

Effect of NaCl and isoosmotic polyethylene glycol stress on gas exchange in shoots of the C₄ xerohalophyte *Haloxylon aphyllum* (Chenopodiaceae)

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Abstract

The effects of NaCl (200 mM) and osmotic stress generated by polyethylene glycol (PEG) on PSII maximal quantum efficiency, photosynthetic CO₂/H₂O gas exchange at two CO₂ concentrations, content of chlorophyll, proline, and malondialdehyde were investigated in shoots of C₄ xerohalophyte *Haloxylon aphyllum* (Chenopodiaceae). The PEG treatment induced a low water osmotic potential (−0.4 MPa) and inhibited photosynthesis (by a factor of 2) and transpiration (by a factor of 4). The NaCl treatment, at equal osmoticity conditions, reduced transpiration (by a factor of 2) and stimulated photosynthesis (by a factor of 2.5). Only the PEG-treated plants showed osmotic stress effects, which were demonstrated by an increase in proline and malondialdehyde contents in the shoot tissue. The data indicated that the halophilic character of this species was essential for maintaining the plant water status and photosynthesis under osmoticity induced by NaCl treatment. Herewith, the presence of C₄-type photosynthesis appeared to be just an auxiliary mechanism, because this xerohalophyte did not reveal the efficiency in water use typical for C₄ plants under osmotic stress, in the absence of a saline substrate.

Additional key words: net photosynthetic rate; osmotic stress; oxidation; proline; salinity; transpiration.

Introduction

Soil salinity may influence photosynthetic CO₂/H₂O gas exchange negatively in two ways. It prevents osmotic absorption of water by the leaves due to a decrease in water potential of the soil with increasing salinity. Also, transpiration may lead to the accumulation of toxic salt concentrations. In fact, physiological plant responses to soil salinity include maintaining of osmotic and ionic homeostasis in leaf tissues.

Under osmotic stress, photosynthesis is suppressed with diminished relative water content (RWC) and water potential (Lawlor 1995, Cornic and Massacci 1996) in C₃ and C₄ plants. However, the role of stomatal and nonstomatal factors in photosynthesis limitation remains unclear (Lawlor 2002).

Halophilic plants have a different ability to accumulate salt in their leaves without affecting photosynthesis (Balnokin *et al.* 2005). This trait permits them to overcome

negative osmotic potential of salty soil by adjusting the water potential in leaves lower than that in the soil.

The effect of salinity on photosynthetic efficiency is species-specific (Eshghizaden *et al.* 2012). Drought and salinity can hamper considerably the process of photosynthesis by altering the ultrastructure of organelles, changing concentrations of various pigments (Eshghizaden *et al.* 2012), the quantum yield of PSII (Sudhir and Murthy 2004) and metabolites, as well as modifying enzymes involved in photosynthesis (Eshghizaden *et al.* 2012) and stomata opening (Ashraf and Harris 2013). At the same time, Lu *et al.* (2002) showed that salinity does not affect PSII photochemistry and photosynthetic pigment composition of the halophilic plant *Sueda salsa*.

Some cellular mechanisms that can help ameliorate the effects of environmental stresses (salt and drought) include the accumulation of compatible osmolytes, such as proline

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Abbreviations: Chl – chlorophyll; DM – dry mass; *E* – transpiration rate; FM – fresh mass; F₀ – minimal fluorescence yield of dark-adapted state; F_m – maximal fluorescence yield of dark-adapted state; F_v – variable fluorescence = F_m – F₀; F_v/F_m – maximal quantum yield of PSII photochemistry; MDA – malondialdehyde; P_N – net photosynthetic rate; PEG – polyethylene glycol; ROS – reactive oxygen species; RWC – relative water content; WUE – water-use efficiency (= P_N/E); ψ_s – osmotic potential of the nutrient solution.

