Short Communication

Seed filling and fatty acid changes in developing seeds of castor bean (*Ricinus communis* L.)

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Abstract

A detailed knowledge of storage reserve accumulation in developing seeds can provide vital information to create strategies for improving the lipid content as well as the natural value of castor (*Ricinus communis* L.) crop. After hand pollination (female flowers-tagged), seed filling (lipid and protein accumulation) in castor bean is initiated as early as 7 days after pollination (*DAP*). Accumulation of proteins (0.129–0.152 mg/seed) and lipids (0.01–0.051 mg/seed) in developing castor bean seeds increased rapidly up to 35 *DAP*, followed by a decline in both the storage reserve accumulations at the maturation stage of 63 *DAP*. Nile red (10 g/ml dissolved in glycerol) fluorescence imaging technique was used to visualize the oil body synthesis (seed sections) from 7 to 63 *DAP*. Significant variations (P< 0.05) in fatty acid compositions were observed concurrent with the lipid accumulation pattern at different developmental stages. The unsaturated fatty acids (UFAs) content comprised 97.5% of the total fatty acids composition. Ricinoleic acid content was the predominant fatty acid component (over 85%) with other fatty acids such as palmitic, stearic, oleic, linoleic, and linolenic present in minimal amounts. Current findings entrust vital information on endogenous protein and lipid deposition in this potential oil crop in an innovative approach for physiological modifications in its oil seed.

Keywords: Fatty acids, ricinoleic acid, oil body, protein.
Abbreviations: DAP_Days after pollination, TAG_Triacylglycerol, FA_Fatty acids, FW_Fresh weight, DW_Dry Weight, SFA_Saturated fatty acids, UFA_Unsaturated fatty acids.

Introduction

Seed storage reserves are essential supplements for plant development as well as for commercial applications, therefore it is necessary to understand and improve the genetic, biochemical and physiological mechanisms favoring the high rate of incorporation of the main reserve components in seeds (Coelho et al., 2008). Lipids and proteins are considered as the major storage reserve components in several seeds (Da silva, 1998). The physiological modification of lipid and protein reserve determines the seed quality. Plant derived lipids in the form of fatty acids are of wide industrial importance (Goldberg et al., 2008). Previous investigations on storage reserve accumulation in developing seeds have evaluated differential distribution of fatty acids and proteins (Kaushik et al., 2010). In oilseeds, fatty acid reserves are synthesized mainly during the seed-filling phase of seed development (Ruuska et al., 2002). The fatty acid composition of oil seeds are determined by the accumulated amount of each fatty acid in its biosynthetic pathway during the seed filling period. Seed filling is a phase defined by morphological, cellular, and metabolic changes in the endosperm and embryo that coincide with rapidly increasing storage reserves, such as fatty acids and protein (Norton and Harris, 1975; Ruuska et al., 2002). In relation to this, castor bean (*Ricinus communis* L) an important industrially acclaimed oil crop has rich source of storage reserves (oil and protein). In castor oil seeds, about 40-50% by seed weight consists of lipid and l5-20% comprise proteins (Ramos et al., 1984), which are deposited in membrane-bound organelles, lipid (fatty acid complex) and protein bodies, respectively. The

most frequent fatty acids in oils are lauric, palmitic, estearic, linoleic and linolenic, although others may also be present. It is important to note that seed oils differ in their content of fatty acids. For instance, oleic acid is the main component of olive oil, whereas it is linoleic acid in soybean oil and in linseed oil it is linolenic acid. Similarly, ricinoleic acid is the major fatty acid proportion of castor oil (Vieira1 et al., 2000) and this ricinoleic acid is widely used in nylon, cosmetic, lubricate, plastic, pharma industries and recently ricinoleate derived methyl esters are used as raw material for biodiesel production (Goodrum and Geller, 2005; Puthli et al., 2006). In detail, ricinoleic acid is a naturally occurring 12-hydroxy fatty acid which constitutes about 90% of the total fatty acids in castor oil. Ricinoleic acid can serve as a substrate for the synthesis of conjugated linoleic acids. It is considered safe and non-toxic as a food constituent in humans, up to a daily intake of 2.5 grams per day. Ricinoleic acid methyl ester is a neutral, more lipophilic form of the free acid that can be used as an analytical source for biodiesel production (Da Silva et al., 2010). With continuing advances in global protein analyses, there have been several proteomic studies of seed filling in oilseeds, including soybean (Agrawal et al., 2008), rapeseed (Hajduch et al., 2005), and *Medicago truncatula* (Kumar et al., 2009). In relation to this, several oil seeds have been explored for their rich sources of storage reserves and fatty acids (Chung et al., 1995; Gecgel et al., 2007; Kaushik et al., 2010; Ishikawa et al., 2001). In recent years, several molecular and breeding techniques have been implemented for an improvised and increased content of castor oil i.e., fatty acid source. Recently, interest in castor

