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Article

Sensitivity and Regulation of Diel Photosynthesis in Red-Fleshed Pitaya (*Hylocereus polyrhizus*) Micropropagules under Mannitol-Induced Water Stress/Rehydration Cycle In Vitro

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Abstract: Climate change-induced prolonged water stress (WS) affects crassulacean acid metabolism photosynthesis in pitaya (*Hylocereus*), limiting crop productivity through insufficient photosynthate. To document how WS/rehydration affects diel photosynthesis, red-fleshed pitaya (*H. polyrhizus*) micropropagules were studied for 5 weeks in a mannitol-induced water potential gradient replaced with moderate (MWS; −1.0 MPa in week 2; −0.5 MPa for the rest) or intensified (IWS; −1.0 and −1.5 MPa in weeks 2 and 3; −0.5 MPa for the rest) WS in vitro. Net photosynthetic rate (Pn) and integrated net CO_2 uptake (INCU) were measured using an Arduino-based photosynthesis system. Micropropagules under MWS had similar Pn in weeks 5 and 1, whereas the control (−0.5 MPa) increased. Pn recovery did not occur after IWS. The average relative INCU was similar in the control and MWS, but lower in IWS. The Pn difference increased with WS, becoming more evident at dawn (Phase II), evening (Phase IV), and predawn the next day (Phase I), and occurred earlier in Phases IV and I under IWS. MWS did not reduce photosynthesis, demonstrating that the photosynthetic regulation could respond to short-term WS in pitaya and indicating the potential of watering for Pn recovery at evening and predawn under IWS.

Keywords: pitahaya; crassulacean acid metabolism; diel CO_2 uptake; photosynthetic pattern; phase; water stress; water potential

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1. Introduction

Water content (WC) of plants depends on the soil–plant–atmosphere continuum [1,2], where soil-water absorbed by roots via cohesion [3] transpires through stomata under moderate vapor pressure deficit (VPD). Soil-water availability and water potential (WP) regulate WC, leaf area, and photosynthesis, affecting crop production and food security [4–6]. However, global warming-induced drought, heavy precipitation, and floods inhibit stomatal conductance [4,5,7], affecting plant photosynthesis.

Taiwan experienced drastic flood/drought alternation, with a 30% precipitation deviation and a difference of 5–10 rainy days in 2022 compared with 1991–2022 [8]. Moderate water management and irrigation for adaptation and cultivation may mitigate such risks [4,5].

Well-watered crassulacean acid metabolism (CAM) plants, such as *Hylocereus* spp., *Kalanchoe pinnata*, *Opuntia basilaris*, and *Agave deserti* [9–12], develop robust succulent shoots with abundant mucilage [13,14], organic acids, and C-assimilation. Although CAM plants can grow in arid/semi-arid regions [15,16], continuous water stress (WS) disrupted C-uptake, as observed in *H. undatus* and *K. daigremontiana* [17–19], probably due to the oxidative damage of chlorophyll and photosynthetic apparatus [5,20,21], also reported in C_3 and C_4 plants [22].

CAM involves four-phased diel photosynthesis, identified based on CO_2 exchange and activation of metabolism-related enzymes [23]. Phase I begins at night with cytoplasmic phosphoenolpyruvate carboxylase (PEPC)-regulated CO_2 fixation and open stomata, formation of malic acid by malate dehydrogenase, and storage in vacuoles [16,24]. However, Phase III occurs during the day, with the release of HCO_3^- from malic acid in the cytoplasm and assimilation of 3-phosphoglycerate in the chloroplasts via ribulose bisphosphate carboxylase/oxygenase (Rubisco) with closed stomata [16,25]. Phases II (dawn) and IV (evening) are distinguished by open stomata during the day; CO_2 is internalized and assimilated via PEPC (cytoplasm) and Rubisco (chloroplast) [23,26,27].

CAM is measured via closed or open-flow systems using an infrared gas analyzer, with either single shoots or whole plants [28]. One-year-old field-grown *H. undatus* plants had a maximum net photosynthetic rate (Pn) of 8.0 μmol/m^2/s and diel integrated net CO_2 uptake (INCU) of 232 mmol/m^2/day under well-irrigated conditions [29], with excellent chlorophyll fluorescence (F_v/F_m = 0.9) and titratable acid content [17,29].

However, long-term WS-induced stomatal closure and impaired photosynthesis decreased diel INCU by 78% [14,17,21,30]. Similarly, Pn reduced by 48% in *H. undatus* micropropagules after a 14-day drought [31] that was reversed by 7-day irrigation [18,29]. *H. undatus* is a constitutive CAM plant showing increased diel INCU during maturation [12,17], which has not yet been confirmed after rehydration. Photosynthesis is a crucial indicator of stress-induced damage because of its moderate sensitivity to low WS [22]. Monitoring photosynthesis, especially during CAM phase shifts under WS and rehydration, may improve irrigation management.

H. polyrhizus 'Da Hong' is a popular, red-fleshed, non-facultative CAM pitaya related to white-fleshed *H. undatus* [14,29,32]. *H. polyrhizus* is popular because of its abundant betalain (antioxidant) in the peels [33]. origin, rib morphology [34–36], self-pollination capacity [37], long flowering duration (April to October) in Taiwan [38,39], and red flesh differentiate *H. polyrhizus* from *H. undatus*. These variations in physiology, photosynthesis, and reproduction require further investigation. However, this cultivar, which is prominent in Taiwan and southern Asia [32,40], is prone to insufficient photosynthate and water in fruiting shoots after cycling harvest [41]. Micropropagules planted on well-hydrated Murashige and Skoog (MS) medium had 2.63 μmol/m^2/s Pn under a 16-h light/dark photoperiod at 25 °C [32]. Information on the photosynthesis-related differences between in vitro- and field-grown plants during WS and after rehydration is limited.

This study elucidates the effects of dynamic watering (solution replacement) on photosynthesis in 'Da Hong' to understand the in vitro diel INCU sensitivity at a particular WP, using a previously established Arduino-based whole-plant continuous open-flow photosynthesis system [32], which has advantages of accuracy, noninvasiveness, and real-time detection [6,42,43]. It is unlikely that the elimination of WS restores debilitated photosynthesis after WS treatment below a critical WP; hence, we hypothesize that temporary moderate WS with rehydration may restore photosynthesis in 'Da Hong' micropropagules owing to the ability of diel photosynthetic regulation to respond to short-term WS. Understanding photosynthetic changes during various forms of low-water-induced stress would reveal an essential picture of CAM-related phase shift and the relationship between CO_2 uptake and water use efficiency.

