Trees (2020) 34:891–902
https://doi.org/10.1007/s00468-020-01968-x

ORIGINAL ARTICLE

A comparative study of seed reserve accumulation in five *Styrax* species with potential for biofuel production

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Received: 8 October 2019 / Accepted: 27 February 2020 / Published online: 17 March 2020

Abstract

Key Message **Carbon competition between starch and oil biosynthesis during seed development of *Styrax tonkinensis* has been reported, but reserve dynamics indicate an absence of significant carbon competition in other *Styrax* species.**

Abstract The accumulation of different storage reserves during seed development can be competitive. Carbon competition (i.e., carbon flow into starch instead of oil) has been reported in *Styrax tonkinensis* (Pierre) Craib ex Hartwich. In this study, developmental patterns in morphology and the accumulation of major biochemical fractions of five other *Styrax* species (*S. calvescens* Perkins, *S. dasyantha* Perk., *S. faberi* Perk., *S. japonicus* Sieb. et Zucc., and *S. odoratissimus* Champ. ex Benth.) were analyzed over 50 days. In all species, kernel fresh matter and dry matter increased during development, while the percent water content trended downwards to lower than 40% at the last sampling date. The time to maturity varied with species, as did the evolution of oil, fatty acids composition, sugar, starch, and protein. Contents of these major reserve fractions per kernel increased with development and were strongly correlated with each other. Final oil concentration ranged from 489 to 548 mg g^{-1}, and the proportions of major fatty acids (palmitic, oleic and linoleic acid) were similar across species (11–15%, 39–41% and 43–47%, respectively). In contrast, starch concentrations never exceeded 7–45 mg g^{-1}, depending on species and developmental stage. Final protein concentration ranged from 165 to 237 mg g^{-1}. A slowdown in the speed of oil accumulation midway during development may occur in *S. faberi* but was not present in the other species. None of the five *Styrax* species examined here presented evidence for significant carbon competition between starch and oil accumulation.

Keywords Seed development · Seed reserves · Seed oil · Biodiesel feedstock · Fatty acid composition

Introduction

During seed development, carbon, nitrogen and other nutrients are transported from maternal tissues to the seed, and then stored in the form of lipid, starch and protein. There are large species-specific differences in resource investment into

Communicated by Buckeridge.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00468-020-01968-x) contains supplementary material, which is available to authorized users.

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these components (Lonien and Schwender 2009; Ruan et al. 2012). While sucrose is typically the common carbon source (Schwender et al. 2006; Zhang et al. 2018a), the metabolic pathways associated with the biosynthesis of different seed storage reserves interact with each other. Partitioning of carbon and nitrogen into the major storage products within the developing seed is an important process. Carbon partitioning involving glycolysis and the oxidative pentose phosphate pathways determines the eventual starch/oil balance (Baud and Lepiniec 2008; Lin et al. 2004). Carbon competition during seed development and storage lipid breakdown has been reported in many species, such as *Arabidopsis thaliana* (L.) Heynh. and *Brassica napus* L. (Boud et al. 2002; Eastimond and Rawthorne 2000).

Styrax, a genus of about 130 species of large shrubs or small trees in the family *Styracaceae*, has a widespread but disjunct distribution across the Americas, eastern Asia, and the Mediterranean region (Fritsch 2001). This genus is the source of benzoin rosin, and has great biofuel potential because the seed kernels have a high oil content and excellent fatty acid composition that imparts good fuel properties (Kar and Sen 2012; Wang et al. 2015; Cesur 2019; Wu et al. 2019). *Styrax* species have mature kernel oils that satisfy the biodiesel standards of China (GB/T 20828), the European Union (EN 14214), Germany (DIN V51606), and the United States of America (ASTM D6751) with good density, kinematic viscosity, cetane number, iodine value, and cold filter plugging point (Wu et al. 2019). The existence of a period of oil accumulation speed slow down (OASD) during *Styrax* seed development, with more than a 30 day downward trend in crude oil concentration at middle to later stages of seed development, was first reported in *Styrax tonkinensis* (Pierre) Craib ex Hartwich, a woody biodiesel species (Shi et al. 2013). The phenomenon was analyzed and discussed by Zhang et al. (2017), who attributed it to a reduction in lipid deposition resulting from a shift in carbon flow towards starch rather than oil accumulation between 80 and 120 days after flowering (DAF). For the development and utilization of other *Styrax* species as biodiesel feedstock, it is important to know whether carbon competition during seed kernel development is universal in *Styrax*.

In this study, we selected five *Styrax* species, i.e. *Styrax calvescens* Perkins var. *cinerascens* Rehder., *Styrax dasyantha* Perk., *Styrax faberi* Perk. var. *faberi*, *Styrax japonicus* Sieb. et Zucc., and *Styrax odoratissimus* Champ. ex Benth., to observe patterns in morphology and accumulation of major biochemical fractions over an extended time course during seed development. The aims of the study were to answer the following questions: (1) How does seed development in different *Styrax* species vary in terms of changes in morphology and storage compound composition? (2) Does an OASD exist in *Styrax* species other than *S. tonkinensis*? (3) If so, can the OASD be attributed to carbon competition between starch and oil? Answers to these questions may help us further understand oil accumulation in *Styrax* species to further explore and potentially enhance their feedstock potential in biodiesel applications.

Materials and methods

Plant materials

Five-year-old *Styrax* trees sampled in this study were growing without fertilizer application at the *Styracaceae* Germplasm Repository, Luhe District, Nanjing, China (32° 32′ N, 118° 50′ E). Fifteen trees of each species (provenance unknown) were tagged for sampling in the spring of 2018. Peak blooming of four of the five species occurred May 3rd (*S. dasyantha* and *S. faberi*) and 4th (*S. calvescens* and *S. odoratissimus*), but flowering in *S. japonicus* was approximately 8 days earlier than the others (April 26th). Beginning July 18th, fresh fruits were randomly harvested from each selected plant every 10 days (i.e., July 28th, August 7th, August 17th, August 27th, and September 6th) then sealed in plastic bags embedded in an insulated ice box (− 10 °C). There were three biological replicates for each time point, with each replicate consisting of material pooled from five different trees. High and low temperatures from July 11th to September 10th in Luhe District (Fig. S1) were obtained from Weather Post (http://www.tianqihoubao.com). Fifteen fruits of each species were cut longitudinally and observed using a SZX16 stereomicroscope (Olympus Co., Tokyo, Japan). For each biological replicate, 30 kernels separated from fresh fruits were weighed immediately (fresh mass (FM)) and after drying at 65 °C for 72 h (dry mass (DM)). The remaining fruits were frozen in liquid nitrogen and then stored at − 80 °C for other analyses.