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(Szabados and Savoure 2010, Aslam *et al.* 2011). Proline plays diverse roles under osmotic stress conditions; it stabilizes proteins, membranes, and subcellular structures, and protects cellular functions by scavenging reactive oxygen species (Kishor *et al.* 2005). The role of proline has been shown in cellular homeostasis, including redox balance and energy status (Szabados and Savoure 2010). Proline can act as a signal molecule to modulate mitochondrial functions, to affect cell proliferation and cell death, and to trigger specific gene expression, which can be essential for plant recovery from stress (Sharma and Dubey 2005, Mishra and Dubey 2006, Szabados and Savoure 2010). Although proline accumulation is commonly used as an indicator for drought tolerance (Vanrensburg *et al.* 1993), its function, regulation, accumulation, and degradation are not yet completely understood, and the correlation between the proline content and abiotic stress tolerance in plants is not always apparent. Inhibition of the photosynthetic apparatus due to ionic or osmotic stresses is usually accompanied by the formation of reactive oxygen species (ROS) (Parvais and Satyawati 2008, Ashraf and Harris 2013). Halophytes possess robust antioxidant mechanisms, including enzymatic and nonenzymatic scavenging pathways, which are able also to compensate the harmful effects of ROS production (Jithesh *et al.* 2006). An increase in malondialdehyde (MDA) and an amplified lipid peroxidation are often used as indicators for ROS production in plants during environmental stress conditions (Jithesh *et al.* 2006).

Materials and methods

Plants and culture condition: The seeds of *Haloxylon aphyllum* (Minkw.) Iljin (Chenopodiaceae) were collected in the south-west of the Kyzylkum desert in the Ecocenter 'Djeiran', Bukhara, Uzbekistan (39°34'N, 64°42'E). Seeds were germinated in distilled water. Seedlings, 4–6 d old, were transplanted to perlite soaked with 25% Hoagland nutrient solution in plastics pots (24 cm in length, 20 cm width, and 10 cm depth). The seedlings were grown under circadian cycle of 10 h dark/14 h light (200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PAR). The plants (35-d-old) were transferred to the experimental solutions in irrigated perlite. After transfer, the experimental solutions in the pots were renewed every 4 d in order to maintain the initial concentration. For the controls, the nutrient solutions (25% Hoagland) in the pots were replaced every 4 d. The experiment lasted 14 d (plant age was 35–48 d during this experiment). During this period, the pot size did not inhibit the growth of the control plants. Solutes of 200 mM NaCl (NaCl) and iso-osmotic PEG 6000 (PEG, 18.8%, m/v) were prepared with a freezing-point osmometer *Osmomat 030* (Gonotec, Germany). Osmotic potential (ψ_s) of the PEG and NaCl solutes was calculated ($\psi_s = \text{as } -0.4 \text{ MPa}$) according to Michel and Kaufman (1973). For each treatment and control measurements four different plants from four different pots were used.

C_4 plants exhibit two times higher water-use efficiency (WUE) than C_3 plants (Ashraf and Harris 2013). Therefore, since the discovery of C_4 photosynthesis in the mid-60s, attempts have been made to transfer genes from the C_4 photosynthetic pathway to C_3 plants (Sage and Zhu 2011, Ashraf and Harris 2013). However, it also has been shown that despite better WUE, C_4 plants are equally or even less resistant to water stress than their C_3 counterparts (Ghannoum 2009, Ripley *et al.* 2010). Thus, relation between the C_4 photosynthetic mechanism and salt accumulation trait and its contribution to osmotic stress resistance remains an open question in halophilic plants.

The tree-like shrub species, *Haloxylon aphyllum* (Minkw.) Iljin, Chenopodiaceae (saxaul leafless, saxaul black), is ecologically one of the main components of the xerophytic community and economically the most important xerohalophytes (soil reinforcer, pasture fodder plant, and a source of fuel for the local population) in the Turan desert (Central Asia). This C_4 plant (Pyankov *et al.* 1992) represents an interesting target for the study of ionic and osmotic stress resistance mechanisms because it shows resistance both to salinity and drought.

In this study, we investigated the role of C_4 photosynthetic mechanism in high WUE formation under low osmotic potential with and without salinity of nutrient substrate. We tested whether C_4 photosynthesis is auxiliary and salt accumulation dependent mechanism during adaptation of C_4 xerohalophyte *H. aphyllum* (Chenopodiaceae) to low soil osmotic potential.

RWC: At the end of the experiment, RWC was assessed in shoots of all experimental groups. Biomass was estimated for shoot fresh (FM) and dry matter (DM). Plant samples were dried at 80°C for 2 d until reaching a constant mass in order to measure quantitatively the DM. The RWC in the shoots was calculated as:

$$\text{RWC} = (\text{FM} - \text{DM})/\text{DM}.$$

Proline: Free proline was determined according to Bates *et al.* (1973). Shoot samples (0.2 g) from each group were homogenized in 2 ml of boiling distiller water, heated at 100°C for 10 min in a water bath, and then the homogenates were centrifuged. The mixtures were heated at 100°C for 1 h in a water bath after adding acid ninhydrin and glacial acetic acid. Reaction was stopped by submerging the sample into an ice bath. The absorbance of mixtures were read at 520 nm using a *Genesis 10 UV Scanning* spectrophotometer (Thermo Scientific, USA). Proline concentrations were determined using a calibration curve and expressed as $\mu\text{g g}^{-1}$ (FM).