2. Materials and Methods

2.1. Plants and Pre-Experimental Conditions

Disinfected areoles from mature joints of potted 'Da Hong', from a commercial orchard in central Taiwan (24°14′33.2″ N, 120°48′21.6″ E), were planted in vitro and incubated at 25 °C with a 16-h photoperiod of 50 mol/m^2/s fluorescent light. Sprouts of 15 mm length were excised and inoculated in pH-adjusted MS medium (M5519; Sigma Chemical Co., St. Louis, MO, USA) supplemented with 3% (*w*/*v*) sucrose, 0.8% (*w*/*v*) agar, and 0.25% (*w*/*v*) activated charcoal [44].

After 2 months of incubation of sprout segments, multiplicated cloning explants were excised into 15 mm lengths and cultured in polypropylene (PP) growth vessels with 25 segments each. Depending on experiments with airflow rate and WS, vessels contained sterile distilled water-supplemented vermiculite or activated charcoal-free MS medium. A sterile PP transparent membrane (10.0 cm × 7.3 cm) with 25 holes was placed over the vermiculite/medium to prevent water loss due to air ventilation. Vessels were placed in a culture environment identical to the multiplication procedure for 8 weeks before the vessel lids were replaced with those drilled with three or four holes (varied with experiments), attached with silicone tubes, and sealed with a transparent plastic film for 1-week adaptation.

Experiments were carried out from August 2021 to August 2022 using an Arduino-based open-flow photosynthesis system [32]. The system was performed under whole-canopy conditions after considering the plant structure and architecture [6,42] and ambient factors in open-flow states (light intensity, airflow rate, temperature and humidity) [28,45,46]. These considerations increased the precise photosynthesis estimation in field-grown plants by a high-resolution long-term measurement [6,47].

A presumed relative air humidity of 99% was maintained using an air pump bubbling outdoor air into water and buffering wetted air in a box. Therefore, VPD was not considered in this study. Inlet-wetted air of vessels was filtered (0.2 µm × 25 mm; Acrodisc syringe filter with a Supor membrane; Pall Corp., Port Washington, NY, USA). Experimental conditions, including an 80 $\mu mol/m^2/s$ fluorescent light under a 12-h day length and 25 °C, were monitored by sensors and collected by dataloggers, as mentioned by Lee et al. [32]. Vessels with pitaya micropropagules were transferred to the conditions described in this paragraph and adapted for 1 week before the experiments were conducted.

2.2. Photosynthetic Responses after Application of Different Flow Rates

As a vital factor in photosynthesis, the airflow rate affects temperature and humidity within vessels that affect stomatal movement, photosynthetic apparatus function, and enzyme activity [6,28,30,46,48,49]. The correct airflow to diminish temperature damage is essential, because CAM plants have a lower optimal temperature (20–30 °C) for photosynthesis than C_3 and C_4 plants [50–52].

Inlet-wetted air was supplied to three-hole-lid growth vessels in serial airflow rates of 200, 400, 600, 800, and 1000 mL/min through flow meters (Dwyer Instruments, Inc., Michigan, IN, USA). Photosynthesis was detected to access an appropriate flow rate for micropropagules grown in vitro under experimental environmental conditions.

Micropropagules of this experiment were cultured in vessels with 100 mL activated charcoal-free MS medium. Four replicate vessels were used for each flow rate treatment, and the experiment was repeated twice.

2.3. A System with Lid-Modified and Vermiculite-Supplemented Growth Vessels

Modified growth vessels with four-hole lids were prepared in advance to replace culture solutions of different WP for convenient photosynthesis evaluation. The vessel lid contained three holes with silicone tubes to facilitate airflow (air inlet and outlet) and sampling [32], in addition to a 0.6-cm diameter hole attached to a silicone tube (inner/outer (I/O) diameter: 0.3/0.6 cm, length: 26.0 cm) wrapped with polytetrafluoroethylene thread seal tape to facilitate solution replacement via aspiration (Figure 1A). The inlet end of the solution-replacement silicone tube was capped with a stuffed silicone tube (I/O diameter: 0.6/1.0 cm, length: 1.0 cm) to prevent infections caused by outdoor microorganisms. Additionally, a silicone tube (I/O diameter: 1.0/1.4 cm, length: 2.0 cm) surrounded with cotton at the bottom end was placed at the corner of the vermiculite-filled vessel by which the replacement tube would be unobstructed during aspiration for solution exchange.

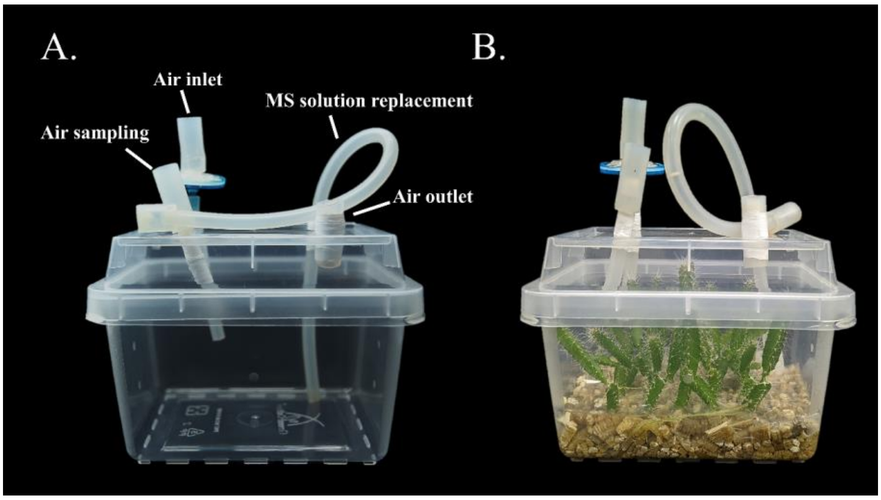

Figure 1. Culture vessels for an open-flow photosynthesis system. (**A**) Experimental set-up showing air inlet, outlet, sampling, and MS solution replacement tubes. (**B**) The vessel with 'Da Hong' micropropagules growing on vermiculite supplemented with mannitol-induced MS solution.

