Structure and photosynthetic metabolism in green prop roots of C₄ sorghum

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ABSTRACT
Plants contain chloroplasts in various organs exposed to sunlight. The C₄ crop, sorghum (Sorghum bicolor), develops prop roots on stems above the soil level with the progression of growth. These roots penetrate in the soil and form green above-ground (AG) and non-green below-ground (BG) portions. We investigated the structure and photosynthetic metabolism in the AG portion of prop roots in comparison with leaf blades and the BG portion. The AG portion lacked stomata on the epidermis and showed typical root structure as in the BG portion. However, they contained granal chloroplasts in the cortex and stele parenchyma. The chlorophyll content was much lower in the AG portion than in leaf blades. Western blot analysis showed that the AG portion accumulates ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) but lacks substantially phosphoenolpyruvate carboxylase and pyruvate, Pi dikinase. An immunolocalization study confirmed that Rubisco is accumulated in the chloroplasts of AG portion. The AG portion accumulated only small amount of malate without diurnal change as in other organs, indicating that crassulacean acid metabolism (CAM) is inactive. The δ¹³C values of all organs including the AG portion were within the C₄ range, suggesting that their tissue carbon is derived from C₄ photosynthesis of leaves. These data suggest that the AG portion of prop roots could re-fix internally respired CO₂ via C₃ cycle, whereas this photosynthetic function may provide O₂ to heterotrophic tissues of prop roots. This study also demonstrates that different photosynthetic types can function in different organs of a single plant.

Introduction
Photosynthesis is one of the most important physiological function for plants. CO₂ is assimilated into carbohydrate using solar energy harvested by thylakoids of chloroplasts. Then, O₂ is simultaneously released during the photosynthetic process. In most plants, the primary photosynthetic organ is leaves (leaf blades in grasses). However, other plant organs containing chlorophyll (Chl) can also perform photosynthesis: for example, husks and leaf sheaths in maize (Langdale et al., 1988; Pengelly et al., 2011); panicles and stems in grasses (Imaizumi et al., 1990; Wakayama et al., 2006); fruits of cucumber (Sui et al., 2017); immature fruits of tomato
The source of CO\textsubscript{2} for their photosynthesis is the atmosphere and internally respired CO\textsubscript{2} (Aschan & Pfanz, 2003; Avila et al., 2014; Brazel & O'Maoileidigh, 2019; Sage & Khoshravesh, 2016). In leaves having many stomata on the epidermis, CO\textsubscript{2} is acquired from the atmosphere through stomata, although it has been recently reported that a part of photosynthetic CO\textsubscript{2} released from mitochondria is re-fixed by adjacent chloroplasts (Busch et al., 2013; Hatakeyama & Ueno, 2016). In non-foliar organs such as fruits and pods, the source of CO\textsubscript{2} is both the atmosphere and internally respired CO\textsubscript{2}, since these organs have a considerable number of stomata on the surface. However, compact chlorenchyma in these organs prevents the inner diffusion of atmospheric CO\textsubscript{2} through stomata. It is generally thought that the significance of re-fixation of respired CO\textsubscript{2} is to improve plant carbon economy by offsetting partially a carbon loss and to provide oxygen to heterotrophic tissues (Aschan & Pfanz, 2003; Avila et al., 2014; Brazel & O'Maoileidigh, 2019; Sage & Khoshravesh, 2016). Therefore, plants develop chloroplasts in almost all organs exposed to sunlight.

Here, we report the structure and photosynthetic metabolism in green prop roots of sorghum. Sorghum is one of the most important grain crops together with maize. They are also important as foliage and energy crops (Carpita & McCann, 2008). These grass crops have large plant bodies and develop adventitiously prop roots on stems above the soil level. The developing prop roots penetrate in the soil. From this morphological feature, it is thought that prop roots serve as additional support of the plant axis (Esau, 1977). On the other hand, the above-ground (AG) portion of prop roots is green, indicating the presence of Chl. In contrast to the below-ground (BG) portion of prop roots lacking Chl, the AG portion of prop roots exposed to sunlight would have photosynthetic function. The stomata serve in gas exchange between the plant body and its environment and occurs on all aerial parts of the plant body, but roots usually lack stomata (Esau, 1977). As far as we know, the structural and photosynthetic traits of prop roots in grasses have not been fully investigated.

