

A comparative analysis of leaf anatomical and photosynthetic characteristics of *Phragmites australis* from two different habitations

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Abstract: To analysis the effect of water perturbation on the leaf anatomical and photosynthetic traits of plant, the reeds (*Phragmites australis*) from two different habitations were selected. Compared to the terrestrial reed, the aquatic reed had higher stomata density, index, leaf thickness, vascular and xylem diameter. The proline and soluble sugar of the aquatic reed were also 34.7%, 63.6% higher than those of the terrestrial reed. Between the two ecotypes, the chlorophyll contents and chlorophyll fluorescence parameters differed considerably. Moreover the analysis of the linear correlation of two ecotypes of reeds showed that leaf stomata density, soluble sugar content and the chlorophyll contents of two ecotypes had significantly linear correlations with relative water content. It can be concluded that there exist remarkable divergences in anatomy and physiology between the natural *P. australis* growing in two different habitats and water is a major factor limiting the productivity of plant.

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1. Introduction

Recently the increasing drought, high temperature and salinity have resulted in great environmental changes (Wardlaw 2002; Chaves et al., 2003). Plants adapt to changing environmental conditions (e.g. water and temperature) by modifying their morphological properties and physiological processes. As a consequence, plants growing in different habitats show significant differences in anatomical characteristics and in physiological traits (Huxman & M 2003; Ripley et al., 2008). To successfully cope with drought stress, plants in arid regions generally minimize water loss by closing stomata, thickening the leaf blade and the epidermal cell dimensions, increasing LMA (leaf mass per unit area) or reducing light absorbance through rolled leaves (Ehleringer & Cooper 1992; Cellier et al., 2000; Pena et al., 2005). Meanwhile other important strategies for plant respond to drought include maximizing water uptake by increasing investment in roots and vessel number, reducing vessels size in stems (Jackson et al., 2000; Sobrado 2007). Natural drought is usually accompanied by other environmental factors, such as high temperature and high irradiance. The combination of these factors thus influence electron flow from PS I to PS II. It was reported that water use efficiency was enhanced and photoinhibition was mitigated under conditions of water stress (Kao & T 1998). Under these conditions, leaves show a transient decrease of photochemical efficiency of PS II (F/F_m), or undergo decline of leaf maximal photochemical efficiency (F_v/F_m) (Osmond

et al., 1999). These strategies for plant successfully to tolerate environmental changes are complex and plants may combine with a mixture of response types (Chaves, et al., 2003; Chen & Wang 2009; Dehesh & Liu 2010). Yet little is know about the divergence and compensatory effects of these strategies especially for natural plants.

Phragmites australis Trin is a cosmopolitan perennial grass with economic and ecological value. It distributes widely in Hexi Corridor of arid and semi-arid temperate desert regions of Northwestern China. There is an oasis-desert transitional zone from swamp via heavy salt meadow and light salt meadow to dune habitats, building a natural soil water potential gradient from wet to dry, which afford an opportunity to clarify the variations of anatomical and physiological traits (e.g. photosynthetic characteristics) within one species in response to changing habitats with different soil water levels (Gong et al., 2011). Thus different aspects of the species such as physiology and ecology have been extensively studied over the past decades (Yang et al., 2003; Eid et al., 2010). Studies on the species demonstrated that the changes of the environmental factor have great effect on the variations of anatomical traits such as interveinal distance (ID), vein mean diameter (VMD) and photosynthetic characteristics such as net CO₂ assimilation rate (A_n), stomatal conductance (g_s), the RuBPCase activity (Wang et al., 1995; D. et al., 2006; Eid et al., 2010; Wang et al., 2010).

There are two ecotypes according to their habitats in Huangshi, Hubei. The aquatic reed distributes in littoral zones of the lakes and rivers, whit typical

habitats of swamps, riversides and lakesides. The terrestrial reed grows in the submontane grasslands which are typical terrestrial habitats. In this paper, the anatomical and photosynthetic characteristics of *P australis* growing in two different habitations were compared. It was hypothesized that there exist remarkable divergences in anatomy and physiology between the natural *P australis* growing in two different habitats and water was a major factor limiting the productivity of the plant.

