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Full Length Article

Ultrastructural Changes in Oil Bodies Accumulation and Fatty Acids Composition during Seed Development of *Styrax tonkinensis*

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Abstract

Styrax tonkinensis is a potential biofuel feedstock due to having high seed oil content and excellent fatty acid composition. This study aims at oil bodies formulation and fatty acids composition of the oil extracts from *S. tonkinensis* seeds. Oil bodies acquired through transmission electron microscopy (TEM) at 40 days after flowering (DAF), but the peak of oil bodies was observed after 100 DAF. Then, the size of the oil bodies generally increased, while their number decreased. The highest crude oil content (68%, w/w of dry seed) was recorded in seeds harvested in early October. The time-dependent process of oil accumulation during seed development showed a two-phase framework, with a high rate of increase of oil content during 20–40 and 80–100 DAF. GC/MS analysis showed that the main fatty acids (FAs) were linoleic acid, oleic acid, palmitic acid and stearic acid and their contents were 44.31–63.59%, 37.1–43.65%, 6.92–20.89% and 1.73–8.13%, respectively. And the ratio of unsaturated/saturated fatty acids was affected by climatic temperature significantly. This revealed that the ultrastructure of the endosperm and embryo significantly changed with the accumulation process of the oil bodies and fatty acids composition during the development of *S. tonkinensis* seeds. © 2020 Friends Science Publishers

Keywords: Biofuel; Seed development; Fatty acids composition; Oil body

Introduction

During seed development, fat accumulates in storage tissues such as cotyledons, endosperm or shield (Huang 1996; Murphy and Vance 1999), which percentage varies widely between species, ranging from 1 to 60% (w/w of total dry weight) (Ohlrogge and Browse 1995). Some researchers found that lipids in oil-seeds accumulated to comparatively high levels from early to late stages of seed development (Archana *et al*. 1999; Neuhaus and Emes 2000; Özcan 2013; Yadav and Singh 2003). And some exist in the form of triacyglycerides in fat or oil bodies (OBs) for seed germination and seedling growth (Chapman and Ohlroggen 2012; Chapman *et al*. 2012), others may distribute in more complex structures such as membrane phospholipids. Furthermore, the sizes of the OBs are related to the content of triacylglycerol (TAG) (Tzen *et al.* 1993), and most of it may be the result of the fusion of small OBs and coalescences of smaller ones (Ariotti *et al.* 2012). Concerning the type of fatty acids (FAs) produced, Carvalho *et al*. (2010) reported that the three most abundant FAs in seeds of thirteen species of *Artemisia* were palmitic acid, linoleic acid and α-linolenic acid. In particularly, these latter ones unsaturated FAs (UFAs) are predominated in all *Artemisia* species. Rao *et al.* (2008) suggested that the high level of α-linolenic acid may largely be controlled by the level of some gene expression.

The deciduous tree *Styrax tonkinensis*, a member of the *Styracaceae* family, is characterized by rapid growth, a straight trunk and white fragrant flowers (Xu and Yu 2015). Its wood is suitable for the manufacture of handicrafts, pulp and paper and so on (Luo *et al*. 2007). It also produces benzoin which is a resin composed of fragrant fatty acids or triterpenoids that is utilized as a source of a complex spice or as a medicine (Wang *et al.* 2006; Burger *et al.* 2016). Its seeds have high oil content (aprox. 54.86% w/w, Wu *et al.* 2019) and nutritional components (oil, soluble sugar, starch, free amino-acid and soluble protein) that can be used as biofuel due to its excellent fatty acid composition and good fuel properties (Xiao *et al.* 2005; Liu *et al.* 2007; Zhang *et al.* 2017; Wu *et al.* 2019). Therefore, the species has high potential commercial value and can make an important contribution to the local economy (Ounekham 2009).

