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The long-term response of photosynthesis in walnut (*Juglans regia* L.) leaf to a leaf-to-fruit ratio

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Abstract

For clarifying the relationship between a leaf-to-fruit ratio (LFR) and photosynthesis, LFR manipulation was performed with *Juglans regia* cv. Xinxin2 in order to test the photosynthesis response to LFR in source leaves. Results showed that LFR with one and two leaves was positively correlated with net photosynthetic rate (P_N), chlorophyll content, and specific leaf mass, implying extremely low LFR inhibited the leaf development. However, LFR with five leaves was negatively correlated with P_N, positively correlated with starch, but not related to intercellular CO_2 concentration, indicating the high LFR caused the nonstomatal limitation and feedback inhibition of photosynthetic production. No significant differences in P_N between LFRs (with three and four leaves) probably indicated a balanced state of coordinated supply and demand between the source leaf and sink fruit. The above results indicated that the response of photosynthesis in the source leaves to LFR depends on the variation range of LFR.

Additional key words: gas exchange; leaf anatomical structure; leaf traits; nut; source-sink relationship.

Introduction

The pivotal influence of leaf-to-fruit ratio (LFR) on photosynthesis has been widely established in many higher plant species, including apple (*Malus pumila* Mill.) (Naor *et al*. 2008, Zhang *et al*. 2009, Naschitz *et al*. 2010), olive (*Olea europaea* L.) (Trentacoste *et al*. 2011, Bustan *et al*. 2016), plum (*Prunus domestica* L.) (Duan *et al*. 2016), citrus (*Citrus reticulata* Blanco) (Nebauer *et al*. 2011), mango (*Mangifera indica* L.) (Léchaudel *et al*. 2005), and grape (*Vitis vinifera* L.) (Bobeica *et al*. 2015). In the leaves of most plants, the increase in net photosynthetic rate (P_N) after LFR reduction (mainly defoliation) has been typically documented (Urban *et al*. 2004, Li *et al*. 2007, Bobeica *et al*. 2015). However, the negative correlation between LFR and P_N mentioned above depends on a degree of defoliation. For example, data from sour cherry (*Prunus cerasus*) leaves, which was monitored after defoliation, shows that removal of 30% of leaves decreased P_N, but when defoliated by 20%, the remaining leaves showed a rise in P_N (Layne and Flore 1993). The similar result was also observed in other plants (Poston *et al*. 1976, Proctor *et al*. 1982). In addition, some studies have shown that LFR has no significant effect on P_N (Sams and Flore 1983, Plaut *et al*. 1987). Collectively, this conflicting information has tended to obscure the precise nature of the effect of LFR on photosynthesis. As a solution to this problem, we put forward a hypothesis that the response of P_N in source leaves to LFR could depend on the variation range of LFR. When LFRs change in a different range, the effect of LFR on photosynthesis is different.

What is the cause of the effect of LFR on photosynthesis? It has been proposed that the downregulation of P_N in leaves of plants with a high LFR is largely the result of carbohydrate accumulation in source leaves (Urban *et al*. 2004, DaMatta *et al*. 2008), although this has not always been observed (Bustan *et al*. 2011). In addition, research on kiwifruit suggests that an extreme deficiency in photosynthetic products caused by an extremely low LFR could inhibit photosynthesis and fruit development (Fang *et al*. 2001). It has been clearly established that leaf traits, including leaf age, stomatal characters, chlorophyll (Chl) content, and specific leaf mass (SLM), are closely related to photosynthesis. The photosynthates produced by plant leaves are initially utilized to meet the leaf own physiological needs, and then the surplus photosynthates are exported to other sinks (Ramos 1985). A question arises whether the extremely low LFR could have an adverse effect on the growth and development of the leaf itself. Despite a relatively large number of studies, which have

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Abbreviations: Chl – chlorophyll; C_i – intercellular CO_2 concentration; DAF – days after full bloom of female flowers; g_s – stomatal conductance; FL – LFRs with five leaves; LFR – leaf-to-fruit ratio; LT – leaf thickness; OTL – LFRs with one and two leaves; P_N – net photosynthetic rate; P_N-PAR – photosynthetic rate response to PAR; PTT – palisade tissue thickness; SLM – specific leaf mass; Sta – starch; STT – spongy tissue thickness; TFL – LFRs with three and four leaves; TSS – total soluble sugar.
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examined LFR in many fruit trees, little is known about the extent to which LFR can modulate photosynthesis and the relationship among LFR, P_N, and leaf traits in walnut. Thus, it is necessary to determine the optimum LFR for walnut and its precise cultivation management.

Among nuts, walnut is one of the most important species from an economic and botanical point of view, and in many countries, it has a rich cultural heritage. Today walnut is grown in over 60 countries around the globe, and China has the highest production (Avanzato *et al*. 2014). Walnut is also the second largest tree species after jujube in Xinjiang. *Juglans regia* L. cv. 'Xinxin2' is the main cultivar walnut variety in southern Xinjiang basin. The hypothesis proposed in this paper is that the relationship between photosynthesis and LFR depends on the range of LFR. In order to verify this hypothesis, a larger range of LFRs should be designed. According to the investigation, the LFRs of *Juglans regia* L. cv. 'Xinxin2' are relatively rich in natural conditions, which provides an opportunity to test the hypothesis proposed in this paper. According to literature, there were many studies on walnuts, such as walnut flowering (Hassankhah *et al*. 2018), bud dormancy (Gholizadeh *et al*. 2017), *ex vitro* acclimation of walnut plantlets (Maleki Asayesh *et al*. 2017a,b), *etc*. Previous studies showed that photosynthesis of walnut leaves was affected by many factors, including kaolin (Rosati *et al*. 2007, Gharaghani *et al*. 2018), rootstock varieties (Li *et al*. 2017), fertilizers (Nicodemus *et al*. 2008, Liu *et al*. 2010), acclimation (Chenevard *et al*. 1997), light (Dean *et al*. 1982, Atanasova *et al*. 2003), salinity (Zhang *et al*. 2002), and water (Scartazza *et al*. 2001). Despite previous studies reporting photosynthetic response to changes in source-sink relationships, a systematic research on long-term response of photosynthesis and physiological characteristics of walnut leaves under different source-sink relationship is very limited (Wang *et al*. 2010, Moscatello *et al*. 2017).