2. Materials and methods

Plant materials and study sites

The research areas are located at the Hubei Normal University, Huangshi City, Hubei Province. The mean annual precipitation is 1386 mm, and the annual temperature is 17 °C. Two ecotypes of reeds were selected as the plant materials. One was collected from an aquatic habitat near the Qinshan Lake. And the other from the site in the vicinity of Zaigong mountain which is typical terrestrial habitation. A more detailed description of the soil properties in the two sites can be found in Table 1.

Table 1. Locations, soil properties, climate and topography between two sample sites in which two populations of *P australis* were compared.

	Terrestrial reed	Aquatic reed
locations	30°14' N, 115°03' E	30°13' N, 115°03' E
Annual precipitation (mm)	1382.6	1382.6
Annual temperature (°C)	17	17
Elevation (m)	35	25
Habitation	wetland	Lake
pH	8.66	8.50

Areas within each site with even distribution of *P australis* were selected. 6-10 quadrats (1 m×1 m) for *P australis*, about 5-10 m apart from one another, were established as sampling plots. All the quadrats at each site experienced same light regimes and precipitation. Samplings were carried out in June, 2011.

Leaf relative water content measurement

Mature plants were randomly selected within two sites and 2nd fully expanded leaves (from shoot top, hereafter) were sampled from 5 to 8 mature plants in each quadrat at about 9:00 am, then weighed immediately to obtain fresh weight (FW). They were placed into plastic bags (100 ml) filled with distilled water overnight. The turgid fresh weight (TW) was measured the next morning. Samples were placed in perforated paper bags, oven-dried at 80 °C for 24 h and weighed to measure dry weight (DW). Relative water content (RWC) was calculated as $RWC = [(FW-DW)/(TW-DW)] / 100\%$ (Wang et al., 2008).

Leaf anatomy

Sections of 2 cm×2 cm were cut from the middle of 2nd leaves and fixed in FAA (3.7% formalin, 50% ethanol, 5% acetic acid). Then they were dehydrated progressively in an ethanol series (50-100%) and infiltrated with warm (56–58 °C) paraffin. Leaf slides of 8-10 mm were obtained with a rotary microtome (Leitz, Wetzlar, Germany). The slides were deparaffinized, rehydrated and stained with safranin/fast green (1% aqueous safranin and 0.5% fast green in 95% ethanol). The sections were mounted in glycerol, photographed by a camera (MC80, Zeiss) attached to a microscope (Axioskop, Zeiss) and used to measure leaf thickness, as well as the diameters of all the vessels, xylems and vascular bundles (Cordell et al., 1998; Sobrado, 2007).

Leaf pieces (1cm×1 cm) were treated with bleach (NaClO) to eliminate the mesophyll tissue. Once the two epidermises were separated and the leaf mesophyll remains were eliminated, the pieces were stained with safranin and mounted in glycerol. From these leaf preparations, epidermal cells and abaxial stomata were counted for all treatments (Sobrado, 2007).

Proline and soluble sugar

For proline and soluble sugar analysis, 50g 2nd leaf samples for each ecotype were taken from each quadrat in the two sites. The samples were oven-dried at 80 °C to constant weight and ground using a mortar and pestle to pass through a 100 mesh screen. 0.5 g powder samples were added with sulfosalicylic acid (10 ml, 3%), and the extract was filtered through filter paper. 2 ml aliquots were taken for proline estimation by the acid-ninhydrin method (Bates et al., 1973).

Approximately 50 mg leaf powders of each sample were extracted with 80% ethanol (v/v) at 85 °C for 1 h. The solutions were then centrifuged at 12 000 g for 10 min. Ethanol extraction step was repeated three times and the three resulting supernatants were combined, treated with activated charcoal, and evaporated to dryness in a vacuum evaporator. Residues were redissolved in distilled water, and subjected to soluble sugar analysis using the anthrone-sulfuric acid method (Ebell, 1965).