Lipid analyses

To extract seed oil, 50 seed kernels from each time point were separately weighed and ground after drying at 65 °C for 72 h. Oil content in each sample was measured by the Soxhlet extraction method as described by Zhang (2006) using petroleum ether (30–60 °C) as the solvent. The seed oil was collected on a rotary vacuum evaporator at 65 °C and then methyl-esterified for analysis of fatty acids (FAs). The methyl-esterification reaction system included 0.2 mL seed oil, 2 mL petroleum ether:benzene (1:1, v/v), and 2 mL potassium hydroxide in methanol (0.4 mol/L) in a 10 mL vial. Vials were shaken vigorously during the 15 min conversion at room temperature before adding 5.8 mL deionized water and then centrifuged at 3000 rpm for 5 min at 15 °C. The supernatant was analyzed by gas chromatography-mass spectrometry (GC/MS) using a TRACE DSQ GC/MS

(Thermo Fisher Scientific Inc., Waltham, USA) with parameter settings according to Zhang et al. (2017).

Carbohydrate analyses

To measure soluble sugar and starch contents of kernels, 500 mg samples were ground and homogenized in 8 mL of deionized water and placed in a boiling water bath for 20 min. After cooling to room temperature, the homogenate was centrifuged at 5000 rpm for 10 min at 15 °C to separate the supernatant and residue for measurements of total soluble sugar and starch (Zhang et al. 2017), respectively, using an anthrone colorimetric method at 630 nm (Morris 1948; Li 2006). The supernatants were brought up to 25 mL with deionized water and 0.2 mL aliquots were transferred to test tubes to which 1.8 mL deionized water, 0.5 mL anthrone-ethyl acetate solution (2%, v/v) and 5 mL concentrated sulphuric acid (98%, v/v) were then added. The absorbance was read after 10 min in a Beckman DU 800 UV–visible spectrophotometer (Beckman Coulter Inc., Brea, USA) and compared to glucose standards. The residues were homogenized in 10 mL of deionized water and placed in a boiling water bath for 15 min. After adding 2 mL perchloric acid (9.2 mol/L), the tubes were placed in a boiling water bath for another 5 min, cooled to room temperature, and brought up to 25 mL with deionized water. Aliquots of 0.2 mL were transferred to test tubes for analysis as above but with starch as the standard.

Protein analyses

Total soluble protein content was quantified according to Bradford (1976). 500 mg samples were ground and homogenized in 5 mL of 20 mM phosphate buffer (pH 7.5). After centrifuging at 6000 rpm for 20 min at 15 °C, the supernatant was diluted for measurement with bovine serum albumin as the standard.

Statistical analysis

Values are expressed as mean ± SD ($n = 3$) and presented relative to days after flowering (DAF). One-way analysis of variance (ANOVA) with repeated measures was used to test for changes with DAF for each species separately. Species differences at kernel maturity were also tested by one-way ANOVA. Pearson correlation coefficients were used to examine relationships between nutrient concentrations within species. All statistics were performed using SigmaPlot 14.0 (Systat Software Inc., San Jose, USA). For all parameters measured, α was set at 0.05 and P values less than α were considered significant.

Results

Changes in seed morphology, weight and water content

Representative longitudinal sections of fruits at different time points are presented in Fig. 1. The structural anatomy of each species was clear during the whole observation period, especially the lignified seed coats and milky-white kernels. The endosperm and embryo grew considerably at early stages, especially in *S. dasyantha* and *S. odoratissimus*, filling almost the entire seed by August 27th (115 and 114 DAF, respectively). The fruits were spherical to oblong with an apical style remnant, especially in *S. calvescens*, *S. faberi* and *S. japonicus*. The seeds and kernels were oval or elliptical in section. The fruit sizes of *S. japonicus* and *S. odoratissimus* were larger than for other species, while the kernel size of *S. japonicus* was largest and the pericarp size of *S. odoratissimus* was thickest.

In all five species, the kernel FM and DM both increased over the whole observation period (Fig. 2) and were highly correlated (ρ ranged from 0.88 to 0.99; $P < 0.01$). The kernel FM of *S. calvescens* increased by 225% between 74 and 84 DAF, and then increased slowly to 52.3 ± 2.0 mg at 124 DAF, while DM increased continuously. In *S. dasyantha*, kernel FM and DM increased continuously between 75 and 95 DAF, followed by a 10-day lag phase before they increased again. *Styrax faberi* kernel FM had a continuous increase to 84.2 ± 6.4 mg from 75 to 105 DAF; thereafter, the kernel FM remained stable while the DM continued to increase. *Styrax japonicus* kernel FM and DM increased rapidly before 102 DAF and then maintained a slow increase. In *S. odoratissimus,* kernel FM and DM increased relatively steadily over the whole observation period. Species ranking for final DM, from high to low, was: *S. japonicus* (87.9 ± 3.2 mg), *S. dasyantha* (65.0 ± 0.7 mg), *S. faberi* (58.7 ± 0.4 mg), *S. odoratissimus* (56.5 ± 4.2 mg) and *S. calvescens* (39.6 ± 0.5 mg). Ranking for final FM was: *S. japonicus* (144 ± 9 mg), *S. dasyantha* (95.9 ± 2.5 mg), *S. odoratissimus* (86.0 ± 2.8 mg), *S. faberi* (82.7 ± 1.6 mg) and *S. calvescens* (63.0 ± 1.6 mg). Trends in water content (mg per kernel) depended on species but were generally less marked after the first or second sampling date. Water content was most stable in *S. japonicus*. Overall, percent water content trended downward during the whole observation period, falling below 40% in all species by the last sampling date.

Changes in oil and fatty acids

There were significant changes in kernel oil concentrations during development (Table 1). Concentrations in