In this study, the LFRs of walnut trees were artificially altered using a variety of manipulations (defoliation, fruit thinning, and girdling). After manipulation, the leaf traits, anatomical structure, carbohydrate content, and gas-exchange parameters were investigated to evaluate the long-term response of leaf photosynthesis to LFR during the growing season. The aims of this research were (*1*) to test the hypothesis that the response of P_N in the source leaves of walnut to LFR depends on the variation range of LFR, and (*2*) clarify the cause of the effect of LFR on photosynthesis. This research can provide a theoretical basis for regulating and controlling the reasonable load of walnut in actual production.

Materials and methods

Experimental site and plant materials: The experiment was carried out in a walnut orchard located in the southern Xinjiang, China (41°11'06.31"–41°12'47.74"N, 79°12'12.76"–79°13'57.87"E; 1,394 m a.s.l.). This region experiences a warm temperate continental arid climate, with a mean annual temperature of 9.4°C and an average rainfall of 91.5 mm. A uniform stand of 10-year-old walnut trees (*Juglans regia* L. cv. 'Xinxin2') with ground diameter of about 25 cm and tree height of about 6.5 m was selected for this study. The trees were grown at a spacing of 5.0 × 6.0 m in east-west rows in an anthropogenic-alluvial soil.

Leaf-to-fruit ratio manipulation: LFR is defined as the ratio of the number of leaves to the number of fruits. The LFRs used in this study reflect the bearing habit found in walnut trees under natural conditions. After fruit setting, 50 homogenous trees were divided into A and B groups. Five trees in A group were used for gas-exchange measurements, and 45 trees in B group were used for leaf sample collection. Sun-exposed and girdled shoots with fully expanded leaves and developing fruits on the south of trees were treated by defoliation or defruiting. In the A group, there were 5 trees selected for gas-exchange measurement and each tree was subjected to all of the following 15 LFR treatments (Fig. 1): (*1*) girdled shoots with one fruit and one leaf (1L:1F) (Fig. 1*A*), (*2*) girdled shoots with one leaf and two fruits (1L:2F) (Fig. 1*B*), (*3*) girdled shoots with one leaf and three fruits (1L:3F) (Fig. 1*C*), (*4*) girdled shoots with two leaves and one fruit (2L:1F) (Fig. 1*D*), (*5*) girdled shoots with two leaves and two fruits (2L:2F) (Fig. 1*E*), (*6*) girdled shoots with two leaves and three fruits (2L:3F) (Fig. 1*F*), (*7*) girdled shoots with three leaves and one fruit (3L:1F) (Fig. 1*G*), (*8*) girdled shoots with three leaves and two fruits (3L:2F) (Fig. 1*H*), (*9*) girdled shoots with three leaves and three fruits (3L:3F) (Fig. 1*I*), (*10*) girdled shoots with four leaves and one fruit (4L:1F) (Fig. 1*J*), (*11*) girdled shoots with four leaves and two fruits (4L:2F) (Fig. 1*K*), (*12*) girdled shoots with four leaves and three fruits (4L:3F) (Fig. 1*L*), (*13*) girdled shoots with five leaves and one fruit (5L:1F) (Fig. 1*M*), (*14*) girdled shoots with five leaves and two fruits (5L:2F) (Fig. 1*N*), and (*15*) girdled shoots with five leaves and three fruits (5L:3F) (Fig. 1*O*). In the B group, 45 trees remained for leaf sampling. The remaining 45 trees were divided into 15 groups (each group consisted of three trees), and all the sun-exposed and bearing shoots with fully expanded

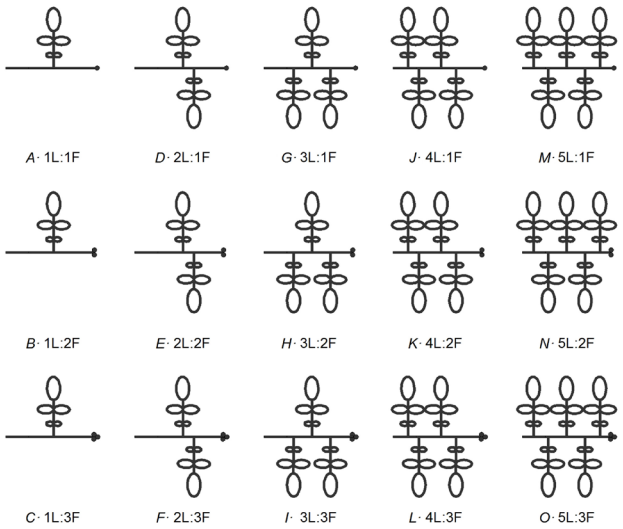

Fig. 1. Different leaf-to-fruit ratio manipulations on the girdled shoots of walnut.

leaves and developing fruits on the south of the three trees (15 bearing shoots per tree) were subjected to one of the 15 LFR treatments. At 10 mm from the base of the bearing shoot, a complete circle of phloem tissues, about 5 mm wide, was removed with a grafting knife, without affecting the xylem. The girdles were preserved for the entire growing season by discarding any scar tissue at 15-d intervals. Immature leaves and the apical and auxiliary buds were removed from the girdled shoots to ensure that assimilates mainly flowed to the fruit.

Gas-exchange measurements: During the growing season in 2017, the actual amount of rainfall received was 57.3 mm, and leaf gas-exchange parameters were measured on 5 trees in the group A once per day (11:00–14:00 h), on five cloudless days at 7 (13 May), 22 (28 May), 52 (27 June), 82 (27 July), and 107 (21 August) days after initiating LFR manipulation (30, 45, 75, 105, and 130 d after full bloom of female flowers, DAF). Using a portable photosynthesis system (*LI-6400XT*, *LI-COR*, USA) with small cylinders filled with carbon dioxide and a broad leaf chamber (2 × 3 cm) to measure two fully developed leaves close to fruit per shoot. Light intensity was set to 1,300 μmol(photon) m^{-2} s^{-1} (according to initial assessment of light-response curves, data not shown), and CO_2 concentration was set to 380 mmol m^{-2} s^{-1}. Leaf temperature and H_2O vapour concentrations were consistent with the values observed in the external environment. The selected leaves were placed in the leaf chamber to measure net photosynthetic rate [P_N, μmol(CO_2) m^{-2} s^{-1}], stomatal conductance [g_s, mol(H_2O) m^{-2} s^{-1}], and intercellular CO_2 concentration [C_i, μmol(CO_2) mol^{-1}].