Photosynthetic contents and Chlorophyll fluorescence parameters

Healthy and intact mature plants within two sites were collected and cultured in the laboratory simulated with the situation they lived, respectively. The samples from Qinshan Lake were planted in box in which the soil and water brought from Qinshan Lake, and the samples from other site were planted in box in which soil brought from the vicinity of Zaigong mountain. They were both cultured under a light intensity of 50 μmol photons m⁻² s⁻¹ and temperature

of 15°C for 1 day, before testing of photosynthetic pigments and chlorophyll fluorescence. The top most recently fully expanded leaves were used to measure chlorophyll fluorescence parameters by a modulated chl fluorometer (FMS2; Hansatech Ltd., King's Lynn, Norfolk, UK) with definition and calculation of fluorescence parameters as described by Maxwell and Johnson (2000) between 11:00 and 13:00 (local solar time). Potential quantum yield of PSII (F_v/F_m) was measured after darkening the plants for 15 min, where F_v is maximum variable fluorescence and F_m is maximum fluorescence of the dark-adapted plants under a light-saturating flash of 0.8 s and $-3,000 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ ($F_v = F_m - F_o$; where F_o is ground fluorescence of the dark-adapted plant) The relative electron transport rate (rETR, arbitrary unit) was assessed as: $\text{rETR} = \text{yield} \cdot 0.5 \cdot \text{photon flux density (PFD)}$. NPQ was calculated by the Stern–Volmer equation as $\text{NPQ} = (F_m - F_m') / F_m'$, where F_m is the value of the predawn measurements in the experiments (Bilgeret al., 1995, Maxwell & Johnson 2000).

For measurement of leaf pigment, blades of samples weighing exactly 0.1 g fresh weight were ground with the substitution of an 80% (v/v) acetone, then kept for 12 h in the dark till all samples were completely decoloured. After centrifugation for 20 min at $44,000 \times g$, pigment contents were measured by the method of Lichtenthaler and Wellburn (Xu et al., 2003).

Statistical analysis

All data were submitted to Bartlett test for the homogeneity of variance and then analysed using

ANOVA with SPSS 13.0 (SPSS for Windows, Chicago, IL, USA). Difference in each parameter between the two ecotypes was tested using independent sample test at 0.05 probability levels. Correlations between parameters were determined using least squares linear correlation.

3. Result

Soil properties

There were no significant differences in annual precipitation, annual temperature, pH, and elevation between the two ecotypes of *P australis*, but the habitats of the two ecotypes of *P australis* differed significantly (Table 1). Aquatic reed grew in the shore of the Qinshan Lake, where the fresh water depth is about 1.1m, yet the terrestrial reed grew at the vicinity of Zaigong mountain, the soil moisture is about 42.4%.

Leaf anatomical traits

Except for vascular and motor cell diameter, there were significant differences in other anatomical traits between the two ecotypes of *P australis*. Just as table 2 showed: Stomata density, index, leaf thickness, vascular and xylem diameter of the aquatic reed were higher than those of the terrestrial reed. The aquatic reed had about 11.1 % , 15.3 % , 35 % , 3.1 % and 41.2 % higher in stomata density, index, leaf thickness, vascular and xylem diameter compared to the terrestrial reed, respectively ($P < 0.01$). However the vessel diameter, vascular and motor cell distance and VD/L (vessel diameter/leaf thickness) of the aquatic reed exhibited different pattern. Those traits of the aquatic reed were 20.1 % , 10.5 % , 19.3% and 24.6% lower than those of the terrestrial reed ($P < 0.05$).

Table 2. Stomata density , Stomata index ,the Vessel, Xylem, Vascular and motor cell diameter, Vascular and motor cell distance, leaf thickness and VD/L (Vessel diameter/leaf thickness) of the two populations of *P australis*. Values are means (\pm SE) of 15 - 30 replications. (* $P < 0.05$, ** $P < 0.01$, ^{ns}not significant).