industrial benefits has increased considerably in many countries. Although several castor plantations have come up in various tropical parts of the world, there is limited information on the metabolite composition of the currently grown cultivars. Proximate and FA compositions of various castor cultivars have been reported by previous workers (Gupta et al., 1951; Ramos et al., 1984); however, there have been no studies on the changes of proximate storage reserve quantification and FA composition in the developing seed of this genotype. Thus, considering the repentance of bioactives and unusual fatty acid composition of castor seeds, and its potential as a biofuel product, we analysed the seed fatty acid compositions, storage reserve accumulation and the intracellular lipid droplets in a potential castor bean cultivar grown in china.

Results and Discussion

Storage reserve changes

To understand the seed-filling changes, we initially classified castor seed development into three stages (7–63 *DAP*) based on the physiological events, seed coat color, seed size, and seed fresh and dry weight. The early stage (7–14 *DAP*) was marked by seeds of Pale green color (Figure 1A) with minimal increase in seed size (Figure 1B). The mid-development phase (21–35 *DAP*) was associated with a rapid increase in seed mass (Figure 1B) and seed coat dry matter (Figure 1B), with the seed color changing from pale yellow to black (Figure 1A). The maturation phase (35–63 *DAP*) was associated with a rapid increase in seed dry mass (Figure 1B) and a change in the seed coat color to brown. Hardened seed coat was observed in mid-development and maturation phase of seed development. During seed development, the total soluble protein content per seed increased sharply during 7–35 *DAP*, followed by a slight decrease at 63 *DAP*. At this stage, the seed is at an advanced stage of maturity and enters into the desiccation phase. Total lipid content increased until 63 *DAP*. After 7 *DAP*, the total lipid content was as low as ca. 0.01 mg/seed, rapid oil filling started only after 14 *DAP*, and the rate of accumulation was maximum during 21–35 *DAP* (Figure 1C). Similarly, Chen et al. (2007) reported a rapid oil deposition rate in a castor variety during the mid-developmental stages owing to the seed fresh and dry weight changes. It is noteworthy that the protein content in each seed was much higher (0.116-0.160 mg) than the total lipid contents (0.01–0.125 mg/seed) at all developmental stages (Figure 1D). The oil body content at 7 *DAP* stage was minimal to be estimated. It is, thus, evident that, although lipid accumulation starts in developing seeds soon after pollination, the active mobilization of lipids into oil bodies takes place only after 14 *DAP*, so that, during subsequent developments, most of the neutral lipid deposits were accounted for by the oil bodies.

Distribution of neutral lipids by Nile red imaging

Nile red, a fluorescent probe for neutral lipids, serves as an ideal agent to monitor changes in intracellular neutral lipid accumulation sites (Greenspan et al., 1985). Nile red fluorescing molecules accumulated at the early stage (7 *DAP*) of seed development in castor (Figure 2A). In contrast, *Arabidopsis* exhibits the synthesis of TAGs in the late heart stage (Siloto et al., 2006). In the transverse sectioning of the developing seeds (current study), the fluorescence due to Nile red increased markedly during transition from 7 to 63 *DAP*; the increase being highest at 14–35 *DAP* (Figures 2B–E). Stabilization of oil bodies was observed during the maturation phase at 35–63 *DAP* (Figure 2F). A rapid accumulation of oil

bodies during seed development is, thus, evident both from the quantitative estimation of lipids in whole seeds as well as fluorescence microscopic analysis of sections.