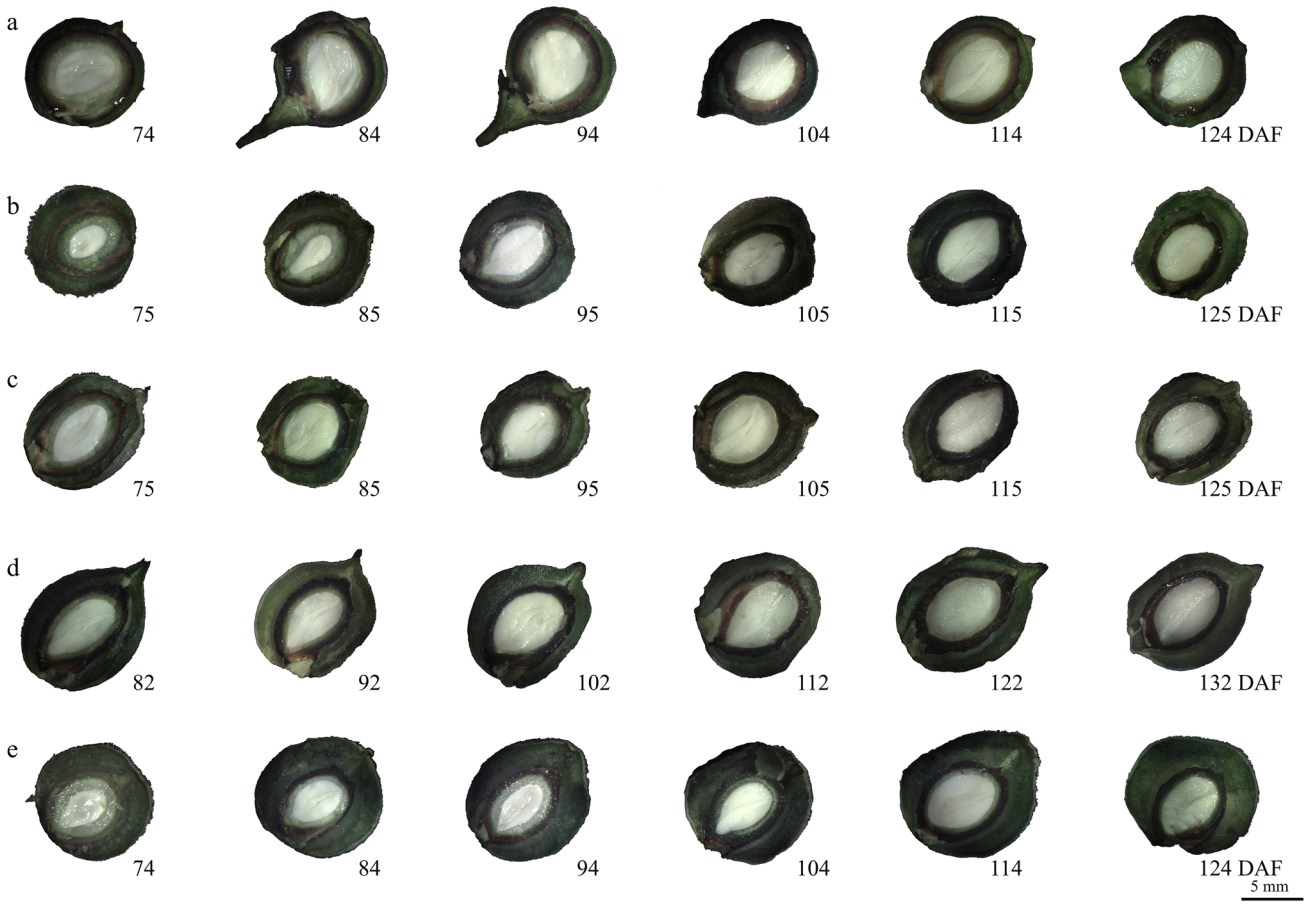

Fig. 1 Longitudinal sections of fruits of five *Styrax* species at different time points during development (2.5×). **a** *S. calvescens*; **b** *S. dasyantha*; **c** *S. faberi*; **d** *S. japonicus*; **e** *S. odoratissimus*. Numbers to the lower right of each image indicate days after flowering (DAF)

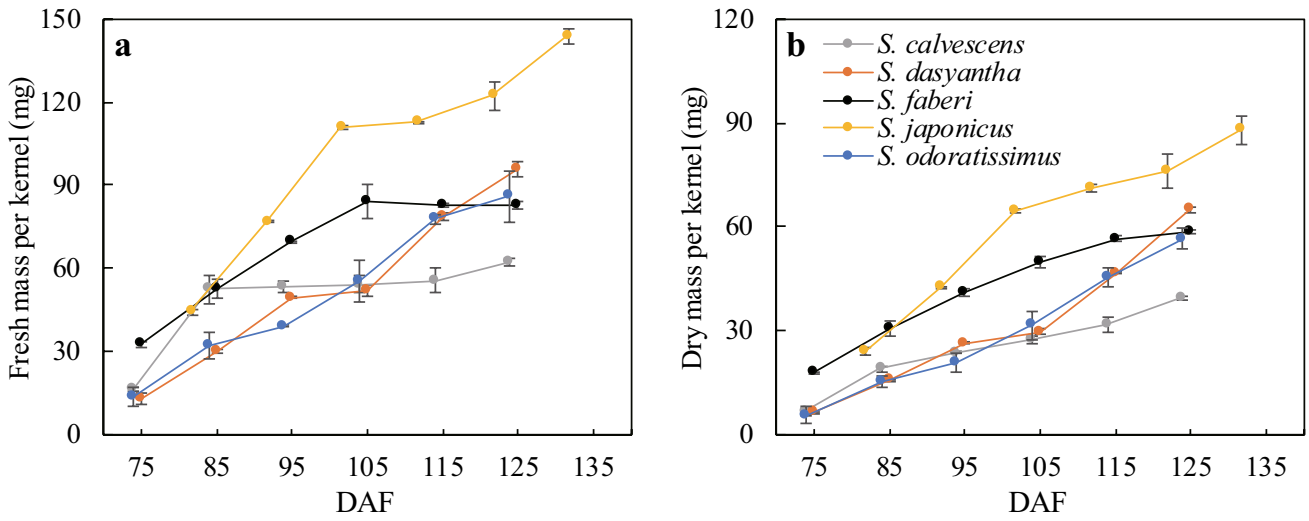

Fig. 2 Changes in fresh mass (FM) and dry mass (DM) in developing kernels of five *Styrax* species relative to days after flowering (DAF). **a** Evolution of FM; **b** evolution of DM. Data points show mean ± SD

Table 1 Summary of statistical differences among dates of sampling for each measured physiological indicator within five species of *Styrax*

