a small amount of quartz sand and 10 ml of a 3% (w/v) aqueous sulfosalicylic acid solution. The homogenate was centrifuged at 3000 g for 20 min. The supernatant was treated with acid ninhydrin (2.5 g ninhydrin/100 ml of a solution containing glacial acetic acid:distilled water:85% *o*-phosphoric acid at a ratio of 6:3:1) and boiled for 1 h, and the reaction was terminated in a water bath at room temperature (25 °C) for 10 min. Absorbance at 520 nm was determined using L-proline as the standard. The amount of proline was expressed as mg g^{-1} dw.

Statistical analyses

The effects of water-level treatments on plant biomass accumulation, leaf membrane permeability, leaf number, leaf length, root length, relative aerenchyma area, ADH, MDA, proline, and WSC were analyzed using one-way ANOVA followed by Tukey's HSD in a completely randomized design with the five levels of treatments. Normality of the data was tested using the Shapiro-Wilk test and the homogeneity was confirmed using Levene's test. The data for WSC were $\log_{10}$-transformed to reduce hetero-

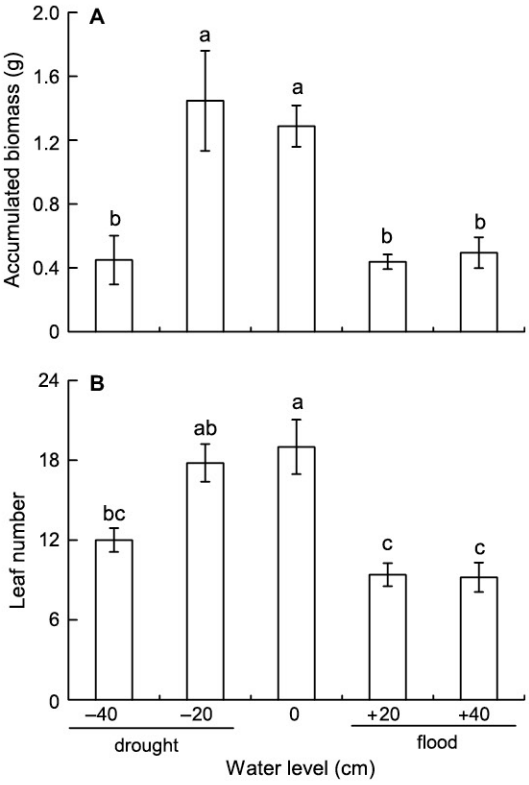

Fig. 1. (**A**) Biomass accumulation and (**B**) leaf number (means ± SEs, n = 5) of *Carex brevicuspis* growing in five water-level treatments. Different letters indicate significant differences between treatments at p = 0.05 (Tukey's test).

geneity of variances. All statistical analyses were performed using the software SAS ver. 8.2 (SAS institute Inc., USA).

Results

Plant growth response

Significantly more biomass accumulated in the –20- and 0-cm treatments (1.29–1.45 g) than in the other treatments (0.44–0.50 g) ($F_{4,20}$ = 8.38, p < 0.001; Fig. 1A). There were no significant differences among +40 cm, +20 cm, and –40 cm treatments (Tukey test: p = 0.828) or between the –20 cm and 0 cm treatments (Tukey test: p = 0.521; Fig. 1A). Leaf number followed a similar pattern (Fig. 1B). Clearly, both flood (+20 cm and +40 cm treatments) and severe drought

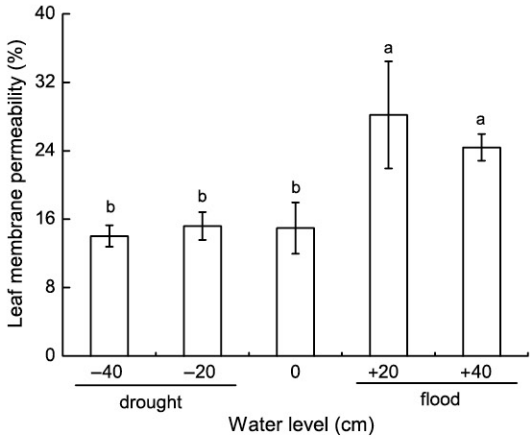

Fig. 2. Membrane permeability (means ± SEs, n = 5) of *Carex brevicuspis* growing in five water-level treatments. Different letters indicate significant differences between treatments at p = 0.05 (Tukey's test).

(–40 cm treatment) affected plant growth negatively.