Vermiculite-related culture conditions for micropropagules have been described in detail (see Section 2.1). Before autoclaving, vessels were filled with 250 mL vermiculite (no compacting) and supplemented with 60 mL distilled water. The vermiculite, covered by a 25-hole PP membrane, was suitable for in vitro planting, as it was porous, had a light texture, and was convenient to replace culture solutions. Sterile vermiculite-filled vessels with a small amount of water inside were mixed with 80 mL sterile distilled water before planting, supporting 'Da Hong' sprout segment rooting and development under a culture environment for 9 weeks, followed by 1-week adaptation in −0.5 MPa of WP culture solution (Figure 1B).

2.4. Mannitol-Induced Dynamic Water Stress Modulation for Micropropagule Photosynthesis Evaluation

Although mannitol accumulates as a photosynthate via mannose-6-phosphate reductase and reduces osmotic stress [7,53], it is scarcely used by vascular plant cells [54,55] and rarely causes ionic toxicity [56,57]. Consequently, a physiological survey of *H. undatus* micropropagules was recently conducted [31].

Therefore, serial WPs in MS solutions regulated by mannitol (M4125; Sigma-Aldrich Co., St. Louis, MO, USA) were used to determine the WS levels. Culture solutions were initially modulated to WP values of −0.5, −1.0, and −1.5 MPa using a Psypro dew point meter (Wescor, Inc., Logan, UT, USA) via a C-52 sample chamber. To assist the decrease in WP without requiring excess mannitol, 3% (w/v) sucrose was added to the culture solution. Vessels treated with −0.5 MPa solution continuously for 5 weeks were considered as controls; vessels for −1.0 MPa in week 2, with −0.5 MPa for the rest were considered as moderate WS (MWS). For intensified WS (IWS), culture solutions at −1.0 and −1.5 MPa were supplied in weeks 2 and 3, respectively, with −0.5 MPa for the rest (Table 1).

The culture solution in vermiculite was removed using the silicone tube for solution replacement before 60 mL of the target solution was injected. The procedure was performed twice to dilute the dose effects of the previous solution. In contrast, the solution exchange from the one with a lower (−1.5 MPa) to a higher (−0.5 MPa) WP was repeated thrice. The duration of all treatments was 5 weeks. Four vessels were used as replicates for each treatment at varying weeks, and experiments were repeated twice in the control and MWS groups. The IWS treatment was conducted once because the Pn dramatically decreased with the solution replacement, and the Pn could not meet the expected results after WS elimination.

Table 1. Treatment of MS solution replacement with different water potentials for 5 successive weeks.

Treatments [1]	Water Potential of MS Solution (MPa)					
	Pre-Treatment for Adaptation [2]	Weeks after Treatment				
		1	2	3	4	5
Control	−0.5	−0.5	−0.5	−0.5	−0.5	−0.5
Moderate water stress	−0.5	−0.5	−1.0	−0.5	−0.5	−0.5
Intensified water stress	−0.5	−0.5	−1.0	−1.5	−0.5	−0.5

[1] Treatments: control, water potentials (WP) of exchanged culture solutions were −0.5 MPa every week; moderate water stress, the WP of the culture solution on week 2 was −1.0 MPa induced by mannitol but −0.5 MPa for the rest of the weeks; intensified water stress, WP of culture solutions on weeks 2 and 3 were −0.5 and −1.0 MPa, and that for the other weeks were −0.5 MPa. [2] Treatments were adapted in −0.5 MPa MS solution for 1 week before the experiment.

2.5. Dynamic Photosynthesis Analysis

To evaluate photosynthesis by gas (CO_2) exchange measurement, an open-flow photosynthesis system calculated the equations presented in a previous study [32]. The detected CO_2 values from each vessel were recorded every 30 s for 5 consecutive weeks. The CAM photosynthesis phases were shifted after signs of three successive positive/negative Pn [58,59].

2.6. Data and Statistical Analysis

The Pn and diel INCU data were collected from three to four vessels in various flow rate treatments. The relative INCU data from different WS were analyzed by Fisher's protected least significant difference (LSD) test after Levene's test and one-way analysis of variance at α = 0.05 using SAS 9.4 software (SAS Institute, Inc., Cary, NC, USA). In addition, relative INCU differences in three to four vessels compared to week 1 were evaluated by the Student's paired *t*-test at α = 0.05. Figures were generated using SigmaPlot 10.0 (Systat Software, Inc., San Jose, CA, USA).

The difference intensity in Pn was calculated as the mean weekly Pn discrepancy (D) compared to week 1 after each WP treatment.

$$D = \left[\sum_{i=2}^{5} (v_i - v_1)/v_1 \times 100\%\right]/m/4, \quad (1)$$

where v is the Pn on week i after treatments, and m is the number of replicate vessels (3–4). The logarithm of absolute value D was transformed under base 10. The relative INCU related to that on week 1 was calculated as follows:

$$Relative\ INCU = \left[\sum_{j=1}^{5} \left(x_j/x_1\right) \times 100\%\right]/n, \quad (2)$$

where x is the diel INCU (starting calculation from 06:00 h) on day 4 (or forward) on week j, and n were vessel replicates (3–4).

3. Results

3.1. Photosynthetic Response Determination after Application of Different Flow Rates

To determine the effects of airflow rate on *H. polyrhizus* 'Da Hong' photosynthesis, Pn was measured at a range of flow rates and compared to a baseline value of 1000 mL/min. The flow rates increased Pn and Pn percentage (Figure 2), where the Pn percentage at a flow rate of 1000 mL/min was significantly higher than that at 600, 400, and 200 mL/min, with values of 68.03%, 67.00%, and 58.20%, respectively, and was similar to 85.30% of Pn percentage at 800 mL/min (Figure 3A).