Sorghum is a C\textsubscript{4} plant of grasses. In most C\textsubscript{4} leaves, two types of photosynthetic cell, mesophyll and bundle sheath (BS) cells, are differentiated (Langdale, 2011). The division of labor between these photosynthetic cells is required for the operation of C\textsubscript{4} photosynthesis (Hatch, 1987; Leegood, 2013). In the mesophyll cells, atmospheric CO\textsubscript{2} is primarily fixed in C\textsubscript{4} acids by phosphoenolpyruvate carboxylase (PEPC). The formed C\textsubscript{4} acids are transferred into the BS cells where they are decarboxylated to supply CO\textsubscript{2} for ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco). On the other hand, the C\textsubscript{3} product of the decarboxylation reaction is returned to the mesophyll cells, where it is converted to PEP, the substrate of PEPC, by pyruvate, Pi dikinase (PPDK) (Hatch, 1987; Leegood, 2013). Then, it is interesting to ask whether the AG portion of prop roots also performs C\textsubscript{4} photosynthesis in two types of photosynthetic cell, as in leaves. The root is a specialized organ for absorption and transportation of water and minerals from the soil solution and anchors the plant body in the soil. In general, the root lacks photosynthetic function. However, roots of some plants are known to have photosynthetic function. The aerial roots of epiphytic orchids perform crassulacean acid metabolism (CAM) as in leaves (Martin et al., 2010; Motomura et al., 2008). The pneumatophores of some mangroves can photosynthesize (Aschan & Pfanz, 2003; Kitaya et al., 2002), although the detailed photosynthetic metabolism remains unclear.

In this study, we investigated the structural, biochemical, and physiological traits involving in photosynthetic metabolism in the AG portion of sorghum prop roots, comparing with those in leaf blades and the BG portion of prop roots. Our special interest is to know whether the differentiation of photosynthetic cells occurs in the prop root and which type of photosynthetic metabolism operates in the AG portion. This study not only provides basic information about physiology of this major crop but also provides a unique opportunity for understanding of the genetic regulation in the expression of photosynthetic type among different organs within a single plant.

**Materials and methods**

**Plant materials**

Seeds of *Sorghum bicolor* (L.) Moench (cv. Metresorghum) were purchased from Takii & Co. Ltd., Kyoto, Japan. They were germinated on a perforated multi-well nursery box filled with loam soil granules in early August 2018 for 1 week in a greenhouse at Kyushu University. The seedlings were then transplanted into 8-L pots (one plant per pot) holding sandy loam soil mixed with chemical fertilizer containing 1.2 g each of nitrogen, phosphorus, and potassium. The plants were grown in the greenhouse...
for 7 weeks. The prop roots that developed on the basal position of stem above the soil level and uppermost fully expanded leaves were sampled from each of three plants for experiments. The prop roots were divided into AG and BG portions. During the growth period, the minimum and maximum temperatures in the greenhouse were 24°C and 35°C, and photosynthetic photon flux density at midday exceeded 1500 µmol m⁻² s⁻¹ at plant top. Plants were watered daily.

**Anatomical and ultrastructural observations**

Samples of prop roots and leaf blades were collected in early morning. Small segments taken from the middle of the AG and BG portions of prop roots and leaf blades were fixed in 3% (v/v) glutaraldehyde in 50 mM sodium phosphate buffer (pH 6.8) at room temperature for 2 h. They were then washed with distilled water. Transverse sections of prop roots and leaf blades were hand-cut and viewed under a light microscope (Eclipse CI-L, Nikon Instech Co. Ltd., Tokyo, Japan). A part of the prop-root samples fixed in glutaraldehyde were washed with phosphate buffer and post-fixed in 2% (w/v) OsO₄ in phosphate buffer for 2 h. They were then dehydrated through an acetone series and embedded in Quetol resin (Nissin EM Co. Ltd., Tokyo, Japan). The embedded tissue samples were transversely cut with a diamond knife. Ultrathin sections were stained with Ti blue (Nissin EM; Inaga et al., 2007) and lead citrate and viewed under a transmission electron microscope (JEM-100CX IIK, JEOL, Tokyo, Japan) at 75 kV.