Plant injury degree

Leaf membrane permeability was significantly higher in the +20- and +40-cm treatments (24.40%–28.18%) than in the other treatments (14.02%–15.20%) ($F_{4,20}$ = 4.12, p = 0.014; Fig. 2), indicating that flooding injured leaf tissues. There were no significant differences among the 0-, –20-, and –40-cm treatments (Tukey test: p = 0.767; Fig. 2), suggesting that the plants could successfully resist drought.

Plant avoidance strategies

Leaves and roots were the longest in the –20-cm treatment and the shortest in the +20- and +40-cm treatments (leaf length: $F_{4,20}$ = 6.32, p = 0.002; root length: $F_{4,20}$ = 24.24, p < 0.001; Fig. 3A and B). The root-to-leaf length ratio was the highest in the –20-cm treatment, intermediate in the 0- and +20-cm treatments, and lowest in the +40- and –40-cm treatments ($F_{4,20}$ = 5.15, p = 0.005; Fig. 3C). The relative aerenchyma area was significantly smaller in the +20- and +40-cm treatments (mean ± SE = 21.02% ± 2.10%, n = 5; and 10.39% ± 1.88%, n = 5; respectively) than

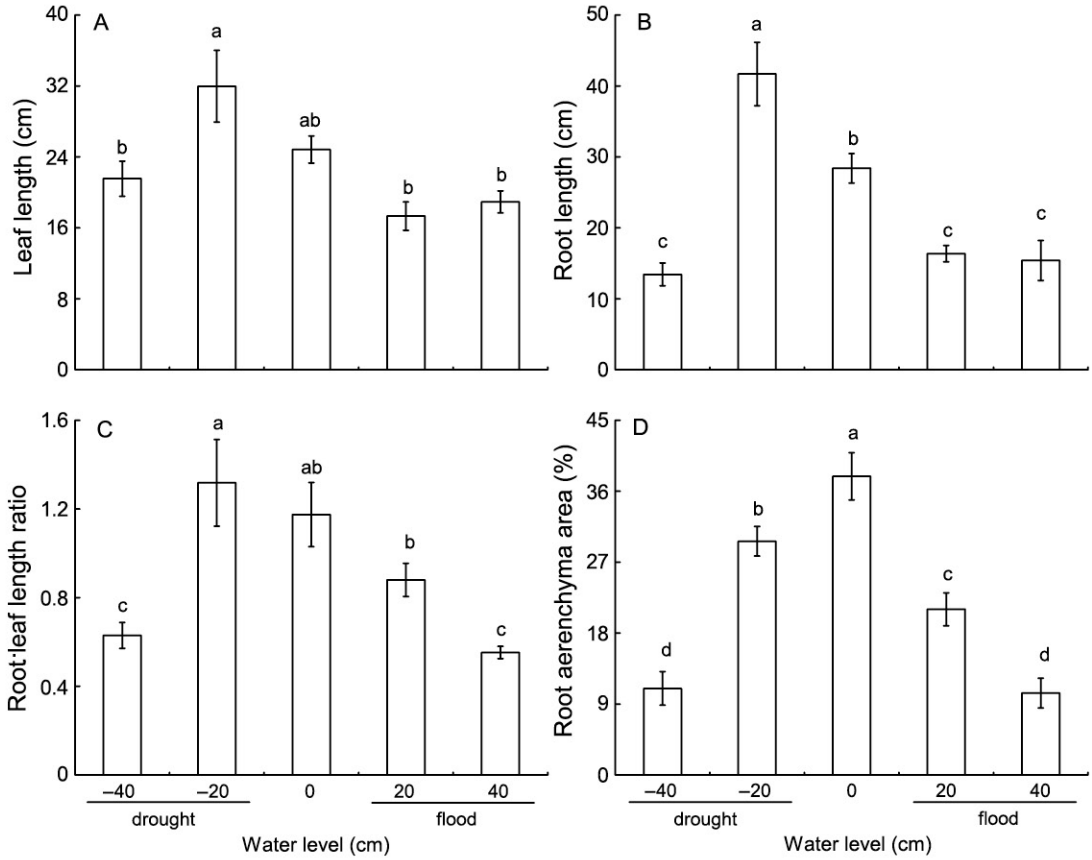

Fig. 3. (**A**) Leaf length, (**B**) root length, (**C**) root:leaf length ratio, and (**D**) relative aerenchyma area (means ± SEs, n = 5) of *Carex brevicuspis* growing in five water levels. Different letters indicate significant differences between treatments at p = 0.05 (Tukey's test).

in the 0-cm treatment (37.90% ± 3.01%, n = 5) ($F_{4,20} = 63.3, p < 0.001$; Fig. 3D).