Lipid peroxidation: The level of lipid peroxidation in plant tissues was determined by measuring the contents of MDA, which is the breakdown product of lipid peroxidation (Heath and Pasker 1968). Shoot samples

(0.2 g) were homogenized in 4 ml of 20% trichloroacetic acid, then centrifuged at $10,000 \times g$ at 4°C for 15 min. Supernatant (1 ml) was then mixed with 4 ml of 20% trichloroacetic acid containing 0.5% of 2-thiobarbituric acid, and the solution was heated for 30 min at 95°C . The samples were cooled on ice for 5 min and recentrifuged for 12 min at $10,000 \times g$. Nonspecific absorbance of supernatant measured by *Genesis 10 UV Scanning* (Thermo Scientific, USA) at 600 nm was subtracted from the maximum absorbance at 532 nm. To estimate MDA concentration, an extinction coefficient of $155 \text{ mM}^{-1} \text{ cm}^{-1}$ was used. Relative changes in MDA content were expressed as percentage of the control.

Pigment extraction and quantification: Chlorophyll (Chl) content was extracted in 96% ethanol using purified glass sand for sample homogenization. After centrifugation at 4°C , the Chl *a* and Chl *b* contents were determined spectrophotometrically at 665 nm and 649 nm by *Genesis 10 UV Scanning* (Thermo Scientific, USA). The concentrations were calculated according to Lichtenthaler (1987).

Chl fluorescence was measured with a pulse amplitude modulated fluorometer *PAM 101* (Walz, Effeltrich, Germany) following the recommendations of the manufacturer (Schreiber 1997). Chl fluorescence of the leaf in the leaf chamber was excited and directed to the fluorometer through a flexible fiber-optic light guide *101F* (Walz, Effeltrich, Germany). Minimal (F_0) and maximal (F_m) fluorescence from the shoots in dark-adapted state were determined during the first and last stages of the combined procedure to measure fluorescence and gas exchange, as described below. The maximum quantum efficiency (yield) of PSII (F_v/F_m) in dark-adapted shoots was calculated as $(F_m - F_0)/F_m$.

CO₂/H₂O exchange: Net photosynthetic rate (P_N) was measured using a single-channel infrared gas analyzer *LI-820* (LI-COR Inc., Lincoln, NE, USA) in an open scheme (Laisk 1977). The concentration of CO₂ and humidity of the air supplied were measured in the empty leaf chamber before and after the experiment. CO₂-exchange measurements (P_N) were subsequently recalculated per

Results

Water content in shoots: The water content measured in shoots of the PEG-treated plants ($\psi_s = -0.4 \text{ MPa}$) was three times lower than that in the controls. However, the RWC measured in shoots after the NaCl treatment was only approximately 30% lower (Fig. 1A). Thus, the NaCl treatment with equivalent osmotic potential affected RWC a much less than the PEG treatment.

Proline and MDA content: The accumulation of proline was used as an indicator of drought tolerance, and MDA

leaf area. Leaf transpiration (E) is traditionally calculated based on the difference in air humidity between the inlet and outlet of the leaf chamber (Laisk 1977). The constant humidity at the air stream entering the leaf chamber was provided by a *LI-610* humidifier (LI-COR Inc., Lincoln, NE, USA). To determine the moisture content at the outlet of the leaf chamber, a psychrometric sensor *HMP50* (Vaisala INTERCAP, Finland) was used. A gas mixing unit allowed reducing CO₂ to a predetermined concentration in the air stream at the input to the leaf chamber.