Many studies on *S. tonkinensis* have focused mostly on ornamental value (Xu and Yu 2015), rules of seed oil accumulation (Zhang *et al*. 2017, 2018; Wu *et al.* 2020), benzoin resin production (Wang *et al*. 2006) and so on. However, little is known about relatively comprehensive research of accumulation of fatty acids, oil body and ultrastructural changes during seed development. The objective of the present study was to monitor the process of lipid accumulation using transmission electron microscopy (TEM), and to measure the oil content and composition in seeds of *S. tonkinensis* during their development and maturation, with the intent to provide data for directional breeding programs aimed at improving oil content and desired FAs composition in *S. tonkinensis* seeds.

Materials and Methods

Plant material

The *S. tonkinensis* trees were grown under natural conditions within the breeding collection of Jiangsu Guoxing Biotechnology Limited, which is located in the Hewang Lake Community, Ma'an street, Luhe District, Nanjing City, Jiangsu Province, China (32°32′ N, 118°50′E). Peak flowering occurred on May 21, 2013. Therefore, data regarding temperature, total precipitation and evaporation at Luhe District from May 21st to October 10th, 2013 were recorded (Fig. 1). In early May, 2013, 10 robust trees (4 years old) that reached at peak fruiting were tagged for sampling. Seed harvesting was collected randomly from June 10, 2013 that was 20 days after flowering (DAF), and thereafter continued every 20–21 days until October 10, 2013 resulting in a total of seven sampling events. Then, fruits were peeled, mixed evenly and stored in a refrigerator (SANYO, Japan) at -70°C before analysis.

Anatomical observation

In order to examine the process of material accumulation (especially lipid) in endosperm and embryo during development stages of *S. tonkinensis* seeds, some samples were observed by stereomicroscopy (Olympus Corporation, Tokyo, Japan) and TEM (JEOL, Tokyo, Japan). To keep the description simple, only one picture was selected for each phase.

After being cleaned with distilled water and drying, samples were selected from the endosperm near the radicle and embryo (hypocotyl cells near the radicle) for TEM analysis. Samples were fixed in 4% glutaraldehyde, cleaned with phosphate buffer (0.1 *M*, pH 7.2), and fixed again in 1% osmic acid (0.1 *M* pH 7.2 phosphate buffer). Before sectioning, the fixed samples were cleaned with phosphoric acid buffer solution and dehydrated in a graded series of ethanol (30-50-70-90-100%) solutions. Then, they were infiltrated, embedded polymerized in Epon812 epoxy resin bought from Electron Microscopy Sciences (E.M.S., U.S.A.), and then repaired, sliced semi-thinly using LKB-5 ultra-microtome (LKB Instruments Inc., Lucerne, Switzerland), positioned, sectioned ultra-thinly (50 nm) and semi-thin section (2 μm) for three times, stained with uranyl acetate and lead citrate, and finally observed and photographed in a JEM-1400 TEM (JEOL, Tokyo, Japan). The TEM images were viewed for measuring in Nano Measurer 1.2 (Fudan University, Shanghai, China, 2008) and calculated the size of oil body diameter (OBD) (Zhang *et al*. 2018).

Oil content

A Soxhlet extraction method (GB/T14772-2008) was used to analyze the crude oil content in seeds. At each developmental stage, 0.5 g of finely ground seed sample was placed into aluminum boxes and dried at 105°C for 2 h. The powdered samples were subsequently placed into individual Soxhlet extractor bottles and 200 mL of petroleum ether (boiling point 60–90°C) was added. The samples were repeatedly extracted at 85°C in a water bath until all of the crude oil was removed. The filter paper bags were then baked at 105°C to completely volatize the petroleum ether, placed into a dryer, allowed to cool, and then weighed until a constant weight was obtained. Each assay was repeated a total of 3 times for each sample.

Lipid analysis

Extraction and esterification of oil

The samples of extracted seed oil, obtained from the different aged seeds, were dried by using a rotary evaporator (RE-3002, Shanghai, China). Then, 4 mL mixture of benzene and petroleum ether (volume ratio 1:1), and 1 mL KOH-CH_3OH solution (0.4 mol/L) were added to each sample, vortexed and left to stand for 15 min. Subsequently, 8 mL distilled water was added, and each sample was sufficiently vortexed until the solution was clear and stratified. The resultant supernatant was collected, which was yellow, clear and emitted a mild oil odor, evaporated by decompressing, dissolved in 5 mL hexane, and then analyzed by gas chromatography and mass spectrometry (GC-MS) for precise determination of both lipid content and fatty acid composition following some reported methods (Sardesai and Manning 1968; Zhang *et al*. 2017).

