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Biochemical Changes during Fruit and Seed Development in Nanjing Linden (*Tilia miqueiana* M.)

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Abstract: In China, the wild population of Nanjing Linden (*Tilia miqueliana* M.) is experiencing a drastic decline, primarily due to high levels of empty seeds. This study aimed to measure the biochemical and physiological changes during fruit and seed development in *T. miqueliana* to determine the developmental mechanism. The weight method and photosynthetic respiration were used to determine the biological aspects of both fruits and embryos, while transmission electron microscopy and the anthrone colorimetric method were used to determine the endosperm content, including sugar, starch, protein, and fat. Enzyme-linked immunosorbent assays were conducted to determine the levels of endogenous plant hormones such as indole-3-acetic acid (IAA), gibberellic acid 3 (GA_3), zeatin riboside (ZR), and abscisic acid (ABA). The nonlinear least-squares method was used to fit the model of nutrient and hormone levels, revealing that fruit size expanded from the 5th to the 65th day and that fruit moisture content exhibited a downward trend, along with a decrease in fruit respiration intensity. Embryos were found to be fully developed between 35 DAF and 65 DAF, while the nutrients in the endosperm, i.e., sugar, starch, protein, and fat, continuously accumulated after 50 DAF. Additionally, ABA, IAA, GA_3, and ZR contents were found to synergistically regulate seed development and maturation.

Keywords: cell structure and morphology development process; endogenous hormones; endosperm contents; maturation; moisture content; seed embryo

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1. Introduction

The distribution range of Nanjing linden (*Tilia miqueiana* M.), a species used for timber, honey, and ornamental trees, covers most of the southeastern parts of China. In recent decades, the populations of *T. miqueiana* have dramatically declined in size and number throughout its natural distribution regions. Now, *T. miqueiana* is an endangered tree species in China. The life history of a seed begins with flowering and pollination and proceeds through fertilisation, development, maturation, scattering, dormancy, and germination. This series of steps translates into a subtle, unique, and interconnected life course [1]. The embryos of *Ginkgo biloba* L. begin to grow on the 140th day after flowering (140 DAF) [2]. However, embryo development is closely related to seed nutrients and endogenous hormones in the developing endosperm. During seed development, the nutrients in the plant flow to the seed in a dissolved state and accumulate inside the seed. With the development of the seed, these nutrients are gradually converted into non-dissolved dry matter, mainly starch, protein, and fat with high molecular weights [3,4]. When the seeds of *Brassica napus* L. mature, soluble sugars first form and are later transformed into starch, protein, and fat [5], and rutin, protein, and starch contents gradually rise when *Fagopyrum tataricum* G. seeds mature [6].

In addition, the plant hormone auxin is closely involved in many aspects of plant development, including seed formation [7], such as the embryo, endosperm, and seed coat.

In fact, after anthesis and seed setting, as the embryo develops, there is an initial stage of cell division and tissue differentiation [8]. Especially after dormancy, the contents of ABA and GA are very important for seed germination [9]. The maturation period of *Araucaria angustifolia* (Bert.) O. seeds can be shortened by increased ABA synthesis and accumulation, whereas GA rapidly declines during seed development and maturation [10]. Additionally, it is possible that other plant hormones also play a vital role in the development and maturation of seeds. For instance, during the wheat (*Triticum aestivum* L.) seed maturation process, the levels of IAA and SA gradually decrease [11]. The seed-filling phase then begins with a constant or nearly constant gradient until the end and can therefore be represented by a straight-line relationship [12]. During seed filling, the embryo of most tree species gradually grows. Sometimes, the beginning and end of embryo development greatly differ between plant species.

The stamens of *T. miqueiana* are precocious and almost sterile, and they have to be cross-pollinated. Additionally, the flowering phase is brief—only a week—which frequently leads to differences in florescence, subsequent years of low fertility, and a low seed setting rate. The seed of *T. miqueiana* is abortive, and the seed setting rate is generally less than 10%, which happens when development stops midway due to unknown reasons after pollination and fertilisation, resulting in a low full-seed percentage [13]. Additionally, the seed of *T. miqueiana* has a hard and difficult-to-penetrate seed coat, causing deep mechanical and physiological dormancy, and it is difficult for seeds to sprout in the year they were sown without any treatment. After two or three years of sowing, the majority of seeds begin to sprout. So, deep seed dormancy has made it extremely difficult to reproduce naturally, putting it on the verge of extinction [14]. These factors seriously affect the *T. miqueiana* seed yield and seedling propagation. In addition, due to the lack of knowledge of the early stage and the influence of human activities on the living environment of *T. miqueiana* in the state of nature, there are only 2000 wild *T. miqueiana* trees in China [13]. Thus, it is an endangered and rare species protected under a national program. Previous research has focused on breaking the seed dormancy of *T. miqueiana* and assumed that the coat permeability might be an important cause of the seed dormancy, with the aim of elucidating the cause of this decline. Nevertheless, few studies have examined fruit and seed development, including the storage of nutrients, moisture content, embryo growth, and endogenous hormones. In this study, the physiological and biochemical changes during seed development in *T. miqueiana* were therefore analysed, and the mechanism of seed development was revealed.

