Limitations in photosynthesis of sugar beets under water deficit and rehydration conditions

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Abstract

Background: Limiting factors in photosynthesis includes stomatal limitations, mesophyll limitations, and biochemical limitations, and mesophyll limitations are the leading limiting factors in photosynthesis as shown by many studies. Mesophyll conductance (gm) exerts significant influences on the photosynthetic capacity of crops, playing a decisive role in mesophyll limitations. The limiting factors in photosynthesis for many crops under water deficit and rehydration conditions have been studied, but few studies of this kind on sugar beet can be found. Results: Our study aimed to analyze the dynamic changes of mesophyll limitations, stomatal limitations, and biochemical limitations by examining the influences of water deficit and rehydration on photosynthetic characteristics, electron transport, and leaf anatomical structure. According to the analysis on photosynthetic characteristics, severe deficit irrigation treatment significantly decreased photosynthesis rate, light saturation point, and CO2 saturation point. After rehydration, there was no difference in photosynthetic characteristics between the well irrigated sugar beets and the ones with severe deficit irrigation treatment. The analysis on leaf anatomical structure found that sugar beets dealt with water deficit by increasing leaf (mesophyll) thickness, and the severe deficit irrigation treatment significantly decreased the number of chloroplasts without influencing each one in terms of shape and cross-section area. No significant influence of water deficit was found on the electron transport rate (Jflu) of sugar beets during the process of CO2 assimilation. Conclusions: Therefore, deficit irrigation treatments cannot significantly influence photosynthetic assimilation of sugar beets. The analysis of limitations in photosynthesis showed that the stomatal and biochemical limitations increased while the mesophyll limitations decreased when sugar beets were under water deficit; however, the stomatal and biochemical limitations decreased while the mesophyll limitations increased after rehydration. The photosynthesis of sugar beets was mainly influenced by mesophyll limitations, and the limiting factors changed significantly only for severe deficit irrigation treatment.

Background

The most obvious influence on plants brought by water deficit is the decrease of water availability which is the leading factor limiting the growth process of plants (Chaves et al., 2009; Leufen et al., 2013). The low water availability influences crop carbon balance which depends largely on photosynthesis. Thus, the influence of water shortage on photosynthesis has been the research topic for decades. Meanwhile, leading limiting factors in photosynthesis for crops under water deficit have drawn great attention (Flexas et al., 2009; Flexas et al., 2016; Hernandez-Santana et al., 2017).

Stomatal limitations, mesophyll limitations, and biochemical limitations are the main limiting factors in photosynthesis according to the previous researches (Flexas et al., 2016). Initially, stomatal limitations were categorized as stomatal factors by researchers while mesophyll and biochemical limitations were categorized as non-stomatal factors. As time goes by, categorization varied. Stomatal and mesophyll limitations were categorized as CO2 diffusion factors, while biochemical limitations were categorized as non-CO2 diffusion factors (Flexas et al., 2009; Flexas et al., 2012; Flexas et al., 2014); it was confirmed that limitations in CO2 diffusion might exist in all photosynthetic processes involving CO2, and that the limitations in photosynthetic processes not involving CO2 were biochemical limitations. Many researches had shown that mesophyll limitations (belonging to the CO2-diffusion limitations) significantly decreased photosynthesis rate, and might be the leading limiting factors in photosynthesis process (Flexas et al., 2008; Galmes et al., 2014; Tosens et al., 2016; Peguero-Pina et al., 2017), but research on Oliver trees conducted by Hernandez-Santana et al. (2017) showed that stomatal limitations were the leading limiting factors in photosynthesis. Evans et al. (2009) reported that in most cases, stomatal closure decreased photosynthetic rate, and the pathway (from the ambient air outside the leaves to carboxylation center) for CO2 changed when water deficit occurred. The decrease of CO2 diffusion was because of stomatal closure and the decrease of mesophyll conductance (gm) (Han et al., 2018). Therefore, in most cases, the decrease of CO2 diffusion by water deficit includes: (1) the decrease of CO2 diffusion from the ambient air outside the leaves to substomatal cavities via stoma (stage of stomatal limitations); (2) the decrease of CO2 diffusion from substomatal cavities to carboxylation center (stage of mesophyll limitations) (Chaves et al., 2009; Erismann et al., 2008). gm is a quantitative indicator for CO2 diffusing from stoma to carboxylation sites (Flexas et al., 2012); it is limited and varied, and mainly functions on CO2 diffusion. The
mesophyll conductance varies significantly for crop leaves with different shapes and characteristics, and is closely related to plant species and leaf anatomical characteristics (Tomas et al., 2013; Flexas et al., 2016). It has been proved that mesophyll conductance increases with the increase of leaf thickness while it decreases with the increase of leaf density (Hassiotou et al., 2010). Zhou et al. (2007) reported that the responses of photosynthesis to water deficit contained two stages. With stomatal conductance ($g_s$) as the boundary, the first stage was that when stomatal conductance was greater than 0.05-0.10 mol H$_2$O m$^{-2}$ s$^{-1}$, photosynthesis rate was mainly influenced by CO$_2$-diffusion limitations; the second stage was that when stomatal conductance was lower than 0.05-0.10 mol H$_2$O m$^{-2}$ s$^{-1}$, CO$_2$-diffusion limitations did not significantly affect photosynthesis, but metabolic disorders occurred in plants, especially, in this case, high intensity light was prone to oxidative stress.