Leaf sampling: Two fully developed leaves close to fruit per shoot were sampled on the trees in the group B; it was carried out on three trees per LFR and three shoots per tree. The leaf samples were divided into three parts. The first part was fixed in fixative solution (formalin-alcohol-acetic acid, FAA) for the production of paraffin sections, the second part was used for the leaf trait determinations (Chl content and SLM), and the third part was used for the carbohydrates determination.

Leaf anatomical structure: Leaf samples collected on 45 and 130 DAF were used for anatomical structure observation. Leaves were cut in pieces (1 × 0.5 cm wide), fixed in FAA, embedded in paraffin, and sliced following the protocol of Willey (1971). The section cutting, examination, measurements, and photographing were separately done by rotary microtome *Leica Microsystems* (*RM2265*, *CMS GmbH*, Germany) and digital camera *DFC495* (*Leica,* Germany) with image recording. Reported leaf thickness (LT), thickness of palisade tissue (PTT), and thickness of spongy tissue (STT) were the averages of the two observed samples collected on 45 and 130 DAF.

Leaf traits determination: SLM was measured according to Jumrani *et al.* (2017). Leaf squares (2 cm^2) were oven dried at 70°C for 3 h to obtain dry mass. The ratio of the leaf dry mass to the leaf area was SLM. The method of determining Chl content was slightly changed with reference to Hazratia's (2016) method. The chlorophyll extract was obtained after 0.1 g leaf sample was cut into filaments (about 5 mm × 1 cm) and overnight in 5 mL of 80% (φ) acetone solution (light-proof and sealed). One mL extract was mixed 4 mL of 80% (φ) acetone solution, and then measured by ultraviolet spectrophotometer (*UV1800PC*, *Shanghai Jinghua Science and Technology Instrument Co., Ltd*., China) at 645 and 663 nm, respectively. The Chl content was calculated using the following formula:

$$Chl = (20.2\ D_{645} + 8.02\ D_{663}) \times V/M,$$

where Chl is the total chlorophyll content in leaf samples; D_{645} is the absorbance at 645 nm; D_{663} is the absorbance at 663 nm; V is the total volume; W is the fresh mass of leaf sample.

Leaf carbohydrates determination: Total soluble sugar (TSS) was measured according to Ebell (1969). Crushed leaf sample of 0.05 g was added to 4 mL of 80% (φ) ethanol solution and placed in a water bath at 80°C for 40 min, and then centrifuged for 3 min at 12,000 rpm in a high-speed freezing centrifuge (*5415D*, *Eppendorf,* Germany) to obtain supernatant. Two mL of 80% (φ) ethanol solution was added to the residue for repeating the above water bath and centrifuge operation for 2 times, and the supernatant extracted three times was mixed with 10 mg of activated carbon, and decolorized in a water bath at 80°C for 30 min, and then centrifuged for 3 min at 12,000 rpm. The supernatant was extracted and diluted to 10 mL, and the sample of sugar extract was obtained. One mL of sugar extract was mixed with 3 mL of anthrone reagent and placed in a water bath at 100°C for 10 min. After cooling, it was measured by ultraviolet spectrophotometer at 620 nm (*UV1800PC*, *Shanghai Jinghua Science and Technology Instrument Co., Ltd*., China). The content of glucose (μg·mL^{-1}) was found on the standard curve. The TSS was calculated using the following formula:

$$TSS = (C \times V1 \times N \times 106)/M,$$

where C is the content of glucose (μg·mL^{-1}) found on the standard curve; V1 is the liquid volume when measured by spectrophotometer; N is a dilution multiple; W is the fresh mass of leaf sample.

The measurement of starch content followed the method of Wang *et al*.(1993). The residue after extraction of sugar was added to 20 mL of deionized water, and placed in a water bath at 100°C for 15 min, and then added to 2 mL of 9.2 mol·L^{-1} perchloric acid solution to extract for 15 min. Starch extract obtained after cooling and filtering, was diluted to 50 mL to determine starch content by referencing to TSS content determination method.

Statistical analysis: Data were statistically analyzed using *SPSS version 22.0* statistical software. Firstly, analysis of variance (*ANOVA*) of two factors with multiple response variables was used to examine the effects of LFR, time, and their interaction on the profiles of gas-exchange parameters, Chl, SLM, and carbohydrate, and variance analysis of single factor multiple response variables was used to

Table 1. Multiple response variable analysis of variance. Chl – chlorophyll; C_i – intercellular CO_2 concentration; g_s – stomatal conductance; LFR – leaf-to-fruit ratio; LT – leaf thickness; P_N – net photosynthetic rate; PTT – palisade tissue thickness; SLM – specific leaf mass; Sta – starch; STT – spongy tissue thickness; TSS – total soluble sugar. df – degree of freedom; SS – sum of squares; MS – mean square.