2. Materials and Methods

2.1. Fruit (Seed) Collection

In September 2020, fruits of *T. miqueiana* were collected from a mature tree on Niushou Mountain in Nanjing City, Jiangsu Province, China. Niushou Mountain (118′74 E, 31′92 N) is 248 m high and is located in the northern subtropical monsoon climate zone. The annual average temperature is 16 °C, the frost-free period is 237 days, the annual average humidity is 77%, and average rainfall is 1005.7 mm. The pH value of the mountain's soil is 5.5–6.5 [15]. The fruits collected were staged by days after flowering (DAF) as follows: 5 DAF (15 June), 20 DAF (30 June), 35 DAF (15 July), 50 DAF (30 July), 65 DAF (15 August), 80 DAF (30 August), 100 DAF (20 September), and 120 DAF (10 October). The size, respiration rate, water content of fruits, and embryo length were measured immediately. At the same time, the endosperms were taken from the fruits and stored in a freezer at −80 °C (Forma™ 89000, Thermo Scientific, MA, USA).

2.2. Fruit Size

Three replicates (100 fruits per replicate) of fruits were analysed per sample after fruit collection. The transverse and longitudinal diameters of the fruit were measured with a vernier calliper. Because the fruit is approximately spherical, the following ellipsoid

formula was used to calculate its size: $V = \frac{4}{3} \times a^2 b\pi$, where a = $\frac{1}{2}$ transverse diameter and b = $\frac{1}{2}$ longitudinal diameter.

2.3. Fruit Moisture Content and Respiration Rate

The fruit moisture content (MC) was determined using the high-temperature drying method described by Ista [16]. Three replicates of each of the 100 samples were oven-dried at 130 °C for 4 h to constant weight (AX 60, Carbolite Gero, Germany). MC was calculated using the following equation: $MC = \frac{M2-M3}{M2-M1} \times 100$, where $M1$ = weight (g) of the container and cover; $M2$ = weight (g) of the container, cover, and contents before drying; and $M3$ = weight (g) of the container, cover, and contents after drying.

The respiration rate during fruit development was tested with a photosynthetic respirometer (Li-6400, Lincoln, NE, USA). The respiratory rate Pn ($\mu mol \cdot g^{-1} \cdot min^{-1}$) was expressed as the CO_2 released by the respiration of 1 g of seeds in 1 min. One gram of randomly selected seeds from each treatment group was examined at 5, 20, 35, 50, 65, 80, 100, and 120 days. The moisture on the surface of the seeds was absorbed with absorbent paper. Then, the seeds were placed in the sample chamber. The two ends of the sample chamber were connected to the air inlet and the air outlet. The measurement time was 2 min, and measurements were recorded every 2 s. The final figure is the average. The photosynthesis respirometer was in analytical mode (light intensity = 0, ambient temperature = 25 °C).

2.4. Embryo Growth

Embryos were taken out from the fruit, and their lengths were defined under a stereo microscope (Eclipse Ci; Nikon, Tokyo, Japan) with three replicates (100 seeds per replicate) of seeds during their development.

2.5. Ultrastructural Observation of Endosperm Cells

The endosperm cell morphologies of five fruits randomly selected from each test sample were observed using transmission electron microscopy (TEM) (HT7700, Hitachi, Japan). The endosperms were cut into 1 mm^3 pieces, immediately fixed in 2.5% glutaraldehyde aqueous fixative solution (Servicebio) for 2 h, then transferred to 1% OsO_4 for 5 h, dehydrated in an ethanol series, and embedded in acetone blocks. Acetone blocks were sectioned to a thickness of 60 nm on a microtome with a glass knife, and sections were stained with 5% uranyl acetate and 0.5% lead citrate and viewed under an HT7700 transmission electron microscope. TEM image processing and analysis were performed using Image J software (Java-based public image processing software developed by the National Institutes of Health).

2.6. Embryo Germination Test

The embryo germination test included immature and mature embryo groups. There were four replicates in each group with 100 embryos. The immature embryos were derived from the fruits at 50 DAF, while the mature fruit embryos were obtained from the fruits at 65 DAF. All taken embryos were placed on soaked cotton in a germination box and put in an incubator (25 °C, 1000 Lx for 12 h) (RH-500A, RunHua, JS, China) for 30 days. When the embryo grew into a normal seedling, the embryo was considered to have germinated. The embryo germination percentage was calculated using the formula proposed by ISTA [16]: $G = \frac{N}{A} \times 100$, where G = % germination, N = number of embryos germinated, and A = total number of embryos tested. The final percent germination represents the mean (±standard deviation) of four replicates.

2.7. Assessment of Sugar, Starch, Protein, and Fat Contents

Total starch and soluble sugar contents were assessed using the anthrone colorimetric method [17]. Soluble protein content was assessed using the method described by Bradford [18], and fat content was determined using Soxhlet extraction [19].

2.8. Quantification of Endogenous Hormones

We used enzyme-linked immunosorbent assays to extract, purify, and quantify the levels of endogenous gibberellic acid (GA), abscisic acid (ABA), indole-3-acetic acid (IAA), and zeatin riboside (ZR) following the protocols described by Zhao et al. [20] and Wang et al. [21]. The kit utilised in this study was kindly provided by the Laboratory of Plant Endogenous Hormones, China Agricultural University. To extract the hormones, we used 10 mL of cold 80% (v/v) methanol extraction medium containing 1 $mmol^{-1}$ butylhydroxytoluene as an antioxidant. The extract was then incubated at 4 °C for 4 h and centrifuged × g at 4 °C for 15 min. The resulting supernatant was prewashed with 10 mL of 100% (w/v) and 5 mL of 80% (v/v) methanol, respectively, through a Sep-Pak C18 column (Waters Corp., Milford, MA, USA). A total of 10 mL of 100% (v/v) methanol and 10 mL of ether-eluting hormone components were then extracted and dissolved in 2 mL of phosphate-buffered saline (PBS) containing Tween 20 and 0.1% (w/v) gelatin (pH 7.5) for ELISA detection. We repeated all experiments three times to calculate the mean and standard error.

