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Full Length Research Paper

The dynamics of fat, protein and sugar metabolism during walnut (*Juglans regia* L.) fruit development

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Walnut (*Juglans r*egia L.) is named the "super food" in the 21st century. In this study, the 9-year-old precocious walnut cultivar 'Xiangling' were used to exam the developmental process, and the dynamics of fat, protein and sugar content in the fruit, and the activities of enzymes related with sugar metabolism were further determined and analyzed. The result shows that the developmental process of walnut fruit could be divided into four stages: Slow growth {within 30 days after florescence, (DAF)}, fast growth (30 to 60 DAF), fat accumulation (60 to 100 DAF) and fruit maturity (100 to 140 DAF). Fat content in walnut fruit increased continuously and the maximum increment occurred within 60 to 90 DAF. It amounted to 50.11% at 100 DAF and maximized with 63.13% at 140 DAF when the fruit ripens. Sucrose and glucose content showed similar trend. They were both low at earlier stage, then gradually increased and reached the peak at 90 DAF, followed by gradual descending. Fructose content topped at earlier stage and then gradually decreased. Acid invertase (AI, EC 3.2.1.26), neutral invertase (NI, EC 3.2.1.26), sucrose phosphate synthase (SPS, EC 2.4.1.14) and sucrose synthase (SS, EC 2.4.1.13) activity were higher during the earlier stage and then decreased until the minimum point at 80 DAF, thereafter these enzyme activities turned to ascend the crest value at 90 to 100 DAF, and then kept decreasing until the late maturity stage. Our further analysis revealed soluble sugar content had significant positive correlation with fructose and glucose content ($P<0.05$). Sucrose content positively correlated with glucose content at $P<0.01$, with SS and SPS activity at$P<0.05$. Protein content negatively correlated with NI and AI activity at $P<0.01$, with fructose content at $P<0.05$. Our result suggests that sucrose played an important role during carbohydrate metabolism of walnut fruit.

Key words: Walnut (*Juglans regia* L.), fruit development, sugar, fat, protein, invertase.

INTRODUCTION

The 'hidden hunger' caused by nutrient imbalance is an invisible threat to human health. Currently, over three billion people are micronutrient malnourished (Welch and Graham, 2004; WHO, 2002). Since 20th century, it has reached broad consensus that rich nutrition and balanced food by cultivating and modifying crops will do good to human health (Pinstrup-Andersen, 2000). According to the reports of USDA National Nutrient Databank, the main nutrient in walnut (*Juglans regia* L.) is fat, which accounts for 65.21% of the whole substances; polyunsaturated fatty acid (47.17%) is one of the main constituent of fatty acid, containing 38.09% linoleic acid and 9.08% linolenic acid. Apart from fat, walnut also contains protein (15.23%) and carbohydrate (13.71%), in

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which carbohydrate is constituted of dietary fiber (6.7%), sucrose (2.43%), glucose (0.08%), fructose (0.09%) and starch (0.06%). The nutrition is very suitable for human health, so FDA (Food and Drug Administration) passed the permission of regarding walnut as a healthy food. Walnut is thus considered to be the "21st century super food", just like the important 'woody grain and oil' tree in China (Li et al., 2009). The walnut embryo developed from fertilization of inferior syncarpous ovary, and green peel developed from bract and perianth. The edible part of nuts is pip formed by embryo cotyledon (Xiao et al., 2009). Zhang et al. (2001) studied the dynamic changes of main nutrient's content such as lipid and mineral elements during the process of fruit ripening, and found that the rate of accumulation of lipids in the kernel and potassium in the shuck was exponential. There exists very significant positive correlation between the level of potassium in the shuck and of the lipids in the kernel. The contents of potassium in the shuck were significantly higher than that in the kernel and the shell. Of the mineral elements in the shuck, the contents of potassium were highest. Wu et al. (2003) studied the changes of sugar metabolism and related enzymes activities of pulp and green peel in walnut fruit development and found that the highest content of total sugar was in the late developmental stage, with the starch content being varied during fruit development and the contents of soluble sugars changing according to the kinds of sugars.

In this study, we studied the dynamic changes of fat, protein and sugar content and the related enzyme activities such as acid invertase (AI, EC 3.2.1.26), neutral invertase (NI, EC 3.2.1.26), sucrose phosphate synthase (SPS, EC 2.4.1.14), sucrose synthase (SS, EC 2.4.1.13) and amylase (EC 3.2.1.1) in fruit development of precocious walnut cultivar 'Xiangling'. The purpose of this study was to understand the dynamics of nutrition metabolism of walnut fruit and to provide scientific basis for reasonable cultivation measures.

MATERIALS AND METHODS

9-year-old plants of precocious walnut cultivar 'Xiangling' were used as plant materials, which were planted with row spacing 5 × 6 m in the garden of the Shandong Institute of Pomology, city Tai'an, province Shandong, China (N 36°09'59", E 117°13'30"). The altitude of experimental site is 150 m; the annual average temperature is 12.8°C. There are about 186.6 frost-free days a year. The annual average rainfall is 600 to 800 mm, occurred mainly in summer. The rainfall of July to August accounts for 53% and June to September accounts for 74%. The annual average relative humidity is 65%. Soil was loamy in texture under sprinkler irrigation 1 to 2 times per day, each for 20 min. Thus, good water and fertilizer conditions are provided.

The dynamics of fruit development

The walnut flowers bloomed on 22th April and florescence ended on 12th May at 2009. With flowers blooming, the hypogynous ovary began to expand and gradually developed into fruit. We randomly marked 10 blooming flowers from the four directions of the center of canopy with 10 similar trees, and then measured the vertical diameter, horizontal diameter and horizontal diameter of suture line of per fruit on 23th April, 2009. From that day to fruit ripened on 10th September, we measured these parameters (the vertical diameter, horizontal diameter and horizontal diameter of suture line of per fruit every 10 days. At the same time, picked another 10 fruits and weighed single fruit fresh weight every 5 days until 2nd June.