	Aquatic reed	Terrestrial reed	P value
Stomata density	419.8 \pm 6.031	378 \pm 7.679	4.509E-04**
Stomata index	0.32 \pm 0.005	0.2773 \pm 0.008	3.618E-05**
Vessel diameter (μm)	23.85 \pm 0.496	29.87 \pm 0.782	6.46E-05 **
Xylem diameter (μm)	58.71 \pm 6.96	31.57 \pm 1.69	0.001836 **
Vascular diameter (μm)	80.408 \pm 11.81	78.01 \pm 1.75	0.831634 ^{ns}
Vascular distance (μm)	77.365 \pm 0.51	86.48 \pm 6.96	0.014441**
Motor cell diameter (μm)	59.413 \pm 1.99	56.05 \pm 2.43	0.318592 ^{ns}
Motor cell distance (μm)	194.17 \pm 9.53	240.52 \pm 13.98	0.022926*
Leaf thickness (μm)	171.83 \pm 4.55	127.25 \pm 2.76	3.05E-06**
VD/L	0.46 \pm 0.062	0.61 \pm 0.022	0.033816*

Proline, soluble sugar and RWC

The soluble sugar content showed significant difference between two ecotypes of *P australis* (Fig 1). The soluble sugar content of the aquatic reed was $558.46 \mu\text{g g}^{-1}\text{DW}$, about 63.6 % higher than that of the terrestrial reed ($P < 0.01$).

Similar with the soluble contents, the contents of the proline and RWC of the aquatic reed were both higher than those of the terrestrial reed. But there were no significant difference in those traits between the two ecotypes of *P australis* ($P > 0.05$).

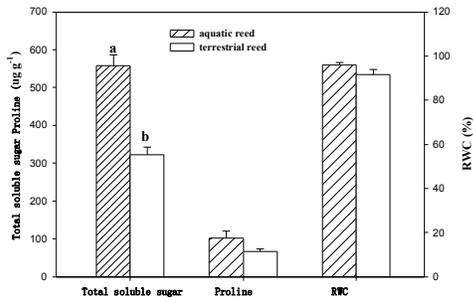


Figure 1. Total soluble sugar, Proline and RWC (relative water content) of the two populations of *P australis*. Bars are the means of 6 replications. ecotypes with different lowercase letters indicate statistically significant differences ($P < 0.05$).

Chlorophyll contents and chlorophyll fluorescence parameters

Between the two ecotypes, the chlorophyll contents differed considerably. Chla, Chlb, Chla+b and Chla/b of the aquatic reed were 1.59µg g⁻¹, 0.43µg g⁻¹, 2.02µg g⁻¹ and 3.69µg g⁻¹, which had 47.2 %, 18.9 %, 40.1 % and 23.7 % higher than those of the terrestrial reed, respectively ($P < 0.05$, Fig 2).

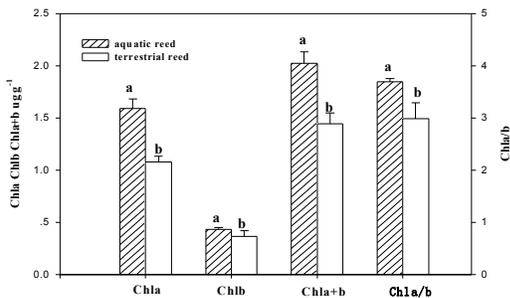


Figure 2. Chlorophyll contents (Chla, Chlb, Chla+b, Chla/b) of the two populations of *P australis*. Bars are the means of 6 replications. ecotypes with different lowercase letters indicate statistically significant differences ($P < 0.05$).