Determination of fatty acid composition

Chromatographic analyses were conducted by a TRACE-DSQ GC-MS (Thermo Electron, U.S.A.) using a DB23 column. Injector temperature was 250°C, oven temperature program was from the initial temperature of 40°C (for 1 min) to 195°C (for 2 min), to 205°C (for 2 min) with an increasing temperature rate of 2°C/min and finally raised to 230°C at a rate of 8°C/min, which was maintained for 1 min.

The carrier gas was helium with a flow rate of 1.0 mL/min. The amount of sample injected was 1 μL and the split ratio was 50:1. The time of solvent delay was 6 min and the ion source was electron impact ion source (EI). The temperature of the source was 230℃, the emission current was 34.6 μA, the ionization voltage was 70 eV, the interface temperature was 250℃, the scanning speed was 2.72 amu/s and the scanning range was 12–550 amu.

Data analysis

The seed oil components were determined by a comparison of the obtained mass spectra with a mass spectral library retrieved from NIST06. The spectrum analysis was also combined with an analysis of the related literature using the data processing system of the MSD workstation (Agilent Technologies 689OGC/5973MSD). The relative percentage of the components (FAs) was calculated using the area normalization method and the following formula:

$$\% \text{ FA} = (\text{FA/total peak area of FAs}) \times 100$$

Where FA is linoleic acid, oleic acid, palmitic acid or stearic acid, and the total peak area of FAs was calculated by summing their area.

The unsaturated index and degree of FA was calculated as described by Zhou *et al.* (2013), that is

$$\text{Index of unsaturated fatty acid (IUFA)} = [\sum_{i=1}^{n}(Si \times Ti)] \times 100$$

In the formula "Si" is the relative content of unsaturated fatty acid (UFA) and "Ti" is the number of unsaturated double bonds in the UFA.

Instead, the degree of UFA= UFAs relative content /saturated fatty acids (SFAs) relative content.

The above data were averaged by three independent experiments, and figures were computed in Microsoft excel. The correlation of fatty acids and OBD with environmental factors was analyzed by S.P.S.S. 19.0 (I.B.M., Armonk, N.Y., U.S.A.) using Pearson's correlation analysis.

Results

Anatomy structural observations of endosperm and embryo

Some characteristics during different development stages of *S. tonkinensis* seeds observed by stereomicroscopy showed that seed developed gradually, the color changed from green to yellow, the substance in the seed altered from liquid state to semi-solid and solid state, and the development of embryo was later than that of endosperm (Table 1). Preliminary differentiation of embryo and dicotyledon are not obviously visible until 100 and 120 DAF, respectively. Therefore, in our study, the observation of the micro-structure of endosperm near the embryo was monitored by TEM from 0 to 140 DAF (Fig. 2a–g) and embryo from 100 to 140 DAF (Fig. 2 h–j).