Combined procedure of Chl fluorescence and gas-exchange measurements: Detached shoots were placed into a darkened, specialized leaf chamber (previously described by Pärnik *et al.* 1987) aerated at a constant rate with an air at temperature of $23 \pm 1^{\circ}\text{C}$ and a relative air humidity of 65–70% (steady-state regime). After leaving the shoot to acclimate to darkness for 20 min, their minimal fluorescence (F_0) was recorded. Then, their maximal fluorescence (F_m) was measured using saturation pulses ($5,000 \mu\text{mol m}^{-2} \text{ s}^{-1}$ PAR, 800 ms duration) of *102-L* (Walz, Effeltrich, Germany). Then the shoots in the leaf chamber were exposed to a PPFD of $2,000 \mu\text{mol m}^{-2} \text{ s}^{-1}$, which was determined with a *LI-250A* light meter (LI-COR Inc., Lincoln, NE, USA). After the 15-min light period, the stabilized CO₂/H₂O exchange was recorded at 360 ppm of atmosphere CO₂ concentration. At the final stage of the experimental procedure, the gas system was switched to 180 ppm of CO₂ concentration, and the steady-state CO₂/H₂O exchange was measured once again. To confirm the absence of photoinhibition at the end of the experiment, Chl fluorescence quantum yield was measured again for the dark-adapted state (F_v/F_m).

WUE was estimated as the ratio between P_N and transpiration (P_N/E) and expressed in $\text{mmol}(\text{CO}_2) \text{ mol}^{-1}(\text{H}_2\text{O})$.

Statistical analysis: All of the measurements were performed four times. The means and calculated standard deviations (SD) were calculated using *Sigma Plot 12.0* statistical program. Comparisons of parameters were made between treatments using analysis of variance (ANOVA) with a post hoc *Tukey's* test for pairwise comparison. Differences were considered significant at $P < 0.05$.

content in shoot tissues was used to assess lipid peroxidation. A significant increase of the proline (four–five times) and MDA (more than two times) contents was detected after the PEG treatment only (Fig. 1B,C). According to both indicators, NaCl did not cause any significant stress in the plants.

Chl fluorescence and pigment content: The content of Chl (*a+b*) and F_v/F_m in shoots of *H. aphyllum* did not change significantly (Table 1).

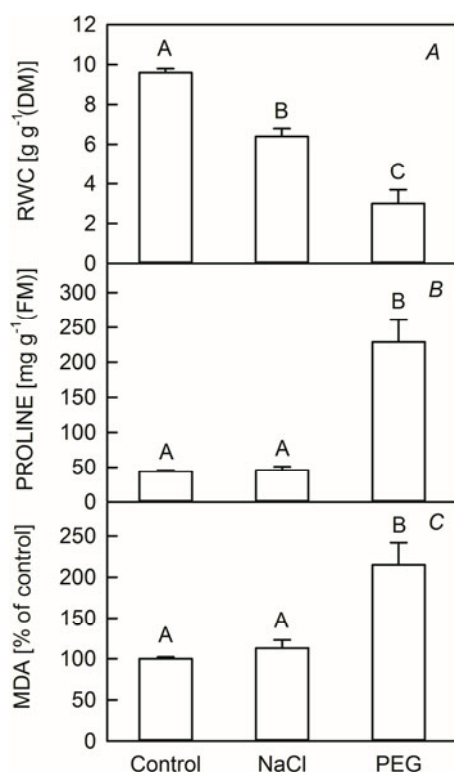


Fig. 1. Effect of osmotic (PEG) and salt (NaCl) treatments on (A) relative water content (RWC), (B) proline, and (C) malondialdehyde (MDA) in shoots of *Haloxylon aphyllum*. The values are means (\pm SD) of four replicates. Letters above the bars represent significant differences at $P < 0.05$ (Tukey's pairwise comparison).

Table 1. The effective quantum yield (Φ_{PSII}) and the contents of chlorophyll (Chl) ($a+b$) in the shoots of *Haloxylon aphyllum* under the different concentrations of NaCl and PEG. The maximum quantum yield of PSII photochemistry (F_v/F_m) of plants was measured following 20 min of dark adaptation. Mean \pm SD ($n = 4$) is shown for each treatment. Superscript letters represent significant differences at $P < 0.05$ (Tukey's pairwise comparison).

Parameter	Control	NaCl	PEG
Chl ($a+b$) [mg m ⁻²]	30.27 \pm 9.81 ^a	24.20 \pm 1.19 ^a	24.47 \pm 0.27 ^a
F_v/F_m	0.90 \pm 0.03 ^a	0.87 \pm 0.005 ^a	0.88 \pm 0.02 ^a

CO₂/H₂O exchange: The NaCl treatment doubled the P_N in the plants. In case of the PEG, the P_N declined to a half of the control (Fig. 2A). In shoots from the NaCl-treated

Discussion

It is generally known that C₄ plants exhibit higher WUE than C₃ plants (Ashraf and Harris 2013). However, according to our results, WUE of C₄-plant *H. aphyllum* may be dependent on soil salinity (Fig. 2C).