The methods of determining nutrient content

When walnut embryo was from colloidal nodular to solidification after 12th June (50 DAF), the content of fat, protein, sugar and the related enzyme activities were measured every 10 days. Every 10 fruits were picked from four directions of the center of crown of 10 sample trees randomly, then we removed embryos from nuts and put them into liquid nitrogen immediately, and then brought back to the laboratory and stored at -70°C until use. Three replications were set.

The fat content was determined by Soxhlet extraction method (Merlo and Passera, 1991). The protein content was determined by Kjeldahl determination. The sample was boiled with H_2SO_4-H_2O_2 mixture, and then determined by KDY-9820 Kjeldahl apparatus (Beijing Huawei Science and Technology Co., Ltd., China). The content of starch, soluble sugar, sucrose, fructose, glucose was determined as described by Xue (1985). The determination of amylase activity was performed as described by Merlo and Passera (1991). The activity of acid invertase (AI), neutral invertase (NI), sugar synthase (SS) and sucrose phosphate synthase (SPS) was detected by improved method of Xue (1985).

Statistical and correlation analyses

The dynamic changes of fruit development were investigated according to the changes of vertical diameter, horizontal diameter and horizontal diameter of suture line per fruit and single fruit fresh weight and their relative increase rate (RGR). The RGR of these parameters was calculated using the following formula (Aanderud et al., 2003; Huang et al., 2011):

$RGR = (V_2 - V_l) / [V_1 \cdot (T_2 - T_1)] \times 100\%$.

Where, V_2, V_l, is the measure value at two measure time; $T_2 - T_1$ is the days between two measure time.

The relative increase rate of fat was also calculated using the above formula. The data were analyzed using EXCEL 2007 and SAS 9.0. The correlation among the content of fat, protein, starch, sucrose, glucose, fructose, soluble sugar, and the activity of amylase, SS, SPS, NI and AI were tested using Pearson correlation coefficient.

RESULTS

The dynamics of walnut fruit development

The fresh weight per fruit increased slowly at the early growth period of fruit (within 30 DAF), then increased sharply reaching the peak (41.122 g) until 100 DAF (1st August). After that green peel cracked and fruit weight decreased a little (Figure 1). The RGR of mean fresh weight per fruit increased speedily after florescence and reached the summit (25.4% per day) 25 DAF (13th May). After that RGR decreased gradually, but after 30 to 40 DAF, RGR decreased sharply from 19.7 to 5.2%.

The RGR was lower but steady until fruit maturation

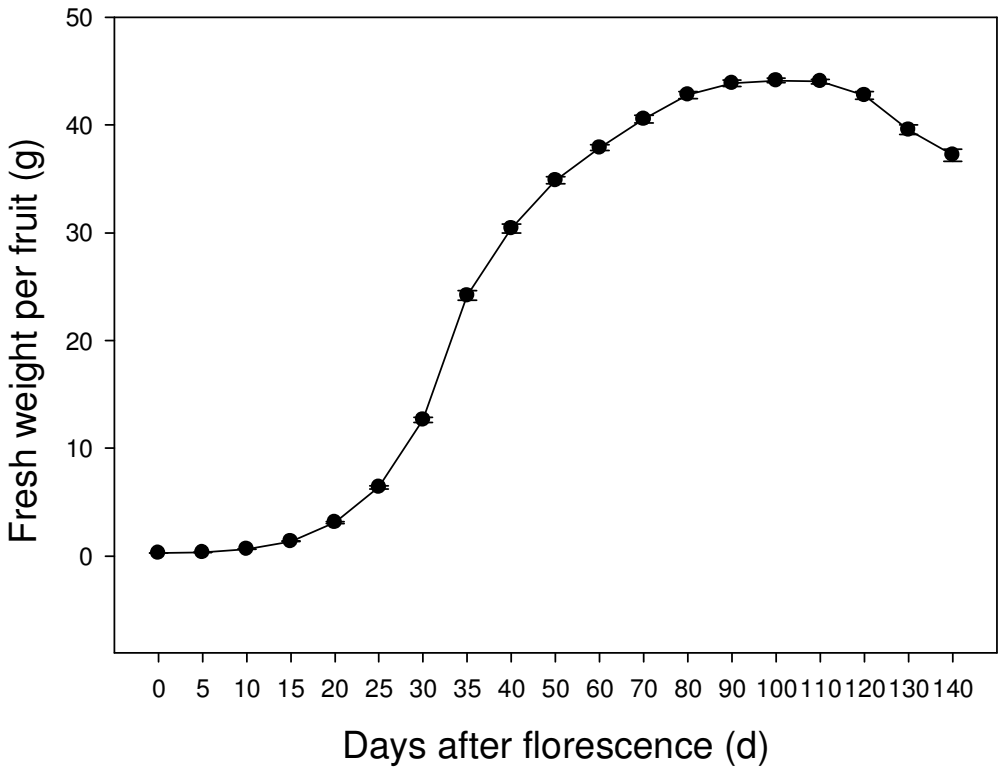

Figure 1. The fruit growth curve of walnut. Error bars indicate standard deviation, n=10.

(Figure 2). The vertical diameter, horizontal diameter and horizontal diameter of suture line of per fruit grew slowly within 25 DAF, and then increased to maximum rapidly at 60 DAF, but the volume per fruit did not increase (Figure 3). However, the RGR of vertical diameter, horizontal diameter and horizontal diameter of suture line increased rapidly within 25 DAF and reached maximum at 20 DAF, then decreased gradually until 60 DAF (Figure 4). The growth dynamics of single fruit weight, vertical diameter, horizontal diameter and horizontal diameter of suture line approached sigmoid pattern.