Acclimation usually occurs after water deficit lasting a certain period of time; it includes changes in plant physiology, morphology, and even genes (Flexas et al., 2009; Flexas et al., 2014). In the process of leaves adapting to water deficit, acclimated leaves tend to exhibit a higher photosynthetic assimilation rate, which are related to the anatomical structure of acclimated leaves as well as their higher electron transport rate compared with those of the non-acclimated leaves (Galme’s et al., 2006; Flexas et al., 2009). In leaves, CO$_2$ diffusion involves two stages: the intercellular gas stage and the cellular liquid stage. The gas stage pathway through intercellular air spaces has less influence on CO$_2$ diffusion compared with liquid stage pathway generally (Evans et al., 2009; Tomas et al. 2013). Within the intercellular gas stage, the CO$_2$ diffusion through intercellular air spaces is influenced by leaf structural characteristics (Han et al., 2018). Cellular liquid stage is consisted of cell and chloroplast, especially the cell wall and chloroplast membrane have the largest limitations for CO$_2$ diffusion (Terashima et al., 2011), besides, the chloroplast size and surface area exposing to intercellular air spaces of mesophyll and chloroplasts ($S_m$/S and $S_c$/S) also have significant influences (Han et al., 2018). Moreover, the effect of rehydration after water deficit on crops and the possibility for photosynthetic rate to be fully restored are also research topics. Generally speaking, the CO$_2$ assimilation rate for crops under severe water deficit can restore 40% - 60% of the maximum rate on the next day after rehydation, but the maximum photosynthetic rate cannot be restored despite it keeps on restoring in the following days (Flexas., 2009). Gomes et al. (2008) pointed out in his research on limitations in photosynthesis that the restoring of CO$_2$ assimilation rate was subjected more to mesophyll limitations than stomatal limitations. This might be due to the great limitations in restoring mesophyll conductance after rehydration.

Currently, few researches can be found on limiting factors in photosynthesis for sugar beets under water deficit and rehydration conditions, especially on the changes of stoma, mesophyll, and biochemical limitations during rehydration. Therefore, the purpose of this study was to analyze how photosynthesis was influenced by the increase of water deficit degree, acclimation, and restoration, and to determine the limitations in photosynthesis during water deficit and rehydration for sugar beets, including: (i) to analyze the influences of water deficit and rehydration on leaves’ anatomic characteristics in terms of acclimation and restoring; (ii) to relate anatomical difference of leaves to structural characteristics such as mesophyll conductance; (iii) to quantify the stomatal, mesophyll, and biochemical limitations in photosynthesis for sugar beets during water deficit and rehydration.

**Results**

**Chlorophyll and chloroplasts**

Before irrigation (on Day 1), the content of chlorophyll (a+b) and chlorophyll a/b, and the number and length of chloroplasts increased with the increase of water deficit degree (Table 2). The contents of chlorophyll (a+b) for moderate and severe deficit irrigation treatments decreased by 21% and 52%, respectively compared with that for well irrigated treatment. The chloroplast thickness for severe deficit irrigation treatment was the largest. The cross-section areas of chloroplasts for moderate and severe deficit irrigation treatments decreased by 22% and 17%, respectively compared with that for well irrigated treatment (Table 3). After rehydration (on Day 3, 4, and 5), the content of chlorophyll (a+b) and chlorophyll a/b for severe deficit irrigation treatment significantly decreased by 32%, 30%, 5%, and 22%, respectively compared with those for well irrigated and moderate deficit irrigation treatments on Day 3, however, they were 10%, 12.5%, 20%, and 4% higher than those for well irrigated and moderate...
deficit irrigation treatments on Day 4, respectively. The number of chloroplasts decreased with the increase of water deficit degree on Day 5. The length, thickness, and cross-section area of chloroplasts for severe deficit irrigation treatment were 15%, 12%, and 23% greater than those for well irrigated treatment, respectively, and were 22%, 35%, and 48% greater than those for moderate deficit irrigation treatment, respectively, on Day 5.

**A\textsubscript{N}-PAR curves**

The net CO\textsubscript{2} assimilation rate of sugar beets increased with increase of photosynthetically active radiation firstly, and then remained stable (Fig. 2.). Before irrigation (on Day 1), the curve for moderate deficit irrigation treatment was higher than those for well irrigated and severe deficit irrigation treatments. The light saturation point for severe deficit irrigation treatment appeared at approximately 500 µmol m\textsuperscript{-2} s\textsuperscript{-1}, earlier than those for well irrigated and moderate deficit irrigation treatments, and A\textsubscript{N} stopped rising at about 10 µmol CO\textsubscript{2} m\textsuperscript{-2} s\textsuperscript{-1}. After rehydration (on Day 3, 4, and 5), the light saturation point for severe deficit irrigation treatment shifted to 1500 µmol m\textsuperscript{-2} s\textsuperscript{-1}, and A\textsubscript{N} was at 20µmol CO\textsubscript{2} m\textsuperscript{-2} s\textsuperscript{-1}. Till Day 5, the curve for severe deficit irrigation treatment was higher than that for moderate deficit irrigation treatment.

**A\textsubscript{N}-C\textsubscript{i} curves**

The net CO\textsubscript{2} assimilation rate of sugar beet leaves increased with the increase of intercellular CO\textsubscript{2} concentration, and stopped rising at a certain concentration (Fig. 3.). Before irrigation (on Day 1), the curve for moderate deficit irrigation treatment was higher than those for well irrigated and severe deficit irrigation treatments. The CO\textsubscript{2} saturation point for severe deficit irrigation treatment appeared at 300 µmol mol\textsuperscript{-1} firstly, and A\textsubscript{N} stopped rising after reaching 10 µmol CO\textsubscript{2} m\textsuperscript{-2} s\textsuperscript{-1}. After rehydration (on Day 3), the curves for moderate and severe deficit irrigation treatments were higher than that for well irrigated treatment, and the CO\textsubscript{2} saturation point rose to 800 µmol mol\textsuperscript{-1}; while the curve for well irrigated treatment was higher than those for moderate and severe deficit irrigation treatments on Day 4 and 5. When the CO\textsubscript{2} saturation point rose to 1,000 µmol mol\textsuperscript{-1} on Day 5, A\textsubscript{N} reached its maximum, 50 µmol CO\textsubscript{2} m\textsuperscript{-2} s\textsuperscript{-1}.