**Antisera**

The following antisera were used for an immunohistochemical study and Western blot of the C₃ and C₄ enzymes: anti-pea leaf Rubisco large subunit (LSU) antiserum (courtesy of S. Muto, Nagoya University, Nagoya, Japan) and anti-maize leaf PEPC and PPDK antiserum (courtesy of T. Sugiyama, RIKEN). These antisera were the same as those used in previous studies (Wakayama et al., 2006; Wakayama et al., 2013). The antisera were used at a dilution of 1:1000 for Rubisco LSU and 1:500 for PEPC and PPDK.

**Immunohistochemistry**

Samples of prop roots and leaf blades were also collected in early morning. Small segments taken from the middle of the AG and BG portions of prop roots and leaf blades were fixed in 3% (w/v) paraformaldehyde/0.2% (v/v) glutaraldehyde in 50 mM sodium phosphate buffer (pH 6.8) in 4°C for 5 h. They were then dehydrated through an ethanol/butanol series and embedded in Paraplast Plus (Sigma-Aldrich Inc., St Louis, MO, USA), as described by Hatakeyama and Ueno (2016). Transverse section (ca. 10 µm thick) were cut on a rotary microtome (PR-50, Yamato Kohki Industrial Co. Ltd., Saitama, Japan) and mounted on slides coated with poly-L-lysine (Sigma-Aldrich Inc.). Immunostaining for PEPC, PPDK, and Rubisco LSU was made as described by Hatakeyama and Ueno (2016).

**Western blot**

Samples (0.1 g FW for leaf blades and 0.4 g FW for the AG and BG portions of prop roots) were collected during the day, frozen in liquid nitrogen immediately, and stored at −80 °C. Extraction of soluble proteins, SDS-PAGE, and Western blotting were performed as reported by Ueno (2004) with the antisera against PEPC, PPDK, and Rubisco LSU.

**Chl and malate contents**

For determination of Chl content, samples (0.1 g FW for leaf blades and 0.4 g FW for the AG and BG portions of prop roots) were collected in the morning and ground with a pestle in a mortar with 80% acetone. Homogenates were centrifuged at 10,000 × g for 5 min at 4°C. Extraction of Chl from tissue debris was repeated several times. The Chl content in supernatants was determined according to Porra et al. (1989). For determination of malate content, prop roots and leaf blades were sampled at 05:00 (the end of night) and 17:00 (the end of day). These samples were frozen in liquid nitrogen immediately and stored at −80 °C. The malate content of these samples (0.1 g FW for leaf blades and 0.4 g FW for the two portions of prop roots) were determined as described in Kondo et al. (2001).

**Carbon isotope ratio**

The two portions of prop roots and leaf blades were air-dried at 80 °C and ground with a pestle in a mortar. The ¹²C and ¹³C contents in tissues (2 mg dry weight) were determined as described in Ueno (2013). The isotope ratio was expressed as a δ¹³C value, in parts per thousand, after reference to the Pee Dee belemnite standard.

**Statistical analysis**

Data for Chl and malate contents and δ¹³C values were presented as means ± SD (n = 3 plants). These data were analyzed using Statce14 software (OMS Publishers, Tokorozawa, Saitama, Japan). The statistical significance of differences in Chl and malate...
contents and $\delta^{13}$C values among leaf blades and the AG and UG portions of prop roots was assessed by Tukey's multiple comparison test at $p < 0.05$. That in malate content between the end of night and the end of day in each tissue was assessed by Student’s t-test at $p < 0.05$.

Results

Structure of prop roots

The sorghum plants began to develop prop roots on the stems above the soil level after about 1 month of transplanting of seedlings. The tips of prop roots soon began to penetrate in the soil, whereas the basal position was exposed to sunlight (see the Graphical Abstract of the first page). The length of the BG and AG portions was 10–15 cm and 4–6 cm, respectively, and their diameter was ca. 4–7 mm. Both BG and AG portions of prop roots showed an inner structure typical of roots, in which the cortex surrounded inner stele that was composed of vascular tissues and parenchyma cells (Figure 1(a,d)). The epidermis of both portions lacked stomata. The sclerenchyma that was composed of several layers of thick-walled cells developed inside the epidermis (Figure 1(a,b,d,e)). The cortex was composed of spherical parenchyma cells with intercellular air spaces (Figure 1(b,e)). In the AG portion, chloroplasts were present mainly in the cortex parenchyma cells and slightly in the stele parenchyma cells (Figure 1(a–c)). An electron microscopic observation revealed that the chloroplasts have well-developed grana (Figure 2). The length of long axis of chloroplasts was $5.2 \pm 0.5 \mu m$ (mean ± SD, $n = 10$). In the cortex and stele of BG portion, no green cells were observed (Figure 1(d–f)). The leaf blades showed Kranz type of leaf anatomy characterized by the differentiation of mesophyll and BS cells (Supplementary data 1), whereas the AG portion did not show such differentiation of chlorenchyma cells (Figure 1(a–c)).