Like the chlorophyll contents, the chlorophyll

fluorescence parameters of the two ecotypes differed considerably (Fig 3). qP of the aquatic reed was 0.970, which had 6.2% higher than that of the terrestrial reed. Similarly, rETR of the aquatic reed was significantly higher than that of the terrestrial reed ($P < 0.01$). Fv/Fm of the two ecotypes also followed the same pattern as qP and rETR, but the difference of the Fv/Fm between two ecotypes was no significant ($P > 0.05$). Otherwise NPQ of the aquatic reed was 0.027, which was lower than that of the terrestrial reed ($P < 0.05$).

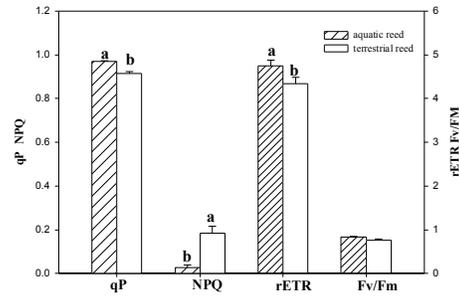


Figure 3. Chlorophyll fluorescence parameters (qP, qN, ETR, Fv/Fm) of the two populations of *P australis*. Bars are the means of 6 replications. ecotypes with different lowercase letters indicate statistically significant differences ($P < 0.05$).

Correlation of RWC with leaf anatomical traits, soluble sugar and proline contents

Based on the correlation of the RWC with leaf anatomical traits, soluble sugar and proline contents for samples from two ecotypes, leaf stomata density was negatively correlated with the RWC (Fig 4A), and stomata index also follow the same pattern as stomata density. It decreased with the increasing of the RWC, and the linear relationship was significant ($R^2=0.53$, Fig. 4B). Unlike the stomata density and index, soluble sugar and proline increased with the increasing of the RWC, meanwhile soluble sugar were significantly correlated with the RWC ($R^2=0.51$, Fig.4C). But the linear relationship of proline with RWC was no significant ($R^2=0.297$, Fig. 4D).

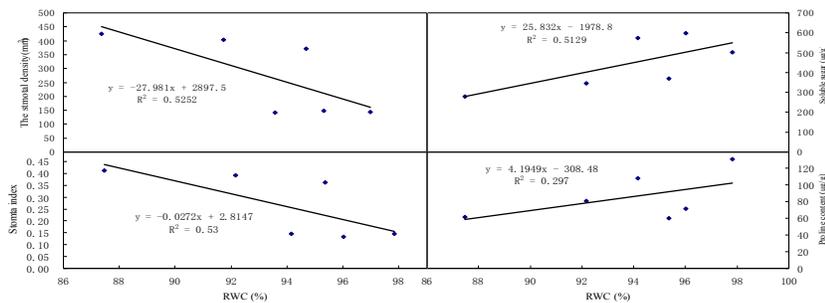


Figure 4. the regression of RWC (relative water content) with the stomatal density (A), stomatal index (B), the soluble sugar (C) and the proline content (D) in two populations of *P australis*.

Correlation of RWC with leaf chl_a, chl_a+b and chl_a/b contents

Figure 5 illustrates the relationship between leaf chlorophyll contents and RWC of two ecotypes of reeds. Leaf chl_a/b was positively correlated with the RWC ($R^2=0.58$, Fig 5A). Similarly chl_a and total chl (chl_a+b) also increased with the increasing of RWC, and significant linear relationship was found between the chl_a, chl_a/b and RWC ($R^2=0.59$, Fig 5B,C). however, there were no significant linear relationship between the leaf chlorophyll fluorescence parameters and RWC (data not shown), which suggesting that the change of RWC had great effect on the photosynthetic efficiency by the mean of Chlorophyll contents instead of chlorophyll fluorescence parameters.