GA antibodies were developed using GA_3 as a hapten. It cross-reacts with other GAs, such as GA_1, which has a similar structure to GA_3. Thus, the detected GA content is the sum of the GA content of GA_3 and other GAs that cross-react with GA antibodies.

3. Results

3.1. Morphological Characteristic Changes in the Fruit and Embryo

3.1.1. Changes in Fruit Size, Moisture Content, Respiration Rate, and Embryo Length

During fruit development, the size of the fruit clearly changed. In the early phase of development, the fruit size increased rapidly from 0.1 ± 0.02 cm^3 at 5 DAF to 0.39 ± 0.04 cm^3 at 35 DAF. After that, the fruit size exhibited a slow upward trend, although the difference in size was not significant until the fruits were fully mature (Figure 1).

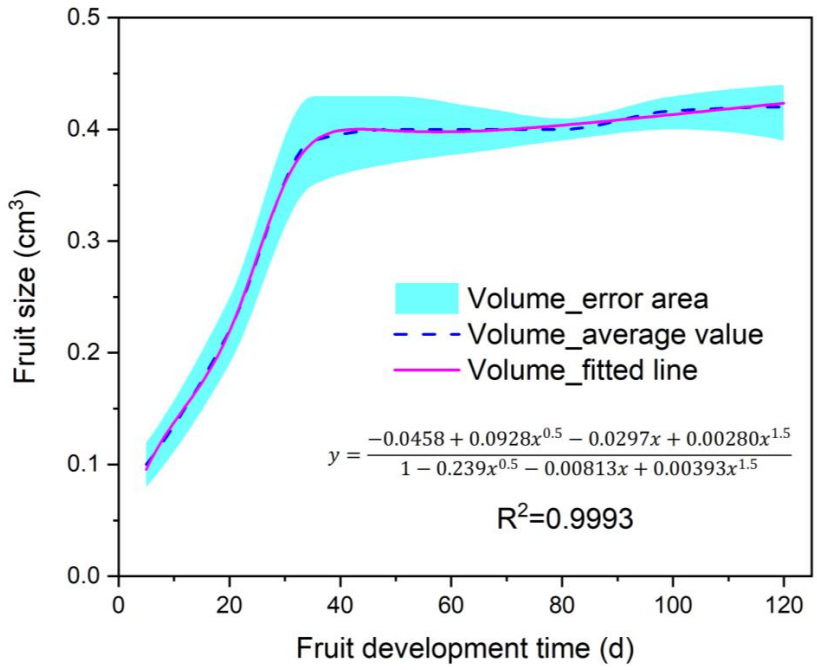

Figure 1. Changes in fruit size during fruit development process.

The fruit moisture content (MC) displayed a downward trend during fruit development (Figure 2), decreasing from 72.21% at 5 DAF to 13.98% at 120 DAF. There were two significant rapid water-loss stages: in the first stage from 5 DAF to 20 DAF, the MC dropped from 72.20% to 47.49%, and in the other stage from 50 DAF to 65 DAF, the MC dropped from 38.05% to 19.9%. On the other hand, the respiration rate of fruits basically had the same changes as the moisture content of fruits. Early in fruit

development (before 20 DAF), the respiration rate of fruits began to fall sharply from $38.72 \pm 1.02 \mu moL \cdot g^{-1} \cdot min^{-1}$ at 5 DAF to $13.46 \pm 0.63 \mu moL \cdot g^{-1} \cdot min^{-1}$ at 20 DAF. It gradually declined to $7.01 \pm 0.99 \mu moL \cdot g^{-1} \cdot min^{-1}$ at 65 DAF. After that, the fruit respiration rate remained basically unchanged until the fruit was fully mature (Figure 2).