Changes in fatty acid compositions

Gas chromatography analysis showed that palmitic acid, stearic acid, oleic acid, linoleic acid, and ricinoleic acid were the major fatty acid components distributed in castor seed oil, of which, ricinoleic acid contributed ca. 85% of the total fatty acids with palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2), and linolenic acids (18:3) distributed meagerly. The UFAs (ricinoleic, oleic, linoleic, and linolenic acids) comprised over 82% of all the fatty acids in the whole-seed lipids at any stage of seed development. Among the saturated fatty acids, the relative proportion of palmitic acid was 15.2% at 7 *DAP*. It showed a gradual increase until 21 *DAP*, followed by a steady decrease in all other stages of seed development (Table 1). Relative content of stearic acid was lower than that of palmitic acid at all stages of seed development. Proportions of stearic acid in whole seeds varied from 1.17% at 7 *DAP* to 0.2% at 63 *DAP* (Table 1). Changes in UFAs were more prominent than those in saturated fatty acids. The relative proportions of oleic acid declined from 28.2% at 7 *DAP* to 13.6% at 35 *DAP* in whole-seed lipids, preceded by a rapid increase in their proportion during the maturation stage 63 *DAP* (Table 1). Insignificant changes in palmitic and stearic acid content indicates that SFA synthesis is not influenced by the seed age. Interestingly, linoleic acid content rapidly increased (76%) during the early stage (7–21 *DAP*), after which, a slow decline in fatty acid levels was noted during mid-mature developmental stages. Similar pattern of linoleic acid changes was also noted in developing seeds of a potential castor variety (Brown et al., 2011). Linoleic acid content remained almost constant (ca. 54%) between 35 and 63 *DAP* (Table 1). Linolenic acid content showed declining patterns from 14 to 63 *DAP*. This was in accordance with the observation of Brown et al. (2011) in lipids from whole seeds of castor. Contrary to this, ricinoleic acid increased from the early to maturation stage, and the highest content was observed during the mid-developmental stage (21–35 *DAP*). In concordance with our results, Chen et al. (2007) also observed rapid synthesis of ricinoleate in developing endosperm seed tissues of castor bean. This difference in the pattern of oleic and linoleic acid and ricinoleic acid accumulation implies the variational activation of desaturase and FAH 12 enzymes with respect to synthesis and storage of neutral lipids. This observation is in accordance with earlier observed high oleate desaturase activity 21 *DAP* in a castor variety (Chen et al., 2007). The continuous increase in ricinoleic acid content until 63 *DAP* suggests that once ricinoleate activity is induced, it extends until seed maturity. Therefore, after 28 *DAP*, ricinoleic acid showed a decreased proportion owing to preferential synthesis of TAG species. Thus, seeds of castor bean cultivar (CS3) under investigation are high in ricinoleic and linoleic acid content. Some of the minor fatty acid components showing an inconsistent pattern of accumulation observed were myristic acid (14:0), arachidic (20:0), erucinoic acid (20:1), behenic (22:0), erucic (22:1), and lignoceric acid (24:0) (data not given). Finally, almost all comparable fatty acid composition of whole seeds indicates that there is no preferential partitioning of any particular fatty acid into the oil bodies. Although these changing patterns were observed in other oilseeds (Ichihara and Noda, 1980; Norton and Harris, 1975; Rubel et al., 1972), the rates of synthesis of fatty acid species were different (P<0.05) at specific developmental stages. This difference can be attributed to biochemical and molecular regulation with more detailed

Table 1. Fatty acid composition of castor bean seeds during seed development (as area percentage).