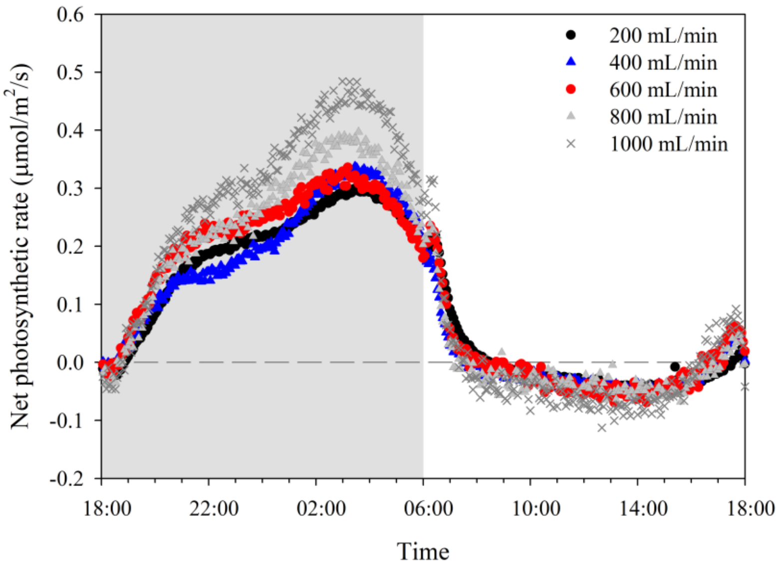

Figure 2. Diel photosynthetic patterns in micropropagules grown at various airflow rates. 'Da Hong' micropropagules grown on MS medium at 25 °C under a 12-h photoperiod with 80 μmol/m^2/s light intensity. Data from each vessel under different flow rates were collected every 30 s, and the mean of 3–4 replication vessels was calculated. The gray block represents the dark period of the experiments.

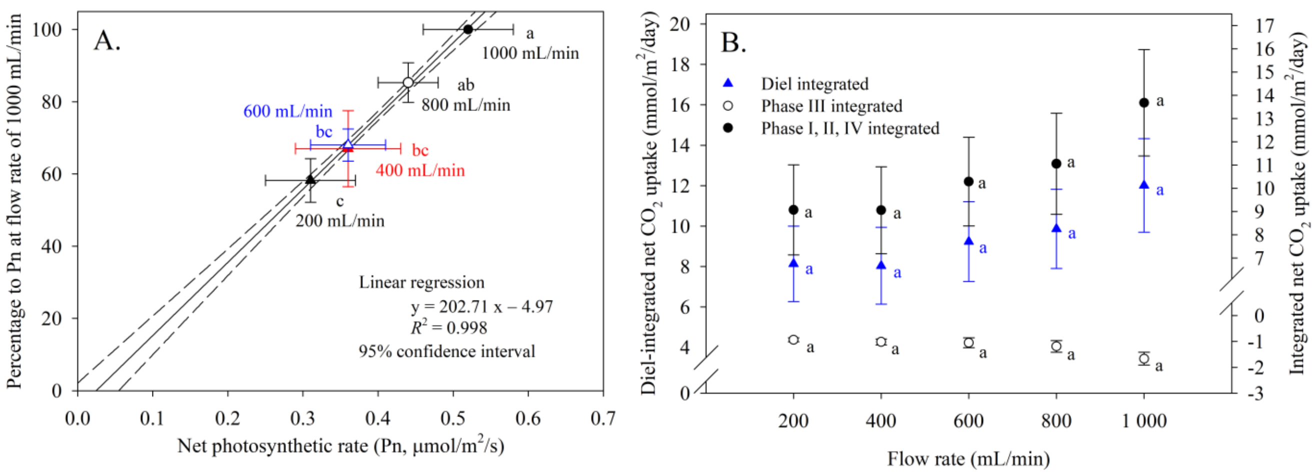

Figure 3. Net photosynthetic rate (Pn) and integrated net CO_2 uptake (INCU) of 10-week-old 'Da Hong' micropropagules at various flow rates. Micropropagules grew on MS medium at 25 °C under a 12-h photoperiod with 80 μmol/m^2/s light intensity. (**A**) Relevance of Pn to Pn percentage at a flow rate of 1000 mL/min. (**B**) INCU of a diel period and four phases under various flow rates. Pn percentage and INCU labeled in the same letters beside symbols in plates A and B indicated no difference after the flow rates were supplied. The replicates of vessels were 3 to 4.

In comparison, the diel INCU of 10-week-old micropropagules grown in the 12-h photoperiod increased with flow rates. However, it was not different (Figure 3B) compared to where CO_2 uptake amounts were 8.13, 8.04, 9.23, 9.86, and 12.01 mmol/m^2/day, respectively, at serial flow rates of 200 to 1000 mL/min.

In summary, 600 mL/min was the critical flow rate with similar INCU and Pn compared to lower flow rates, concerning the power of an air pump in a photosynthesis system (gas exchange capacity). The gas exchange rate was 0.94 vessel/min.

In addition, to determine a particular and stable diel pattern of CO_2 uptake used to evaluate the influence of WS on pitaya in vitro, a 7-day pattern was monitored after weekly MS solution replacements. Micropropagule photosynthesis performance remained steady after 4-day balancing, for instance, from a WP of −0.5 to −1.0 MPa. The photosynthesis of the four phases was similar to that on day 3 when the performance before and after solution replacement was compared (Figure 4). Therefore, photosynthesis after day 4 was used to evaluate photosynthesis behavior.