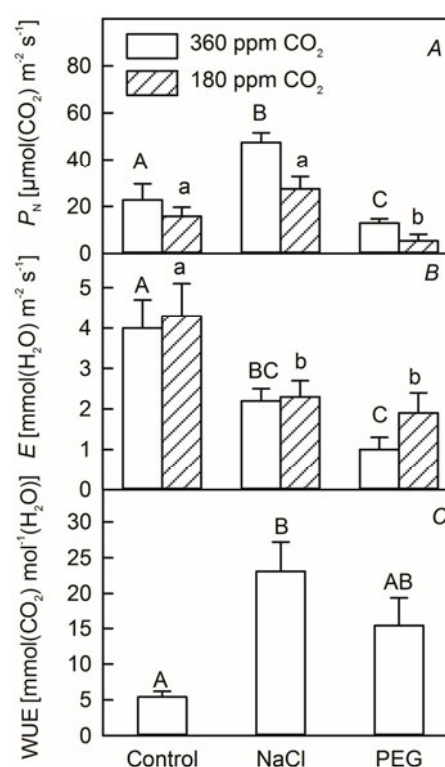


Fig. 2. Effect of osmotic (PEG) and salt (NaCl) treatments on (A) net photosynthetic rate (P_N), (B) transpiration rate (E), and (C) water-use efficiency (WUE) of *Haloxylon aphyllum* shoots. The values are means (\pm SD) of four replicates. The various capital and lowercase letters indicate a significant difference at $P < 0.05$ (Tukey's pairwise comparison) under different CO₂ concentrations.

plants, E decreased by a factor of two. The highest (ca. four times) decrease in E was detected in the PEG-treated plants (Fig. 2B).

The experimental procedure also included gas-exchange measurements at half of the CO₂ concentration. Reduced photosynthesis was observed under lower CO₂ concentration during the treatments. It is known that CO₂ decrease induces stomata opening (Robinson *et al.* 1997). Hence shoot E in an atmosphere with lower CO₂ should be higher than that one under normal condition. However, this effect was observed only after the PEG treatment, when the increased E was statistically significant (Fig. 2B). In other cases, such enhancement was not detected at all.

WUE: The ratio of P_N/E increased significantly by a factor of 4.3 only after the NaCl treatment (Fig. 2C).

Water content in shoots: The NaCl and PEG treatments were accompanied by a decrease in the leaf water content in *H. aphyllum* (Fig. 1A). However, the sharpest decline (ca. three times) in water content was observed after the

PEG treatment. This finding is consistent with results previously reported (Ghannoum 2009, Ripley *et al.* 2010).

Halophytes are known to usually maintain their leaf water status due to salt accumulation (Balnokin 2005, Robinson *et al.* 1997). Under the NaCl treatment, this halophilic species was able to lower the shoot water potential effectively in order to prevent significant tissue dehydration. We could conclude that the absence of NaCl in the medium had the most negative effect on the water status in shoots of *H. aphyllum*.

Proline content and lipid peroxidation: It is known that proline plays a primary role as a powerful osmoprotector, and its biosynthesis depends on salinity (Szabados and Savoure 2010). Indeed, the greatest decrease of RWC after the PEG treatment was accompanied by the highest (fivefold) increase of the proline content in the shoots (Fig. 1B). Despite the equivalent osmoticity of NaCl and PEG treatments, its content in the NaCl-treated shoots remained at the control levels, while the shoots were partly dehydrated. (Fig. 1B). Consequently, under NaCl treatment, the salt accumulation in shoots might represent the main osmoprotective mechanism for *H. aphyllum*.

Toxicity of ions and osmotic stress stimulate the formation of ROS, which may damage membranes and proteins (Zhu 2001). We used MDA to detect lipid peroxidation. In this study, we found a significant MDA increase (by a factor of 2) in shoots of the PEG treated plants, indicating osmotic stress occurrence (Fig. 1C).