The dynamics of fat and protein content during walnut embryo development

From the beginning of embryo development to the stage of fruits fully ripe, the content of fat kept continual accumulating (Figure 5). The embryo of walnut at the early developmental stage was colloidal, and then began to solidify at 50 DAF with fat content accounting for 3.81%. In the stage of 60 to 90 DAF, fat content increased sharply from 5.1 to 42.01%; meanwhile the RGR of fat content increased sharply to 11.0% per day at 70 DAF. The RGR of fat content decreased quickly from 10.0 to 2.0% per day within 90 to 100 DAF (Figure 6). Fat content increased from 50.11% (100 DAF) to 63.13% (nature maturation, 140 DAF), but in the last 10 days of fruit maturation, fat content only increased by1.11%.

In the development of embryo, protein content fluctuated greatly (Figure 7). It was lower at the early stage, but with the development of embryo, protein synthesized quickly, especially at 80 DAF. Protein content increased quickly to 23.04% at 100 DAF, and then decreased gradually to the lowest and balanced level (140 DAF).

The dynamic of sugar content during walnut embryo development

With the development of walnut embryo, the dynamic of soluble sugar content is the unimodal curve of slow-quick-slow. The soluble sugar accumulated slowly at early stage, then maximized to 43.36 $mg \cdot g^{-1}$FW at 90 DAF. After that the soluble sugar content decreased gradually and at later stage of fruit maturation, the soluble sugar content changed slightly (Figure 8). Judging from sugar species, fructose content increased to 11.15 $mg \cdot g^{-1}$FW at 50 DAF, then decreased gradually and slightly. Glucose content, as well as sucrose, increased slowly at early stage, then reached a peak of 90 DAF. After that it decreased gradually. The change of sucrose content was greater ranging from 1.73 $mg \cdot g^{-1}$FW at 50 DAF to 14.16 $mg \cdot g^{-1}$FW at 90 DAF, and then decreased until 120 DAF. In contrast, starch content was steady and the maximum was 1.69 $mg \cdot g^{-1}$ FW and the least was 1.33 $mg \cdot g^{-1}$FW.

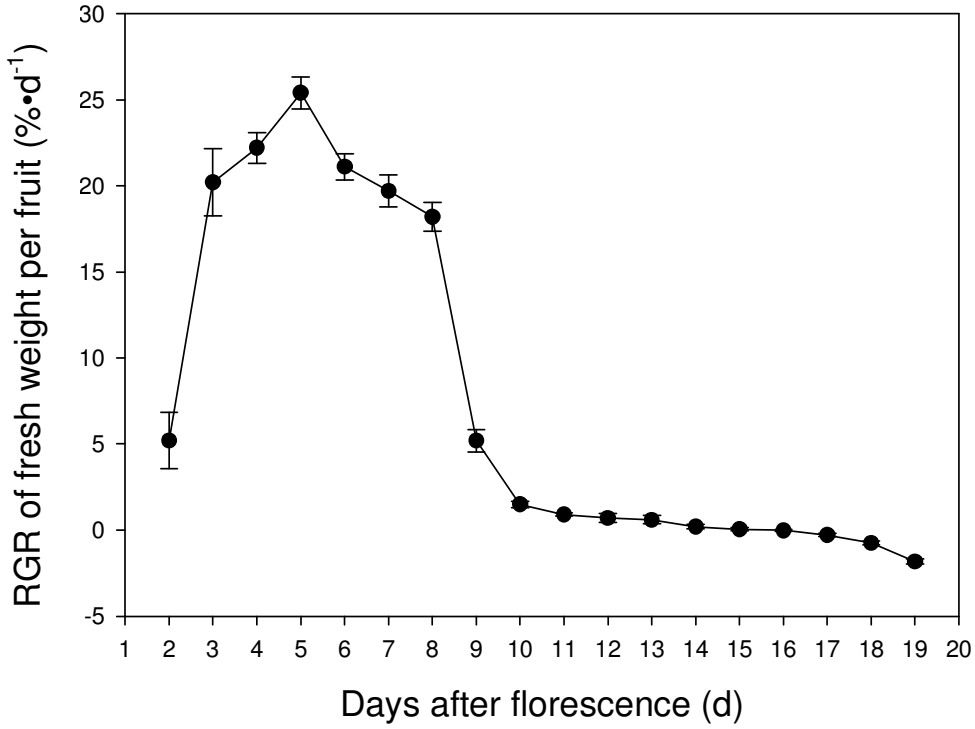

Figure 2. The relative growth rate of fresh weight per fruit. Error bars indicate standard deviation. n=3.