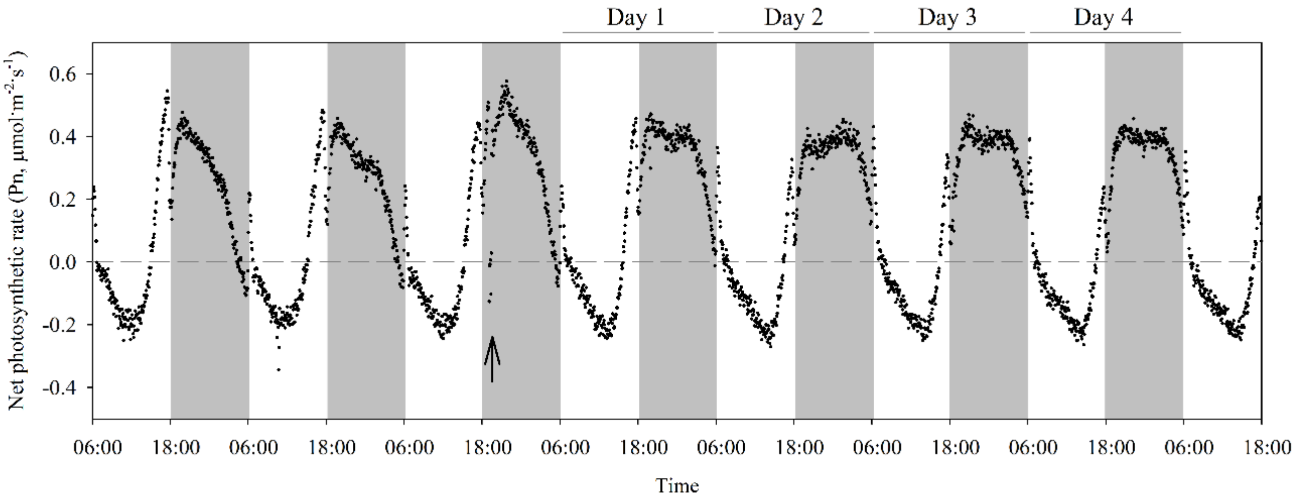

Figure 4. Diel photosynthetic pattern of 10-week-old 'Da Hong' micropropagules grown on MS solution with a water potential (WP) of −1.0 MPa. The culture environment was 25 °C under a 12-h photoperiod with 80 μmol/m^2/s light intensity. The WP of MS solution in vessels was changed from −0.5 to −1.0 MPa at 19:00 h (see arrow). Gray blocks represent the dark periods of experiments.

3.2. Mannitol-Induced Dynamic Water Stress Fluctuated Diel Photosynthetic Pattern and Value

Three WS treatments for 5 successive weeks were conducted to evaluate the effects of WS on diel photosynthetic responses. All photosynthetic data from micropropagules grown in mannitol-induced MS solutions were transformed into relative percentages to uniform Pn for comparison. The photosynthetic patterns of each treatment from day 4 after exchanging MS solutions are shown in Figure 5.

The peaks of the four phases fluctuated during solution replacement. Maximum Pn was amplified to 133.20% (no significant difference between weeks 1 and 5; t(3) = −1.16, $P_{\text{upper-tailed}}$ = 0.8357) after 5 successive weeks of replacement with −0.5 MPa solution (control) and 70.80% and 91.63% of Pn on weeks 3 and 4 (Figure 5A). Although the suppressed Pn was 76.68% and 59.30% on weeks 3 and 4, respectively, a similar trend of Pn fluctuation was observed in the MWS. A 3-week −0.5 MPa solution replacement recovered photosynthesis decrease by a WP of −1.0 MPa, where Pn on week 5 (100.02%) was similar to that on week 1 (Figure 5B).

IWS treatment reduced 'Da Hong' micropropagule photosynthesis. Pn decreased from 100% to 27.85% after 5 weeks of manipulation [t(3) = 5.05, $P_{\text{upper-tailed}}$ = 0.0075 between weeks 1 and 5]. Moreover, the final Pn did not recover during 2 weeks of recuperation under −0.5 MPa solution (Figure 5C).

MWS treatment did not decrease 'Da Hong' photosynthesis, whereas IWS treatment dramatically decreased photosynthesis expression, with no repair.

3.3. Relative Integrated Net CO_2 Uptake after Water Stress Application

The weekly relative INCU compared to week 1 was presented in percentages to recognize a relationship of carbon assimilation oscillation among treatments (Table 2). Compared to the CO_2 uptake of control with a trend of increase (not significant) by weekly −0.5 MPa solution replacements, plants under MWS treatment exhibited relatively stable carbon assimilation after a 5-week incubation, despite a calculated peak on week 2. There was a significant decrease in the relative INCU in IWS after 5 weeks of incubation, with 46.03% (t(2) = 1.69, $P_{\text{upper-tailed}}$ = 0.1167), 59.92% (t(2) = 1.5, $P_{\text{upper-tailed}}$ = 0.1357), 39.34% (t(2) = 6.56, $P_{\text{upper-tailed}}$ = 0.0112), and 32.27% (t(2) = 16.18, $P_{\text{upper-tailed}}$ = 0.019) from weeks 2 to 5 compared to week 1, respectively.