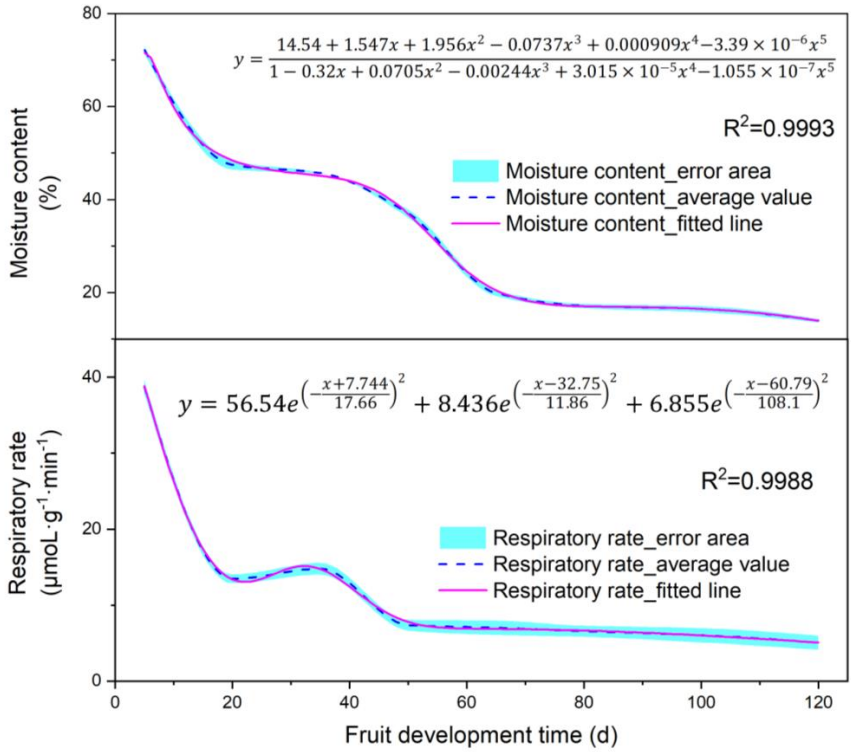

Figure 2. Changes in moisture content and respiratory rate during fruit development process.

The embryo began to substantially grow at 35 DAF. The average length of the immature embryo was 0.28 cm at 50 DAF (Figure 3), and its germination percentage was $23 \pm 0.02\%$ (Figure 4). At 65 DAF, the embryo was fully developed and reached 0.51 cm long. At this time, $97 \pm 0.01\%$ of isolated embryos were able to germinate. This result indicates that between 35 DAF and 65 DAF was the key period of embryo development. In only 30 days, the embryo had completed physiological maturation.

3.1.2. Changes in Fruit Structure and Embryo Morphology

During *T. miqueiana* fruit development, the morphological and anatomical structure of the fruit changed significantly (Figure 5). At 5 DAF, 10 seeds in each fruit arranged in two layers in a total of five chambers were clearly visible.

Young seeds containing a liquid substance were white and transparent and covered with a layer of a young seed coat. At 9 DAF or 12 DAF, the number of seeds in the fruit gradually decreased. At 20 DAF, the percentage of fruits with one seed decreased by up to 30%. This indicates that seed abortion in *T. miqueiana* mainly occurred from 9 DAF to 20 DAF. At 35 DAF, the fruit turned yellowish brown in colour. The mesocarp became a cork layer and was difficult to remove by hand. The seeds were enlarged with a white coat containing liquid substances. At 50 DAF, the pericarp was highly lignified and hard. The thickness of the seed coat became thinner, and the contents were pulpy. Young green embryos appeared. At 65 DAF, there were no further significant changes in fruit morphology, but endosperms became milky yellow, bulky, and solid. At 80 DAF, the fruit was completely mature and nut-like. The pericarp was highly lignified, the seed coat was black and thin, and the endosperm was milky white.

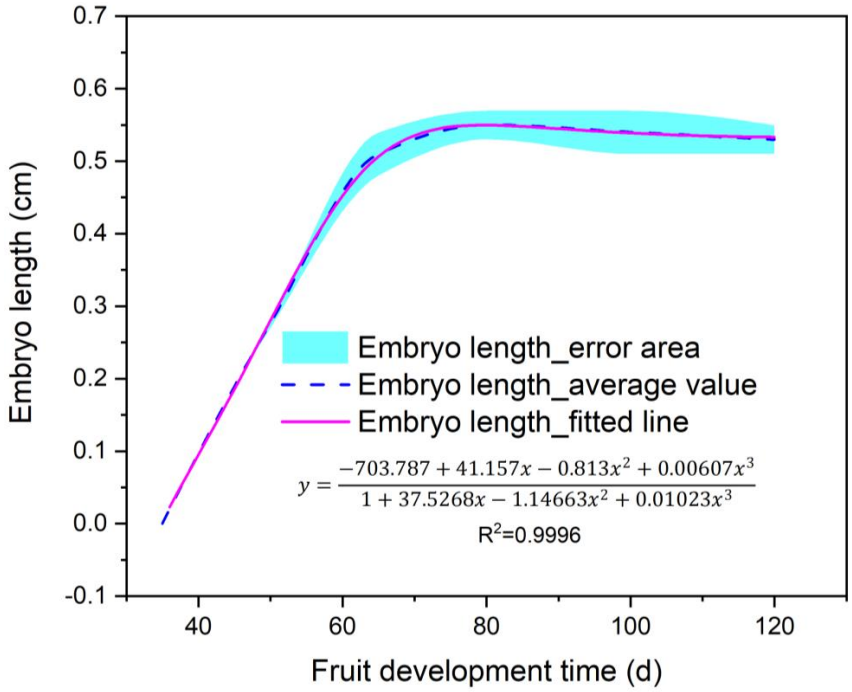

Figure 3. Changes in embryo length during fruit development process.