Effect of salinity and osmoticity on photosynthesis and transpiration: Previous research showed a negative influence of salinity on growth and development of both halophytes and nonhalophilic plant species (Grigore *et al.* 2012), suggesting that photosynthesis could also be damaged by salinity. Drought and salinity stress can cause substantial damage to photosynthetic pigments (Kannan and Kulandaivelu 2011, Ashraf and Harris 2013). However, in our experiments the NaCl and PEG treatments did not induce significant changes in the Chl content and PSII quantum yield (Table 1). The results suggested that primary photochemical reactions in shoots of *H. aphyllum* were stable even under stress. These data are also consistent with previously published results (Lu *et al.* 2002, Maricle *et al.* 2007, Eshghizaden *et al.* 2012).

The dark stage of the photosynthesis depends primarily on the degree of stomata opening. Stomata closure in response to drought and salinity stress generally occurs due to a decrease in soil water potential (Chaves *et al.* 2009), *i.e.*, the drought and salinity are always accompanied by a reduction in soil water potential followed by decreased stomatal conductance (Willmer and Fricker 1983).

In our experiments, the PEG-treated plants lowered simultaneously P_N (*ca.* two times) and E (*ca.* four times) (Fig. 2A,B). However, the gas-exchange response of the shoots to the NaCl treatment was very different. In this case, a significant twofold reduction of E occurred, while

the P_N increased by 2.5 (Fig. 2A,B).

Thus, in *H. aphyllum*, the salinity conditions stimulated the photosynthesis (Fig. 2A) probably by nonstomatal means (Lawlor 2002), and in spite of the E suppression under the NaCl treatment (Fig. 2B).

To find out the reasons for such an effect we applied the following approach: during the measurement of gas exchange, the concentration of CO₂ in the leaf chamber was reduced to half. It is known that in these condition stomata are artificially opened (Robinson *et al.* 1997), and do not limit gas exchange.

Stomatal and nonstomatal limitation of photosynthetic CO₂/H₂O exchange: P_N depends on the stomata opening and other nonstomatal factors, including light and dark biochemical processes (Lawlor 2002). In our experiments, a half of the atmospheric CO₂ concentration was followed by the almost proportional reduction in photosynthesis suggesting a CO₂-limiting condition under all treatments (Fig. 2A). Photosynthetic activity regulation under low CO₂ concentration is known to be related to a nonstomatal mechanism in C₃- (Lawlor 2002) and C₄ plants (Ghannoum 2009). This suggests that stomata opening induced by the low CO₂ concentration did not regulate photosynthesis in the *H. aphyllum* at any treatment. Thus, one may conclude that the observed suppression of photosynthesis after the PEG treatment, and the enhancement during the NaCl treatment, were most likely controlled by nonstomatal factors. In addition, the specificity of the photosynthetic regulation (*i.e.*, NaCl-dependent photosynthesis activation) may be determined by inherent ability of the *H. aphyllum* to accumulate salt.

While a decrease to half of the ambient CO₂ concentration induced stomata opening, the E did not change under the NaCl treatment or the control conditions (Fig. 2B). This suggests that the stomata did not limit water exchange in these cases, and the NaCl-exposed plants sustained successfully their water status. Indeed, no stress-induced proline or MDA production was detected (Fig. 1B,C). However, the E decrease found in the NaCl-treated plants in comparison with the controls was not a consequence of stomatal limitation, because E did not increase after stomata opening induced by experimentally lowered CO₂ concentration. Thus, we concluded that the E decrease observed in this case was probably due to lowering of water potential in shoots of the NaCl-treated plants.

A completely different response of E was found after the PEG treatment. At normal CO₂ concentration, the PEG treatment induced a threefold tissue dehydration (Fig. 1A) resulting in stomata closure followed by decline in E (Fig. 2B).

At the half CO₂ concentration, the shoot E increased by a factor of 2 because of the stomata opening (Fig. 2B), indicating that the stomata limited the shoot E , at least at normal CO₂ concentration. It suggested that the PEG treatment limited directly E by the stomatal conductance.

Hence, only the strong shoot dehydration induced by the PEG treatment was the most significant stress factor in *H. aphyllum*, followed by stomata closing (Figs. 1A, 2B). This stressful condition was also confirmed by a substantial increase in proline and MDA contents in shoots (Fig. 1B,C). Therefore, salt-free substrate combined with PEG osmoticity ($\psi_s = -0.4$ MPa) represented the highly stressful condition for *H. aphyllum*. This also implied that the by inherent ability of the *H. aphyllum* to accumulate salt was a key factor to maintain its water status and photosynthetic CO₂ assimilation under high osmoticity of salty substrate.

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