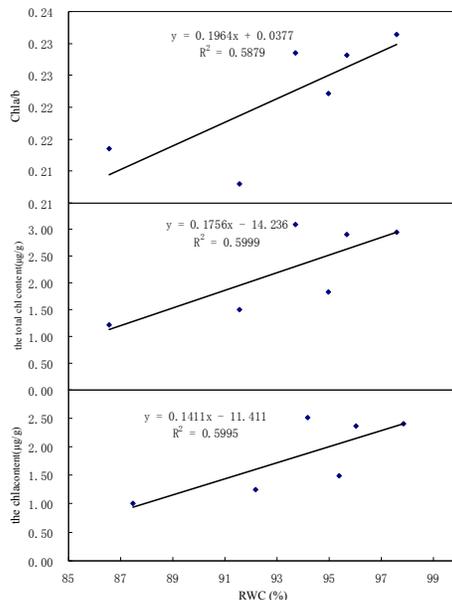


Figure 5. the regression of RWC (relative water content) with the chl_a, total chl and chl_a/b content in two populations of *P. australis*.

4. Discussion

Many studies on plant responses and adaptations to environmental stresses were developed over the past couple of decades, covering subjects from plant plasticity to physiological and biochemical processes under control water and salt stresses. *Phragmites australis* Trin is a cosmopolitan perennial grass which distributed from the wetland to desert region. In this paper two ecotype of reeds grewed in similar habitats except for water moisture (Table 1), but their divergence of the anatomy and physiology suggested they had different strategies in response to water perturbation.

The leaf stoma is a pivotal gate controlling the exchange of CO₂ and water vapour, which enable

plant to minimize water loss by the regulation of stomata size and density (Buckley 2005; Xu & Zhou 2008). Different species or ecotypes had complex flexibilities in stomata density in response to a large range of water status. Several reports have shown that the stomata density and its index increase with the decreasing of the precipitation (Gazanchian et al., 2007; Yang et al., 2007). However some reported that the stomata density of *L. chinensis* at the drier site showed relative lower stomata density (Chen & Wang 2009). Similarly in this paper the aquatic reed also had higher stomata density compare to the terrestrial reed (Table 2), which further proved that relative lower stomata density allowed plants to reduce their transpiration, thus increasing water use efficiencies (WUE) (Paˆaˆkkoˆnen et al., 1998). Moreover Salisbury EJ reported that the distribution of the plant stomata density was determined by the relative water content (RWC) of leaf, and the leaf with higher RWC had higher stomata density which resulted in higher transpiration rate to emission more water vapour (Salisbury 1927). In this paper, the leaf stomata density and index were both significantly positively correlated with RWC (Fig 4, A,B) which was consistent with the result of Salisbury EJ.

Aerenchyma is a spongy tissue with large air-filled spaces, which is typical of aquatic plants and allows the circulation of the gases. Hong et al reported that the number of cavitations was determined by the vascular number due to the alternating arrangement of vascular and cavitations in the leaf of reed (Hong et al., 2008). In this paper (Table 2), the vascular distance of the aquatic ecotype was significantly shorter than that of the terrestrial ecotype which suggested that the aquatic ecotype had much more vascular and cavitations (i.e. more developed aerenchyma system) (Liu et al., 2008) compare to the terrestrial ecotype. Meanwhile the larger vascular and xylem diameter of aquatic ecotype also afforded larger gas-chamber to facilitate gas exchange under the water-logged condition (Table 2). Generally water deficit would result in narrow vessel diameter and increasing vessel density to maximize the water uptake (Lu et al., 1994; Sobrado 2007), But Sobrado also reported that a higher density of narrow vessels would not compensate for large vessels in terms of potential for water flow, and large vessels allowed for low investment in xylem structures while maintaining high permeability. Moreover in some cases, leaves had been found to be less vulnerable to embolism than stems, which resulted in large vessel under water stress (Sack & Holbrook 2006; Chen & Wang 2009). Just as table 2 showed, the terrestrial ecotype had significant larger vessel diameter and higher percentage of leaf thickness occupied by vascular diameter (VD/L), which further confirmed their

observation and explanation.