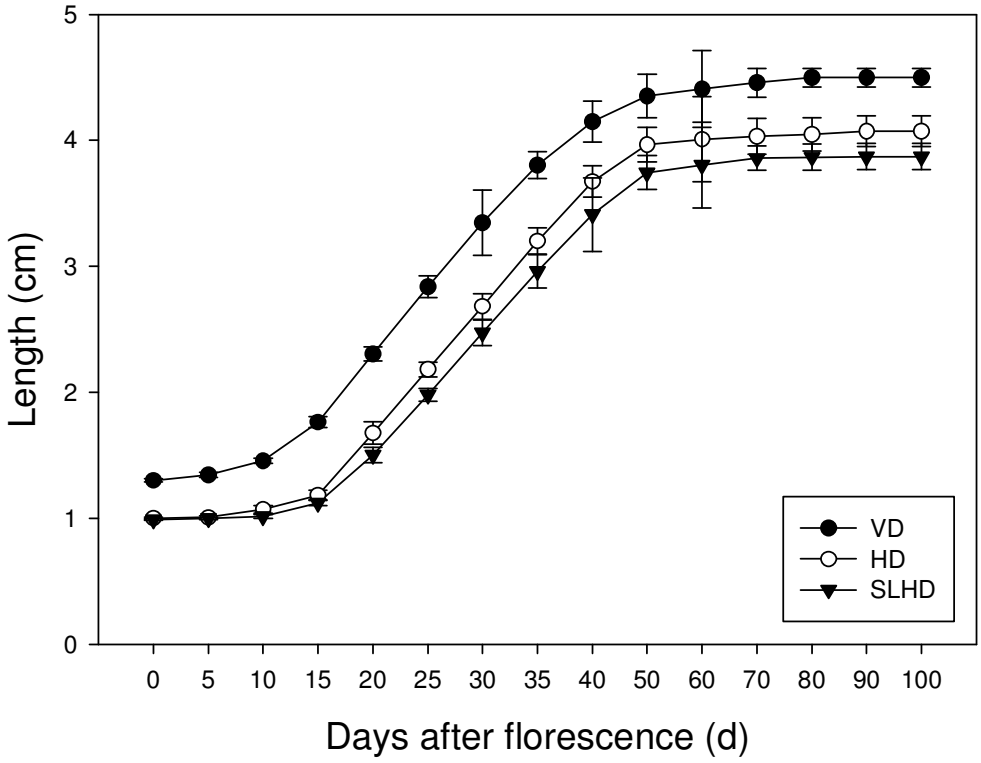

Figure 3. The dynamics of vertical diameter, horizontal diameter and horizontal diameter of suture line per fruit during fruit development.VD: vertical diameter; HD: horizontal diameter; SLHD: horizontal diameter of suture line; Error bars indicate standard deviation, n=10.

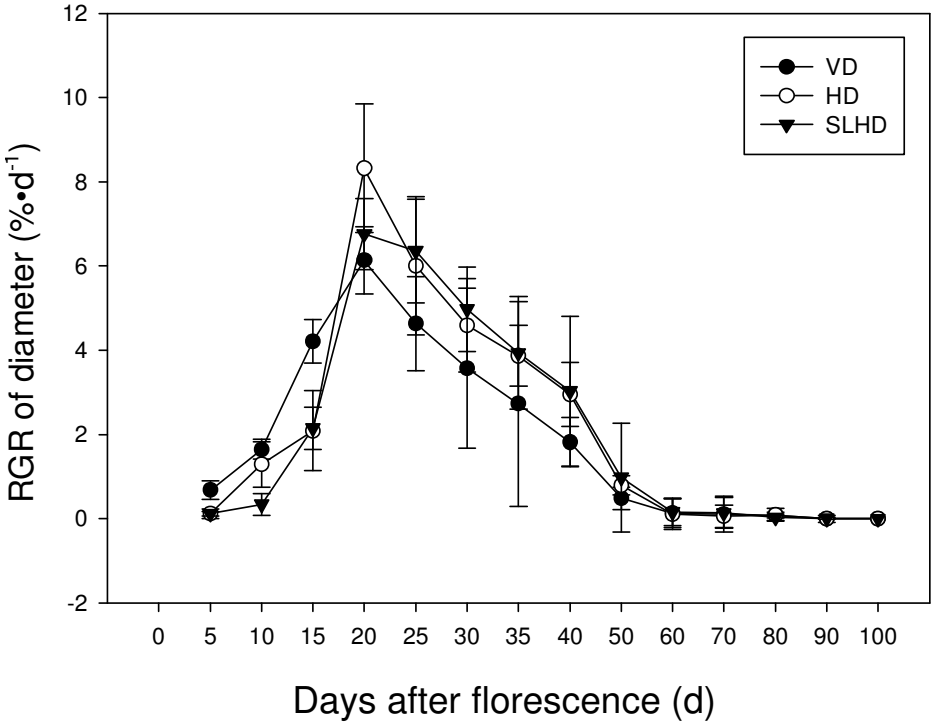

Figure 4. The relative growth rate of vertical diameter, horizontal diameter and horizontal diameter of suture line of per fruit during fruit development. VD: vertical diameter; HD: Horizontal diameter; SLHD: horizontal diameter of suture line; Error bars indicate standard deviation, n=3.

