



Accumulation of ricinoleic acid in developing seeds of castor (*Ricinus communis*) from India

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ABSTRACT

Plant oils serve as a major resource of food, feed, as well as raw materials for various industrial applications. Castor bean (*Ricinus communis* L.) is a plant of *Euphorbiaceae* family which have high level of ricinoleic acid (over 85%) in its seed. Ricinoleic acid is a hydroxy fatty acid (HFA) which is accumulated in seeds as Triacylglycerols (TAGs). Castor oil has a very high demand all over the world and India is the country who majorly fulfils world's demand. Due to its wide industrial usages and associated undesirable characters such as low genetic variability, presence of toxin ricin and allergens, various approaches such as conventional and transgenic approaches are being undertaken for production of ricinoleic acid. For later, understanding of genes involved in the metabolic pathways for the synthesis and accumulation of such fatty acids are crucial, so that they can be engineered to alternate oilseed crops. Present research work deals with the cloning of fatty acid hydroxylase gene from high oil yielding varieties of castor. This study majorly discussed the accumulation of major fatty acids in developing seeds of castor variety (48-1) which describes storage reserve accumulation. Ricinoleic acid accumulation started between 20-30 days after flowering (DAF) and continued to increase till 40 DAF with a slight decrease during maturation stage. This study can be further utilized for biotechnological interventions for production of ricinoleic acid.

Key words: Fatty acid hydroxylase, Fatty acids, Oilseed crops, Ricinoleic acid, *Ricinus communis*, TAGs

Plant contains various fatty acids stored in their seeds that have great nutritional, pharmaceutical as well as industrial value. These FA are stored in seed storage lipids mostly as triacylglycerols (TAGs). Each carbons of glycerol unit are linked to different FA that is helpful from an industrial perspective. Castor bean (*Ricinus communis* L.) which belongs to *Euphorbiaceae* family, is indigenous to the Ethiopian-East African region, India, North-West and South-West Asia and China. Castor oil was used to treat or prevent constipation primarily, so this was also known as medicinal oil (Scarpa and Guerici 1982). Castor oil has application used in veterinary industries as emollient and applied as soothing medium for the eyes of animals. Now a days it has become an important oilseed crop and its derivatives have various application in industrial purposes such as adhesives, dyes, lubricants, inks, dyes and biodiesels (Dwivedi and Sapre 2002). The seeds of castor plant have

high level of naturally occurring ricinoleic acid (over 85%) which is a hydroxy fatty acid (18:1-OH ;12-hydroxyoctadec-cis-9-enoic acid) (Gupta *et al.* 1951; Akpan *et al.* 2006).

Biosynthesis of ricinoleic acid is catalysed by the enzyme oleate 12-hydroxylase (*FAH12*) (Loo *et al.* 1995). A hydroxy group (-OH) is added to the 12th carbon of oleic acid (18:1) present on the sn-2 position of phosphatidylcholine (PC) in the endoplasmic reticulum (Bafar *et al.* 1991). Modified FAs such as ricinoleic acid are incorporated into diacylglycerol (DAG) by removing phosphocholine headgroup from PC (Bates and Browse 2012). De novo-synthesized DAG or PC-derived DAG produce TAG (Bates and Browse 2012) by the activity of acyl-CoA:diacylglycerol acyltransferases (DGAT) enzyme (Hobbs *et al.* 1999; Zou *et al.* 1999). Alternatively, TAG can be formed by an acyl-CoA independent phospholipid: diacylglycerol acyltransferase (PDAT) which guides direct transfer of HFA from PC into DAG (Dahlqvist *et al.* 2000).

India is the country which fulfils world's demand of castor oil and it currently produces about 1 t/ha castor seeds per year. There are number of varieties and hybrids which are grown across the country and still research is going as there is a potential for increase in yield of oil seeds. There are various known varieties and hybrids of castor as per ICAR-IIOR (Indian Institute of Oilseeds Research) and DOD (The Directorate of Oilseeds Development). Jyothi, JC-24,

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Jwala (48-1), Kiran (PCS-136), Pragathi, JC-4, Haritha (PCS-124), DCS-107, GAC-1, YTP-1, GC-2, GC-3, etc. are few varieties of castor. GCH-7, YRCH-1, DCH-177, GCH-4, HCH-6, DCH-32, GCH-6, GAUCH-1, GCH-9, PCH-111, GNCH-1, etc. are few examples of castor hybrids.