Species	Physiological indicators	*P*	7-18	7-28	8-07	8-17	8-27	9-06
S. calvescens	Fresh weight	< 0.001	c	b	b	b	b	a
	Dry weight	< 0.001	f	e	d	c	b	a
	Water content	< 0.001	a	a	b	c	d	e
	Oil concentration	< 0.001	d	d	c	ab	b	a
	Total oil content	< 0.001	f	e	d	c	b	a
	Sugar concentration	< 0.001	a	a	a	bc	c	c
	Total sugar content	< 0.001	e	d	c	c	b	a
	Starch concentration	0.002	b	a	bc	bc	c	b
	Total starch content	< 0.001	c	b	b	b	b	a
	Protein concentration	0.005	bc	bc	d	ab	a	cd
	Total protein content	< 0.001	d	c	c	b	a	a
S. dasyantha	Fresh weight	< 0.001	e	d	c	c	b	a
	Dry weight	< 0.001	f	e	d	c	b	a
	Water content	< 0.001	a	b	b	c	c	d
	Oil concentration	< 0.001	d	bc	c	a	b	a
	Total oil content	< 0.001	f	e	d	c	b	a
	Sugar concentration	< 0.001	a	a	b	c	d	e
	Total sugar content	< 0.001	d	c	b	b	a	a
	Starch concentration	0.006	a	b	b	b	b	a
	Total starch content	< 0.001	c	c	c	c	b	a
	Protein concentration	0.020	c	ab	c	bc	a	ab
	Total protein content	< 0.001	e	d	cd	c	b	a
S. faberi	Fresh weight	< 0.001	d	c	b	a	a	a
	Dry weight	< 0.001	f	e	d	c	b	a
	Water content	< 0.001	a	b	b	b	c	d
	Oil concentration	< 0.001	d	b	d	e	c	a
	Total oil content	< 0.001	f	e	d	c	b	a
	Sugar concentration	< 0.001	a	b	b	b	d	e
	Total sugar content	< 0.001	e	d	bc	a	b	c
	Starch concentration	0.015	bc	abc	c	a	ab	a
	Total starch content	< 0.001	c	c	c	ab	b	a
	Protein concentration	0.001	c	ab	b	ab	ab	a
	Total protein content	< 0.001	f	e	d	c	b	a
S. japonicus	Fresh weight	< 0.001	e	d	c	bc	b	a
	Dry weight	< 0.001	e	d	c	b	b	a
	Water content	0.005	a	a	a	b	b	b
	Oil concentration	< 0.001	e	e	d	b	c	a
	Total oil content	< 0.001	f	e	d	c	b	a
	Sugar concentration	< 0.001	b	b	a	c	c	d
	Total sugar content	< 0.001	e	d	a	bc	ab	b
	Starch concentration	0.002	bc	a	c	ab	b	a
	Total starch content	< 0.001	d	c	c	b	b	a
	Protein concentration	< 0.001	b	ab	c	c	c	a
	Total protein content	< 0.001	d	c	c	b	b	a

Table 1 (continued)

Species	Physiological indicators	*P*	7-18	7-28	8-07	8-17	8-27	9-06
S. odoratissimus	Fresh weight	< 0.001	f	e	d	c	b	a
	Dry weight	< 0.001	f	e	d	c	b	a
	Water content	< 0.001	a	b	bc	c	c	d
	Oil concentration	< 0.001	d	c	b	b	b	a
	Total oil content	< 0.001	f	e	d	c	b	a
	Sugar concentration	< 0.001	a	a	a	b	c	d
	Total sugar content	< 0.001	e	d	c	b	a	a
	Starch concentration	0.005	a	a	b	a	a	a
	Total starch content	< 0.001	e	d	d	c	b	a
	Protein concentration	< 0.001	b	c	c	b	a	a
	Total protein content	< 0.001	e	d	d	c	b	a

In each row the *P* value obtained by ANOVA is followed by the results of Duncan's Multiple Range Tests where different letters indicate significant differences between sampling dates at $\alpha = 0.05$. Sampling dates were July 18th (7-18), July 28th (7-28), August 7th (8-07), August 17th (8-17), August 27th (8-27), and September 6th (9-06), 2018

all five species trended upwards with the development of the embryo and endosperm and were more similar at kernel maturation than earlier in the season (Fig. 3a). *Styrax calvescens* had the steepest increase over any 10-day period, from 320 ± 27 to 503 ± 5 mg g^{-1} between 84 and 94 DAF. In contrast, *S. faberi* had the highest oil concentrations early on, exceeding 500 mg g^{-1} at 75, 85 and 95 DAF, but showed the smallest increase in concentration over the full period of observation. Oil accumulation in this species was marked by a significant decrease in concentration at 105 DAF (to 488 ± 3 mg g^{-1}). Oil concentrations of the other four species tended to plateau towards maturation. There appeared to be slight but statistically weak decreases in *S. calvescens*, *S. dasyantha* and *S. japonicus* at the August 27th sampling (114, 115 and 122 DAF, respectively). There were significant differences between all five species for both oil concentration and content at kernel maturity (Table 2). Species-ranking for final oil concentration relative to kernel DM, from high to low, was: *S. faberi* (548 ± 8 mg g^{-1}), *S. dasyantha* (547 ± 5 mg g^{-1}), *S. odoratissimus* (533 ± 4 mg g^{-1}), *S. calvescens* (503 ± 5 mg g^{-1}) and *S. japonicus* (489 ± 2 mg g^{-1}). Because increases in kernel size during development were more pronounced than changes in oil concentration, total oil content per kernel rose relatively steadily in all five species (Fig. 4), including *S. faberi*. Ranking for final total oil content per kernel was: *S. japonicus* (43.0 ± 0.2 mg), *S. dasyantha* (35.6 ± 0.3 mg), *S. faberi* (32.2 ± 0.4 mg), *S. odoratissimus* (30.0 ± 0.2 mg), and *S. calvescens* (19.9 ± 0.2 mg).

In all five species, there was no obvious change in FA composition during the whole observation period (Fig. 5). Eleven FAs were detected, but profiles were dominated by palmitic acid (C16:0), oleic acid (C18:1) and linoleic acid (C18:2). The proportions of these major FAs were steady; the relative abundance of C18:1 and C18:2, added together, remained near or above 80% during the whole observation period, while C16:0 and other minor FAs always accounted for 10–16% and below 5%, respectively. The final proportions of the three major FAs were similar across species (10.8–14.7%, 39.6–40.7% and 41.9–47.1% for C16:0, C18:1 and C18:2, respectively).

Changes in carbohydrate

Except for a significant peak in *S. japonicus* at 102 DAF (118 ± 3 mg g^{-1}), all five species showed significant (Table 1) downward trends in kernel soluble sugar concentrations during development (Fig. 3b). For example, the soluble sugar concentration of *S. odoratissimus* kernels dropped from 151 ± 5 mg g^{-1} DM to 95.8 ± 0.9 mg g^{-1} DM, but remained higher than the other species at every time point. There were significant species differences for sugar concentration and total sugar content at kernel maturity (Table 2). Species-ranking for final soluble sugar concentration relative to kernel DM, from high to low, was: *S. odoratissimus* (95.8 ± 0.9 mg g^{-1}), *S. calvescens* (89.6 ± 4.2 mg g^{-1}), *S. japonicus* (78.0 ± 1.8 mg g^{-1}), *S. dasyantha* (67.7 ± 5.3 mg g^{-1}) and *S. faberi* (70.5 ± 6.7 mg g^{-1}). Combined with increases in kernel size, total soluble sugar content per kernel generally increased with development, but decreased again at later developmental stages in *S. faberi* and *S. japonicus*. Ranking for final total soluble sugar content per kernel, from high to low, was: *S. japonicus* (6.86 ± 0.16 mg),