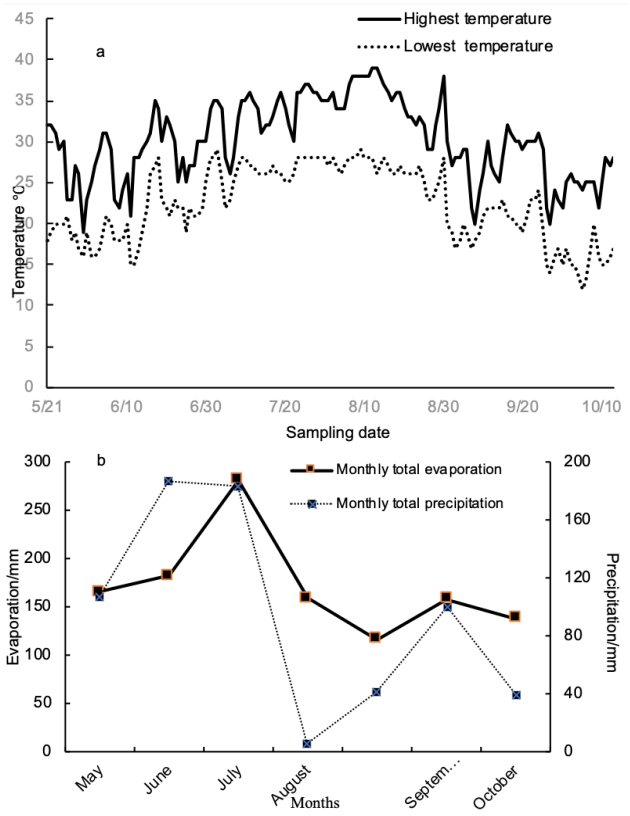

Fig. 1: The records of the highest and lowest temperatures (**a**), and monthly total precipitation and evaporation (**b**) in Luhe District, Nanjing City, Jiangsu Province, China from May 21st to October 10th, 2013 (data from the public service website of Chinese Meteorological Administration at http: //www.weather.com.cn/)

Ultrastructural observations of endosperm cells indicated the formation and accumulation of some substances especially lipids during seed development. At 20 DAF (Fig. 2a), nucleus and nucleolus were obvious in some endosperm cells, and there were more mitochondria providing energy for endosperm cell division, and annulate lamellas in different periods. At 40 DAF (Fig. 2b), there were many high electron dense materials near the cell membrane, vesicles along with a few starch grains. At 60 DAF (Fig. 2c), large vacuole and vesicles appeared with high electron dense materials and a small amount of cytoplasm were distributed close to cell membrane, and more mitochondria occurred. At 80 DAF (Fig. 2d), large vacuole, more mitochondria and endoplasmic reticulum occurred and extracellular space was big. At 100 DAF, many different sizes of gradually accumulated OBs and a lot of high electron dense materials could be obviously observed (Fig. 2e). At 120 DAF (Fig. 2f), in endosperm cells, there were also many different sizes of vesicles with high electron dense materials and gradually grew OBs. At 140 DAF (Fig. 2g), there were many large and small OBs

Table 1: Seed characteristics during different developmental stages of *S. tonkinensis*

Sampling times/DAF	Length/ mm	Width /mm	Weight/g/per seed (FW)	Color of seed coat	Endosperm	Embryo
20	1.09 ± 0.28	0.92 ± 0.17	0.012 ± 0.002	Green	Green liquid	Not found
40	6.04 ± 0.65	4.11 ± 0.41	0.045 ± 0.007	Green	Green and oily semisolid	Embryonic form, lumpy
60	8.05 ± 0.52	5.24 ± 0.39	0.451 ± 1.451	Green	Green and oily semisolid	Gradually growing, white and transparent, lumpy
80	8.25 ± 0.57	6.01 ± 0.71	0.611 ± 1.578	Green	Green and oily semisolid	Gradually growing, white, lumpy
100	8.38 ± 0.74	6.04 ± 0.63	0.683 ± 2.422	Green	White and oily solid	Gradually growing, white, preliminary differentiation
120	9.28 ± 0.48	6.04 ± 0.46	0.717 ± 0.898	Green	White, polished and oily solid	Relatively shorter cotyledon and longer hypocotyl
140	9.53 ± 0.54	6.12 ± 0.18	0.717 ± 0.501	Yellow	White, semitransparent and oily solid	Spoon shape, surrounded by endosperm.