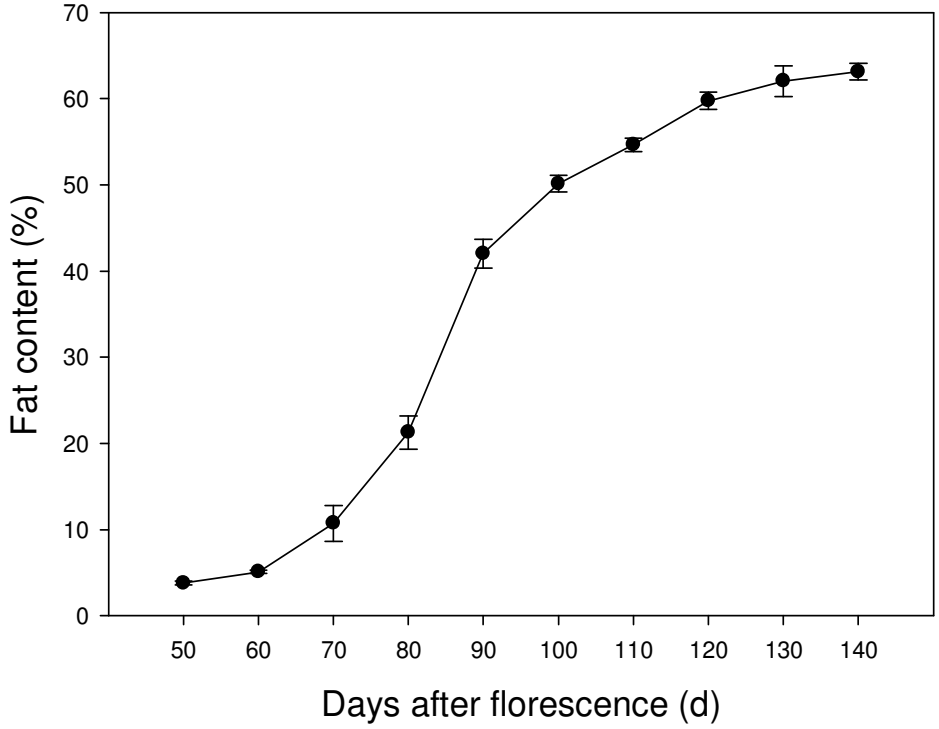

Figure 5. The dynamics of fat content during embryo development. Error bars indicate standard deviation, n=10.

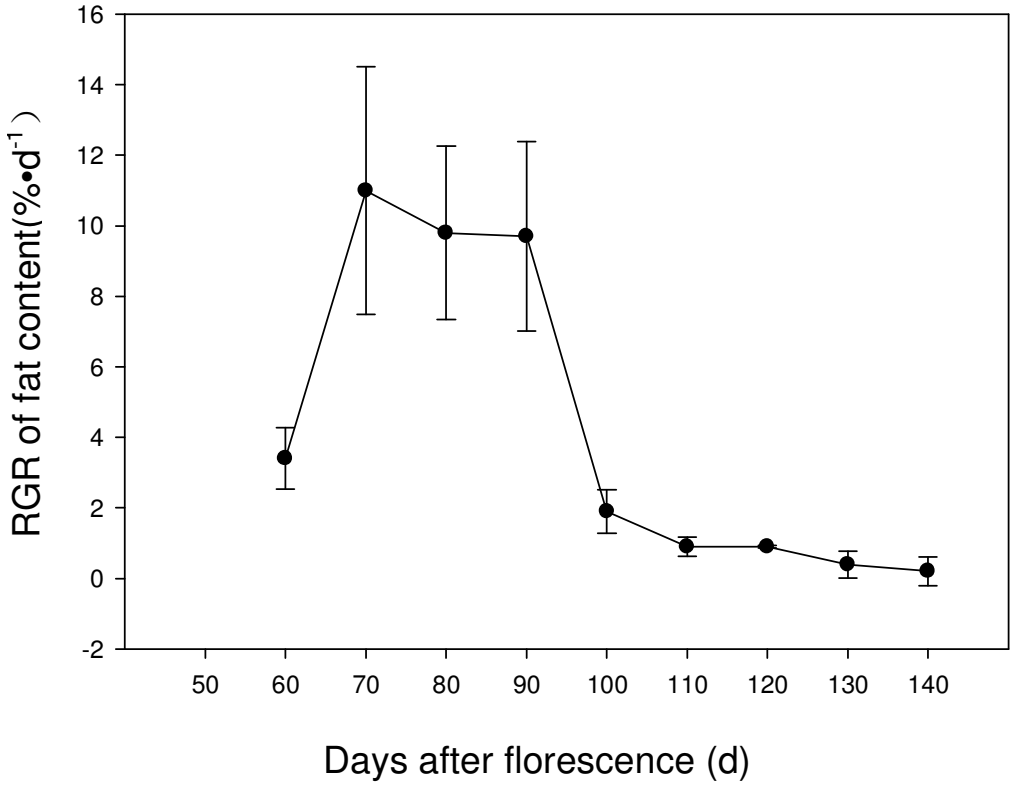

Figure 6. The relative growth rate of fat content during embryo development. Error bars indicate standard deviation, n=3.

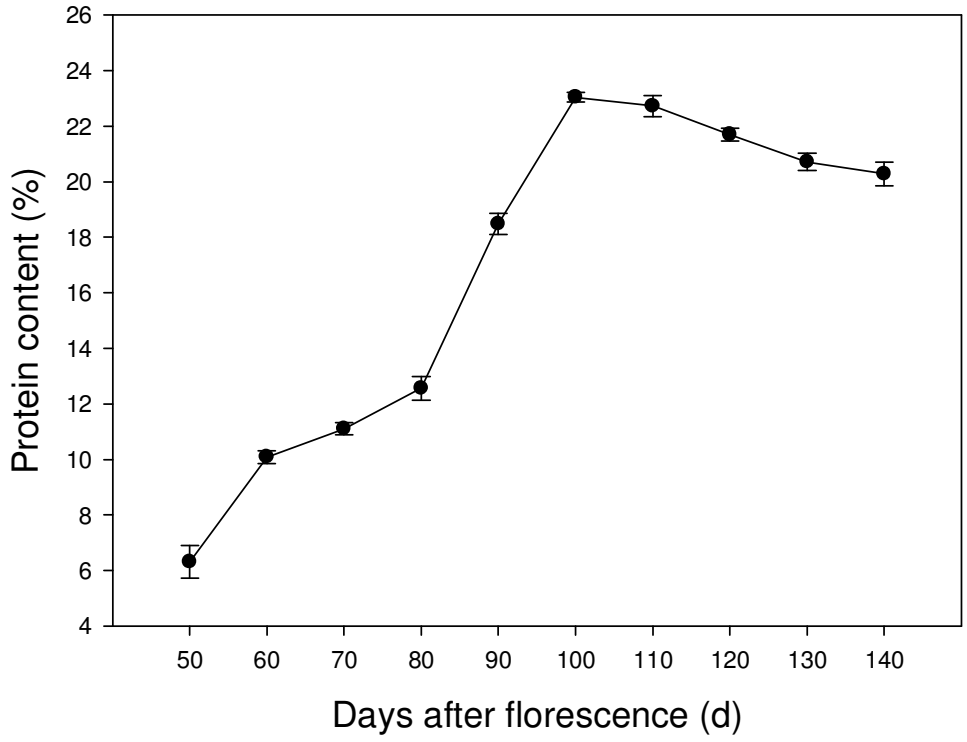

Figure 7. The dynamics of protein content during embryo development. Error bars indicate standard deviation, n=10.