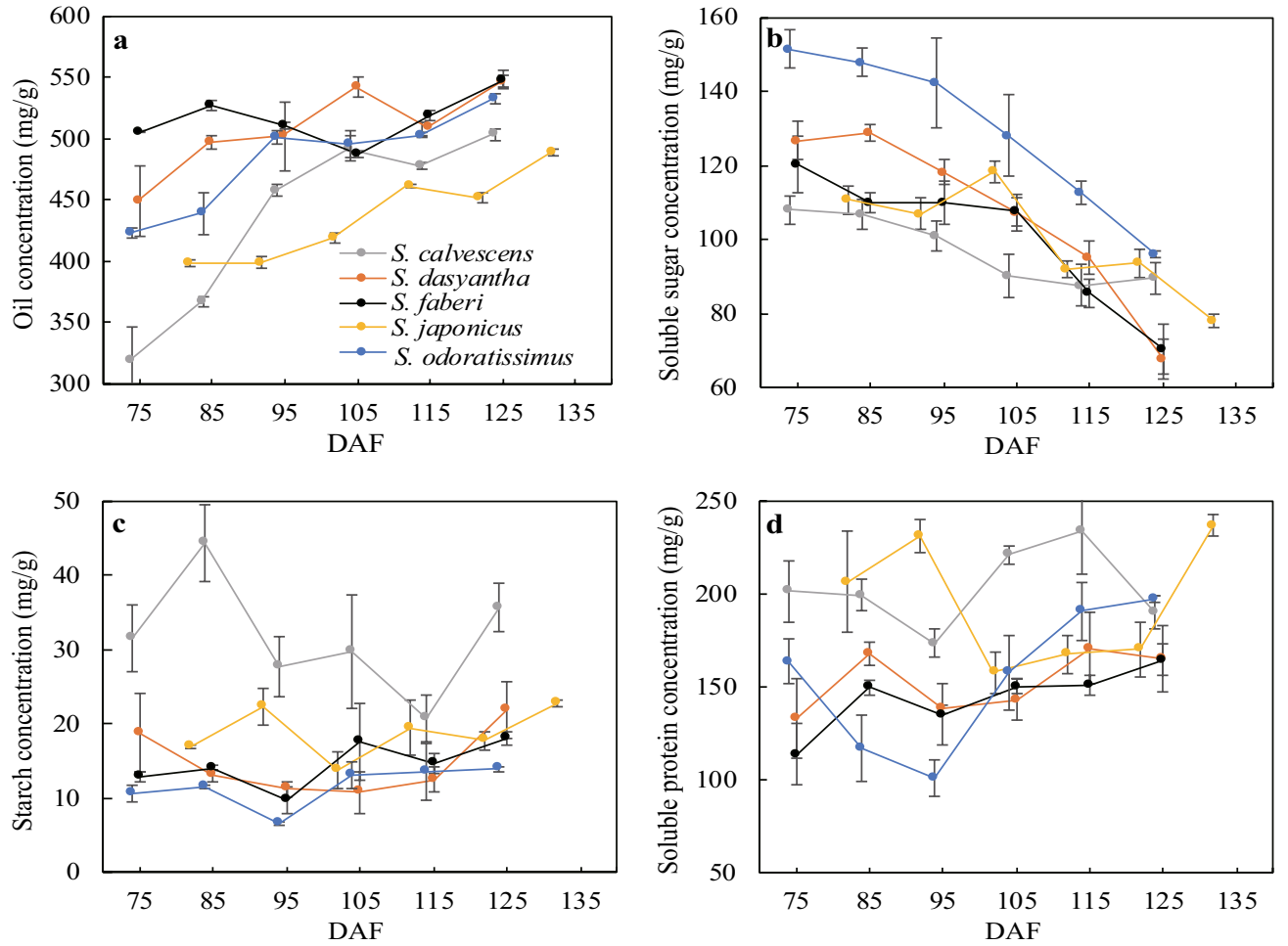

Fig. 3 Changes in nutrient concentrations during kernel development according to days after flowering (DAF) in five *Styrax* species. **a** Kernel oil concentrations; **b** Kernel soluble sugar concentrations; **c** Kernel starch concentrations; **d** Kernel soluble protein concentrations. Data points show mean ± SD

Table 2 Summary of statistical differences among species at kernel maturity for each measured physiological indicator

Physiological indicator	*P*	*S. calvescens*	*S. dasyantha*	*S. faberi*	*S. japonicus*	*S. odoratissimus*
Fresh mass	0.009	b	ab	ab	a	ab
Dry mass	< 0.001	e	b	c	a	d
Water content	0.008	ab	ab	b	a	ab
Oil concentration	< 0.001	d	b	a	e	c
Total oil content	< 0.001	e	b	c	a	d
Sugar concentration	< 0.001	a	c	bc	b	a
Total sugar content	< 0.001	d	c	c	a	b
Starch concentration	0.014	a	ab	ab	ab	b
Total starch content	0.012	ab	ab	ab	a	b
Protein concentration	< 0.001	b	c	c	a	b
Total protein content	< 0.001	e	cd	d	a	bc

In each row the *P* value obtained by ANOVA is followed by the results of Duncan's Multiple Range Tests where different letters indicate significant differences ($\alpha = 0.05$) between species on the last date of sampling (September 6th, 2018; 124-132 DAF, depending on species)