Osmotic adjustment has been considered one of the crucial processes in plant adaptation to drought, and the osmotic compounds synthesized include soluble sugar and amino acids (like proline, aspartic et.al) (Hamilton & SA 2001; sakamoto 2002). In general there are positive correlations between the capacity for proline and soluble sugar accumulation and water deficit (Hare & Cress 1997; Babu et al., 2001).. Meanwhile the accumulation of proline and soluble sugar enable cell to maintain turgor and resulted in relative higher RWC for plant response to drought. However the osmoprotection mechanisms are probably not functional until severe dehydration occurs (Chaves, et al., 2003). In our study whatever aquatic reeds or terrestrial reeds, they grew in environment without severe water stress (Table 1), consequently the soluble sugar and proline probably act as metabolize component or energy storage instead of the role in osmotic adjustment. The higher soluble sugar and proline contents implied much better vegetal statue of the aquatic ecotype than the terrestrial reeds (Fig 2). Furthermore in the present experiment as soluble sugar and proline contents both increased with the increasing of RWC (Fig 4 C,D), that supported the explanation that water was a major factor limiting the productivity of plant (Campbell et al., 1997).

Chlorophyll a, Chlorophyll b are main compositions of leaf pigment contents which can absorb light energy and transfer it to chlorophyll. Thus the change of chlorophyll a, chlorophyll b and chlorophyll a/b has great impact on the plant photosynthetic efficiency. Some reported that the environmental factor (water, salt) would give change to the chlorophyll contents. For instance, under submerged condition, the chlorophyll contents of *Lersia herxandra* declined to an extent low lever with the time extension of the stress. Thus the chlorophyll of *L herxandra* was destroyed by longer submerging (Zhu & Yi 2009). However unlike the *Lersia herxandra*, *P australis* showed different pattern. With the increasing of depth groundwater, the *P australis* enhanced light capture by the mean of chlorophyll contents increase (Liu et al., 2008). Similarly in our study, the aquatic ecotype had higher RWC (relative water content) and showed significantly higher chlorophyll a, chlorophyll b and chlorophyll a/b compared to the terrestrial ecotype (Fig 3). Consequently it was suggested that at some extent the increasing of the RWC which resulted from the water-logged condition would improve the photosynthetic efficiency. Moreover the positive correlation of RWC with Chlorophyll a, Chlorophylla/b and total Chlorophyll content further proved our conclusion (Fig 5).

Photosystem II (PSII) plays a key role in the response of leaf photosynthesis to environmental perturbation (Wu et al., 2012). Chlorophyll fluorescence parameters is the frequently used indicator of PSII in response to stresses. Liu et.al reported that with the decrease of the depth groundwater, maximal photochemical quantum yield (F_v/F_m), relative photosynthetic electron transport rate ($rETR$) and photochemical quenching coefficient (qP) decreased significantly and non-photochemical quenching (NPQ) increased, which suggested that continuing lower groundwater depth made the PSII destroyed (Liu et al., 2008). In our study compared to the aquatic ecotype, the terrestrial ecotype showed lower light energy conversion efficient, photosynthetic electron transport rate, and caused more loss of the excitation energy collected at the antenna as heat (Fig 3). Consequently the terrestrial ecotype had lower photosynthetic efficiency which further supported the result of leaf pigment contents comparison.

In conclusion, there exist distinct divergence of the anatomical and physiological traits between two different ecotypes, and it was because of these divergences that the reeds had different strategies in response to the water perturbation. Furthermore our results showed that these traits were significantly correlated with RWC which suggested that the water might be the important limiting factor for reed growth (Fig 4 ,5). Consequently though two ecotypes of reed showed tolerance to water fluctuation, the aquatic ecotype was in better vegetal statue compare to the terrestrial ecotype.

Abbreviation:

RWC-relative water content; LMA-leaf mass per unit area; VD/L-vessel diameter/Leaf thickness; F_v/F_m -potential maximum quantum yield of PSII; qP -photochemical quenching; NPQ -nonphotochemical quenching; $rETR$ -relative electron transport rate of PSII

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