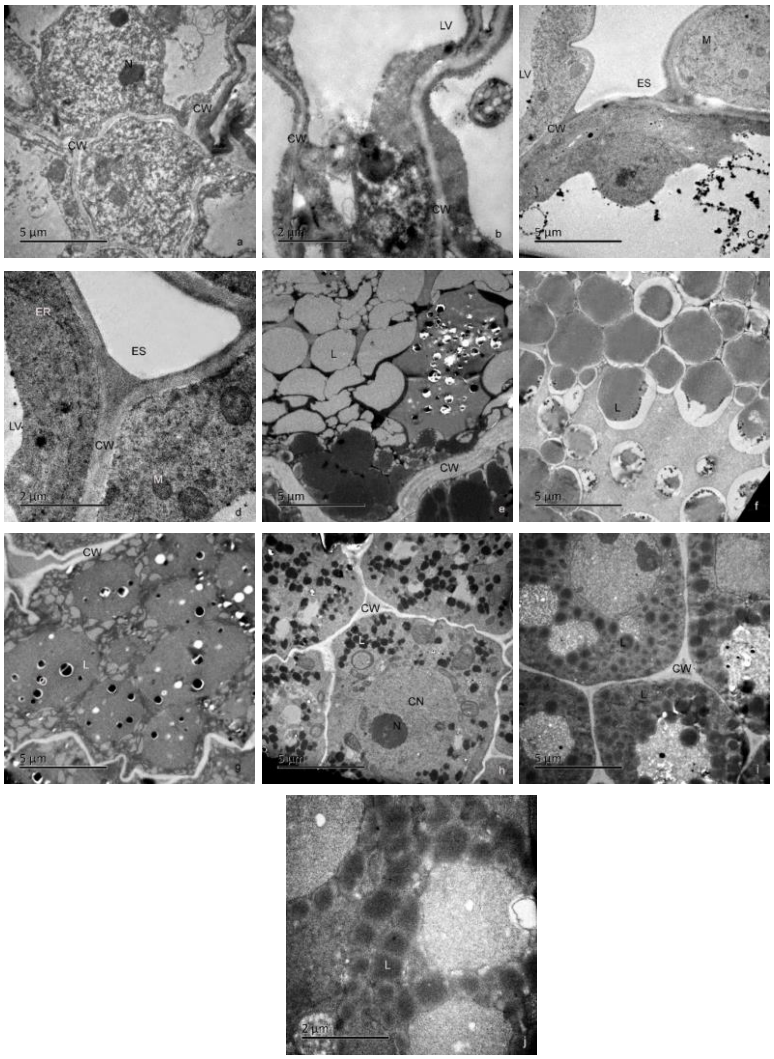

Fig. 2: TEM images of endosperm and embryo of *S. tonkinensis.* Endosperm at 20 (**a**), 40 (**b**), 60 (**c**), 80 (**d**), 100 (**e**), 120 (**f**) and 140 (**g**) DAF. Embryo at 100,120 and 140 DAF, respectively (**h-j**). CN: cell nucleus, CW: cell wall, ER: endoplasmic reticulum, ES: extracellular space, L: oil body or lipid body, LV: large vacuoles, M: mitochondria, N: nucleolus, O: osmiophilic globule

and some high electron dense materials in endosperm cells.

In embryo cells, at 100 DAF (Fig. 2h), there were many high electron dense materials, mitochondria and some lipids except large nuclei and nucleoli. At 120 DAF (Fig. 2i), in addition to the larger cell nucleus and nucleolus, the quantity of OBs appeared more than those at 100 DAF and the sizes were also larger. At 140 DAF (Fig. 2j), nuclei and nucleoli were still observed; different sizes and shapes of high electron dense materials were found; and there were some cavity bubbles, annulate lamellas (not shown), and the dense larger oil body which distributed surrounding organelles in the cells.

In endosperm cells, the mean value of OBs was 2.08 μm at 120 DAF (Fig. 3a). At 140 DAF, the largest diameter of the spherical bodies was about up to 5.41 μm, the smallest one 3.59 μm and the mean value increased up to