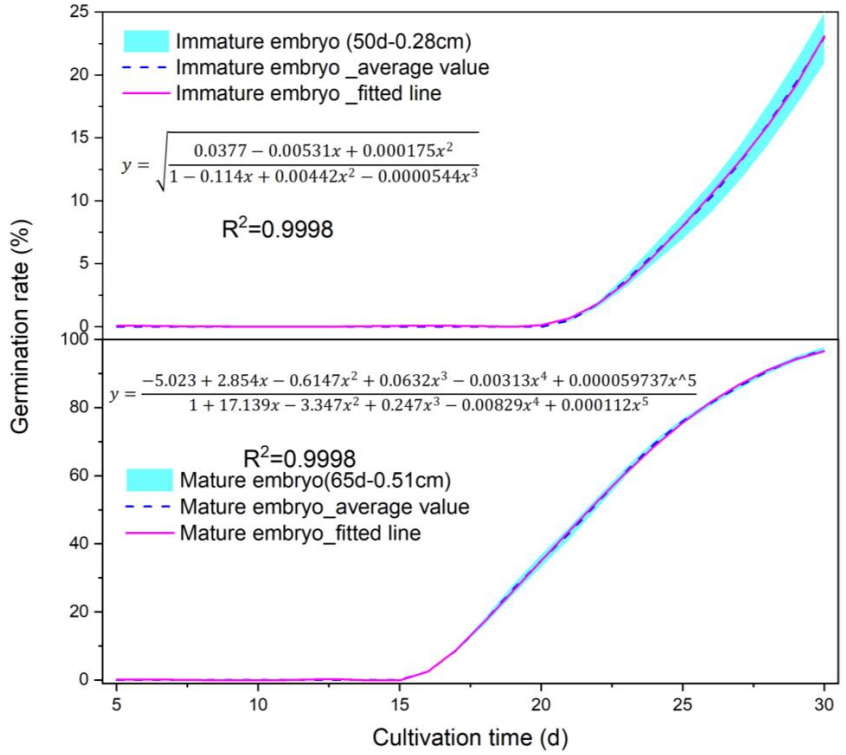

Figure 4. Germination of immature and mature embryos during cultivation time.

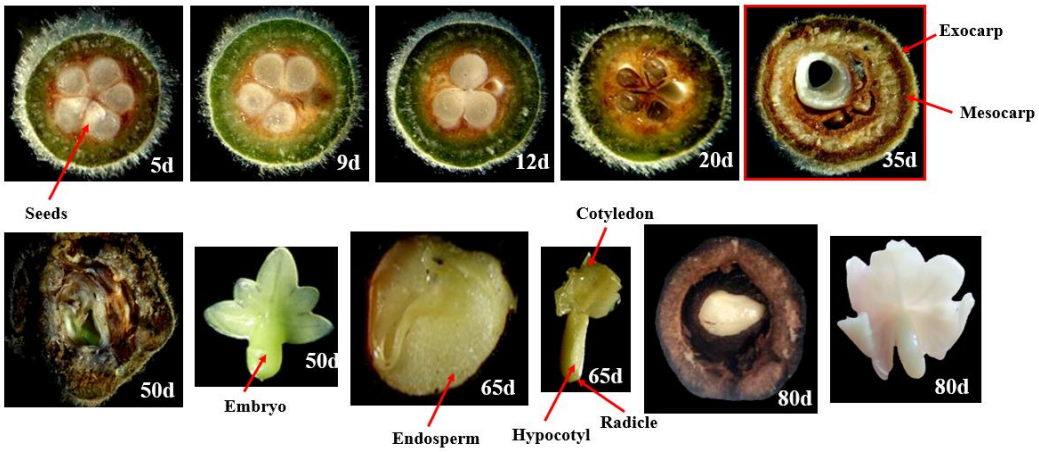

Figure 5. Cross-section of the fruit and embryo at 5 DAF, 9 DAF, 12 DAF, 20 DAF, 35 DAF, 50 DAF, 65 DAF, and 80 DAF.

3.2. Changes in the Morphology of Substances Contained in Endosperm Cells

The endosperm cells were composed of lipid droplets, a dense substance (covered with small cavities), and white vacuoles at 35 DAF and 50 DAF (Figure 6). At 65 DAF and 80 DAF, the number of lipid droplets increased markedly. Additionally, the pores on the dense substance became larger, and the white vacuoles disappeared completely. At 100 DAF and 120 DAF, the cells were densely covered with lipid droplets. Starch granules became larger, and the colour and size of the dense substance were considerably different.

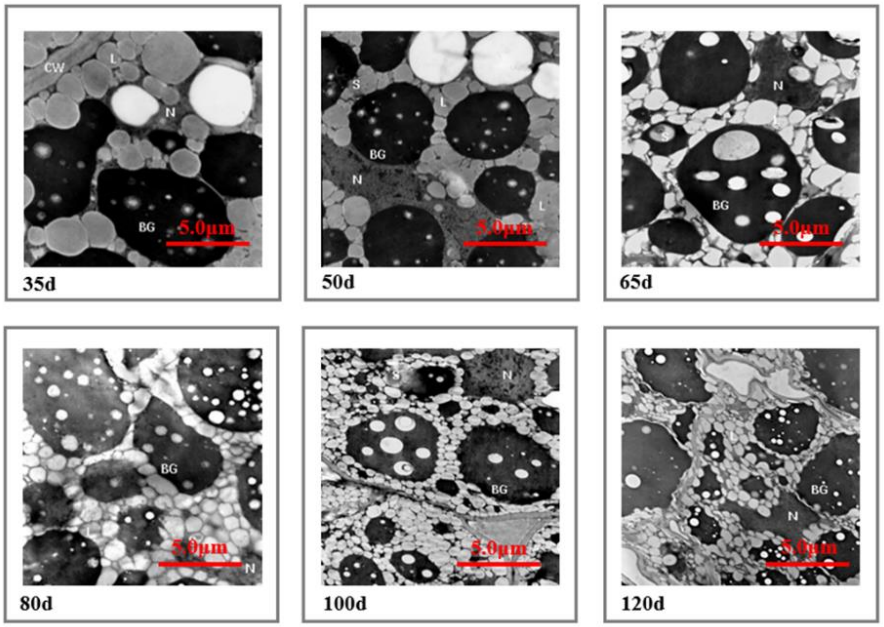

Figure 6. Morphological changes in the contents of endosperm cells. CW—cell wall; BG—dense substance; L—lipid droplet; N—cell nucleus; S—starch granule.