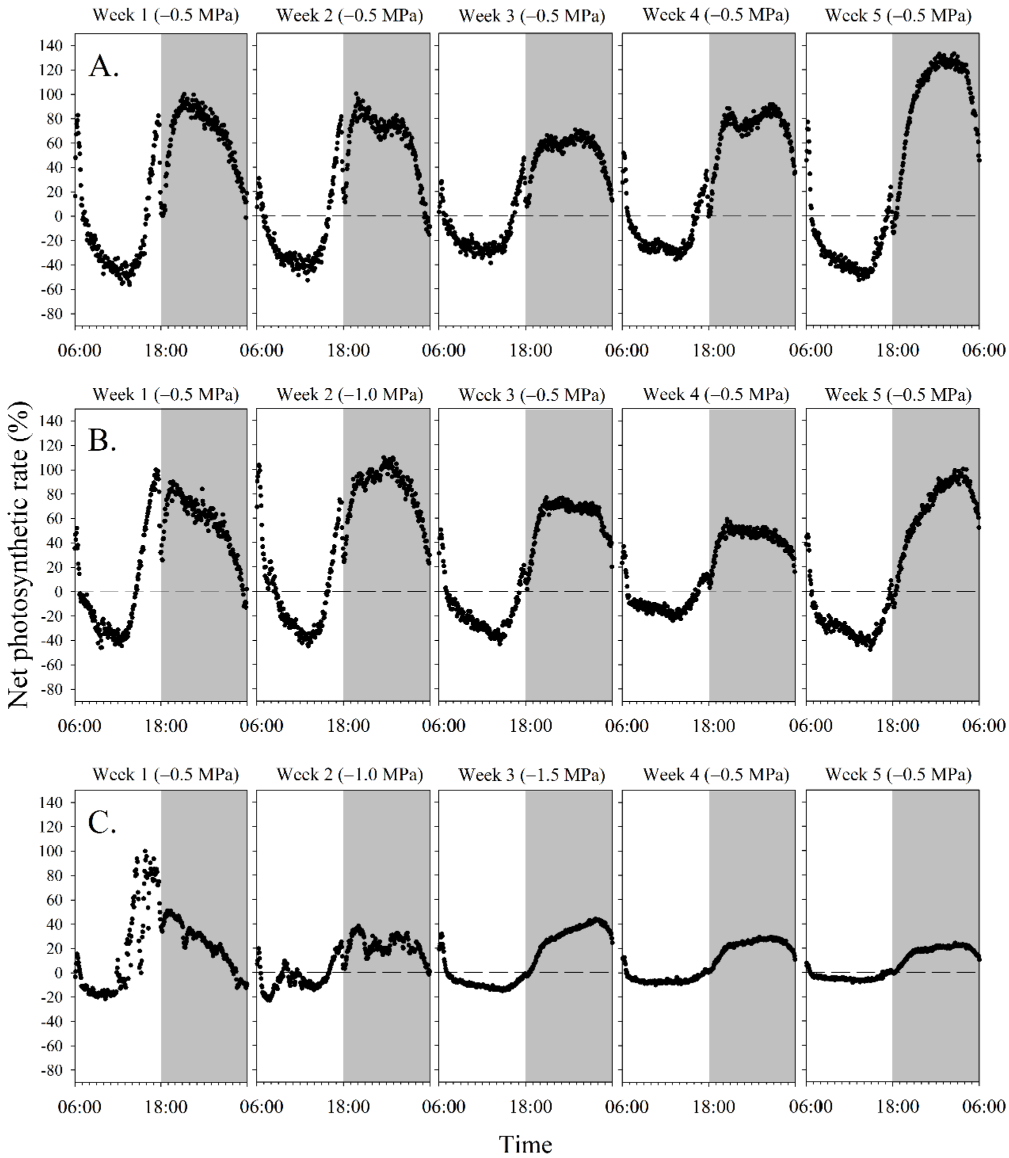

Figure 5. Serial diel photosynthetic patterns of 'Da Hong' micropropagules on day 4 (or forward) after culture solution replacement with different water potentials. The relative Pn of control (**A**), moderate water stress (**B**), and intensified water stress (**C**) were compared to week 1. Gray blocks represent the dark period of experiments.

Table 2. Relative integrated net CO_2 uptake of in vitro 'Da Hong' micropropagules under mannitol-induced water stress treatments.

Treatments [1]	Relative Integrated Net CO_2 Uptake to Week 1 (%)						
	Weeks after Treatment					Mean	
	1	2	3	4	5		
Control	100 ± 0	99.80 ± 35.13	87.55 ± 41.38	120.43 ± 39.44	158.21 ± 96.73	113.20 ± 12.43	A [2]
Moderate water stress	100 ± 0	143.21 ± 16.60	69.14 ± 23.49	71.76 ± 38.26	88.51 ± 21.61	94.52 ± 13.41	A
Intensified water stress	100 ± 0	46.03 ± 21.82	59.92 ± 18.29	39.34 ± 7.04 *	32.27 ± 3.16 *	55.51 ± 12.02	B

[1] Treatments: control, water potentials (WP) of exchanged culture solutions were −0.5 MPa every week; moderate water stress, the WP of the culture solution on week 2 was −1.0 MPa induced by mannitol but −0.5 MPa for the rest of the weeks; intensified water stress, WP of culture solutions on weeks 2 and 3 were −0.5 and −1.0 MPa, and that of the other weeks were −0.5 MPa. [2] Means followed by the same letters within the same column were not significantly different at α = 0.05 via Fisher's protected LSD test. In addition, * represents a significant difference between the treating week and week 1 in the same row at α = 0.05 via the Student's paired *t*-test. n = 3–4 (mean ± SE).

The average INCU of MWS treatment was similar to that of control but was significantly low in IWS treatment (df(2,12) = 5.43, $LSD_{0.05}$ = 38.93, P = 0.021), with values of 94.52%, 113.20%, and 55.51%, respectively. An increasing Pn trend was displayed in weekly −0.5 MPa solution treatments, whereas WP recovery diminished MWS effects on INCU compared with IWS treatment.

3.4. Photosynthetic Difference Intensity to Water Stress Application

To clarify the WS effects on diel photosynthetic pattern status, the Pn difference was compared with that on week 1 and displayed after solution replacement. In addition, previous data were transformed into logarithm base 10 to amplify difference expressions.

The Pn difference intensity of the control (−0.5 MPa) was 1–2 under logarithmic transformation (Figure 6A,B), whereas the variation in MWS treatment increased from 1 to circa (ca.) 2.5 (Figure 6C,D). Both control and MWS treatments manifested intensities in the differences at dawn (Phase II), evening (Phase IV), and predawn the next day (Phase I), where the evening expression after MWS treatment was earlier, i.e., from ca. 18:00 h to ca. 15:30 h, indicating that Pn decrease was earlier in MWS treatment.