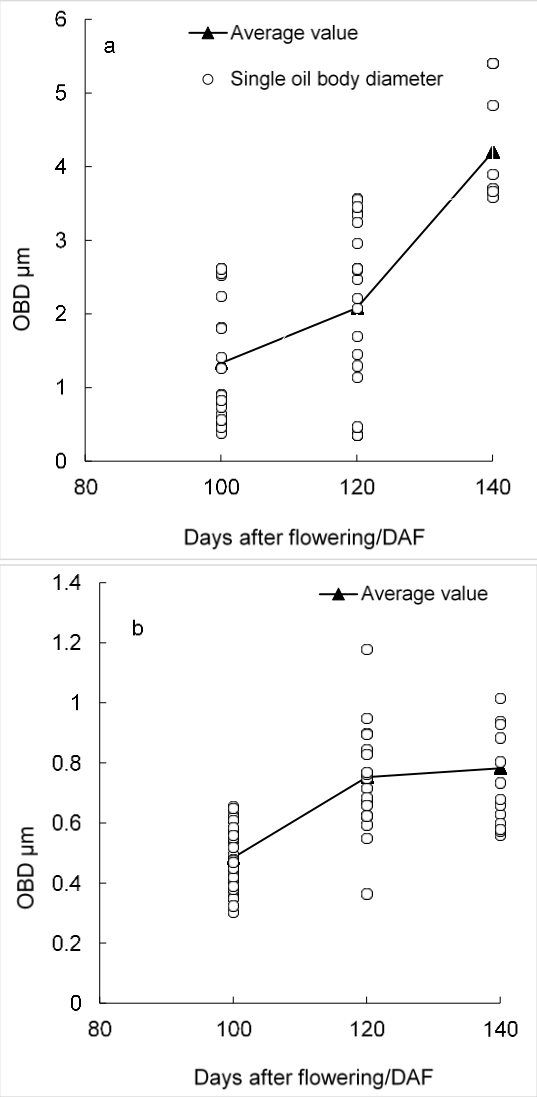

Fig. 3: Changes of oi body diameter (OBD) in endosperm (**a**) and embryo (**b**) cells of *S. tonkinensis.*

4.20 μm (Fig. 3a), indicating that the value of OBD increased rapidly during the stage of seed ripening at which fruit pulp became from green to yellow and endosperm structure was whole and the sizes of fruit and endosperm did not change.

During the embryo development, the OBD mean value at 100 DAF was 0.48 μm (Fig. 3b). And the value at 120 DAF ranged from 0.37 to 1.18 μm and the mean value was 0.73 μm. And the mean value at 140 DAF was 0.78 μm (Fig. 3b), larger than those at 100 and 120 DAF.

Changes in crude oil content during seed development

Crude oil accumulation in developing seeds of *S. tonkinensis* showed a biphasic pattern, which could be characterized by four stages fast-slow-fast-slow (Fig. 4).

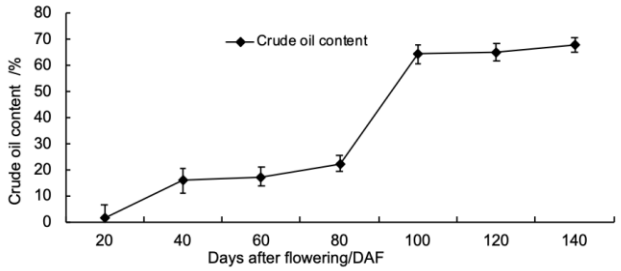

Fig. 4: Changes of crude fat contents during seed development of *S. tonkinensis.*
(Mean of 3 determinations, bars correspond to the standard deviation.)

The first and the second rapid stage occurred during the period of June 11–30 and August 10–30, 2013 and the crude oil content rose from 1.20 to 15.75% and from 22.28 to 64.14%, respectively. The highest level of oil content was observed at the last sampling time, October 10, 2013, when oil content reached 68%.

Changes in fatty acid composition during seed development

The composition of each sample of oil obtained from the *S. tonkinensis* seeds at different DAF was very complex and it changed with the different development stages, as shown by numerous peaks observed in the total ion flow chromatograms of methyl esterified oil samples (Fig. 1S a–g). The polyunsaturated linoleic acid was the most abundant FA during seed development, followed by, in the order, oleic acid, palmitic acid and stearic acid (Fig. 5). Interestingly, the variation of linoleic acid and of oleic acid showed an opposite trend after 40 DAF. Furthermore, linoleic acid reached the highest relative content at 80 DAF.