**Electron transport**

The electron transport rate of chlorophyll fluorescence of sugar beet leaves increased with the increase of PPFD firstly, and stopped rising till a certain value of PPFD (Fig. 4.). Before irrigation (on Day 1), the PPFD-J\textsubscript{fl} curve for well irrigated treatment was higher than those for moderate and severe deficit irrigation treatments. J\textsubscript{fl}s for moderate and severe deficit irrigation treatments reached the maximum (150 µmol m\textsuperscript{-2} s\textsuperscript{-1}) firstly, when PPFD was approximately 180 µmol CO\textsubscript{2} m\textsuperscript{-2} s\textsuperscript{-1}. After rehydration, the curve for severe deficit irrigation treatment almost overlapped that for well irrigated treatment on Day 3, and almost overlapped that for moderate deficit irrigation treatment on Day 4. All curves overlapped on Day 5.

**Quantitative analysis of limitations in photosynthesis**

Mesophyll limitations were the main limitations in photosynthesis of sugar beets for the three treatments (Fig. 5.). Before irrigation (on Day 1), mesophyll limitations accounted for 60% and 70% of all limitations for well irrigated and moderate deficit irrigation treatments, respectively, while mesophyll limitations and stomatal limitations accounted for 30% and 35%, respectively for severe deficit irrigation treatment. After rehydration (on Day 3, 4, and 5), stomatal limitations for well irrigated treatment decreased; stomatal limitations increased but biochemical limitations decreased for moderate deficit irrigation treatment; and stomatal and biochemical limitations decreased but mesophyll limitations increased for severe deficit irrigation treatment.

**Discussion**

There is a urgent need for simultaneously improving photosynthesis approaches that consist of agronomical and physiological, among which one is to improve the photosynthetic rate of plants leaves (Flexas et al., 2016), and it is necessary to learn about
what limits the photosynthetic rate. Some researchers concluded that the decrease of chlorophyll content resulted in the changes of light distribution on canopy, leading to the changes of CO₂ assimilation rate of leaves and canopy, consequently, the yields decreased (Zhu et al., 2010; Ort et al., 2011; Drewry et al., 2014; Slattery et al., 2017). However, in a two-year experiment on beans and other crops, Slattery et al. (2017) showed that even if chlorophyll concentration decreased by more than 50%, it had little effect on dry matter accumulation and yield. In this study, only the chlorophyll concentration for severe deficit irrigation decreased significantly before irrigation (Day 1), and the CO₂ assimilation rate for severe deficit irrigation treatment reached its maximum value at a low photosynthetically active radiation, which may be related to the decrease of chlorophyll concentration (Slattery et al., 2017); while the CO₂ assimilation rate for moderate deficit irrigation and well irrigated treatments reached its maximum value at a high photosynthetically active radiation, and there was no significant difference in chlorophyll concentration between the two treatments. The numbers of chloroplasts for moderate and severe deficit irrigation treatments significantly decreased, but no significant influence on the cross-section area of chloroplasts was found. This also confirmed the study results of Sade. (2017) that environmental factors, including water deficit, affected the photosynthetic apparatus of crops, and decreased photosynthetic rate by accelerating chloroplast degradation. After rehydration (Day 5), the chlorophyll concentration for severe deficit irrigation was significantly higher than that for moderate deficit irrigation and well irrigated treatments. At this time, the light saturation point of A_N-PAR curve for severe deficit irrigation treatment shifted to the right, approaching the light saturation points for moderate deficit irrigation and well irrigated treatments. There was no significant difference in the number and cross-section area of chloroplasts among the three treatments. Therefore, regardless of the degree of water deficit and rehydration, there was no significant relationship between CO₂ assimilation rate and chlorophyll content, chloroplast number, and cross-section area (Slattery et al., 2017), so chlorophyll contributed little to the limitations in photosynthesis of sugar beets under deficit irrigation treatments.