Index	Source of variation	df	SS	MS	*F* value	*P* value
P_N	time (T)	4	457.379	114.345	221.820	<0.05
	leaf-to-fruit ratio (LFR)	14	415.732	29.695	57.606	<0.05
	T × LFR	56	47.808	0.854	1.656	<0.05
g_s	time (T)	4	0.295	0.074	599.051	<0.05
	leaf-to-fruit ratio (LFR)	14	0.172	0.012	100.048	<0.05
	T × LFR	56	0.051	0.001	7.395	<0.05
C_i	time (T)	4	43515.947	10878.987	93.399	<0.05
	leaf-to-fruit ratio (LFR)	14	80951.867	5782.276	49.642	<0.05
	T × LFR	56	9936.309	177.434	1.523	<0.05
Chl	time (T)	4	30.752	7.688	354.997	<0.05
	leaf-to-fruit ratio (LFR)	14	20.532	1.467	67.720	<0.05
	T × LFR	56	1.977	0.035	1.630	<0.05
SLM	time (T)	4	6897.743	1724.436	269.334	<0.05
	leaf-to-fruit ratio (LFR)	14	1956.924	139.780	21.832	<0.05
	T × LFR	56	771.414	13.775	2.152	<0.05
TSS	time (T)	4	0.937	0.234	206.024	<0.05
	leaf-to-fruit ratio (LFR)	14	0.758	0.054	47.631	<0.05
	T × LFR	56	0.257	0.005	4.038	<0.05
Sta	time (T)	4	1.240	0.310	3479.864	<0.05
	leaf-to-fruit ratio (LFR)	14	0.083	0.006	66.238	<0.05
	T × LFR	56	0.050	0.001	9.928	<0.05
LT	leaf-to-fruit ratio (LFR)	14	16636.091	1188.292	26.205	<0.05
PTT	leaf-to-fruit ratio (LFR)	14	4623.398	330.243	15.148	<0.05
STT	leaf-to-fruit ratio (LFR)	14	3723.152	265.939	9.156	<0.05

examine the effect of LFR on leaf anatomical structure. If the effect of LFR was significant, the *ANOVA* and multiple comparisons were used to identify whether there was a significant difference in response to different leaf and fruit treatments.

Results

According to the result of multiple response *ANOVA* (Table 1), the effects of LFR, time, and their interaction on gas exchange, leaf traits, carbohydrate content, and anatomical structure were statistically significant. The results indicated that P_N, g_s, C_i, Chl, SLM, TSS, starch, LT, PTT, and STT varied greatly among girdled shoots with different LFRs and time. According to the values of sum of squares, time had greater effects on Chl, SLM, TSS, starch, P_N, and g_s than those of LFRs, which indicated that the general pattern of these variables mainly depended on the time rather than on LFR. But the general pattern of C_i mainly depended on LFR.

Gas exchange: In the girdled shoots with one and two leaves, P_N decreased significantly with decreasing LFR (Fig. 2*A*,*B*). In the girdled shoots with three and four leaves, LFRs with three fruits (3L:3F and 4L:3F) showed significantly lower P_N values than those for LFRs with one fruit (3L:1F and 4L:1F), which did not differ significantly from LFRs with two fruits (3L:2F and 4L:2F) (Fig. 2*C*,*D*). A trend of higher P_N with decreasing LFR was found in the girdled shoots with five leaves (Fig. 2*E*).

In the girdled shoots with one and two leaves (Fig. 2*F*, *G*), g_s decreased with the decreasing LFR. In the girdled shoots with three and four leaves, g_s of LFRs with three fruits was lower than that of LFRs with two fruits, which were higher than or equal to the g_s values of LFRs with one fruit (Fig. 2*H*,*I*). In the girdled shoots with five leaves, increasing LFR led to a reduction in g_s, and the lowest g_s was observed in 5L:1F, but there was no significant difference between 5L:2F and 5L:3F (Fig. 2*G*).

In the girdled shoots with one and two leaves, the C_i values increased without differing significantly as the LFR decreased (Fig. 2*K*,*L*). In the girdled shoots with five leaves, C_i remained unchanged in response to a change in LFR (Fig. 2*O*). In the girdled shoots with three and four leaves, compared to the LFRs with one and two fruits, LFRs with three fruits had consistently higher C_i values, and the lowest annual mean values were obtained respectively in 3L:1F and 4L:2F (Fig. 2*M*,*N*).

Leaf traits: The Chl content was responsive to the changes in LFR. In the girdled shoots with one and two leaves (Fig. 3*A*,*B*), Chl decreased significantly with decreasing