Castor being one of the plants which produces a very high concentration of such important fatty acid has been explored to increase its oil content by using several molecular and breeding techniques. However, there is limited information of metabolite composition of currently grown cultivars and FA composition of developing seeds. In recent years, the industrial application of ricinoleic acid has also attracted the interest of many countries. Due to undesirable attributes such as presence of ricin and an allergen in its seeds, it has been desirable to look at various alternative sources for production of these usual FAs. So, various genetic engineering techniques are being applied for increasing production of ricinoleic acid in other oilseed crops to fulfil the increase demand in global market. For that understanding of genes involved in metabolic pathway and finding allelic variants of such genes is an important step. Present study reports the accumulation of ricinoleic acid during seed development in a high yielding castor variety and cloning of *fatty acid hydroxylase* gene with an aim to find allelic variant of this gene, if any.

MATERIALS AND METHODS

Plant material

Seeds of castor were collected from Regional Research Station, HAU, Bawal, Haryana. Mature seeds of two high yielding variety of castor DCH-177 and 48-1 were collected and stored for further work. Developing seeds of castor variety (48-1) were also collected with 10 days interval from flowering.

Isolation of total RNA and cDNA synthesis

Total RNA was isolated from developing seeds of castor using Pure Link RNA Mini Kit from Ambion (Cat# 12183018A) following the manufacturer's instructions. DNaseI treatment was done to isolated RNA for removal of any trace of DNA as per manufacturer's instructions Ambion® DNA-free™ kit. 2µg of DNase-treated RNA were used to synthesize the first strand cDNA using superscript III first strand cDNA synthesis kit (Invitrogen, Lot# 1610358) according to manufacturer's instructions.

Cloning of fatty acid hydroxylase gene

The cDNA synthesized was used as a template for amplification of coding region of *fatty acid hydroxylase* gene using gene specific forward (ATGGGAGGTGGTGGTCGCATGTCCAC) and reverse primer (TTAATACTTGTTCCGGTACCAGAAAAC) designed using sequence information from reported sequence NM_001323721.1 (NCBI). PCR reaction was performed using Ex-Taq (Takara) with annealing temperature of 54°C. PCR amplification was carried out in thermal cycler

(Veriti 96 well; Applied Biosystem). Amplified product along with ladder was loaded on 1.2% agarose gel and viewed under Gel Documentation System. Once size was confirmed, it was ligated to pGEMT-Easy vector (Promega) as per manufacturer's instructions and transformed into *E. coli* DH5α cells. Screening of clones were performed by blue white screening and then by colony PCR using appropriate primers for confirmation of insert. Plasmids were isolated from positive clones using G-sure Plasmid Mini Prep Kit (GCC biotech, Cat # G46121). Sequencing of plasmids outsourced using M13 Forward and Reverse primers. The Basic Local Alignment Search Tool (BLAST) was used to find the similarity between sequences and allelic variation amongst different sequences from database.

Fatty acid analysis of developing seeds of castor variety (48-1)

Fatty acid methyl esters (FAMES) were prepared by making fine powder of 1 g seeds with the help of mortar and pestle in the presence of liquid nitrogen. Heptadecanoic acid (17:0) was used as internal standard. 2 ml of 2.5 % H₂SO₄ (v/v) in methanol solution was added to the mixture and were kept at 85°C in water bath for one hour. After incubation and subsequent cooling at room temperature, deionised water (3 ml) and hexane (400 µl) was added. Vortex for mixing of contents for 20-30 seconds followed by centrifugation to separate phases (Ichihara and Fukubayashi 2010). 150 - 200 µl of upper organic phase was taken for GC-MS analysis (Shimadzu's GCMS-QP2010ULTRA series). An electron ionization system operated in electron impact form for GCMS detection with ionization energy of 70eV. Helium gas (99.9%) with constant flow rate of 1 ml/min was used as carrier gas. Sample (1 µl) was injected (with 10:1 split ratio) and injector and ion source temperatures were maintained at 270°C and 230°C respectively. Mass spectra run were taken at 70 electron voltage for total run time of 50 min. Mass to charge ratio for start was 40.00 and for end 60.00. Mass spectrum was analysed using National Institute Standard and Technology (NIST) database.