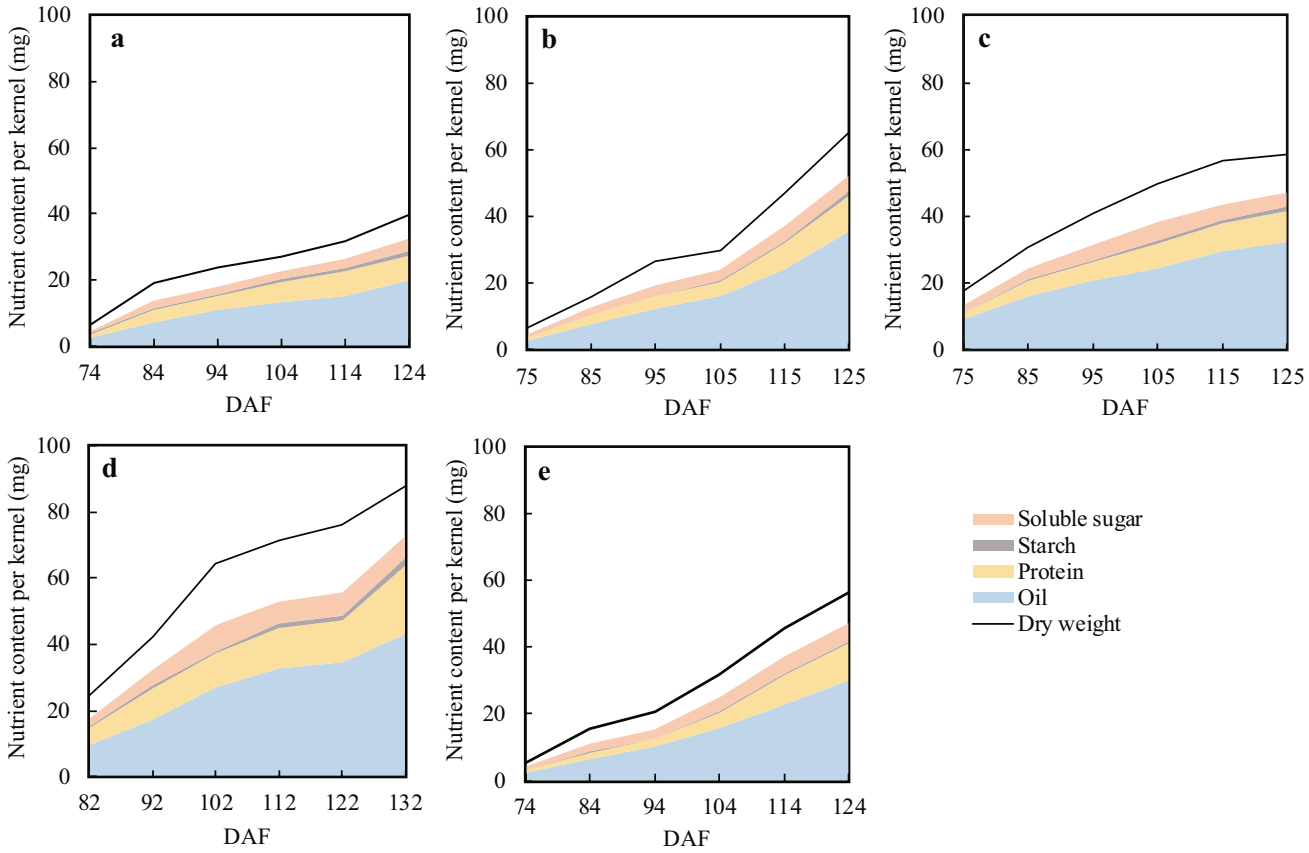

Fig. 4 Overall per kernel accumulation of nutrient contents (lipid, starch, sugar and protein) in five *Styrax* species. **a** *S. calvescens*; **b** *S. dasyantha*; **c** *S. faberi*; **d** *S. japonicus*; **e** *S. odoratissimus*. DAF is days after flowering

S. odoratissimus (5.41 ± 0.05 mg), *S. dasyantha* (4.40 ± 0.34 mg), *S. faberi* (4.13 ± 0.40 mg) and *S. calvescens* (3.52 ± 0.17 mg).

All species had a significant decrease in starch concentration on one or more dates of sampling (Fig. 3c, Table 1). In *S. calvescens* there was also a significant peak in starch concentration at 84 DAF. Nonetheless, in every species, the final kernel starch concentration was similar to the initial concentration. There was a significant difference in starch concentration at kernel maturity only between *S. calvescens* and *S. odaratissimus* (Table 2). Species-ranking for final starch concentration relative to kernel DM, from high to low, was: *S. calvescens* (35.6 ± 3.2 mg g^{-1}), *S. japonicus* (22.7 ± 0.5 mg g^{-1}), *S. dasyantha* (21.9 ± 3.8 mg g^{-1}), *S. faberi* (18.0 ± 0.9 mg g^{-1}) and *S. odoratissimus* (13.9 ± 0.3 mg g^{-1}). *Styrax calvescens* had a higher starch concentration than any of the other species at every time point, but *S. japonicus* had the highest starch content per kernel because of its larger kernel size (cf. Fig. 2b). Ranking for final total starch content per kernel, from high to low, was: *S. japonicus* (2.00 ± 0.05 mg), *S. dasyantha* (1.42 ± 0.25 mg), *S. calvescens* (1.40 ± 0.13 mg), *S. faberi* (1.06 ± 0.05 mg) and *S. odoratissimus* (0.79 ± 0.02 mg), but only *S. japonicus* and *S. odaratissimus* were significantly different from each other (Table 2).

Changes in soluble protein

As was the case with starch concentration, some species showed a significant decrease in protein concentration midway through development, followed by a recovery (Fig. 3d, Table 1). This pattern was most obvious in *S. japonicus*, where protein concentrations rose from 171 ± 19 mg g^{-1} DM to 237 ± 6 mg g^{-1} DM between 122 and 132 DAF. *Styrax calvescens* showed a significant decrease in protein concentration between 114 and 124 DAF. In all species, however, the per kernel protein content increased continuously throughout development, demonstrating no actual decrease in the amount of protein (Fig. 4). Except for *S. calvescens*, final protein concentrations were marginally higher than initial concentrations. There were significant species differences in protein concentration and content at kernel maturity (Table 2). Species-ranking for final soluble protein concentration relative to kernel DM, from high to low, was: *S. japonicus* (237 ± 6 mg g^{-1}), *S. odoratissimus* (198 ± 2 mg g^{-1}), *S. calvescens* (190 ± 9 mg g^{-1}), *S.*