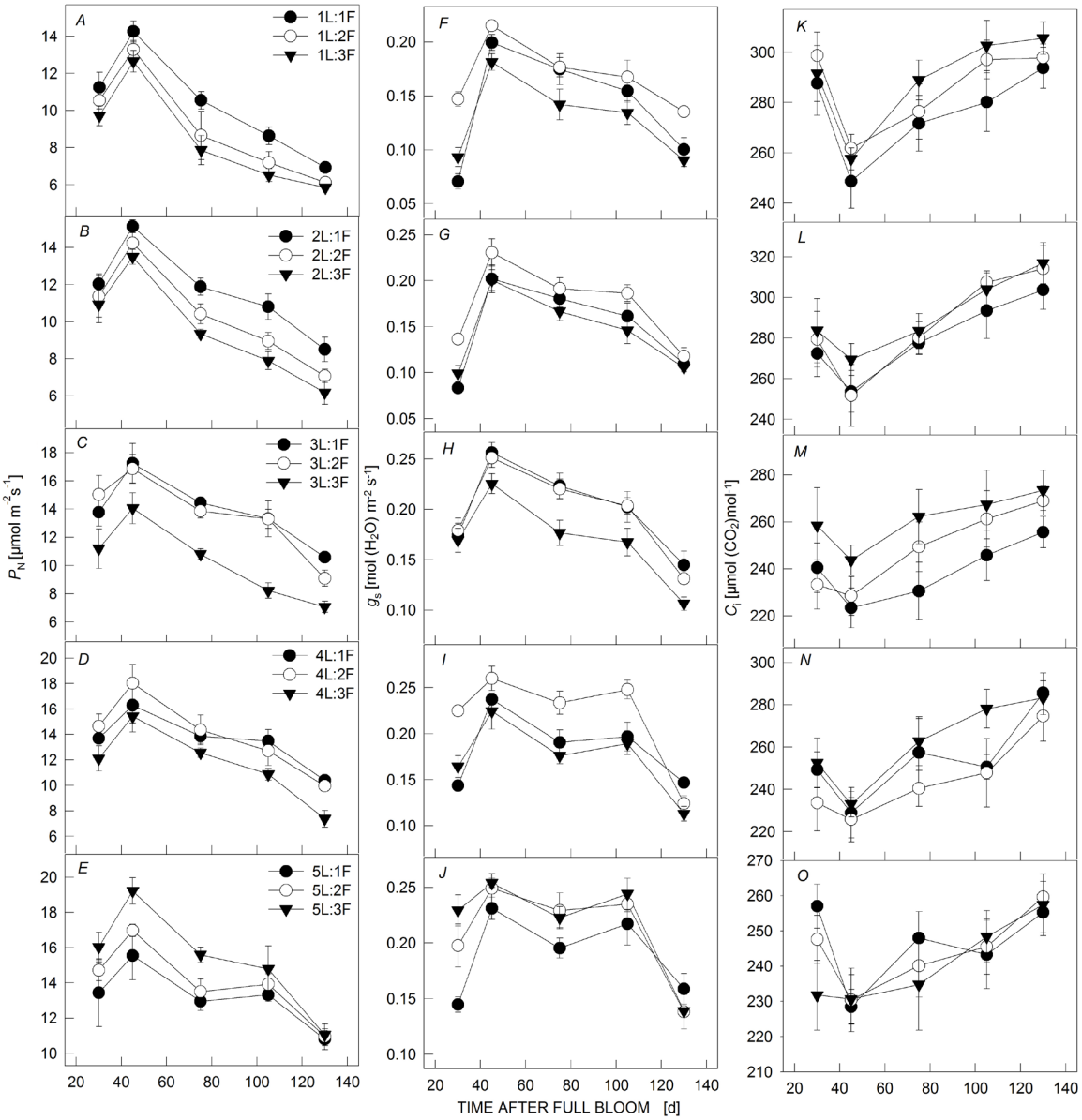

Fig. 2. Seasonal variation of net photosynthetic rate (P_N) (*A–E*), stomatal conductance (g_s) (*F–J*), and intercellular CO_2 concentration (C_i) (*K–O*) in the leaves of girdled walnut shoots with different leaf-to-fruit ratios.

LFR. LFRs with one fruit (1L:1F and 2L:1F) had the higher Chl content than those with two and three fruits (1L:2F, 1L:3F, 2L:2F, and 2L:3F). In the girdled shoots with three and four leaves, the Chl content of LFRs with one and two fruits was con-sistently significantly higher than that of LFRs with three fruits (Fig. 3*C*,*D*). There was no significant difference in the Chl content between 5L:2F and 5L:3F, although the Chl content for these two ratios was significantly different from that for 5L:1F (Fig. 3*E*). In the girdled shoots with one and two leaves, SLM significantly increased with the increasing LFR (Fig. 3*F*,*G*). In the girdled shoots with three, four, and five leaves, SLM was unresponsive to leaf-to-fruit ratio manipulation (Fig. 3*H–J*).

Carbohydrates: In the girdled shoots with one and two leaves, TSS decreased significantly with decreasing LFR (Fig. 4*A*,*B*). There was no significant difference in TSS values between different LFRs with three and four leaves (Fig. 4*C*,*D*). In the girdled shoots with five leaves, 5L:3F showed the highest TSS value (Fig. 4*E*). Starch was also responsive to the changes in LFR, and the starch content increased with the increasing LFR (Fig. 4*F–J*).

Anatomical structure of leaves: There were significant differences in thickness of leaf, palisade tissue, and spongy tissue between LFRs. The thickness of leaf, palisade tissue, and spongy tissue increased with the increasing LFR (Figs. 5, 6). The thickness of leaf (209.38 ± 8.67 μm), palisade tissue (104.99 ± 6.76 μm) and spongy tissue (79.36 ± 6.95 μm) in 5L:1F were significantly higher than

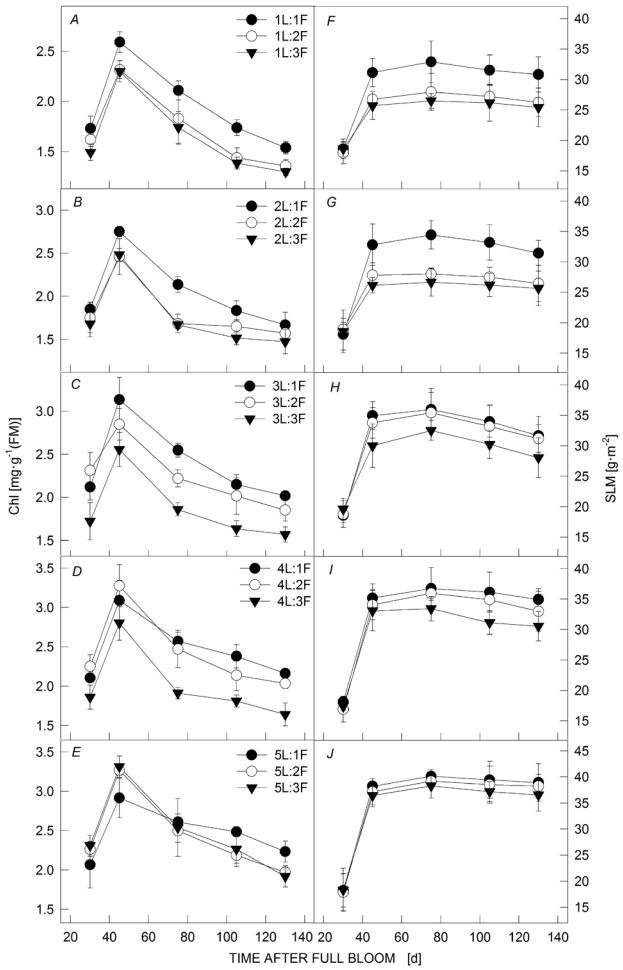

Fig. 3. Seasonal variation of chlorophyll (Chl) content (*A–E*) and specific leaf mass (SLM) (*F–J*) in the leaves of girdled walnut shoots with different leaf-to-fruit ratios.