RESULTS AND DISCUSSION

Castor is an important non-edible oil crop as distinct chemicals and products are obtained from its oil. It is being utilized as starting material for various bio-based products in agriculture, cosmetic, pharmaceuticals and other industries. One of the most promising application of castor oil is utilization as biofuel in aviation. Castor seed oil has been characterized for its physical and bio-chemical properties and transesterification was done for biodiesel production (Madankar *et al.* 2013; Nangbes *et al.* 2013; Omari *et al.* 2015; Khaliq *et al.* 2017; Keera *et al.* 2018). Previous studies on castor seed collected from various locations, reported major FAs that includes palmitic, stearic, oleic, linoleic, ricinoleic, pentadecanoic (Ramos *et al.* 1984; Zeng *et al.* 2009; Ramanjaneyulu *et al.* 2013; Huang *et al.* 2015a; Huang *et al.* 2015b) and ricinoleic acid was the major FA present in mature seeds.

Presence of high ricinoleic acid makes this crop even more valuable source of various raw materials for several industries. Castor accumulates ~85% ricinoleic acid in its seed, it is a good source for studying genes involved in biosynthesis of such industrially important FAs. Two varieties of castor which were used in this study to clone *FAH12* gene, has high yield of oil and are very different in morphology and other characteristics. DCH-177 is a hybrid for all states which was developed and released by Directorate of Oilseed Research, Hyderabad for commercial purpose. It is resistant to *Fusarium* wilt and whitefly. It has yield of 1550-2130 kg/ha and oil content is 49%. It generally takes 90-100 days for maturity (Fig 1). The second variety selected; 48-1 (Jwala) is a mutant which has spineless capsules. It is best suitable for drought prone areas. It is resistant to wilt, capsule borer and is found to be tolerant to jassid and *Botrytis*. It has yield of 1100-2000 kg/ha and oil content of 48%. Plants are taller in size and produce a greater number of effective spikes per plant when compared to other varieties. Capsules of this variety are spineless which make this plant less prone to capsule diseases and pests and due to absence of spine, excess water are not retained on capsule due to heavy rainfall (Fig 1).

Cloning of fatty acid hydroxylase gene

The aim was to obtain sequence information of *FAH12* gene from high yielding Indian castor varieties and investigate the natural variation occurring in the castor that could be used or have the potential for identity preservation of *FAH12* gene and germplasm conservation. Total RNA was isolated from mature fresh seeds of castor of two varieties and analysed on 2% agarose gel to check for integrity (Fig 2). Coding region of *FAH12* was synthesized using gene specific forward and reverse primers designed using sequence information from previously reported sequence NM_001323721.1. The product of size around 1.2kb was amplified (Fig 3) and was cloned followed by sequencing. Preliminary analysis of sequence

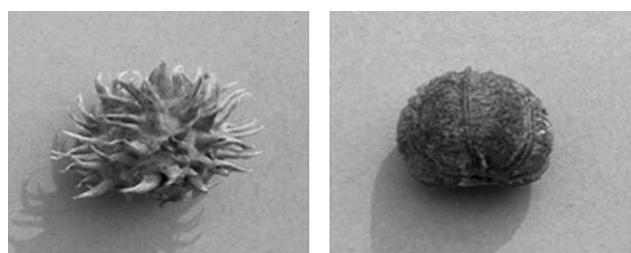


Fig 1 Fruits of castor: DCH-177 (left); 48-1 (right)

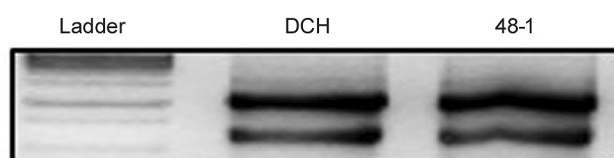


Fig 2 Total RNA

of clones of DCH-177 and 48-1 showed 99% similarity with already reported predicted sequence of *R. communis phosphatidylcholine 12-monooxygenase* (LOC8267537), transcript variant X1, mRNA (XM_002528081.2). Translated amino acid sequence showed 100% similarity to above mentioned sequence. Nucleotide sequences of the two variety have been submitted to NCBI with accession id MN480319 and MN480320.