Leaf anatomical structure plays a decisive role in photosynthesis of plants, and how plant tissues respond to water deficit is also determined by anatomical characteristics of leaves (Haffani et al., 2017). Recently, a new method for detecting plant water has been reported, which predicts water deficit according to the change of leaf thickness; it indicates the relationship between leaf thickness and water availability of plants (Afzal et al., 2017). Thick leaves and mesophyll tissues can increase water availability, which is an adaptive mechanism for crops to cope with water deficit (Boughalleb et al., 2015; Haffani et al., 2017). Because thick leaves and abundant mesophyll tissues can increase the carboxylation sites of CO₂ and help maintain the CO₂ assimilation rate and stomatal conductance (Das et al., 2015). It is concluded that in most cases, water deficit decreases the mesophyll thickness and tissues of crops (Makbul et al., 2011; Osman et al., 2011). In our study, however, on Day 1, the thickness of leaves and mesophyll increased with the increase of the degree of water deficit, especially, the thickness of leaves and mesophyll for severe deficit irrigation treatment were significantly greater than those for well irrigated and moderate deficit irrigation treatments (Table 1). The leaf thickness increased while the CO₂ assimilation rate remained unchanged for severe deficit irrigation treatment, which could be justified by the quantitative analyse of limitations in photosynthesis. Although the leaf thickness for severe deficit irrigation treatment increased, but it was greatly influenced by stomatal and biochemical limitations. The area and volume of mesophyll cells for moderate deficit irrigation treatment decreased, leading to the significant increase of stomatal conductance and volume fraction of intercellular air space (f_ias), the area and volume of mesophyll cells for severe deficit irrigation treatment increased, leading to the increase of mesophyll conductance and S_m/S as well as the significant decrease of g_s and f_ias. This indicted that for severe deficit irrigation treatment, sugar beets cope with water deficit by increasing the area and volume of mesophyll cells to increase the thickness of leaves and mesophyll tissues, thus increasing mesophyll conductance and S_m/S, while for moderate deficit irrigation treatment, stomatal conductance and f_ias were increased to cope with water deficit. Onoda et al. (2017) and Peguero-Pina et al. (2017) reported that S_m/S, S_f/S, and f_ias had great influence on mesophyll conductance and CO₂ assimilation rate. After rehydration (on Day 3, 4, and 5), the thickness of leaves and mesophyll tissues, the area and volume of mesophyll cells, g_uf, g_m, and S_m/S decreased with the increase of water deficit degree, while F_ias showed an opposite trend (Table 1). Ennajeh et al. (2010) showed that water deficit increased the thickness of leaves and mesophyll tissues of Oliver trees, and a high CO₂ assimilation rate was retained by increasing CO₂ carboxylation sites, but the decrease of stomatal conductance due to water deficit was overlooked. Flexas et al. (2009) d that once the water deficit occurred and remained for a period of time, g_s tend to be more stable than g_m. g_s halved
and remained stable for a week, while \( g_m \) was not influenced at the initial stage of water deficit, but became unstable at the later stage. It does not completely comply with our findings, because Flexas et al. conducted rehydration after the desirable water deficit degree was achieved and remained for a certain period of time. However, in our study, rehydration was carried out immediately after reaching the desirable degree of water deficit. Besides, the influence of water deficit and rehydration is related to crop species and varieties (Ennajeh et al., 2010; Slattery et al., 2017).

A\(_N\)-C\(_i\) curve plays an important role in evaluating the limiting factors of CO\(_2\) assimilation rate (Sharkey et al., 2016; Han et al., 2018), and indicates the utilization of low and high concentration CO\(_2\) at different conditions. Shibuya et al. (2017) showed that the decrease of C\(_i\) did not cause a decrease in the CO\(_2\) assimilation rate. The possible reason was that the decrease of C\(_i\) did not reach the extent to decrease the CO\(_2\) assimilation rate, and he showed that A\(_N\)-C\(_i\) curves differed little from each other under different water conditions, and the carboxylation efficiency was not influenced. J\(_{flu}\) is an important indicator of leaf photochemical activity which is reflected by CO\(_2\) assimilation rate. Differing from carboxylation efficiency, photochemical activity tends to remain stable and is subjected to water conditions (Flexas et al., 2009). Flexas et al. (2009) proved that moderate water deficit decreased the CO\(_2\) assimilation rate via diffusion limitation, and J\(_{flu}\)s were similar regardless of water deficit. In this study, when sugar beets were in water deficit (on Day 1), the utilization of sufficient CO\(_2\) for severe deficit irrigation treatment was only close to 500 \( \mu \text{mol m}^{-2} \text{s}^{-1} \), while those for well irrigated and moderate deficit irrigation treatments ranged from 1000 to 1200 \( \mu \text{mol m}^{-2} \text{s}^{-1} \). The maximum CO\(_2\) assimilation rate for severe deficit irrigation treatment was about 20 \( \mu \text{mol m}^{-2} \text{s}^{-1} \), while those for well irrigated and moderate deficit irrigation treatments were 40-50 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) (Fig. 2.). On Day 1, J\(_{flu}\)s increased with the increase of PPFD and remained stable when reaching a certain value. In the range of 0-80 \( \mu \text{mol m}^{-2} \text{s}^{-1} \), J\(_{flu}\)s for the three treatments were the same; when it was above 80 \( \mu \text{mol m}^{-2} \text{s}^{-1} \), J\(_{flu}\) for well irrigated treatment was greater than those for moderate and severe deficit irrigation treatments. After rehydration (on Day 3, 4, and 5), A\(_N\)-C\(_i\) curves changed significantly compared with those on Day 1, especially, the CO\(_2\) concentration greater than 800 \( \mu \text{mol m}^{-1} \) could be utilized for severe deficit irrigation treatment, increasing by 60% or more compared with the previous 500 \( \mu \text{mol m}^{-1} \). As time goes by, A\(_N\)-C\(_i\) curves decreased with the increase of water deficit degree. Flexas et al. (2009) reported that photosynthesis progressively declined in well irrigated plants, which was not due to diffusional limitations, but rather to decreased photosynthetic capacity, as reflected by decreased J\(_{flu}\). In our study, the difference in J\(_{flu}\) among the three treatments was smaller after the water deficit was gone. All curves for the three treatments overlapped till Day 5, which was different with the results of Flexas. et al. (2009) on grape, the reason might be due to the different deficit irrigation methods and different growth stages (Cui et al., 2009).