3.3. Changes in Contents of Sugar, Starch, Protein, and Fat in Endosperm

Especially early in fruit (seed) development, sugar showed a sharp upward trend and reached 66.3 mg·g^{-1} at 35 DAF (Figure 7). Then, it gradually decreased to 30.2 mg·g^{-1} at 80 DAF, during which much sugar was consumed for fast embryo growth. However, when the embryo was fully developed, the sugar remained at a relatively stable level. This indicates that in early seed development, sugars participated in the synthesis of storage substances, and their content increased. Shortly after, small-molecule sugars transformed into macromolecular substances, and their content decreased. Before 35 DAF, the contents

of starch, protein, and fat gradually rose. After that, starch and fat did not significantly change. However, based on fruit (seed) development, the protein slightly decreased when the embryo started to grow. It was presumed that during the development of the embryo, the degradation of the produced proteins provided materials for building the embryo structure. Thereafter, the protein, as seed storage material, began to increase to 32.42 mg·g^{-1} at 80 DAF. The fat content increased rapidly, but it markedly decreased to 13.2 mg·g^{-1} with embryo development. Afterwards, it increased slowly from 0 mg·g^{-1} to 202.6 mg·g^{-1} (at 120 DAF). Both of them increased slowly in the early stage (before 35 DAF) and then rapidly in the later stage (from 35 DAF to 120 DAF).

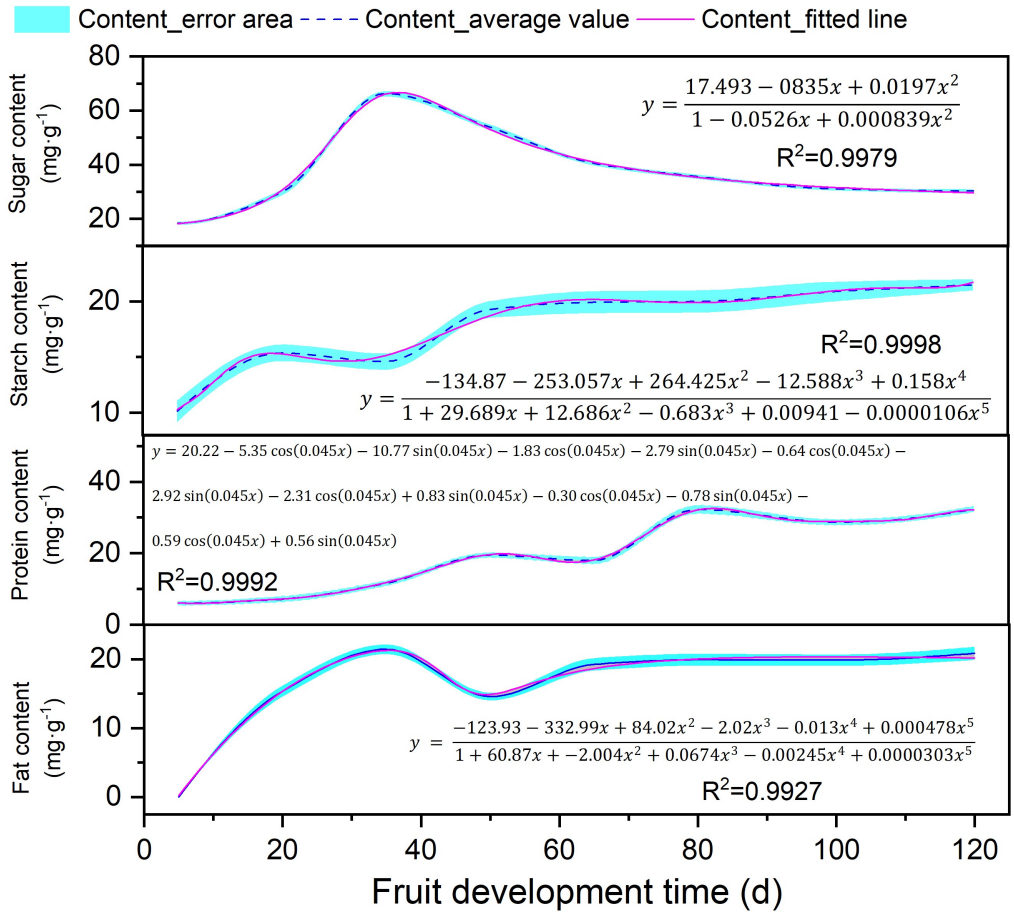

Figure 7. Changes in sugar, starch, protein, and fat contents during fruit development.