As illustrated in Fig. 6, fluctuations occurred also in the unsaturated index and unsaturated degree of fatty acid during seed development. The two indices rapidly increased during the first 60 days after flowering, reaching maximum values of 10.6 and 153.9 for the unsaturated degree and index, respectively. Following this time point, the unsaturated degree rapidly decreased to 4.4 at 100 DAF then gradually to 3.9 at 140 DAF; while the unsaturated index gradually decreased to the minimum values of 123.7 at 120 DAF.

Correlation analysis

The Pearson's correlation analysis between fatty acids and OB ultra-structural variables during development stages indicated that the content of linoleic acid was greatly correlated with monthly mean maximum and minimum temperatures (Table 2), while negatively with palmitic acid (R= -0.840, $P < 0.05$) and OBD (R= -0.997, $P < 0.05$). The degree of unsaturated FAs was positively correlated with monthly average total evaporation, while index of unsaturated FAs negatively correlated with OBD. The

Table 2: Correlation analysis among some index in developing seeds of *S. tonkinensis* (Pearson coefficients, two-tailed, *0.01<P < 0.05, **P < 0.01)

	palmitic acid	degree of UFA	OBD in embryo	monthly highest temperature	monthly lowest temperature	relative humidity of soil at 10 cm depth	relative humidity of soil at 20 cm depth
stearic acid	0.832*	-0.803*	0.811	-0.599	-0.614	-0.105	-0.797*
oleic acid	0.118	-0.187	0.985	-0.710	-0.684	0.814*	0.368
linoleic acid	-0.840*	0.849*	-0.997*	0.903**	0.900**	-0.420	0.351
index of UFA	-0.940*	0.929**	-1.000**	0.823*	0.827*	-0.226	0.528
monthly average total evaporation	-0.294	0.782*	0.883	0.267	0.384	-0.294	0.355

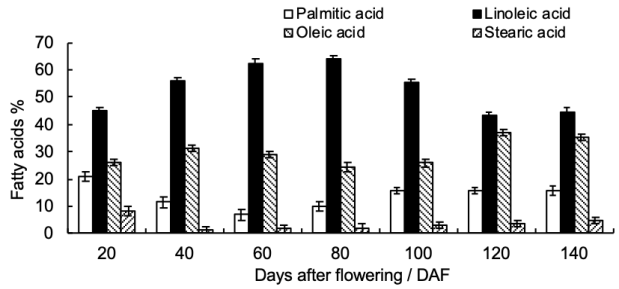

Fig. 5: Content changes of the main fatty acids from *S. tonkinensis* seeds during development. Mean of 3 determinations, bars correspond to the standard deviation

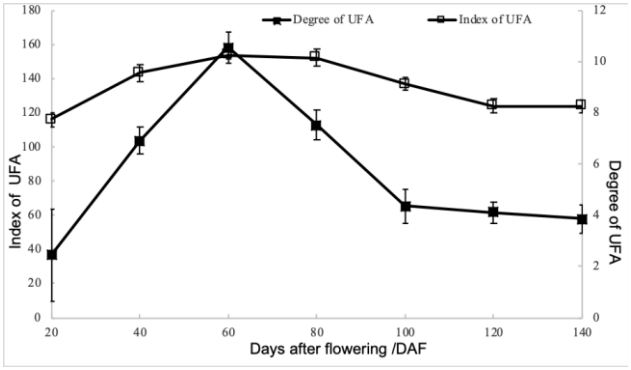

Fig. 6: Changes of unsaturated degree of fatty acids and unsaturated index of fatty acids during seed development of *S. tonkinensis* (Mean of 3 determinations, bars correspond to the standard deviation)

content of stearic acid was positively correlated with palmitic acid, while negatively correlated with relative humidity of soil at 20 cm depth (R= -0.797, P < 0.05). The content of oleic acid was positively correlated with relative humidity of soil at 10 cm depth.