Immunohistochemical localization of photosynthetic enzymes

In leaf blades used as a control, a dense staining for PEPC occurred in the mesophyll cells but lacked in the BS cells (Figure 3(a)). For PPDK, an intense staining occurred in the mesophyll cells, whereas a weak staining was found in the BS cells (Figure 3(b)). As expected, a dense staining for Rubisco LSU exclusively occurred in the BS cells of leaf blades (Figure 3(c)). In prop roots, staining patterns for photosynthetic enzymes were essentially similar in both the cortex with abundant chloroplasts and the stele with

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Figure 1. Structure of (a-c) above-ground and (d-f) below-ground portions of sorghum prop roots. (a, d) Transverse sections of low magnification view. (b, e) Transverse sections of the epidermis and cortex. (c, f) Transverse sections of the endodermis and stele. Co, cortex; En, endodermis; Ep, epidermis; S, stele; Sc, sclerenchyma; V, vascular tissue. Bars = 100 µm for a and d. Bars = 50 µm for b, c, e and f.
a few chloroplasts. In Figure 4, therefore, staining data for the cortex (Figure 4(b–f)) were mainly shown together with one for the stele (Figure 4(a)). In the AG portion of prop roots, almost no staining for PEPC was found in the stele (Figure 4(a)) and cortex. In the BG portion, only weak staining for PEPC occurred in the parenchyma cells of cortex (Figure 4(b)) and stele. In both AG and BG portions of prop roots, no staining for PPDK was found in the cortex (Figure 4(c,d)) and stele. In the AG portion of prop roots, an intense staining for Rubisco LSU was found in the chloroplasts of cortex (Figure 4(e)) and stele. In the BG portion, however, no staining for Rubisco LSU was found in the cortex (Figure 4(f)) and stele.

Western blots of photosynthetic enzymes
A dense band for PEPC occurred in leaf blades (Figure 5). Almost no band for PEPC was found in the AG portion of prop roots, whereas weak bands for PEPC were detectable in the BG portion. Although a dense band for PPDK also occurred in leaf blades, there were no bands in both AG and BG portions of prop roots (Figure 5). Dense bands for Rubisco LSU occurred in both leaf blades and AG portion of prop roots, whereas a very weak band was found in the BG portion (Figure 5).

Chl and malate contents
The leaf blade contained a large amount of Chl, whereas the AG portion of prop roots did only small amount of Chl (Table 1). The amount of Chl in the latter was ca. 4% of that in the former. On the other hand, the BG portion of prop roots substantially lacked Chl (Table 1). The leaf blades and the two portions of prop roots contained only small amounts of malate at both the ends of day and night, and there was no significant difference among them (Table 1). In each tissue, there were no significant differences in malate amounts between the end of day and the end of night.

Carbon isotope ratios
The leaf blades and the two portions of prop roots showed δ13C values typical of C4 plants (Ehleringer & Osmond, 1991). There was no significant difference in δ13C values among the three tissues (Table 1).