In general, photosynthesis is often co-limited by stomatal, mesophyll, and biochemical limitations which are in a well-balanced proportionality (Carriquí et al., 2015). The increase in single kind of limiting factors may results in only a modest increase of A\(_N\) because the other limiting factors will increase accordingly, and the increase may be greater than the decrease (Flexas et al., 2016). Water deficit broke the well-balanced proportionality. There are many reports on limiting factors in photosynthetic rate of crops under water deficit (Flexas et al., 2009; Tomás et al., 2013; Tosens et al., 2016). Gomes et al. (2008) reported that the main limiting factor was the \( g_m \) at restoring period after rehydration. But Flexas et al. (2009) showed that stomatal limitations played the leading role in delaying the restoring of CO\(_2\) assimilation rate, however, on the following day after rehydration, the mesophyll limitations were also significant (even more significant than stomatal limitations for crops under moderate water deficit); mesophyll limitations disappeared 2-3 days later while stomatal limitations remained at least one week. In our study, on Day 1, limiting factors in CO\(_2\) assimilation rate varied for the three treatments. For well irrigated treatment, mesophyll limitations were the leading factors, followed by stomatal and biochemical limitations; for moderate deficit irrigation treatment, mesophyll limitations played the leading role, followed by biochemical and stomatal limitations; for severe deficit irrigation treatment, the three limiting factors were similar though stomatal limitations was slightly greater. After rehydration (on Day 3, 4, and 5), mesophyll limitations were still the greatest for well irrigated treatment, even greater than those on Day 1; while stomatal and biochemical limitations decreased; for moderate deficit irrigation treatment, mesophyll limitations were the greatest, while stomatal limitations increased and biochemical limitations decreased compared with those on Day 1; for severe deficit irrigation treatment, mesophyll limitations increased to be the leading limiting factors while stomatal and biochemical limitations decreased (Fig. 4.). Therefore, there were different limiting factors for different treatments. For well irrigated and
moderate deficit irrigation treatments, mesophyll limitations were the leading factors in reducing photosynthetic assimilation rate regardless of water deficit degree, while for severe deficit irrigation treatment, each kind of limitations can decrease the photosynthetic assimilation rate, but after rehydration, mesophyll limitations played a leading role. Above all, only when the water deficit reached a certain degree, is the photosynthetic assimilation rate of sugar beets subjected to the limitations other than mesophyll limitations. Otherwise, general photosynthetic assimilation rate can be maintained even the crops are under water deficit.

Conclusion

In summary, severe deficit irrigation significantly reduced the CO$_2$ assimilation rate of sugar beet, but returned to the same level as moderate deficit irrigation and well irrigated treatments. Only severe deficit irrigation treatment showed a significant decrease in chlorophyll content, but after rehydration, the chlorophyll content of moderate deficit irrigation and severe deficit irrigation were significantly higher than that of well irrigated irrigation. Severe deficit irrigation significantly reduced chloroplast number, but did not affect chloroplast shape and cross-section area, while it increased the mesophyll (leaf) thickness, which was proven by the increased mesophyll conductance and the decrease of mesophyll intercellular space. The three treatments in this study exerts no significant influence on the electron transport rate during the CO$_2$ assimilation process. Therefore, severe deficit irrigation treatment had no significant influence on the CO$_2$ assimilation for sugar beets. For different deficit irrigation treatments, limiting factors for CO$_2$ assimilation rate varied in time (at the time under water deficit or after rehydration). For well irrigated treatment, mesophyll limitations were the leading factors when undergoing water deficit and rehydration; for moderate deficit irrigation treatment, mesophyll limitations also played the leading role, but stomatal limitations increased while biochemical limitations decreased after rehydration; for severe deficit irrigation treatment, the difference of the three kinds of limitations was small, and mesophyll limitations increased while stomatal and biochemical limitations decreased after rehydration. Generally, mesophyll limitations play a leading role in influencing CO$_2$ assimilation rate of sugar beets, but water deficit can decrease mesophyll limitations and sugar beets can restore in a short period of time after rehydration. Therefore, only severe deficit irrigation treatment can increase the non-CO$_2$ diffusion limitations, but severe deficit irrigation treatment is mainly subjected to CO$_2$ diffusion limitations after rehydration. Except for severe deficit irrigation treatment, the CO$_2$ diffusion limitations are the leading limiting factors reducing photosynthetic assimilation rate of sugar beets.

Methods

Plant material

Pot trials were conducted at Agricultural College of Shihezi University in Xinjiang, China in 2016. The climate is an arid continental climate, with mean daily ET$_o$ and precipitation of 4.8 mm and 0.8 mm, respectively. Seedlings were transplanted from paper tube to 75 L plastic pot (60 cm in height and 40 cm in diameter) containing 80 kg of Calcaric Fluvisol soil after emergence (Table 1). Sugar beets (cv. Beta 356) were transplanted on 18th April and harvested on 1st October. The seeds were bought from BETASEED company. Pots were placed outdoors when there was no rain, and shelters were built when it rains to prevent rainwater from entering the pots, each treatment had eight replications.

Deficit irrigation managements

Sieved soil (80 kg; 3 mm in diameter) was filled into each pot (10 cm soil layer was filled each time 60 cm), and irrigation was conducted to make the soil subside for 1 day. Fertilizers (5 kg organic fertilizers; 10 g N, 12 g P$_2$O$_5$, and 8.7 g K$_2$SO$_4$) were applied to the top 20 cm soil layer without irrigation and subsidence. Soil water content in pots was 17% (soil water content of the 0-20 cm, 20-40 cm, and 40-60 cm soil layers were determined, and then the average value was calculated), soil water capacity was 19% (the method is same as the calculation method of soil water content), soil saturated water content was 26%, and dry soil weight in each pot was calculated to be 68 kg. During the experiment, irrigation amount was controlled by weighing daily and drip irrigation was employed (Supplementary material 1).
Three field capacity treatments were set up during canopy development stage (leaf: 9-28), which were 70% FC for well irrigated group, 50% FC for moderate deficit irrigation treatment, and 30% FC for severe deficit irrigation treatment. As canopy development stage finished, normal irrigation (70% FC) was applied until harvest. During the deficit irrigation stage, when the soil water content dropped to the lower limit of the target FC, the pots were then irrigated to saturated water content. The amount of irrigation water applied to each pot was recorded and used to calculate total water consumption. In this study, the 70% FC treatment was used as the control. On the one hand, 70% FC was the most suitable soil water content for most crops (Chai et al., 2016); on the other hand, sugar beet was a drought-resistant crop (Rytter et al., 2005). Therefore, the 70% FC treatment was used as the control in this experiment, and moderate and severe deficit irrigation treatments were set up on this basis.