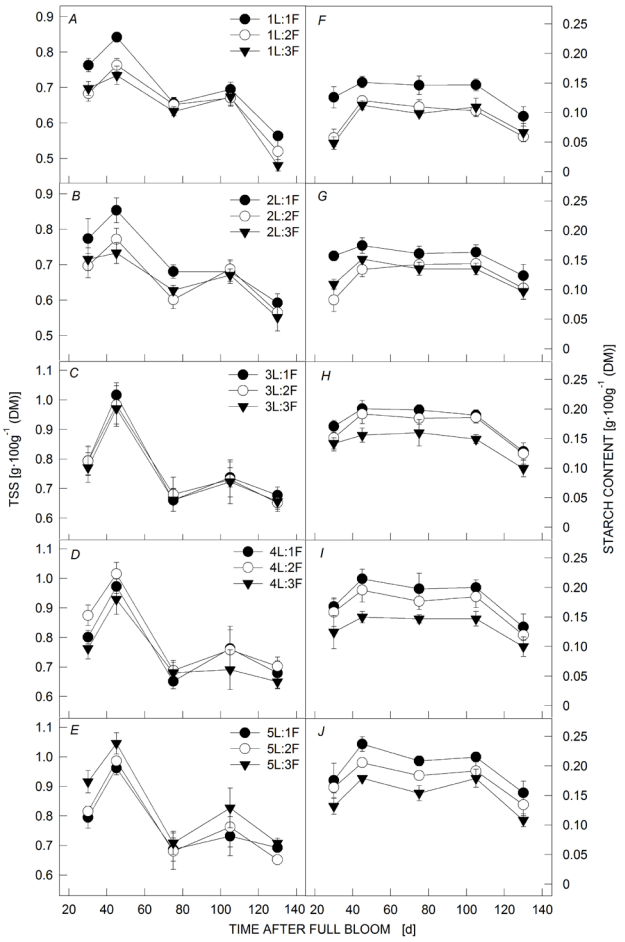

Fig. 4. Seasonal variation of total soluble sugar (TSS) (*A–E*) and starch (*F–J*) content in the leaves of girdled walnut shoots with different leaf-to-fruit ratios.

that of other LFRs. In contrast, 1L:2F, 1L:3F, and 2L:3F showed the lowest thickness of leaf (respectively, 153.01 ± 4.17, 152.84 ± 4.70, and 154.63 ± 7.01 μm) and palisade tissue (respectively, 71.70 ± 3.04, 73.86 ± 2.52, and 72.17 ± 2.04 μm). At the stage of rapid growth of fruit (45 DAF) (Fig. 5), the leaf of LFRs with one and two leaves was light in color and had a low chloroplast number, and its palisade tissue cells were arranged into two layers, accounting for less than 1/2 of the leaf thickness. However, the leaf of LFRs with three to five leaves was dark in color and had the high chloroplast number, and its palisade tissue cells accounted for more than 1/2 of the leaf thickness, although they were also arranged into two layers. In the mature period of fruit (130 DAF) (Fig. 6), the leaf of LFRs with one to three leaves senesced faster, since its epidermis cells were partially distorted, and some mesophyll cells began to disintegrate, and large air space appeared in sponge tissue area accompanied by more free and damaged cells in the mesophyll cells. What is more, the lower epidermis cells began to disintegrate and burst. These phenomena were not obvious in 4L:1F and 5L:1F.

Correlation among variables as affected by LFRs: According the matrix of correlation coefficients among the evaluated variables for the main effects of LFR with one and two leaves (OTR) (Table 2), LFR was significantly and positively correlated with Chl, SLM, TSS, starch, P_N, and g_s. P_N was positively and highly correlated with Chl, SLM, TSS, and starch, but negatively correlated with C_i. LFRs with three and four leaves (TFL) were positively correlated with Chl and starch, but showed no significant correlation with P_N. P_N was significantly correlated with Chl, SLM, and TSS. The main effects of LFRs with five leaves (FR) on the evaluated variables showed that LFR was negatively correlated with P_N and g_s, and positively correlated with starch, and there was a positive and high correlation between P_N and g_s.

Discussion

The relationship between P_N and LFRs with one and two leaves: The decrease in P_N resulting from decreasing LFR with one and two leaves (1L:1F, 1L:2F, 1L:3F, 2L:1F, 2L:2F, and 2L:3F) in the present experiment (Table 2) is in agreement with the findings for chestnut (Xiong *et al*. 2012) and kiwifruit (Fang *et al*. 2001). Results for chestnut

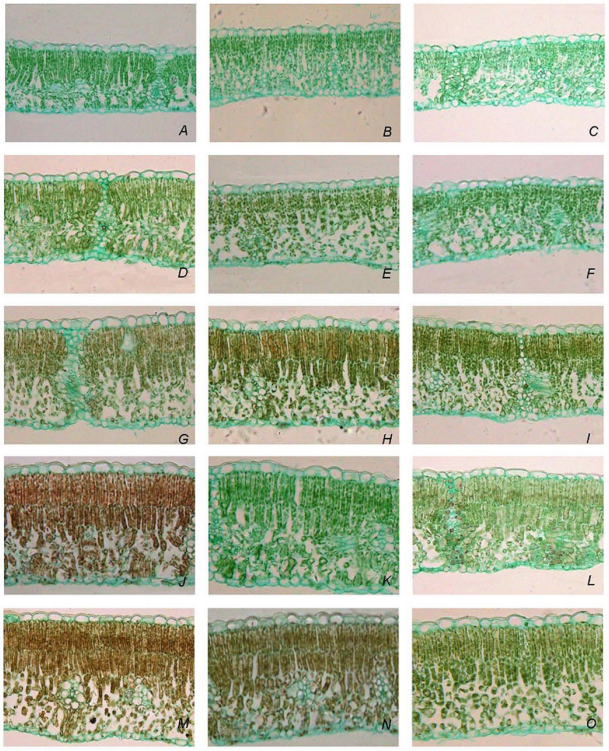

Fig. 5. Anatomical structure of leaves in girdled walnut shoots with different leaf-to-fruit ratio in the fast-growing period. 1L:1F (*A*), 1L:2F (*B*), 1L:3F (*C*), 2L:1F (*D*), 2L:2F (*E*), 2L:3F (*F*), 3L:1F (*G*), 3L:2F (*H*), 3L:3F (*I*), 4L:1F (*J*), 4L:2F (*K*), 4L:3F (*L*), 5L:1F (*M*), 5L:2F (*N*), and 5L:3F (*O*).