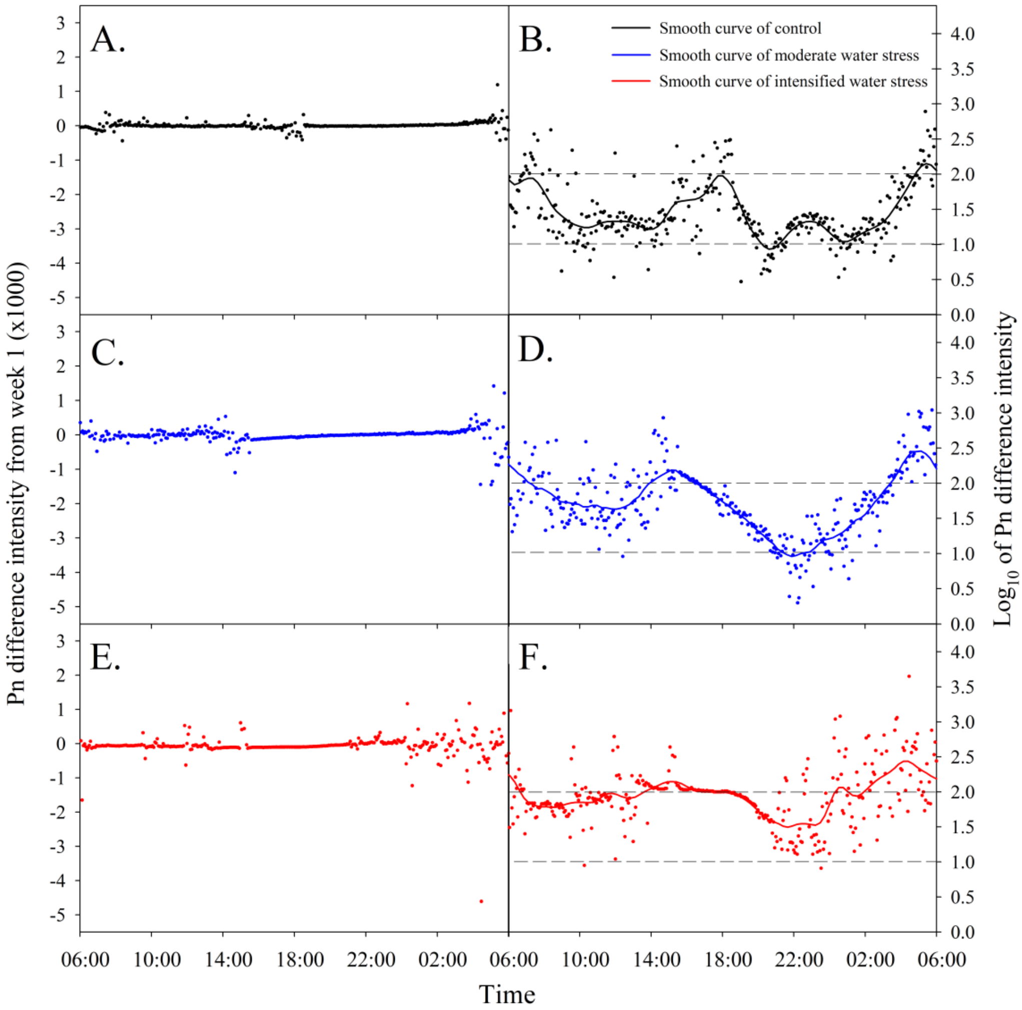

Figure 6. Differences in the net photosynthetic rate (Pn) intensity in 'Da Hong' after various water potential (WP) treatments compared to week 1. The relative Pn and logarithm of Pn difference intensity of the control (**A**,**B**), moderate water stress (**C**,**D**), and intensified water stress (**E**,**F**) are shown, respectively. The intensity difference in Pn was calculated as the mean weekly discrepancy in Pn compared with week one after treatment with solutions at different WP.

Micropropagules grown in IWS condition dramatically decreased Pn during nighttime, especially from 01:00 h to 06:00 h. The difference intensity of Pn in logarithm was advanced from ca. 05:30 h to ca. 04:30 h (Phase I) compared to the control and MWS treatments (Figure 6E,F). In summary, Pn expression of 'Da Hong' was sensitive at Phases II, IV, and I pre-decreased with WS.

4. Discussion

CO_2 uptake response to WS elimination depends on species and CAM types [12,19]. However, little information is available from the literature on red-fleshed pitaya 'Da Hong,' which is cultivated at >50% in Taiwan [40,60]. To discover photosynthetic pattern fluctuations after dynamic WS induced by mannitol, this study investigated 'Da Hong' responses under in vitro conditions. The results indicated that Pn was not influenced by MWS treatment.

4.1. Photosynthesis Expression Response to Airflow Rates

A gas exchange rate of 0.94 vessel/min at a flow rate of 600 mL/min was used in this study. The maximum Pn at the previous rate was 0.33 μmol/m^2/s under a 12-h photoperiod, and a Pn percentage to that at 1000 mL/min was 68.03%, lower than 3.8 μmol/m^2/s under a 16-h photoperiod in the identical system described by Lee et al. [32]. Irradiation increased with a prolonged photoperiod at a similar light intensity. Previous results proved that lower irradiation decreased Pn and diel carbon assimilation in CAM plants [61,62].

In general, an increased exchange rate increases air disturbance and decreases temperature accumulation within the plant architecture inside the vessels; however, it causes insufficient gas exchange between the plant and the environment, which decreases photosynthesis expression [28,63,64]. Therefore, a 35% Pn in *Malus domestica* at ca. 5.5 vessel/min of the exchange rate compared to leaf performance [65] and a similar reduction of photosynthesis was reported in *Prunus avium* at a flow rate situation of 1–3 vessel exchange/min [66]. Thus, an appropriate exchange rate provided favorable photosynthesis expression. Because similar Pn and diel INCU were discovered between 200 and 600 mL/min (ca. 0.31–0.94 vessel/min) in this study, a proper exchange rate (0.94 vessel/min) was used after considering the system capacity under constant cooling conditions. Regarding further field verification of results in the climate change situation and photosynthetic pattern on field-grown plants, intertwined shoots of field-grown pitaya require trimming and pruning to increase air circulation. In addition, a proper transparent vessel size suits the canopy of a target plant, promoting gas exchange and decreasing air stagnation [67].

This study showed a diel pattern of CO_2 uptake change on day 4 after mannitol-induced MS solution replacement. The offset performance of the gas exchange pattern might respond to the circadian clock, which is modulated by PEPC kinase [68] and light quality [59,69]. PEPC kinase is upregulated by phosphoenolpyruvate and downregulated by cytosolic malate abundance, where the nighttime accumulation of malate decreases the activities of PEPC kinase and PEPC, affecting CO_2 uptake [68,70]. A WS-related stomatal closure decreased CO_2 uptake via phosphoenolpyruvate, influencing diel pattern fluctuation [15,71]. However, this study provided little information on the delayed transition of diel pattern upon replacement with solutions at various WP. Experiments on stomatal conductance and photosynthetic enzyme activity [17] are essential to elucidate this change in response to the WS environment in the climate change situation.

4.2. Response of Photosynthesis to Dynamic Water Stress

In in vitro conditions, a Pn of 2.63 μmol/m^2/s in 'Da Hong' was similar to that of *H. undatus* with 2.26 μmol/m^2/s in a water-sufficient environment [31,32]. However, this study demonstrated that the Pn of 'Da Hong' decreased with WP gradients, where IWS treatment dramatically decreased Pn compared to plants grown in the medium with high WP condition. Consistent with these results, *H. undatus* cultured in MS medium with a WP of −0.92 MPa had 25.2% Pn compared to the WP of −0.54 MPa under a 16-h photoperiod [31]. On the field scale, potted *H. undatus* limited 63% of stomatal conductance and 57% of Pn during 12 days of water withholding, and the soil WP was −4.2 MPa [18]. These results supported the proposal that sufficient WP of medium promotes adequate carbon assimilation to plants, as interpreted by titratable acid accumulation in pitaya succulents [14,17].