The soil weight of a pot corresponding to the target field capacity (FC) = the dry soil weight in the pot + the dry soil weight in the pot × the target soil water content. Therefore, the soil weight of the pots corresponding to 70% FC = 68 + 68 × 19% × 0.7 = 76.8 kg; the soil weight of the pots corresponding to 50% FC = 68 + 68 × 19% × 0.5 = 74.8 kg; and the soil weight of the pots corresponding to 30% FC = 68 + 68 × 19% × 0.3 = 72.1 kg. During the first rehydration, the soil irrigated to the saturated water content of 26%, and the soil weight for each pot was 85.7 kg. Then, when the soil weight for the 70% FC treatment decreased to 76.8 kg, 8.9 kg (85.7 - 76.8) water was irrigated; when the soil weight for the 50% FC treatment decreased to 74.8 kg, 10.9 kg (85.7 - 74.8) water was irrigated; and when the soil weight for the 30% FC treatment decreased to 72.1 kg, 13.6 kg (85.7 - 72.1) water was irrigated. Considering the increasing weight of sugar beet plants over time, the linear relationship between the leaf number and the plant weight under different treatments was fitted (Fig.1) to simulate the plant weight in each pot. The plant weight was subtracted during weighing. The total irrigation amounts during deficit irrigation stage for the 70, 50, and 30% FC treatments were 0.07, 0.06, and 0.04 m³/pot, respectively.

**Instantaneous gas exchange and chlorophyll fluorescence**

From 11:00 h to 14:00, gas exchanges for young and fully expanded main leaves were measured simultaneously using an open gas-exchange system (Li-6400xt; Li-Cor, Inc., Lincoln, NE, USA), and light-response curves were obtained with the light intensities of 0, 11, 36, 69, 106, 146, 203, 368, 624, 986, 1165, and 1391 μmol m⁻²s⁻¹. CO₂-response curves were obtained with different leaves of a plant, with the CO₂ concentrations of 400, 300, 200, 100, 50, 0, 400, 600, 800, 1000, 1200, 1500, and 1800 μmol CO₂ mol⁻¹ and the light intensity of 1800 μmol m⁻² s⁻¹. Once the steady-state of CO₂ concentration was appeared (about 30 min) at 30 °C, a CO₂ response curve was obtained. Chlorophyll fluorescence was measured after 30 min of dark adaption using an imaging pulse amplitude modulated fluorimeter (IMAGMAXI; Heinz Walz, Effeltrich, Germany). Measurement was initiated with dark-adapted leaf tissues characterized by a low fluorescence emission signal (F₀). Then a strong flash of light (4000 μmol m⁻² s⁻¹) by a 0.8 s pulse was exposed to the leaves to measure maximal fluorescence (Fₘ). The maximal photochemical efficiency of PSII (Fᵥ/Fₘ) was calculated using F₀ and Fₘ and Fᵣ is the difference between F₀ and Fₘ. For determining Fₛ (steady-state fluorescence yield), an actinic light source (600 μmol m⁻² s⁻¹) was applied to achieve steady-state photosynthesis, after which a second saturation pulse was applied for 0.7 s to obtain Fₘ' (light-adapted maximum fluorescence). The photosynthetically active radiation (PAR) - electron transport rate (Jₘ) curves were obtained with the light intensities of 0, 11, 36, 69, 106, 146, 203, 368, 624, 986, 1165, and 1391 μmol m⁻² s⁻¹, and then the Jₘ/PFFD curves were obtained.

Chlorophyll, including chlorophyll a (Chl a) and chlorophyll b (Chl b), was extracted with 80% acetone, and their contents (μg g⁻¹ FW) were determined calorimetrically according to the method of Lichtenthaler and Wellburn (1983).

**Estimation of gₘ with Aₙ-Cᵢ and chlorophyll fluorescence**

The curve-fitting method introduced by Sharkey (2016) was used to obtain an alternative estimate of gₘ. This method is based on the changes in the curvature of Aₙ-Cᵢ response curves due to a finite gₘ. By non-linear curve fitting minimizing the sum of
squared model deviations from the data, $g_m$ can be estimated from observed data. The quantum efficiency of PSII photochemistry ($\Phi_{\text{PSII}}$) was calculated as follows (see Equation 1 in the Supplementary Files):

The electron transport rate ($J_{\text{flu}}$) was then calculated as follows: (see Equation 2 in the Supplementary Files)

where $PPFD$ is the photosynthetically active photon flux density, $\alpha$ is leaf absorptance, and $\beta$ reflects the partitioning of absorbed quanta between photosystems II and I (PSI and PSII). $\alpha$ and $\beta$ were assumed to be 0.85 and 0.5, respectively. $g_m$ was estimated by the variable $J$ method (Harley et al., 1992a) : (see Equation 3 in the Supplementary Files)

where $\Gamma^*$ is the CO$_2$ compensation point in the absence of mitochondrial respiration. (See Equation 4 in the Supplementary Files)

where $T_L$ is the leaf temperature (°C). $R_d$ is day respiration. $A_N$ and $C_i$ were obtained from gas exchange measurements at saturating light. The calculated values of $g_m$ were used to covert $A_N$-$C_i$ curves into $A_N$-$C_c$ curves using the following equation: (see Equation 5 in the Supplementary Files)