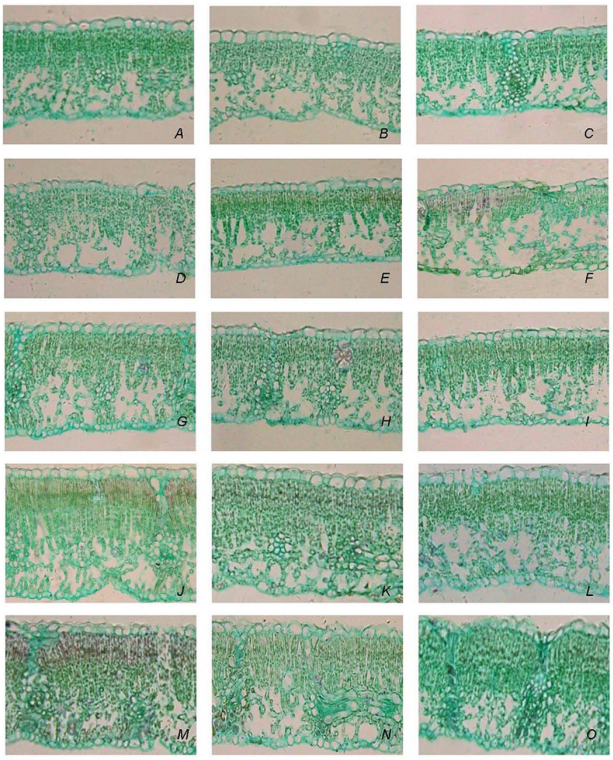

Fig. 6. Anatomical structure of leaves in girdled walnut shoots with different leaf-to-fruit ratio in mature period. 1L:1F (*A*), 1L:2F (*B*), 1L:3F (*C*), 2L:1F (*D*), 2L:2F (*E*), 2L:3F (*F*), 3L:1F (*G*), 3L:2F (*H*), 3L:3F (*I*), 4L:1F (*J*), 4L:2F (*K*), 4L:3F (*L*), 5L:1F (*M*), 5L:2F (*N*), and 5L:3F (*O*).

showed that within a certain range of leaf-to-involucre (10 ~ 33:1), P_N increased with an increasing leaf-to-involucre ratio, and the leaves in treatments with the highest leaf-to-involucre (28 ~ 33:1) showed a higher Chl content. In the present study, LFRs of 2L:1F and 1L:1F had higher P_N and Chl content than other LFRs with a few leaves (Figs. 2*A*,*B*; 3*A*,*B*). It was also found in our study that 1L:2F, 1L:3F, and 2L:3F showed the lowest thickness of leaf and palisade tissue (Fig. 5*B*,*C*,*F*), which indicated that extremely low LFR had an adverse effect on the growth and development of leaf. The downregulation of P_N at extremely low LFR was largely the result of a severe deficiency in photosynthates caused by the extremely few leaves, which could be explained by the lower content of total soluble sugar and starch at low LFR (1L:2F, 1L:3F, 2L:2F, and 2L:3F) (Fig. 4*A*,*B*,*F*,*G*). In addition, the effect of girdling on photosynthesis should not be ignored. Girdling blocks the flow of transported nutrients from other parts of the tree through phloem to the leaves, which is not conducive to the development of leaves (Fang *et al*. 2001). It has been proven that girdling reduced maximal quantum yield of PSII photochemistry (F_v/F_m) (Nebauer *et al*. 2011) and extremely few leaves results in a lower value of P_N (Quentin *et al*. 2013). Similar to other reports (Marini and Barden 1981, Thompson *et al*. 1996, Jumrani *et al*. 2017), the P_N measured in the present study was positively correlated with SLM (Table 2), which is an important index of leaf development. The reduction in the P_N of shoots with low LFRs (2L:3F and 1L:3F), along with the low Chl content and SLM values indicated that girdled shoots with extremely few leaves produced insufficient photosynthetic products to meet both their own requirement and a high fruit load, and that girdling blocked the transport of organic nutrients from other parts to the girdled shoot. The combination of these two factors affected leaf development, which could be reflected in the lower Chl content and SLM (Fig. 3*A*,*B*,*F*,*G*), and the lower thickness of leaf and palisade tissue.

The relationship between P_N and LFRs with five leaves: At LFRs with five leaves (5L:1F, 5L:2F, and 5L:3F), P_N was significantly reduced at high LFR (Table 2), which is consistent with the reports for many plants (Urban *et al*. 2004, DaMatta *et al*. 2008). In this study, since C_i values in leaves changed little with the decreasing P_N and g_s (Fig.2*O*), we deduced that the decrease in P_N was probably attributable to nonstomatal factors. Previously, Farquhar (1982) has suggested that in the case of a reduction in P_N and g_s, an increase or invariability in C_i indicates that the decrease in P_N is mostly caused by nonstomatal limitation. Accordingly, other photochemical or biochemical activities (*e.g.*, Rubisco activity) might be weakened in the leaves of shoots with a high LFR, which contributes to an increase in C_i after stomatal closure (Guo *et al*. 2005). In order to

Table 2. Matrix of correlation coefficients among the response variables significantly affected by leaf-to-fruit ratio (LFR). Chl – chlorophyll; C_i – intercellular CO_2 concentration; g_s – stomatal conductance; FL – LFRs with five leaves; OTL – LFRs with one and two leaves; P_N – net photosynthetic rate; Sta – starch; SLM – specific leaf mass; TFL – LFRs with three and four leaves; TSS – total soluble sugar. ** – extremely significant correlation at the 0.01 level; * – significant correlation at the 0.05 level.