Plant tolerance strategies

The amounts of MDA and proline were the highest in the –40-cm treatment, intermediate in the –40-, –20-, and +20-cm treatments, and the lowest in the 0-cm treatment (proline: $F_{4,20} = 6.34$, $p = 0.002$; MDA: $F_{4,20} = 23.26$, $p < 0.001$; Fig. 4A and B). ADH activity was significantly higher in the +40- and +20-cm treatments (631–797 U mg^{-1} protein) than in the 0-, –20- and –40-cm treatments (201–270 U mg^{-1} protein; $F_{4,20} = 5.97$, $p = 0.002$). However, ADH activity did not differ significantly among the 0-, –20- and –40-cm treatments (Tukey test: $p = 0.635$). The amount of WSC was the highest in the 0- and –20-cm treatments, intermediate in the –40-cm treatment, and lowest in the +20- and +40-cm treatments ($F_{4,20} = 18.02, p < 0.001$; Fig. 4D).

Discussion

A lower aerenchyma area, lower amount of WSC, higher level of ADH activity, and higher levels of MDA and proline in the +20-cm and +40-cm water-level treatments indicated that the preferred strategy of *C. brevicuspis* in response to flood was tolerance rather than avoidance. This result is consistent with our initial hypothesis regarding severe flood but inconsistent with our hypotheses regarding mild flood, which predicted that under mild flood plants would respond with a high level

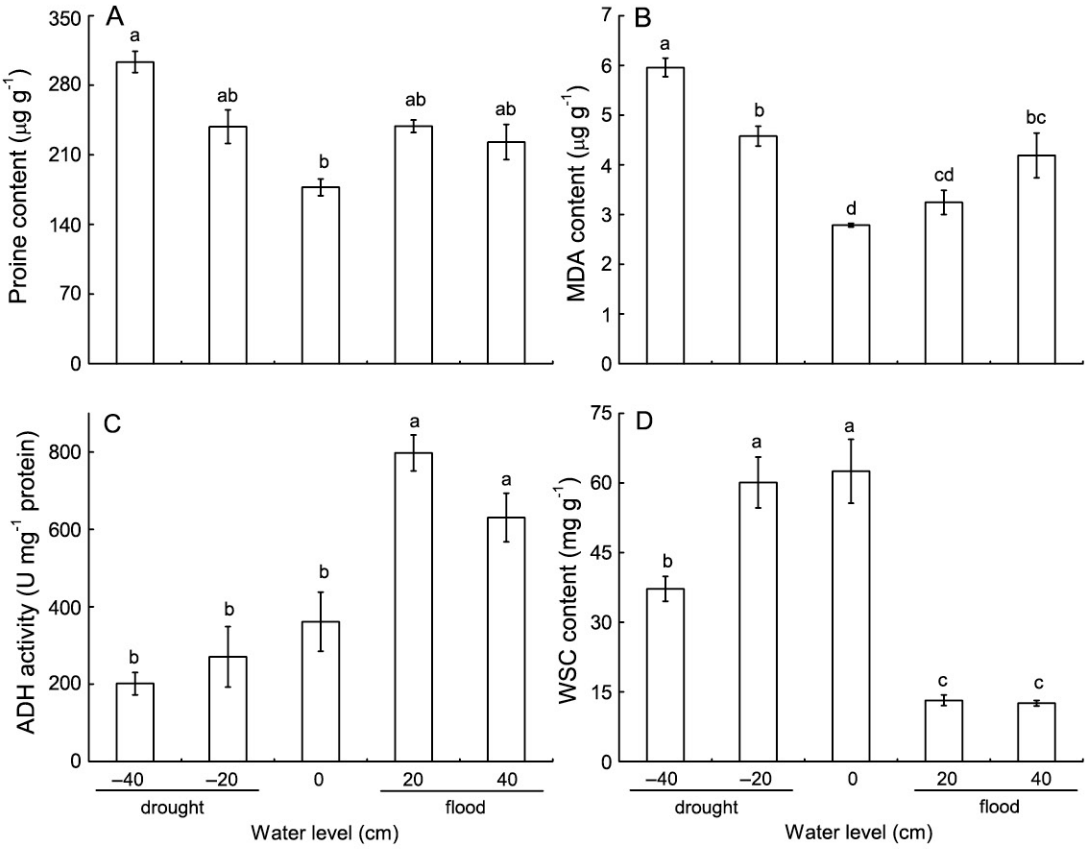

Fig. 4. (**A**) Amount of proline, (**B**) amount of MDA, (**C**) ADH activity, and (**D**) amount of WSC (means ± SEs, $n = 5$) of *Carex brevicuspis* growing in five water levels. Different letters indicate significant differences among treatments at $p = 0.05$ (Tukey's test).

of avoidance. The main reason for this result may be that *C. brevicuspis* is a non-stem plant, so in flooded environments its leaves cannot stand up in the air as do those of caulescent plants (Colmer & Voesenek 2009, Manzur *et al.* 2009). Consequently, a +20-cm water level may cause significant anoxic injury to plant tissues, as supported by the higher membrane permeability and lower biomass accumulation in plants in the +20-cm water level (Figs. 1A and 2).