3.4. Changes in Levels of IAA, ZR, GA$_3$, and ABA in Endosperm

IAA, ZR, GA$_3$, and ABA are the most important during plant development, including fruit (seed) development. Changes in the contents of endogenous IAA, ZR, GA$_3$, and ABA are shown in Figure 8. At the beginning of fruit (seed) development, the contents of ABA in the endosperm were over 120 ng.g^{-1}FW, and the contents of IAA in the endosperm were over 65 ng·g^{-1}·FW. They were significantly higher than those of ZR and GA$_3$. Early in fruit (seed) development, each of them increased to different degrees, especially ABA and IAA. IBA increased from 124.17 ng·g^{-1}·FW to 133.14 ng·g^{-1}·FW, and IAA rose from 69.95 ng·g^{-1}·FW to the maximum value of 112.05 ng·g^{-1}·FW. However, GA$_3$ and ZR only slightly increased. During embryo development, the ABA level declined until the embryo was physiologically mature at 65 ADF, but GA$_3$ rose steadily from 15.23 ± 0.81 ng·g^{-1}·FW to 19.06 ± 0.95 ng·g^{-1}·FW. Furthermore, the ABA level was low and the GA level was high, promoting the rapid growth of the embryo. In addition, ZR and IAA levels had the same changes during embryo growth. For example, during the first half of embryo development, they kept declining, after which they rose until the embryo was well developed.

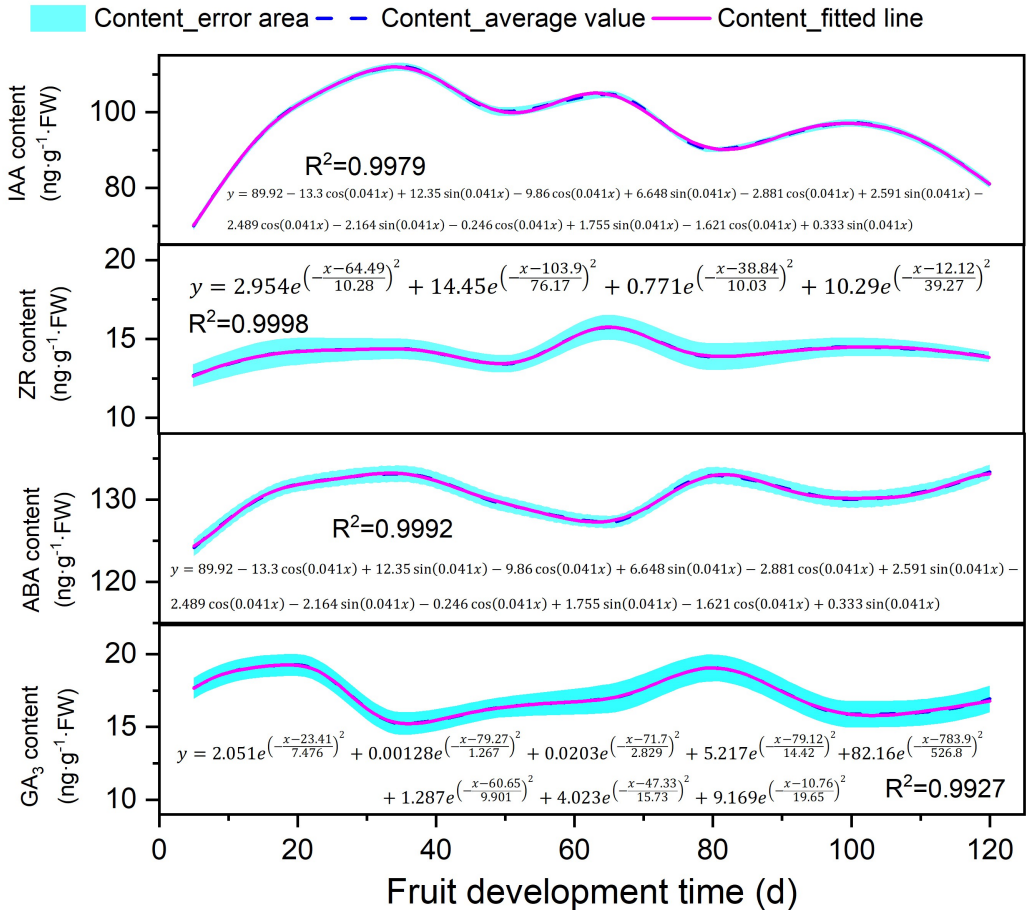

Figure 8. Changes in IAA, ZR, GA_3, and ABA contents of endosperm during fruit (seed) development.

4. Discussion

Seeds are composed of three main compartments, the embryo, endosperm, and seed coat, which originate from different cells of the ovule and have different maternal and paternal genome complements [22]. This study focused on the embryo and endosperm of the *T. miqueiana* seed. The aim was to achieve a better understanding of the mechanisms behind the low full-seed percentage of *T. miqueiana* by researching changes in biological characteristics, endosperm cell morphology, endosperm contents (sugar, starch, protein, and fat), and endogenous hormones (indole-3-acetic acid (IAA), gibberellic acid (GA_3), zeatin riboside (ZR), and abscisic acid (ABA)) during the fruit (seed) development phase. The most striking result is the progressive and drastic changes in fruit (seed) biological characteristics based on the study of the fruit (seed) different development phase.

4.1. Biological Characteristics of Fruit Development

During the fruit development process, dehydration was extensive, as the MC dropped from 72.2% (5 DAF) to 13.98% (120 DAF). At first, each fruit consisted of 10 seeds clearly arranged in two layers in a total of five chambers; unfortunately, only 30% of the fruits had one seed after 15 days, and the rest of the seeds became hollow kernels. There was significant abortion of *T. miqueiana* seeds before the morphological development of the embryo. Different plants have different phases of embryonic developmental arrest. Yang et al. [23] found that the number of aborted *Sorbus pohuashanesis* seeds significantly differed among different fruit (seed) developmental stages, with a higher abortion rate in the early stage compared to the later stage. According to embryo development in *T. miqueiana*, three phases could be determined: (a) pre-embryo development (35 days); (b) embryo development (30 days); (c) post-embryo development until ready for dispersal (over 60 days).