FA analysis of seeds at different developmental stages of castor variety 48-1

To understand the accumulation of fatty acids during different stages of seed development, GC-MS analyses of FAMES was performed. Focus of this analysis was to determine accumulation of ricinoleic acid during a specific stage of seed development. Fruits of castor variety (48-1) were harvested at different developmental stages which were marked from the day of flowering. 10 days interval was taken between two developmental stages (Fig 4).

Table 1 and Fig 5 shows area percentage of ricinoleic acid along with other major FAs (palmitic, stearic, oleic and linoleic acid). Ricinoleic acid was found to be major constituents (81%) of fatty acids of mature seeds, whereas other fatty acids were distributed meagrely. The relative proportion of palmitic acid (saturated fatty acid) was found to 38.41% at 20 DAF followed by decrease in content with developing seeds and ended at 2.42% during maturation (Table 1). Stearic acid content was found to be lower than palmitic acid throughout the seed development. During initial days (20 DAF) it was found to be 2.75% which decreased later and attained 2.36% at maturity (Table 1).

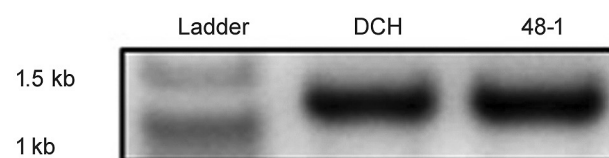


Fig 3 Amplification of *FAH12*

Table 1 FA analysis of seeds of castor variety (48-1) at different seed developmental stages

Fatty acids	20 DAF	30 DAF	40 DAF	50 DAF
Palmitic acid (16:0)	38.41±4.15	10.42±1.48	2.0±0.06	2.42±0.20
Stearic acid (18:0)	2.75±0.33	2.06±0.21	1.63±0.05	2.36±0.26
Oleic acid (18:1)	7.56±1.01	14.91±0.82	6.83±1.20	6.42±1.83
Linoleic acid (18:2)	54.70±2.36	26.72±1.45	6.51±0.04	7.79±1.20
Ricinoleic acid ND (18:1OH)	ND	45.88±3.73	83.01±1.14	81.01±3.60

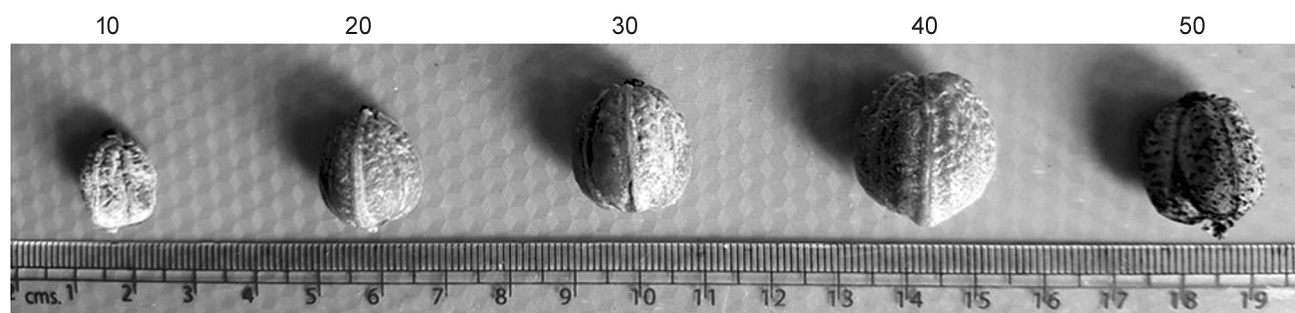


Fig 4 Developing fruits of castor variety 48-1

Oleic acid content decreased from 7.56% (20 DAF) to 6.42% (50 DAF). Linoleic acid content was found to be 54.70% at 20 DAF. However, a gradual decline was observed as seed development progressed and was found to be 7.79% at maturity stage (50 DAF).