**Electron microscopy**

Samples (1×1cm) cut from the upper part of sugar beet leaves were quickly placed in FAA solution (5 ml of formaldehyde, 5 ml of glacial acetic acid, and 90 ml of 70% alcohol), and deposited in refrigerator at 4 ℃. Sections of these samples were made and photographed with an electron microscopy (ZEISS-Imager. M2, Germany); photos were processed by Motic Imagers Advanced 3.2 software. Meanwhile, samples of 1 × 4 mm were cut from the same leaf and placed in 2.5% glutaraldehyde fixative solution. Then it was vacuumed to make samples sink. After 3 h, the samples were washed with 0.1 mol L$^{-1}$ phosphate buffer for 3 times, then transferred into 1% osmium acid for 2 h. The samples were washed with 0.1 mol L$^{-1}$ phosphate buffer for 3 times again and dehydrated with 30%, 50%, 70%, 80%, 90%, and 100% acetone gradient. Then, sections were made using LEICAUC 6 Ultra-thin Slicer and double-stained with uranyl acetate and lead citrate. Sections of each sample were placed on a copper net to be observed and photographed with JEM-1230 transmission electron microscopy. The photos were processed with Motic Imagers Advanced 3.2 software.

The surface areas of mesophyll cells and chloroplasts exposing to leaf intercellular air spaces ($S_m/S$ and $S_c/S$) were calculated as follows (Syvertsen et al., 1995): (see Equation 6 in the Supplementary Files)

Where $L_{\text{mes}}$ is the total length of mesophyll cells facing the intercellular air space in the palisade tissue section, $F$ is the curvature correction factor depending on the shape of cells (Thain, 1983; Evans et al., 1994), and $W$ is the width of the section. (see Equation 7 in the Supplementary Files)

Where $L_c$ is the total length of chloroplast surface area facing the intercellular air space in the mesophyll cells.

The volume fraction of intercellular air space ($f_{\text{iab}}$) was determined as follows: (see Equation 8 in the Supplementary Files)

where $t_{\text{mes}}$ is the mesophyll thickness between the two epidermal layers and $\Sigma S$s is the sum of the cross-section area of mesophyll cells.

Chloroplast length ($L_{\text{chl}}$) and thickness ($T_{\text{chl}}$) were obtained at different positions for each sample at ×30 000 magnifications. For a given section, all characteristics were determined with at least three different fields of view, and at least three different sections were analysed. The cross-section for a chloroplast is assumed to be oval. Then, the cross-section area of chloroplast ($\text{Area}_{\text{chl}}$) in the palisade or spongy tissue sections was calculated as follows: (see Equation 9 in the Supplementary Files)

where $\pi$ is the ratio of the circumference of a circle to its diameter.

**Relative limitation analyses on $A_N$**
The relative limitations on AN were analysed according to Grassi and Magnani (2005), including relative stomatal limitations (ls), mesophyll limitations (lm), and biochemical limitations (lb). lm was calculated using gm calculated from gas-exchange and fluorescence measurements following Harley et al. (1992a). Anatomical characteristics were analysed using the model of Niinemets and Reichstein (2003) modified by Tosens et al. (2016). The relative changes of stomatal, mesophyll, and biochemical limitations were calculated as follows: (see Equations 10-12 in the Supplementary Files)

\[
\text{where } g_{\text{tot}} \text{ is the total conductance for CO}_2 \text{ from the leaf surface to the carboxylation sites (} \frac{1}{g_{\text{tot}}} = \frac{1}{g_s} + \frac{1}{g_m} \text{); } l_s \text{, } l_m \text{, and } l_b \text{ are the corresponding relative limitations (} 0 < l_i < 1, i = s, m, b \text{). Here, was calculated as the slope of } A_N - C_i \text{ response curve over a } C_c \text{ range of 50-100 } \mu\text{mol mol}^{-1} \text{ (Tomas et al., 2013).}
\]

Statistical analysis

The data were analyzed using SPSS 12.0 software, and one-way ANOVA and least significant difference (Duncan) were used to test for differences \((P < 0.05)\). Figures were drawn using Origin 8.5 software.

Abbreviations

\(g_m\): Mesophyll conductance; \(J_{\text{fi}}\): Electron transport rate; \(f_{i\text{as}}\): The volume fraction of intercellular air spaces; \(S_{m}/S\): The chloroplast size and surface area exposing to intercellular air spaces of mesophyll; \(S_c/S\): The chloroplast size and surface area exposing to intercellular air spaces of chloroplasts; \(\text{Chl}\): Chlorophyll; \(A_N\): Net CO\(_2\) assimilation rate; \(\text{PAR}\): Photosynthetically active radiation; \(\text{PPFD}\): Photosynthetic photon flux density; \(C_i\): Intercellular CO\(_2\) concentration; \(\text{FC}\): Field capacity; \(F_o\): Fluorescence emission signal; \(F_m\): Maximal fluorescence. \(\text{PSII}\): Maximal photochemical efficiency; \(F_v\): The difference between \(F_o\) and \(F_m\); \(F_s\): Steady-state fluorescence yield; \(F_m'\): Light-adapted maximum fluorescence; \(\text{PS}\): Photosystems; \(T_L\): Leaf temperature; \(L_{\text{mes}}\): The total length of mesophyll cells facing the intercellular air space; \(L_c\): The total length of chloroplast surface area facing the intercellular air space in the mesophyll cells; \(t_{\text{mes}}\): The mesophyll thickness between the two epidermal layers; \(\Sigma Ss\): The sum of the cross-section area of mesophyll cells; \(L_{\text{chl}}\): Chloroplast length; \(T_{\text{chl}}\): Thickness.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated and/or analysed in this study are included in the article and additional files.

Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions
YL and HF conceived the study. YL, CF, JS, NL, FM and KW performed the experiments. YL and HF analysed the results and wrote the manuscript, with feedback from all authors. All authors have read and approved the manuscript.

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**Tables**

Table 1 Selected soil physicochemical properties at planting in 2016
| Physicochemical Properties | Units         | 2016 |
|---------------------------|---------------|------|
| Sand                      | (%)           | 41.12|
| Silt                      | (%)           | 40.46|
| Clay                      | (%)           | 18.42|
| pH                        | 1:2.5         | 7.30 |
| OM                        | (g kg⁻¹)      | 13.27|
| Total N                   | (g kg⁻¹)      | 0.76 |
| Olsen-P                   | (mg kg⁻¹)     | 22.6 |
| NH₄COOH-K                 | (mg kg⁻¹)     | 157.97|

Table 2 Chlorophyll a+b (chl(a+b)), the ratio between chlorophyll a and chlorophyll b (chla/b), chloroplast number, chloroplast length and chloroplast thickness and the cross-section area of chloroplast (Areachl) in sugar beet leaves by the day each treatment was achieved (Day 1), the first day upon rewatering (Day 3), the second day upon rewatering (Day 4) and the third day upon rewatering (Day 5).

Table 3 Leaf thickness, leaf mesophyll thickness, mesophyll cell area, mesophyll volume, stomatal conductance (gₛ), mesophyll conductance (gₘ), the surface of mesophyll cells and chloroplasts exposed to leaf intercellular airspaces (Sₛ/S and Sₖ/S; μm² μm⁻²) and the volume fraction of intercellular air space (fias) in sugar beet leaves by the day each treatment was achieved (Day 1), the first day upon rewatering (Day 3), the second day upon rewatering (Day 4) and the third day upon rewatering (Day 5).
|                | Day 1                | Day 2                | Day 3                | Day 4                | Day 5                |
|----------------|----------------------|----------------------|----------------------|----------------------|----------------------|
| **Leaf thickness (µm)** | Well irrigated 0.46 ef | Well irrigated 0.47 ef | Moderate stress 0.53 c | Severe stress 0.46 fg | Severe stress 0.49 de |
| **Leaf mesophyll thickness (µm)** | Well irrigated 0.41 de | Moderate stress 0.41 de | Severe stress 0.44 c | Moderate stress 0.38 ef | Severe stress 0.42 cd |
| **Mesophyll cell area (µm²)** | Well irrigated 0.36 bcd | Moderate stress 0.33 cde | Severe stress 0.42 b | Moderate stress 0.31 ef | Severe stress 0.39 bc |
| **Mesophyll cell volume (µm³)** | Well irrigated 0.021 ab | Moderate stress 0.014 cd | Severe stress 0.023 ab | Moderate stress 0.016 bc | Severe stress 0.020 abc |
| **gₛ (µmol H₂O m⁻² s⁻¹)** | Well irrigated 0.36 d | Moderate stress 0.52 b | Severe stress 0.13 f | Moderate stress 0.54 c | Severe stress 0.35 abc |
| **gₛ (µmol CO₂ m⁻² s⁻¹)** | Well irrigated 0.11 abc | Moderate stress 0.09 abc | Severe stress 0.13 a | Moderate stress 0.08 c | Severe stress 0.09 abc |
| **Sₘ/S** | Well irrigated 1.17 bc | Moderate stress 1.15 bc | Severe stress 1.22 b | Moderate stress 0.88 de | Severe stress 1.01 cde |
| **Sₖ/S** | Well irrigated 0.69 ab | Moderate stress 0.53 bcd | Severe stress 0.66 abc | Moderate stress 0.54 bcd | Severe stress 0.60 abcd |
| **Fₖₛ (%)** | Well irrigated 0.58 abc | Moderate stress 0.61 a | Severe stress 0.53 abcd | Moderate stress 0.52 bcd | Severe stress 0.49 d |

### Figures

**Figure 1**

The relationship between leaf number and plant weight of sugar beet
Figure 2

AN expressed on the basis of leaf area as a function of PAR by the day each treatment was achieved (Day 1), the first day upon rewatering (Day 3), the second day upon rewatering (Day 4) and the third day upon rewatering (Day 5).

Figure 3

AN expressed on the basis of Ci by the day each treatment was achieved (Day 1), the first day upon rewatering (Day 3), the second day upon rewatering (Day 4) and the third day upon rewatering (Day 5).
Figure 4

Ju in well irrigated, moderately, and severely water stressed sugar beet by the day each treatment was achieved (Day 1), the first day upon rewatering (Day 3), the second day upon rewatering (Day 4) and the third day upon rewatering (Day 5).

Figure 5

Quantitative limitation of photosynthesis in well irrigated, moderately, and severely water stressed sugar beet by the day each treatment was achieved (Day 1), the first day upon rewatering (Day 3), the second day upon rewatering (Day 4) and the third day upon rewatering (Day 5).

Supplementary Files
This is a list of supplementary files associated with this preprint. Click to download.

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- Equation9.jpg
- Equations1012.jpg
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- Equation1.jpg
- Equation3.jpg