Irrigation is an essential practice that ameliorates the Pn of field-grown plants under climate change circumstances [4,5]. Rewatering recovered 50% Pn of *H. undatus* in 2 days and 100% in 7 days after 10 to 12 days of WS, where the soil WP decreased from −3.2 to −4.2 MPa during water withholding [18,29]. In comparison, this study illustrated that WS with a decreasing WP to −1.5 MPa led to an unrecoverable Pn in 'Da Hong' in vitro, which might result from poorly maintained photosynthesis during WS by unfulfilled succulent shoots of micropropagules [72].

Responses to rewatering after WS on photosynthesis were investigated in herbaceous plants, such as maize and potato in field conditions, illustrating a similar trend that increasing WS decreased the Pn of potato without full recovery by irrigation, and the Pn decrease level was dependent on crop growth stages [73,74]. WS impairing the photosynthetic apparatus [17,21,75] and related enzyme activities [5,21,76] reduces the photosynthetic capacity. Opinions on plant physiological juvenile status [12], high humidity environment, or photoheterotrophic conditions [77] beyond this study refer to in vitro photosynthesis fluctuation that requires consideration.

Compared to Pn detected from a single time point, diel INCU represents a full picture of carbon assimilation. However, information regarding WP recovery is nearly inaccessible for economic crops, including white- and red-fleshed field-grown pitaya. Despite restoring WP, this study obtained a dramatically reduced diel CO_2 uptake during a 3-week progressive increase in WS (from −0.5 MPa to −1.5 MPa). The diel INCU of *H. undatus* decreased from ca. 200 to ca. 50 $mmol/m^2/day$ after >24 days of water withholding [30]. Moreover, only 6% of the 24-h CO_2 uptake was retained after 22 days of drought stress in wild *K. laxiflora* [78]. However, soil WP from both studies were unavailable.

In contrast, 'Da Hong' micropropagules exhibited a similar relative CO_2 uptake to week 1 after treatment with 1-week −1.0 MPa solution and 3-week rehydration (i.e., MWS treatment), paralleling Pn expression. Similarly, a full recovery of diel CO_2 uptake from 22 days of water deficit and 4 days of rewatering was demonstrated in *K. laxiflora* [78]. A 91.1% return to nocturnal malate formation was illustrated in CAM bromeliad *Aechmea* 'Maya' after 40 days of recovery [79]. Because carbon assimilation and Pn recovery are performed in two stages, i.e., stomatal and non-stomatal restriction repair [76,80], and WS sensitivity varies from maturation of leaves/organs [81,82], on-farm experiments on aging plants are further required after considering the results of this study. These results demonstrated that the Pn and diel integrated carbon assimilation of 'Da Hong' micropropagules were undamaged by MWS treatment (i.e., −1.0 MPa for 1 week and followed by 3 weeks of −0.5 MPa).

4.3. Regulated Photosynthetic Pattern to Dynamic Water Stress

Earlier Pn difference intensity from this study illustrated a response to CO_2 deficiency upon stomatal closure. CAM plants under a prolonged WS duration reduced stomatal open period in Phases IV and II [83] and increased CO_2 uptake in Phase I [68]. However, carbon assimilation expression in Phase I was reduced under intensified stress conditions [15,71]. These results exhibited a similar trend in Phase IV (evening) with MWS treatment, where Pn decreased earlier than that in steady watering conditions. However, there was a progressive increase in Pn difference intensity during Phase I (nighttime) after IWS treatment. This study indirectly verified that partially decreased CO_2 uptake in potted *H. undatus* improved from a short-term WS followed by rehydration [14], whereas no information was available on Pn recovery in plants with IWS [18].

Insufficient organic acid accumulation at night influences daytime photosynthate assimilation, affecting plant development and extending to the CAM phase scale of diel

CO_2 uptake [70,82]. Recognizing that the metabolic and biochemical processes during phase transition after plants are subjected to WS and rehydration renders references to water supply on photosynthate amelioration, improving crop yield in the climate-change environment. In this study, the Pn difference intensity compared to week 1 under logarithm transformation expressed CO_2 uptake deficiency during phase shift by gradient WS. Both in vitro- and field-grown plants are required to investigate the dynamic performance of antioxidative metabolism, photosynthetic enzyme activity, and carbon allocation, partition, and utilization in pitaya to evaluate the effects of WS on CAM phase shift.

5. Conclusions

Pn and diel INCU sensitivity to WP gradients were evaluated via an open-flow in vitro system in 'Da Hong' pitaya. WS conditions were created using modified vessels to replace culture solutions, enabling dynamic photosynthesis under long-term monitoring, where mannitol modulated water availability for plants. Pn and diel CO_2 uptake increased at an airflow rate from 200 to 1000 mL/min, and a 600-mL/min airflow rate was used in this study, considering system gas exchange capability. An increased trend of Pn was exhibited in control (−0.5 MPa) compared to MWS conditions (−1.0 MPa on week 2) after 5 successive weeks of manipulation. Predictably, IWS conditions (−1.0 and −1.5 MPa on weeks 2 and 3, respectively) dramatically decreased photosynthesis expression without repair under a WP of −0.5 MPa for 2 weeks.

The relative INCU on week 1 was analogous between the control and MWS treatments during the 5-week treatment period. In contrast, there was a significant decrease in diel CO_2 uptake under IWS treatment. WS intensities increased the magnitude of the diel Pn difference intensity compared to week 1. Similarly, the peak occurrence of Pn difference intensity was in advance, paralleled by WS, in which an earlier decrease of CO_2 uptake occurred in Phase IV.

In conclusion, a temporary MWS (WP of −1.0 MPa at week 2) with 3-week rehydration did not reduce the photosynthesis of 'Da Hong' plants in vitro, indicating the ability of photosynthesis to respond to short-term WS. However, maintaining moderate WP should be critical to the enhancement of photosynthesis. Different metabolic and biochemical processes at different phases of the CAM pathway require further investigation. In this study, the WP of the culture medium would provide a reference for pitaya photosynthesis, and future field-scale validation could assist on-farm water management.

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