Discussion
Structural features
We confirmed that both AG and BG portions of prop roots have the inner structure of roots, although the AG portion is exposed to sunlight (Figure 1). It is important to know whether stomata are present or absent on the epidermis of prop roots. We observed no stomata on the epidermis of both AG and BG portions of prop roots. Furthermore, the sclerenchyma consisting of several layers of thick-walled cells developed under the epidermis and encircled the inner cortex and stele tissues. Accordingly, the atmospheric CO2 could not easily diffuse into the cortex and stele parenchyma of prop roots. Nevertheless, these parenchyma cells in the AG portion contained a considerable number of chloroplasts (Figure 1). These chloroplasts had well-developed grana (Figure 2), as in mesophyll chloroplasts of leaf blades (Makino & Ueno, 2018). The length of long axis of chloroplasts was also similar to those reported in the mesophyll chloroplasts of sorghum leaf blades (5.6 ± 0.8 µm, Makino & Ueno, 2018; 6.1 ± 0.9 µm; Yoshimura et al., 2004). There was no structural evidence of the differentiation of two types of chlorenchyma in the cortex and stele of prop roots. Previous studies have reported that leaf sheath, spikes, husks, and stems of some C4 grasses show Kranz-like anatomy, although the structural features were somewhat or greatly modified from that of leaf blades (Langdale et al., 1988; Pengelly et al., 2011; Wakayama et al., 2006). The structure of chlorenchyma of the AG portion was rather similar to those of the lamina joint and sheath pulvinus of the C4 grass Arundinella hirta, in which the chlorenchyma is composed of a single type of cell (Wakayama et al., 2013). As expected, the BG portion of prop roots, which is not exposed to sunlight, lacked chloroplasts.
Photosynthetic metabolism

Although the AG portion of prop roots contained Chl (Table 1), the amount was much lower than that in leaf blades. The BG portion of prop roots substantially lacked Chl. These data suggest that photosynthetic activity of the AG portion would be very lower than that of leaf blades. Our immunohistochemical study for leaf blades (Figure 3) confirmed that PEPC and Rubisco are localized in mesophyll and BS cells, respectively, and PPDK are localized mainly in mesophyll cells with weak accumulation in BS cells, as reported in previous studies (Edwards et al., 2001; Ueno, 1998). On the other hand, the chlorenchyma of AG portion of prop roots accumulates Rubisco in the chloroplasts but lacks both PEPC and PPDK (Figure 4). The parenchyma of BG portion almost lacked these photosynthetic enzyme proteins, although weak staining for PEPC was detected. Western blot analysis supported these accumulation patterns of photosynthetic enzymes in prop roots (Figure 5). A weak band of PEPC was detected in the BG portion. It has been known that roots have PEPC activity (Kocurek & Pilarski, 2011). Besides C₄ and CAM photosynthesis, PEPC is involved in various physiological tasks such as anaplerotic CO₂ fixation, maintenance of ion balance, and pH regulation (Gowik & Westhoff, 2011; Melzer & O'Leary, 1987). On the other hand, it is interesting to note that a weak band of Rubisco LSU was found in the BG portion of prop roots (Figure 5). It is unknown whether this Rubisco protein is active as an enzyme. Morita et al., (2016) have reported that Rubisco genes are expressed in roots of some plant species. These accumulation patterns of C₃ and C₄ photosynthetic enzymes in prop roots suggest that the AG portion performs C₃ photosynthesis, differing from leaf blades with C₄ photosynthesis.

We also investigated the diurnal pattern of malate accumulation in prop roots to assess the existence of CAM (Table 1). The results showed that the malate contents in prop roots are very low without diurnal changes, as in leaf blades. In typical CAM plants, malate content greatly changes from morning to night. For example, Kalanchoë blossfeldiana shows malate contents of 9–68 and 1–2 µmol g⁻¹ FW at the end of night and the end of day, respectively (Kondo et al., 2001). From these data, it is evident that the AG portion of prop roots do not perform CAM. It has been suggested that, in some plants such as tobacco and celery, malate produced in the roots moves with transpiration stream in vessels to the above-organs, stems and petioles, in which tissues surrounding the vascular bundles contain abundant chloroplasts and high activities of C₄-acid decarboxylases. In these tissues, CO₂ generated by malate decarboxylation would be fixed by the C₃ cycle of chloroplasts (Hibberd & Quick, 2002). However, it seems unlikely that such C₄-like cycle between the roots and above-organs operates between the BG and AG portions of prop roots of sorghum, because their chloroplasts are not closely associated with vascular bundles (present mainly in the cortex) and malate contents in prop roots were generally low (Table 1).