LFR		Chl	SLM	TSS	Sta	P_N	g_s	C_i
OTL	LFR	0.797**	0.622**	0.752**	0.867**	0.880**	0.774**	–0.341
	Chl	-	0.317	0.457	0.885**	0.558*	0.550*	–0.045
	SLM		-	0.953**	0.351	0.843**	0.928**	–0.938**
	TSS			-	0.511**	0.884**	0.960**	–0.821**
	Sta				-	0.689**	0.610**	–0.086
	P_N					-	0.920**	–0.672**
	g_s						-	–0.794**
TFL	LFR	0.703**	0.461	0.162	0.723**	0.373	0.211	–0.286
	Chl	-	0.041	–0.077	0.988**	0.528*	0.393	–0.143
	SLM		-	0.879**	0.006	0.572*	0.631**	–0.810**
	TSS			-	–0.099	0.535*	0.783**	–0.878**
	Sta				-	0.456	0.352	0.120
	P_N					-	0.774**	–0.610**
	g_s						-	–0.863**
FL	LFR	0.008	0.281	–0.618	0.899**	–0.743*	–0.784*	0.218
	Chl	-	–0.923**	–0.599	0.393	–0.639	–0.559	0.913**
	SLM		-	0.416	–0.133	–0.424	0.358	–0.851**
	TSS			-	–0.887**	0.910**	0.891**	–0.821**
	Sta				-	–0.950**	–0.962**	0.615
	P_N					-	0.993**	–0.813**
	g_s						-	–0.766*

confirm whether Rubisco and/or stomatal limitations caused differences between different LFRs, future research needs to compare the P_N-C_i and P_N-PAR curves of the photosynthesis among different LFRs. Although a low LFR was associated with the enhanced Chl content, no significant correlation between P_N and Chl content was observed (Table 2), which suggests that the Chl content was not the main factor limiting P_N. More leaves on the girdled shoot could produce sufficient photosynthetic products to meet the requirement of both leaves and fruits. Previous studies showed that the reduction in photosynthesis after girdling was consistent with a feedback effect of an increased carbohydrate content of the leaves (Moscatello *et al*. 2017). Similarly, in this study, a high LFR also contributed to the accumulation of carbohydrates when the plant's sinks failed to utilize the products of photosynthesis, which could be reflected by the trend for a higher starch content (Fig. 4*G*) and SLM (Fig. 3*G*) with decreasing LFR, and consistent with the feedback inhibition of photosynthetic production (Iglesias *et al*. 2002). Thus, nonstomatal limitation and feedback inhibition of photosynthetic production might be the main factors responsible for the decline in P_N in the long-term response process after changing LFR. The mechanisms underlying nonstomatal limitation and the relationship between photosynthesis and accumulation of photosynthetic products will be the focus of our future research work.

The relationship between P_N and LFRs with three and four leaves: In LFRs with three and four leaves (3L:1F, 3L:2F, 3L:3F, 4L:1F, 4L:2F, and 4L:3F), there was no significant correlation between LFR and P_N (Table 2). Consequently, within a certain LFR range, P_N had only under-gone very slight changes (Fig. 2*C*,*D*), which indicates that when supply-side processes in sources and demand-side processes in sinks reach a balance, leaves have considerable flexibility in their photosynthetic apparatus within the range of LFR. Thus, LFR had no significant effect on the photosynthetic performance of leaves. It was also likely that the small span between two different LFRs led to insignificant difference in P_N between the different LFRs. However, 3L:3F and 4L:3F both showed the lowest P_N, g_s, and higher C_i (Fig. 2*C*,*D*,*H*,*I*,*M*,*N*), which suggests that a decrease in P_N is not caused by lower CO_2 entry through the stomata, but is mainly associated with the diminished ability to capture light and fix CO_2 in leaves (Alves *et al*. 2011), because Chl contents were also lower in 3L:3F and 4L:3F (Fig. 3*C*,*D*). It is also possible that the excessive fruit load affected leaf development, which would further impede the development of the photosynthetic apparatus.

The relationship between P_N and LFRs with different fruits: Meanwhile, we also found that P_N firstly increased and then decreased with the increasing LFR with one and two fruits. However, there was a continuous rise in P_N with the increasing LFR with three fruits, which indicated that when the number of fruits remained unchanged,

the relationship between P_N and LFR showed different variation trend, and the decrease in P_N in 5L:1F, 4L:1F and 5L:2F were also attributable to the feedback inhibition.

In the present study, it was interesting to note that at 105–130 DAF, a reduction in P_N was observed for all the LFRs with three fruits (Fig. 2*A–E*). Wang *et al*. (2010) also found that during the fruit maturity period, the P_N and Chl of leaves on the fruit-bearing shoots were lower than that on the growing shoots. We deduce that the decrease in P_N was probably attributable to the leaf senescence, since the leaves of shoots with the high fruit load showed rapid senescence in the mature period of fruits (Fig. 6), which was accompanied by a decrease in the Chl content (Fig. 3*A–E*). A decrease in the Chl content is the most obvious symptom of leaf senescence (Gao *et al*. 2016). Thus, a reduced P_N is likely to be due to accelerated leaf senescence caused by an excessive fruit load. In this regard, it has been reported that the inhibition of leaf photosynthesis by the high availability of photosynthetic products depends on the growth stage (Gucci *et al*. 1995, Syvertsen *et al*. 2003).

Conclusions: The changes in leaf traits and gas-exchange parameters at different LFRs observed in this study confirmed our hypothesis that the effects of LFR on P_N depend on LFR range. Moreover, the cause of the effect of LFR on photosynthesis is different within the different LFR range. The positive correlation between LFR (with one and two leaves) and P_N could be attributed to the inhibition of leaf development. The nonstomatal limitation and feedback inhibition of photosynthetic production contribute a negative correlation between LFR (with five leaves) and P_N. Under a balanced state of coordinated supply and demand between source leaf and sink fruit, the LFR (with three or four leaves) had no significant effect on the photosynthetic performance. Additionally, the excessive fruit load accelerated leaf senescence resulting in a reduction in P_N. We believe that the above results enrich the theory of photosynthesis of walnut trees under source-sink manipulation and provide a theoretical basis for regulating and controlling the reasonable load of walnut in actual production. For example, in walnut cultivation and management, when the number of leaves on the bearing branch is small, it is advisable to retain one fruit, and it also requires at least five leaves to supply three fruits for normal growth.

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