Discussion

Oil is mainly stored in the oil body in the form of TAG in subcellular structure of seed embryo or endosperm (Huang 1992; Durrett *et al.* 2008). The present finding indicates that the seed endosperm changes obviously from liquid to gel and finally becomes solid (Zhao and Wu 2020), both endosperm and embryo have storage function of nutrients (especially lipids). In 2013, the mean values of OBD in embryo and endosperm remarkably increased and at 140 DAF the OBD was 3.2- and 1.6-fold larger than at 100 DAF in endosperm and embryo, respectively. Interestingly, the OBD in endosperm was larger than the one in embryo which was consistent with the findings of Zhang *et al.* (2018), who reported about two different ways for lipid accumulation in seeds. Furthermore, it is worth pointing out that the number of OBs might be decreased during late mature stage of seeds as a result of small OB fusion, which determined the formation of larger OBs (Miquel *et al.* 2014).

In present study, the structural changes of OBs exactly coincided with dynamic changes of oil content, which could be explained by previous findings (Jolivet *et al.* 2011; Miquel *et al.* 2014) that the oleosins studied have specific functions in the dynamics of lipid accumulation. As well known, the various components of the oil may vary considerably from one year to another one (Salvador *et al.* 2003). These results may be explained by gene expression pattern, proteomics analysis and protein immunodetection (Jolivet *et al.* 2011). Although some researchers have reported about *S. tonkinensis* seed oil (Liu *et al.* 2007; Fu *et al.* 2014), to the best of our knowledge, the changes of its oil content during seed development have not been explored. Herein, the oil content of *S. tonkinensis* seeds, up to 68% of the total dry weight at 140 DAF (Fig. 4) which may reach the maximum oil yield (Zhang *et al.* 2017; Wu *et al.* 2020), was higher than the findings of Wu *et al*. (2019).

Fatty acids (FAs) composition during seed development is also a crucial parameter to monitor for evaluating the best period of seed harvesting (Kaushik *et al.* 2010). It also seemed that the compounds present in the oil and the synthetic rate of FAs changed during early stages of seed development were similar with the report of Yadav and Singh (2003). The percentage of UFAs in the seeds was much higher than those records for most oil-rich seeds, such as *Telfaria occidentalis*, *Jatropha curcas* (Esuoso *et al.* 1998; Adebowale and Adedire 2006). The relative content of UFAs was higher than the saturated ones and changed during all stages of seed development. The main UFAs present in the seeds of *S. tonkinensis* were linoleic acid and oleic acid, both of which are essential FAs for humans (Aguilera *et al.* 2000). Nevertheless, because of a high content of UFAs, when exposed to air it might be susceptible to oxidation, which may have a negative impact on the stability of biodiesel (Mohibbe *et al.* 2005). On the

contrary, the high degree of unsaturation will be an advantage to make *S. tonkinensis* oil interesting for edible purposes (Adebowale and Adedire 2006; Liu *et al.* 2007; Shi *et al.* 2014). In addition, palmitic acid was the main SFAs similar with the oil from *Pistacia lentiscus*, even though the total FA composition was different (Charf *et al.* 2008).

The fluctuation of the degree and index of UFAs can reflect to some extent different membrane fluidity in the endosperm, as suggested by the fact that, at early stages of seed development, endosperm was liquid. It is well known that oil content of seeds varies significantly by genotype and environmental factors (Dwivedi *et al.* 1993). It is also reported that OBs microstructure and suspension stability may be affected by temperature, pH medium and salt presence, and so on (Jolivet *et al.* 2013). Our observed correlations indicated that the FAs composition (*e.g.*, the degree and index of UFA) and OBD were strongly affected by temperature (Wolf *et al.* 1982), total evaporation and relative humidity of soil. Thus, the mechanism of environmental impact on FA synthesis in *S. tonkinensis* seeds needs further study.

Conclusion

In short, the study here reported that both endosperm and embryo in *S. tonkinensis* seeds have the function of storing fat. The changes of oil content, fatty composition and OBD were dynamic that indicate the best period for seed harvesting to obtain an oil with desired characteristics in terms of unsaturation and relative content of linoleic acid. This study provides a scientific basis for the development and utilization of such woody oil plants.

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