Despite that the AG portion of prop roots performs C₃ photosynthesis, it showed C₄-like δ¹³C values as in leaf blades (Table 1). This fact suggests that carbon constituting these tissues is derived from carbon fixed by C₄ photosynthesis of leaf blades. This is the case in the BG...
Physiological consideration

It is unlikely that the re-fixation of respired CO₂ in the AG portion of prop roots contributes significantly to the carbon economy of the sorghum plant, because of much lower Chl content in the AG portion than in the portion of prop roots. Presumably, the chlorenchyma of prop roots would fix respiratory CO₂, which shows C₄-like δ¹³C values, by Rubisco. Their robust surface structure without stomata would become a diffusional barrier and could slow the efflux of respiratory CO₂. As a result, this structural feature would enable Rubisco to fix CO₂ of C₄-like δ¹³C values. This would be analogous to the gastight environment for Rubisco within BS cells of C₄ plants (Hatch, 1987; Leegood, 2013). If Rubisco in the AG of prop roots fixes directly the atmospheric CO₂, the δ¹³C values may become more negative than those of leaf blades (e.g. Yakir, Osmond & Giles, 1991).

Figure 4. Immunohistochemical localization of C₃ and C₄ photosynthetic enzymes in (a, c, e) above-ground and (b, d, f) below-ground portions of sorghum prop roots. (a), (b) PEPC. (c), (d) PPDK. (e), (f) Rubisco LSU. All panels are cortex tissues, except that (a) is a stele tissue. c, chloroplast. Bars = 50 µm.

Figure 5. Western blots of protein extracts from leaf blades and prop roots of sorghum. Total soluble protein (20 µg for PEPC and PPDK and 2.5 µg for Rubisco LSU) was subjected to SDS-PAGE, blotting on nitrocellulose membranes, and identification with antisera against the C₃ and C₄ enzymes. Lane 1, leaf blade; lane 2, above-ground portion of prop root; lane 3, below-ground portion of prop root.

Physiological consideration

It is unlikely that the re-fixation of respired CO₂ in the AG portion of prop roots contributes significantly to the carbon economy of the sorghum plant, because of much lower Chl content in the AG portion than in the
leaf blades. However, O$_2$ released by this photosynthetic process may be supplied to heterotrophic tissues of prop roots. This function may have a significant role to compensate oxygen depletion in the BG portion, when sorghum plants grow under wet soil conditions and then if their prop roots form lacunae in response to hypoxia. Wittmann and Pfanz (2018) have shown that, in stems of trees, the corticular photosynthesis is not only a CO$_2$ recycling mechanism but also a mechanism to raise the cortical O$_2$ concentration and counteract temporal/spatial hypoxia inside stems. Further research will be required to understand the significance of photosynthetic function in prop roots.

Expression of photosynthetic types

The results of this study are also intriguing from a point of view of the expression of photosynthetic types. The expression of photosynthetic type is environmentally and developmentally regulated (Langdale, 2011; Ueno, 2001). In C$_4$ plants, leaf blades perform C$_4$ photosynthesis. However, all other organs containing Chl do not necessarily perform C$_4$ photosynthesis. In C$_4$ eudicots, Salsola gemmascens and S. soda, the cotyledons exhibit C$_3$ photosynthesis (Lauterbach et al., 2017; Pyankov et al., 2000). In another C$_4$ eudicot Cleome angustifolia, the pods exhibit C$_3$ photosynthesis (Voznesenskaya et al., 2018). On the other hand, the lamina joint and sheath pulvinus of the C$_4$ grass Arundinella hirta seem to operate a C$_4$-like cycle in a single type of chlorenchyma (Wakayama et al., 2013). Our study on prop roots of sorghum also demonstrates organ-specific expression of C$_3$ and C$_4$ photosynthetic types in roots and leaves.

Acknowledgments

We thank Dr. Y. Hatakeyama for his help in preparation of the figures and Prof. N. Furuya, Faculty of Agriculture, Kyushu University, for use of an electron microscope.

Disclosure statement

No potential conflict of interest was reported by the authors.

Table 1. Biochemical and physiological traits involved in photosynthesis in leaf blades and prop roots of sorghum.

| Trait                   | Leaf blade | Above-ground | Below-ground |
|-------------------------|------------|--------------|--------------|
| Chl content (mg g$^{-1}$ FW) | 45.7 ± 2.16a | 2.33 ± 0.60b | 0.06 ± 0.02c |
| Malate content (μmol g$^{-1}$ FW) | End of night | 1.6 ± 0.7a | 1.6 ± 0.9a | 0.6 ± 0.2a |
| δ$^{13}$C (%)         | 2.3 ± 0.7a | 1.6 ± 0.9a | 1.6 ± 0.7a |

Values are given as the mean ± SD (n = 3). The different letters indicate a significant difference at p < 0.05.

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