Fatty acids	Seed development *(DAP)*					
	7	14	21	28	35	63
C16:0 (Palmitic acid)	0.25±0.2[a]	0.13±0.2[a]	0.22±0.4[ab]	0.04±0.1[a]	0.01±0.1[c]	0.03±0.2[d]
C18:0 (Stearic acid)	0.13± 0.4[a]	0.04±0.2[a]	0.01±0.1[a]	0.01±0.1[b]	0.005±0.4[c]	0.02±0.2[d]
C18:1 (Oleic acid)	0.5±0.3[a]	0.09±0.1[a]	0.1±0.2[b]	0.06±0.1[a]	0.02±0.2[c]	0.12±0.4[d]
C18:2 (Linoleic acid)	0.1±0.2[b]	0.42±0.3[a]	0.48±0.2[a]	0.14±0.3[c]	0.09±0.1[d]	0.14±0.2[e]
C18:3 (Linolenic acid)	nd	0.11±0.3[a]	0.09±0.01[a]	0.02±0.01[a]	0.01±0.1[b]	0.02±0.1[c]
C18:1-OH (Ricinoleic acid)	nd	0.18±0.5[a]	0.19±0.2[b]	0.66±0.5[c]	0.79±0.6[d]	0.59±0.4[e]
C20:0 (Eicosanoic acid)	nd	nd	nd	0.007±0.1[a]	0.007±0.1[a]	0.01±0.2[b]
Total saturated fatty acids	33	17	22	5	2	6
Total mono-unsaturated fatty acids	42	9	1	6	4	13
Total poly-unsaturated fatty acids	1	72	64	84	91	77

nd- not detected[*] Values (means of triplicates ± SD) with different superscripts (a-e) are significantly different at P<0.05 as determined by Fisher's LSD procedure.

investigation. Altogether, the present study on seed development in castor (CS3) provides new information on the accumulation and partitioning of storage reserves and development stage-dependent modulation of fatty acid composition and spatial distribution of oil bodies. It is crucial to observe that the desaturation of fatty acids occurs after 21 *DAP*, leading to enhanced accumulation of ricinoleic acid during seed filling.

Materials and methods

Plant material

Seeds of castor bean var. CS3 (kindly provided by Zibo Academy of Agricultural Sciences, Shandong, China) were germinated and grown in the greenhouse of Xishuangbanna tropical botanical garden (Kunming branch) with the temperature of day at 24-26°C and night at 18-20°C with the humidity controlled at 60-80%. Mature female flowers were hand pollinated and tagged, and the tagging dates were recorded as 0 day after pollination (*DAP*). Capsules were harvested at a 7 day interval from 7 to 63 days after pollination (*DAP*). Seed samples collected were dissected and frozen immediately in liquid nitrogen and stored at -80°C for further analysis. Seeds dissected at different developing stages were weighted (FW) and kept for vacuum centrifugation over night. The dried seed samples were then weighted (DW).

Lipid analysis

Total lipid was extracted from seed samples using the hexane-isopropanol method as described previously (Xu et al., 2011). Fatty acid methyl esters (FAME) of total lipids were prepared and quantified as described elsewhere (Badling et al., 1988). In detail, a modified method was used for fatty acid methylation. Total lipids were methylated in 1 ml of 2% H_2SO_4 dissolved in MeOH for 1-2 hrs at 85°C.The existents were washed with 1% Kcl and centrifuged at 3000 *g* for the collection of lipid extracts. The fatty acid ester extracts were dissolved in dichloromethane and analysed by gas chromatography immediately.