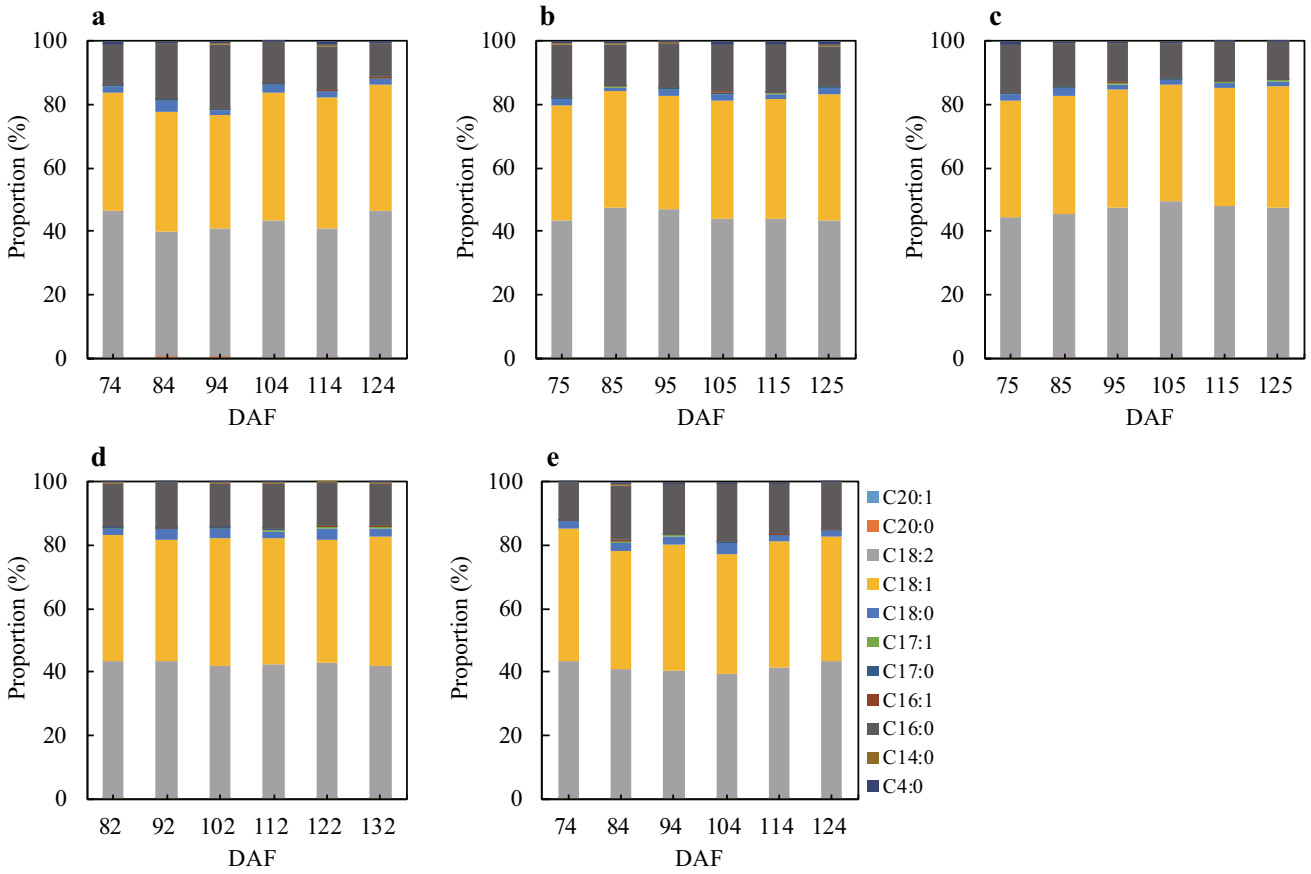

Fig. 5 Fatty acid composition during kernel development in five *Styrax* species. **a** *S. calvescens*; **b** *S. dasyantha*; **c** *S. faberi*; **d** *S. japonicus*; **e** *S. odoratissimus*. DAF is days after flowering

dasyantha (165 ± 18 mg g^{-1}) and *S. faberi* (165 ± 9 mg g^{-1}). Overall trends in soluble protein content were largely determined by growth in kernel size. Ranking for final total protein content per kernel, from high to low, was: *S. japonicus* (20.8 ± 0.5 mg), *S. odoratissimus* (11.2 ± 0.1 mg), *S. dasyantha* (10.7 ± 1.2 mg), *S. faberi* (9.66 ± 0.49 mg) and *S. calvescens* (7.53 ± 0.34 mg).

Correlation between different major reserve fractions

Using mean values for each species at each sampling time, correlations between the concentrations of the major reserves were analyzed (Table 3). There were significant negative linear correlations between oil and soluble sugar concentrations in *S. calvescens*, *S. japonicus* and *S. odoratissimus* (ρ ranged from −0.911 to −0.847; $P < 0.05$) and negative but non-significant linear correlations in *S. dasyantha* ($\rho = -0.734$, $P = 0.097$) and *S. faberi* ($\rho = -0.708$, $P = 0.116$). In other words, sugar concentrations went down

Table 3 Pearson correlations between different mean kernel nutrient concentrations

Species	Oil/Sugar	Oil/Starch	Oil/Protein	Sugar/Starch	Sugar/Protein	Starch/Protein
S. calvescens	−0.911*	−0.422	0.156	0.532	−0.495	−0.411
S. dasyantha	−0.734	−0.070	0.419	−0.481	−0.476	0.095
S. faberi	−0.708	0.192	0.514	−0.579	−0.795	0.673
S. japonicus	−0.899*	0.374	0.003	−0.689	−0.339	0.789
S. odoratissimus	−0.847*	0.261	0.368	−0.676	−0.761	0.823*

*Correlation significant at the level of 0.05

as oil concentrations increased during development. There was also a significant positive correlation between concentrations of starch and protein in *S. odoratissimus* ($\rho = 0.823$, $P < 0.05$). All other correlations were insignificant and were especially weak between oil and starch.

Discussion

Styrax kernels, consisting of the embryo and endosperm, grow gradually but fill almost the whole seed at maturation (Silva et al. 2019; Zhang et al. 2017). As shown in Fig. 1, fruit size was relatively stable from July 18th to September 6th, while kernel size increased. The progression in kernel size (and mass, Fig. 2) varied with species. In *S. dasyantha* and *S. odoratissimus*, kernel size and mass increased steadily during whole observation period, whereas kernel growth plateaued or slowed down in *S. calvescens*, *S. faberi* and *S. japonicas* at 84 DAF, 105 DAF and 102 DAF, respectively. Changes in kernel size and mass in *S. tonkinensis* over a comparable period, as reported by Zhang et al. (2017), were similar to the latter three species. Seed maturation may proceed more slowly in *S. dasyantha* and *S. odoratissimus* relative to other species of *Styrax*.

The desiccation tolerance of seeds increases during development, coincident with a decrease in water content (Schwallier et al. 2011). The percent water content of the five *Styrax* species studied here decreased continuously as dry mass accumulated, but the total water content (mg) of kernels was relatively steady during the latter stages of development, which may be related to the importance of water in the processes of transporting and accumulating assimilates during seed development (Matheus et al. 2011). With more time, as development is completed and the seeds approach hygroscopic balance, total water content would be expected to decrease.