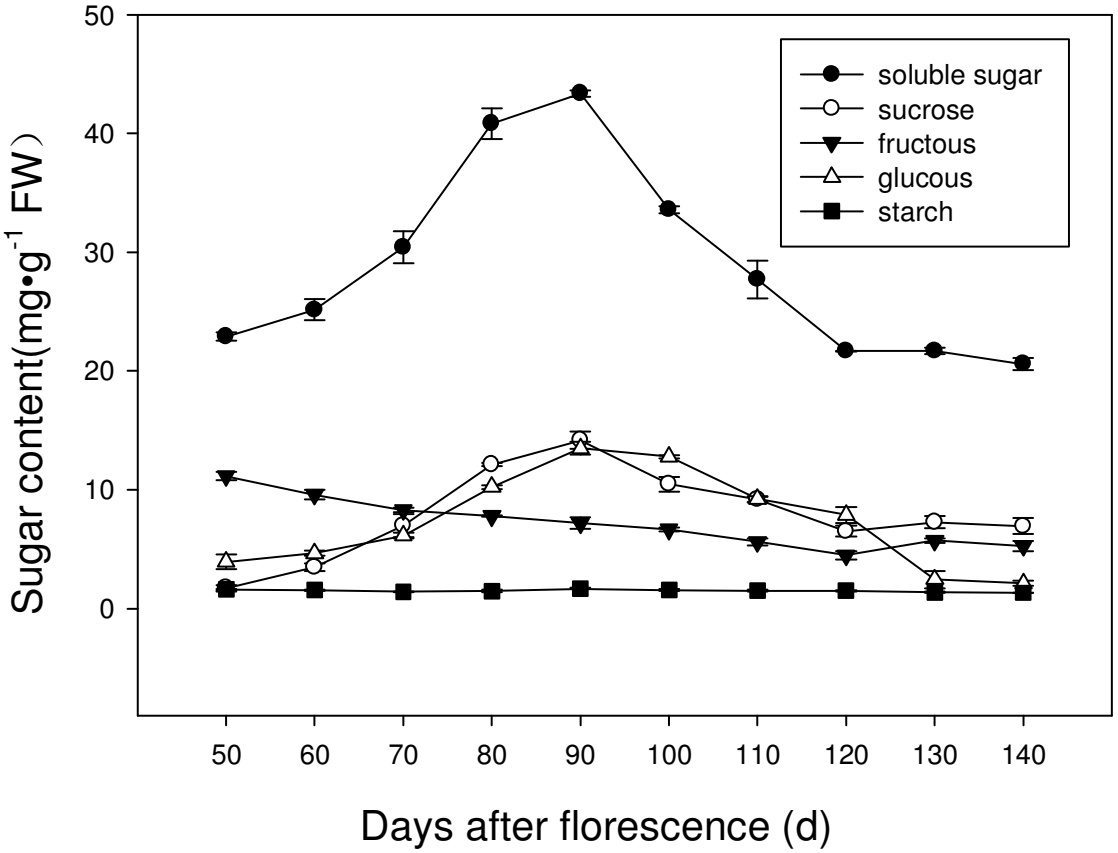

Figure 8. The dynamics of sugar content during embryo development. Error bars indicate standard deviation, n=10.

The dynamics of enzymatic activities during walnut embryo development

Amylase

Amylase activity trends to increase to 80 DAF then decrease (Figure 9). Amylase activity was lower at early stage, then increased rapidly to the maximum of 1.581 μmol glucose·g^{-1}FW·h^{-1} on 80 DAF, and then decreased. Amylase activity increased in frutescence process and was similar with the change of its substrate starch and its product glucose.

Acid invertase (AI) and neutral invertase (NI)

The change of AI activity was in accordance with NI, but AI activity was always higher than that of NI. AI and NI activities were both high at early stage. With the development of embryo, AI and NI activities decreased rapidly to 1.79 μmol glucose·g^{-1} FW·h^{-1} and 1.54 μmol glucose·g^{-1} FW·h^{-1} at 80 DAF, respectively. AI and NI increased rapidly to 2.49 μmol glucose·g^{-1} FW·h^{-1} and 2.24 μmol glucose·g^{-1} FW·h^{-1} at 90 DAF, then began to decrease until 130 DAF (Figure 9).

Sucrose synthase (SS) and sucrose phosphate synthase (SPS)

The change of SS activity was in accordance with SPS, and SS activity was always higher than SPS activity. SS and SPS activities declined at early stage, then decreased rapidly to 6.11 μmol sucrose·g^{-1} FW·h^{-1} and 3.52 μmol sucrose·g^{-1} FW·h^{-1} at 80 DAF, respectively. SS and SPS activities increased rapidly. SS and SPS activities reached maximum 13.04 μmol sucrose·g^{-1} FW·h^{-1} and 10.63 μmol sucrose·g^{-1} FW·h^{-1}, respectively at 100 DAF. Then SS and SPS activities began to decrease till 130 DAF (Figure 9).