Aerenchyma formation is usually considered an effective strategy to cope with waterlogging in wetland plants such as *Deyeuxia angustifolia* (Xie *et al.* 2008), *Paspalum dilatatum* (Vasellati *et al.* 2001) and *Carex* species (Moog 1998, Qin *et al.* 2010). However, in the present study, the relative aerenchyma area in the roots of *C. brevicuspis* was small in submerged plants (21.02% and 10.39% in the +20- and +40-cm treatments, respectively). This finding indicates that lysigenous formation of aerenchyma does not necessarily confer an advantage in plant roots under complete submergence. It has been reported that prolonged maintenance of a juvenile root structure, with an intact cortical tissue and an efficient system of narrow, gas-filled channels, is a basic feature of flood-tolerant *Carex* species (Moog 1998). Higher amounts of MDA and proline and higher levels of ADH activity under flood conditions suggested that *C. brevicuspis* can resist lipid peroxidation and root anaerobiosis (Geigenberger 2003, Sofo *et al.* 2004, Arbona *et al.* 2008). However, the lower biomass accumulation and lower number of leaves in the +20- and +40-cm treatments indicated that plant responses under flood were directed toward survival instead of growth, as seen also in *Lotus tenuis* (Manzur *et al.* 2009).

Higher amounts of MDA, proline, and WSC, combined with longer roots and root-to-leaf length ratio in the –20-cm water-level treatment indicated that the plants responded with high levels of both avoidance and tolerance strategies to mild drought. The shorter roots, greater amounts of MDA and proline, and lower amount of WSC in the –40-cm water-level treatment indicated a high level of tolerance strategy and a low level of avoidance strategy under severe drought. These results are consistent with our second hypothesis, which predicted that plants would respond with higher levels of avoidance under mild drought and higher levels of tolerance under severe drought.

Greater biomass accumulation and higher leaf number in the –20-cm water-level treatment indicated that high levels of avoidance and tolerance strategies in *C. brevicuspis* enable the plants to efficiently resist mild drought. However, a lower biomass accumulation and lower number of leaves in the –40-cm treatment, and no changes in leaf membrane permeability, under mild or severe drought demonstrated that severe drought inhibits plant growth, rather than causing physiological damage. WSC may be depleted to sustain the plant's survival during severe drought (Baruch 1994a). These findings were consistent with our field observation that *C. brevicuspis* can complete its life cycle even at higher elevations (Deng *et al.* 2013).

Our research revealed that *C. brevicuspis* can shift from tolerance strategy to avoidance strategy under mild drought, and *vice versa* under flooding. However, plants also adopted tolerance strategy under mild drought. This result contradicts some previous findings which suggested that a trade-off exists between avoidance and tolerance as a response to environmental stress, independent of the stress type (van der Meijden *et al.* 1988, Fineblum & Rausher 1995). Generally, the trade-off between avoidance and tolerance largely depends on the availability of resources such as nutrients (Katjiua & Ward 2006) or light (Lockhart *et al.* 2013). In our experiment, root elongation (avoidence strategy) in the –20-cm water level treatment can supply the plant with sufficient water for metabolism, while the increase in osmotically-active substances (tolerance strategy) can prevent excessive loss of water. Therefore, both avoidance and tolerance strategies may maximize plant fitness under mild drought, as shown by the significantly higher biomass accumulation in the –20-cm water-level treatment (Fig. 1A).

Avoidance and tolerance are fundamental responses of plants to environmental stress (Ludlow 1980). The response of plants in any given situation might depend on the stress level and type. The non-stem species *C. brevicuspis* responded to flood with a high level of tolerance. However, some caulescent aquatic species, such as *Deyeuxia angustifolia* (Xie *et al.* 2008) and *Rumex palustris* (Chen *et al.* 2009), responded with high levels of avoidance to mild flood. Plant life-form may be another factor affecting the prevalence of avoidance or tolerance. Our research indicated that *C. brevicuspis* adapts better to drought than to flood, which may be the main reason why *C. brevicuspis* reproduces early in seasonally-flooded wetlands (Bernard 1990, Chen *et al.* 2014).

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