During seed development, concentrations of the major reserve fractions had different change trends (Saldivar et al. 2011). Oil concentration showed an upward trend overall (Fig. 3a) while the soluble sugar concentration had a downward trend (Fig. 3b); changes in starch and protein concentrations did not go clearly one way or the other (Fig. 3c, d). By comparison, *S. calvescens* had the highest kernel starch concentrations, but *S. faberi* and *S. odoratissimus* had higher oil and soluble sugar concentrations, respectively. Oil and soluble protein contents per kernel shared similar change trends (Fig. 4), with a significant positive linear correlation between them (ρ ranged from 0.917 to 0.995; $P < 0.01$), which may reflect that oil was stored in oil bodies combined

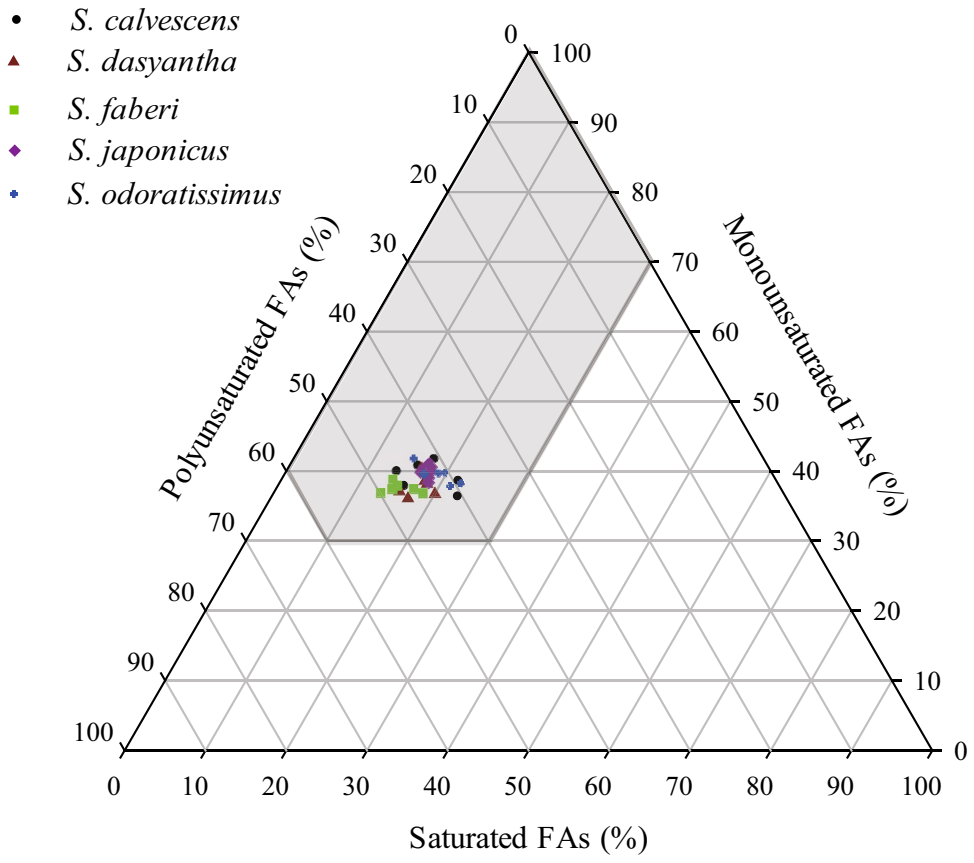

Fig. 6 Placement of kernel oil samples on a chart predictive of biodiesel properties based on fatty acid composition. The gray area delineates fatty acid compositions satisfying limits for biodiesel fuel properties

with proteins (Zhang et al. 2018b). Overall reserve content per kernel kept pace with increases in DM (Fig. 4), but oil accounted for close to half the kernel DM throughout development.

To assess the possibility of using *Styrax* oil as a raw material for the production of biodiesel, a triangular prediction model (Fig. 6, Fig. S2) was used to predict fuel properties from the percentage of saturated, monounsaturated and polyunsaturated FAs (Wang et al. 2012). All samples remained within a region (gray area in Fig. 6, as defined by Wang et al. (2012)) indicating that oils from all five *Styrax* species are likely to fully meet biodiesel feedstock property standards (i.e., cetane number, iodine value, cold filter plugging point, and oxidation stability) regardless of developmental stage.

The other major reserve fractions constitute alternative metabolic sinks that may compete with oil synthesis. For example, starch biosynthesis during seed maturation can affect oil accumulation by reducing carbon flux to the glycolytic pathway (Lin et al. 2006; Weselake et al. 2009). For *S. tonkinensis*, the main evidence for the existence of carbon competition is the existence of an OASD during seed development where the oil concentration decreases by more than 10% (from 556 to 428 mg g^{-1}) and starch begins to rapidly accumulate (Fritsch 2001; Zhang et al. 2017). In the present study, however, an OASD (between 85 and 105 DAF) coinciding with an increase in starch content (at 105 DAF) only occurred in *S. faberi* (Fig. 3a, c). There were no similar indications of possible competition between oil and starch synthesis in the remaining four species. Furthermore, starch content was always very low relative to oil content, throughout development (Fig. 4). This is also true of *S. tonkinensis* (Zhang et al. 2017). Protein and total soluble sugar contents represent much more significant sinks for carbon in *Styrax*.

There were significant negative linear correlations between oil and soluble sugar concentrations in three species across development (Table 3), but without further detail these cannot be interpreted to indicate the possibility of carbon competition between the two reserve fractions. Sugars arriving through the phloem may support the synthesis of oil and/or carbon skeletons for amino acid and protein synthesis, but may also be directed towards trehalose, a disaccharide associated with desiccation tolerance and often found at high concentrations in seeds (Krasensky and Jonak 2012).

Conclusions

In this study, morphological and physiological properties of developing seed kernels from five *Styrax* species were observed from July 18th (74-82 DAF) to September 6th (124-132 DAF). The time to maturity varied with species, as did the evolution of oil, sugar, starch and protein contents, and fatty acid composition. Oil concentration showed an upward trend overall while the soluble sugar concentration decreased during development; changes in starch and protein concentrations were less directional. Final oil concentrations ranged from 489 to 548 mg g^{-1}. *Styrax calvescens* kernels had the highest starch concentration, but *S. faberi* and *S. odoratissimus* had higher oil and soluble sugar concentrations, respectively. The proportions of major fatty acids (palmitic, oleic and linoleic acid) in the accumulated oils were similar across species (11–15%, 43–47% and 39–41%, respectively) and uniformly favorable as feedstock for biofuel production. Rates of kernel development varied with species, which suggests the possibility of planting a mix of species to stagger harvesting. As in *S. tonkinensis* (Zhang et al. 2017), there may be an OASD period during kernel development in *S. faberi*, but not in the other species, and there was little evidence for carbon competition between starch and oil synthesis. The existence of carbon competition during seed development is, therefore, not a universal phenomenon in *Styrax* species.

Author contribution statement QW, ZZ and FY conceived and designed research. QW, YC and XZ conducted experiments. QW, ZZ and RDG analyzed data. QW, FY and RDG wrote the manuscript. All authors read and approved the manuscript.

Acknowledgments We thank Mr. Xiaoyue Ji from Advanced Analysis Testing Center, Nanjing Forestry University for the GC/MS technical assistance.

Funding This work was funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD), National Natural Science Foundation of China (3197140894) and the Doctorate Fellowship Foundation of Nanjing Forestry University.

Compliance with ethical standards

Conflict of interest The authors declare no known or potential conflict of interest.

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