Correlation analysis

The correlation analysis among fat, protein, starch, soluble sugar content and enzyme activities showed that: soluble sugar content had significant positive correlation with sucrose and glucose content (r=0.8323, P<0.01; r=0.8198, P<0.01, respectively) and positive correlation with amylase activity (r=0.7185, P<0.05); Fructose content had significant positive correlation with AI and NI activities (r=0.8886, P<0.01; r=0.8612, P<0.01, respec-

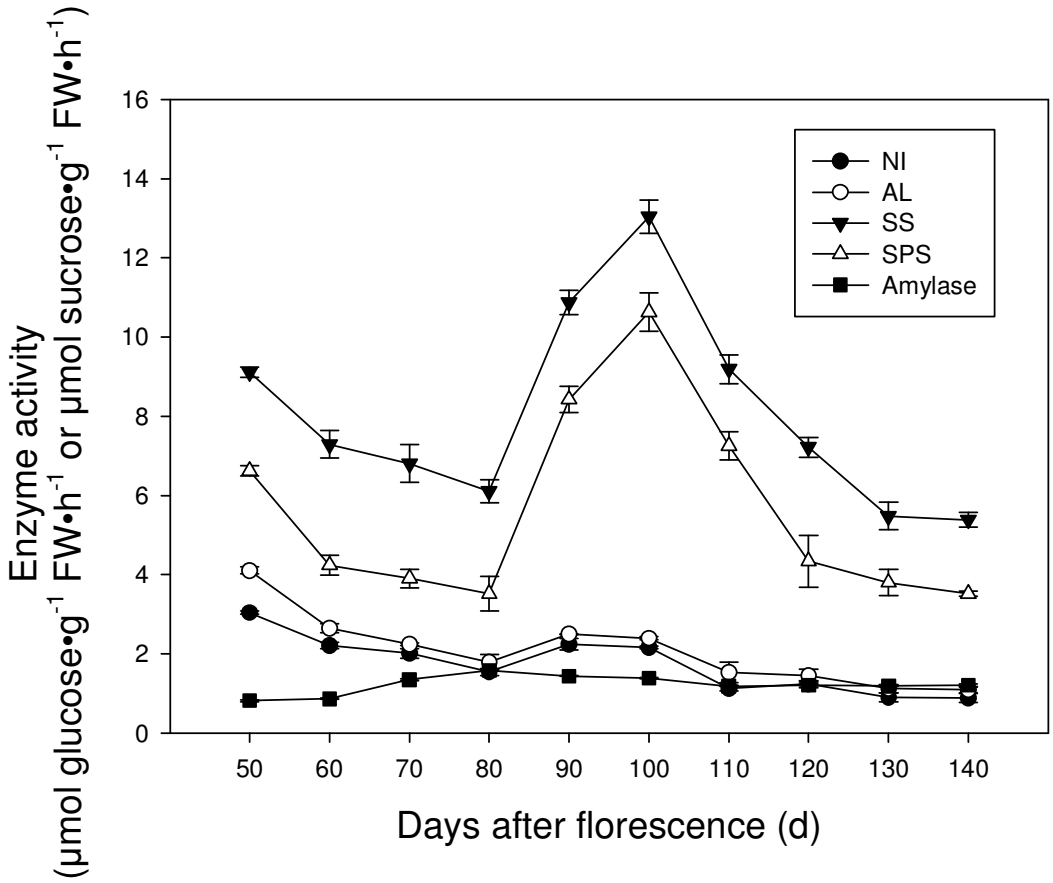

Figure 9. The dynamics of enzyme activities during embryo development. AI: Acid invertase; NI: Neutral invertase; SS: Sugar synthase; SPS: Sucrose phosphate synthase. Error bars indicate standard deviation, n=10.

tively); sucrose content had significant positive correlation with glucose content and amylase activity (r=0.7825, P<0.01; r=0.8968, P<0.01, respectively); glucose content had positive correlation with amylase activity, SS activity and SPS activity (r=0.6325, P<0.05; r=0.7308, P<0.05; r=0.6904, P<0.05, respectively); starch content had positive correlation with the activity of AI, NI, SS and SPS (r=0.7197, P< 0.05; r=0.7509, P<0.05; r=0.7587, P<0.05; r=0.6866, respectively); fat content had significant negative correlation with fructose content and NI activity (r=-0.9376, P<0.01; r=-0.7672, P<0.01, respectively), and negative correlation with AI activity(r=-0.7633, P <0.05); Protein content had significant negative correlation with fructose content (r=-0.9182, P<0.01), and had negative correlation with NI and AI activities(r=-0.7053, P<0.05; r=-0.6649, P<0.05, respectively); Other correlations were not significant (Table 1).

DISCUSSION

In this study, the results of fruit weight, vertical diameter, horizontal diameter and horizontal diameter of suture line of single fruit showed that the growth dynamic of walnut fruit was in a sigmoid pattern (Figures 1 and 3). Shell (episperm) formation is hardening from top to base gradually with the development of fruit and embryo organs. Therefore, according to the study of fruit, embryo and endosperm development (Zhang, 1980, 1983; Luza and Polito, 1991; Tadeo et al., 1994; Sartorius and Stösser, 1997) as well as the results of this study, the developmental process of walnut fruit could be divided into four stages:

1. Slow growth (within 30 DAF): In this period, absolute increment of fruit was lower, but the RGR was the highest. This period was overlapped with florescence, and was the zygote dormancy stage, the original embryo stage and globular embryo period.
2. Fast growth (30 to 60 DAF): In this period, absolute increment of weight and volume of fruit are at maximum; hereafter fruit volume did not increase (Figure 1). And the cotyledons, radicle, embryo and testa of embryo organs were formed in this period.
3. Fat accumulation (60 to 100 DAF): In this period, fat (Figure 5), soluble sugar content (Figure 8) and protein

Table 1. The correlation among the content of sugar, protein, fat and the activities of enzymes related with sugar metabolism.