4.2. Changes in Cell Morphology in Endosperm

A build-up of nutrient reserves occurs in the later half of embryo maturation [24]. The number of lipid droplets increased, white vacuoles disappeared, and dense substances with different colours and sizes increased. This indicates that nutrients in the endosperm cells are constantly accumulating, which determines the size and quality of the seeds [25].

4.3. Changes in Endosperm Contents (Sugar, Starch, Protein, and Fat)

The contents of starch, protein, and fat all increased during the development process. This is consistent with observations for *Festuca arundinacea*, *Momordica charantia* L., and *Ginkgo biloba* L. seeds [26–28]. The sugar content increased during the early phase of development and decreased during the late stage. It is speculated that sugars are transformed into starch, protein, and fat, the contents of which play important roles in seed viability [29]. Insufficient starch accumulation results in low corn seed viability [30]. The integrity of the protein synthesis system is related to seed viability [31].

4.4. Changes in Endogenous Hormone Levels (IAA, ZR, GA_3, and ABA)

The plant hormone auxin is closely related to many aspects of plant development, including seed formation. Among the currently known higher plants, the most important auxin is IAA, which is initially biosynthesised from tryptophan [32]. Meanwhile, auxin levels have been linked to seed growth and development in many plant species. In *arabidopsis thaliana*, seed endosperm cell proliferation was positively correlated with increased auxin signalling, which was confirmed by auxin R2D2 sensor measurements [33]. In corn, the use of the DR5 auxin report gene [34] in the early stages of endosperm development detected the activity of auxin, which is associated with the accumulation of IAA in the maize endosperm; similar results were also observed in rice seeds. The IAA content is higher in the early stage and gradually decreases in the late stage of seed development [35,36]. This is consistent with the upward-downward trend of IAA during seed development in *T. miqueiana*.

Zein (Z) and ZR are the main active cytokinins in plants. Surface analyses in related studies showed that there were very high levels of Z and ZR in maize, rice, wheat, and barley grains [37]. Lulsdorf et al. [38] found that the cytokinin content peaked very early, at 4 days after anthesis, in *Cicer arietinum* and *Caryocolum anatolicum*, when the zygote was just developing into a spherical embryo and the cells were dividing rapidly. By day 8 after anthesis, the concentrations of these hormones were reduced, and by day 12, very little trans-ZR was present, except for trans-ZR in *C. anatolicum*. This is consistent with the upward-downward trend of ZR during seed development in *T. miqueiana*. Emery [39] correlated the predominance of ZR with pod abscission in white lupine. In legume species, flower and pod abortion is common, including in chickpea.

ABA is an important component of plant hormones and plays an important role in many stages of the plant life cycle, including seed development [40]. ABA regulates the accumulation of storage substances in seeds and their tolerance to dry conditions [41]. The ABA content in most seeds exhibits an upward trend during the early stage of embryo maturation and a downward trend during the later stage [42]. Embryo development in *T. miqueiana* was basically completed between days 35 and 65. The content of ABA exhibited an upward-downward trend from days 0 to 65. This is consistent with the trends in ABA content during embryonic development in *Pinus koraiensis* and *Rosa hybrida* [43,44]. An increase in ABA content is the basic characteristic of seed maturation [45]. The maturation of apple and *Rosa roxburghii* seeds is accompanied by an increase in ABA content [46,47]. This is consistent with observations for *T. miqueiana* seeds. High levels of ABA may promote seed dormancy during the mature period [48], which may be an important cause of *T. miqueiana* seed dormancy.

GAs indirectly promote seed development by promoting the synthesis of auxin [49]. The GA content during seed development in Korean pine is always low, exhibiting an upward-downward trend. It is speculated that the effect of GAs is not significant [44].

Accordingly, the role of GAs in *Picea glauca* seed development was not obvious [50]. The GA_3 content during *T. miqueiana* seed development did not change significantly, exhibiting a fluctuating upward-downward trend. It is speculated that GA_3 has no significant effect on seed development.

5. Conclusions

Changes in the biological characteristics, endosperm cell morphology, endosperm contents (sugar, starch protein, and fat), and endogenous hormones (IAA, GA_3, ZR, and ABA) of *T. miqueiana* seeds during their development process were studied, from which the mechanism of seed development was revealed. During seed development, the fruit size expanded from the 5th day to the 65th day. Fruit MC exhibited a downward trend along with the same changes in fruit respiration intensity. Embryos were fully developed between 35 DAF and 65 DAF. Nutrients continuously accumulated in the endosperm. Endosperm starch, protein, and fat exhibited an upward trend, whereas sugar exhibited a downward trend. The contents of the endogenous hormones ABA and IAA were significantly higher than those of GA_3 and ZR. The ABA content exhibited an overall upward trend, whereas IAA exhibited an overall downward trend. Overall changes in GA_3 and ZR contents were not pronounced. To sum up, the first 10 days of October, when the seeds are fully developed, is the best time to harvest *T. miqueiana*. The growth environment has a significant impact on the development of *T. miqueiana*, and the nutritional conditions are also essential for seed development.

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