Protein analysis

Whole seeds at different developmental stages were homogenized in 0.1M Tris-Hcl buffer, pH 7.8 (4 ml for 1 g of fresh weight). The homogenate was centrifuged for 20 min at 22000 *g*. Protein concentration in the supernatant was determined at 280 nm according to (Bradford, 1976), using
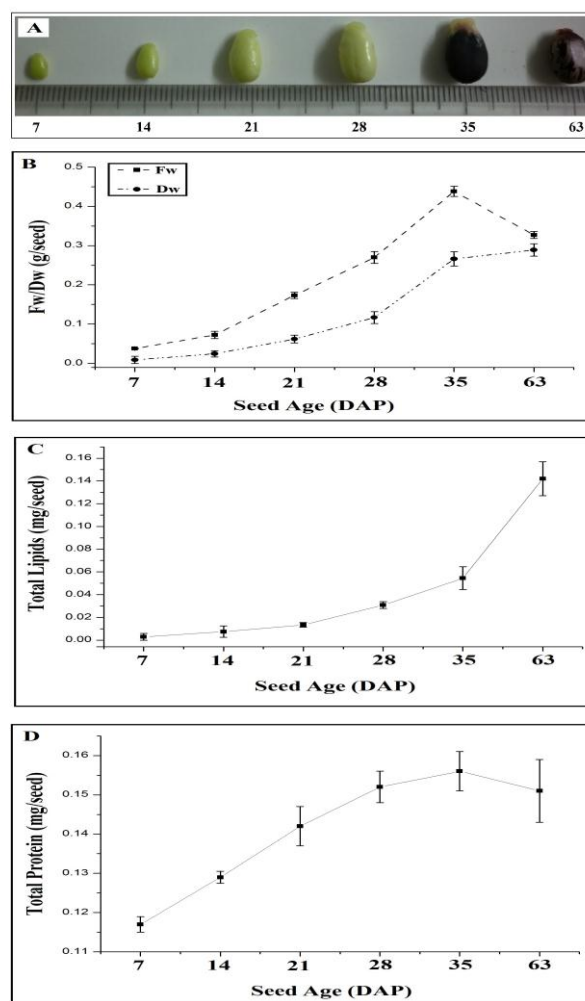

Fig 1. Characterization of castor seed development. A. Castor seeds at six developmental stages. B. Changes in fresh weight and dry weight per seed. C. Total lipid accumulation during seed development. D. Total protein accumulation during seed development.

bovine serum albumin as a standard. Nanodrop spectrophotometer was used to measure the optimum protein concentrations.
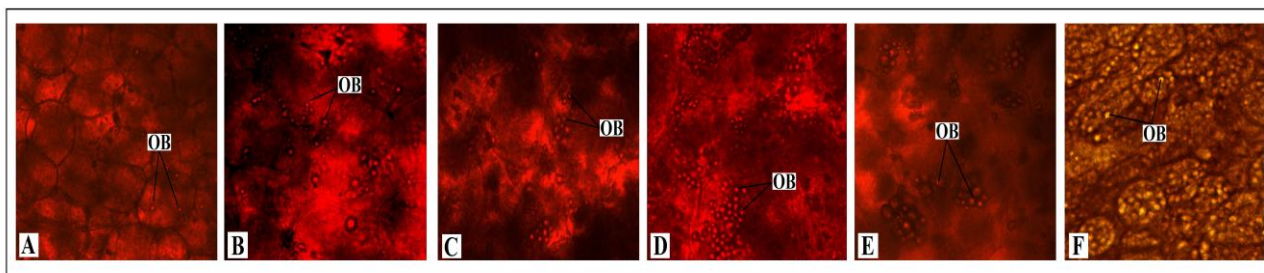
Fig 2. Localization of oil bodies (OB) during seed development (A-7 DAP; B-14 DAP; C-21 DAP; D-28 DAP; E-35 DAP; F-63 DAP). All preparations (seed sections) were treated with Nile red (10 g/ml) solution and observed for fluorescence.

Fluorescence imaging

Dissected seed sections were treated with 10 g/ml of Nile red prepared in aqueous medium and 75% glycerol, respectively. Fluorescence from intracellular neutral lipid accumulating sites (oil bodies) was observed by excitation at 485 nm using fluorescence microscope, leading to fluorescence emission at 525 nm. Fluorescence microscopic observations were made using an 'Olympus BX51' fluorescence microscope (Japan). Images were captured using 'Axiocam' digital camera and analyzed using the Axiovision software.

Statistical analysis

Data obtained (mean ± SD, triplicates) were subjected to a one-way analysis of variance (ANOVA) to determine the statistical significance of the treatment at $P<0.05$. Differences between means were analyzed using Fisher's least significant difference (LSD) procedure.

Conclusion

In this present investigation, the total lipid content, protein content, fatty acid composition, and oil body synthesis (visualization) of castor bean oil (CS3 variety) has been outlined. This castor bean oil is very unique with high ricinoleic acid and has potential for industrial application. Importantly, the study revealed that castor seed oil has been proven as a potential source of ricinoleic acid for further exploration.

Acknowledgements

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