Parameter	Correlation coefficient											
	AI	NI	SS	SPS	Amylase	Fructose	Sucrose	Glucose	Starch	Soluble sugar	Fat	Protein
AI	1.0000											
NI	0.9766**	1.0000										
SS	0.4854	0.5530	1.0000									
SPS	0.3987	0.4492	0.9823**	1.0000								
Amylase	-0.4845	-0.6026	0.0728	-0.0840	1.0000							
Fructose	0.8886**	0.8612**	0.1414	0.0527	-0.4539	1.0000						
Sucrose	-0.3669	-0.2384	0.3208	0.359	0.8968**	-0.4330	1.0000					
Glucose	0.0982	0.2267	0.7308*	0.6906*	0.6325*	-0.1270	0.7825**	1.0000	0.6191			
Starch	0.7197*	0.7509*	0.7587*	0.6866*	-0.1304	0.4722	0.1629	0.6191	1.0000			
Soluble sugar	0.1540	0.2914	0.4430	0.4028	0.7185*	0.1370	0.8198**	0.8323**	0.5032	1.0000		
Fat	-0.7637*	-0.7672**	-0.0022	0.1244	0.3217	-0.9378**	0.3856	0.0832	-0.3707	-0.1965	1.0000	
Protein	-0.7053*	-0.6649*	0.2207	0.3200	0.4067	-0.9182**	0.5024	0.3207	-0.2285	-0.0316	0.9499**	1.0000

*, ** Represent significant at $P<0.05$ and significant at $P<0.01$, respectively.

(Figure 7) metabolism were most active, especially fat content which increased from 5.1% at 60 DAF to 50.11% at 100 DAF (Figure 5).

4. Fruit maturation (100 to 140 DAF): In this period, peel of fruit began to discolor, shrinkage and crack, and the weight per fruit, soluble sugar content and protein content decreased (Figures 1, 7 and 8). The fat content increased, but the RGR of fat decreased.

The division of these four stages was based on the characteristics of fruit organic development and the morphological growth dynamic changes, alone with the characteristics of nutrition metabolism of walnut fruit. In production, rational nitrogen fertilization and irrigation were able to enrich phenolic compounds and content of linoleic acid in walnuts according to the characteristics of development in different periods of walnut embryos (Verardo et al., 2009).

In higher plants, AI and NI could hydrolyze sucrose into glucose and fructose and the reaction was reversible. SS not only can catalyze sucrose synthesis but also can catalyze sucrose disintegration. SPS was the main enzyme in sucrose synthesis (Huber and Huber, 1996). The results of sugar content and enzyme activities at the earlier stage of walnut fruit development showed that the content of AI, NI, SS and SPS had a high peak value and the accumulation of sugar and fat was little. Activities of AI, NI, SS and SPS decreased rapidly within 30 to 60 DAF, followed by a decline reaching a low point at 80 DAF, then increased to a high point until within 90 to 100 DAF, while decrease occurred again until walnut fruit ripened. Krishnan and Pueppke (1990) suggested that highly active invertase enzyme was related with fast growth of tissue.

Within 30 DAF, the original embryo and globular embryo developed and fruit cell division and morphogenesis were exuberant. In the period of fast growth (30 to 60 DAF), AI, NI, SS and SPS activities decreased, sugar content increased except fructose, but fat content is lower. At 60 to 100 DAF, fat increased fast; sucrose, glucose and starch content increased to maximum gradually, but fructose content continue to decline; AI, NI, SS and SPS activities were on a lower level at 60 to 80 DAF, then increased to maximum at 100 DAF. In the period of fruit maturation, sucrose, glucose, starch content declined gradually; AI, NI, SS and SPS activities also declined gradually, but fat content increased till fruit mature naturally.

Fat is an important nutrient in walnut embryos. Fat content of walnut embryo accumulated gradually till fruit naturally mature. In the stage of 60 to 90 DAF, fat content increased sharply; meanwhile the RGR of fat content had a maximum at 70 DAF. Then the RGR of fat content decreased quickly within 90 to 100 DAF (Figure 6). Sigmoid pattern of fat accumulation in

Arabidopsis seeds closely parallels the increase of seed weight, exhibiting a sharp raise between 7th day and 17th day after pollination and reached maximum at 18th day after pollination, then showed a slight fall at the very end of the maturation process (Baud and Lepiniec, 2009).

Plant fat metabolism is quite a complex process, which mainly takes place in plastid and endoplasmic reticulum. Sucrose was imported into the cytoplasm and was then hydrolyzed into hexose by invertase and SS. Glucose and fructose need to be phosphorylated by hexokinases (HXKs) or fructokinases (FRKs) before taking effect in lipid metabolism (Ohlrogge and Jaworski,1997; Nikolau et al., 2003; Damari-Weissler et al., 2006; Chen et al., 2009).

The correlation analysis showed that unloading of sugar, fat synthesis and accumulation were a complex network. Fat content had significant negative correlation with fructose content and NI activity, and had negative correlation with AI activity; protein content had significant negative correlation with fructose content, and had negative correlation with NI and AI activities. On the contrary, fructose content had significant positive correlation with AI and NI activities. Soluble sugar content had significant positive correlation with sucrose and glucose content and positive correlation with amylase activity; sucrose content had significant positive correlation with glucose content and amylase activity; glucose content had positive correlation with amylase activity, SS activity and SPS activity; Starch content had positive correlation with the activity of AI, NI, SS and SPS (Table 1). Our results suggest that the regulatory mechanism of walnut fat metabolism are not clear at present and deserve further study.

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Abbreviations

DAF, Days after florescence; **AI,** acid invertase; **NI,** neutral invertase; **SPS,** sucrose phosphate synthase; **SS,** sucrose synthase.

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