Contrary to this, ricinoleic acid content was not observed till 20 DAF and it increased rapidly from 45.88% at 30 DAF to 83.01% at 40 DAF, whereas a slight decrease to 81.01% was observed at the final maturation stage (Table 1, Fig 5). Thus between 20 to 30 DAF, synthesis and accumulation of HFA was observed. Similar pattern of fatty acid accumulation in developing seeds of castor variety was reported in previous studies. In a study conducted by Chen *et al.* no ricinoleic acid accumulation was detected during early stages (12-19 DAF). It increased rapidly to 77% at 26 DAF and attained its maximum value (90%) at 40 DAF (Chen *et al.* 2007). In another study conducted by Chandrasekaran and Liu, it was found that no accumulation of ricinoleic acid was observed during initial days. It increased from 18% to 66% between 14 to 28 DAF. It attained its maximum percentage (79%) at 35 DAF and decreased to 59% during its maturation phase (63DAF) (Chandrasekaran and Liu 2013). Similar pattern of accumulation of different FAs was also reported in the study conducted by Xiaofeng *et al.* (Xiaofeng *et al.* 2016). Due to variation in activation of desaturase and FAH12 enzymes, such difference in pattern of oleic, linoleic and ricinoleic acid was observed. Current observation is also in accordance with fatty acid into other oil bodies (Norton and Harris 1975; Ichihara and Noda 1980; Rubel *et al.* 1972).

4.78% of total FAME was saturated FA (SFA), whereas

Table 2 FA analysis of mature seeds of castor variety 48-1.

SFA		MUFA		PUFA	RA
Palmitic acid	Stearic acid	Oleic acid	Linoleic acid	Ricinoleic acid	
2.42	2.36	6.42	7.79	81.01	
Total SFA	Total UFA	Oleic/ Linoleic	Oleic/ RA	SFA/UFA	
4.78	95.22	0.82	0.07	0.05	

*SFA saturated fatty acid; MUFA monounsaturated fatty acid; PUFA polyunsaturated fatty acid; RA ricinoleic acid; UFA unsaturated fatty acid

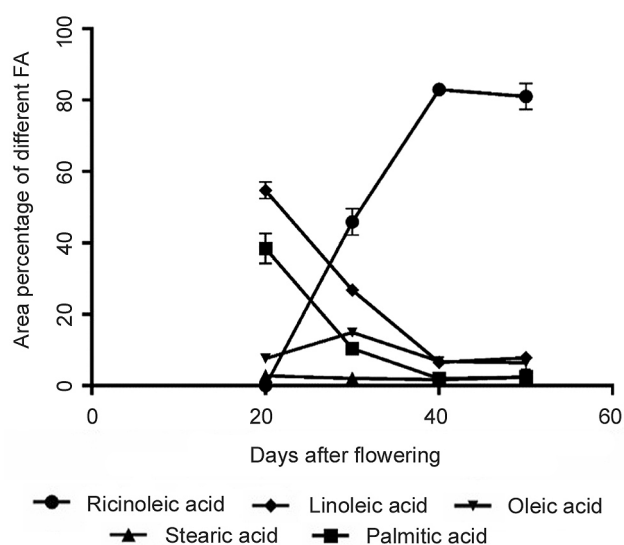


Fig 5 Shows area percentage of different FAs of developing seeds of 48-1 after GCMS analysis.

95.22% of total FAME was unsaturated FA (UFA) in mature seeds of castor variety (48-1). Ratio of oleic and ricinoleic acid (RA) was found to be 0.07 and ratio of SFA and UFA was found to be 0.05 (Table 2). It was comparable to previously reported genotypes of castor (Shah *et al.* 2017). Altogether, the present study on cloning of *FAH12* gene and fatty acid accumulation in seed development in castor provides insight into accumulation and partitioning of storage reserves in Indian castor variety 48-1.

Conclusion

There is a growth in worldwide demand of castor oil due to its low cost, easy availability and non-edible nature. The present study indicates the changes in FA composition especially accumulation of ricinoleic acid in the developing seeds of 48-1 variety of castor which is a widely grown variety of castor. Cloning of *FAH12* gene associated with ricinoleic acid production was undertaken in two distinct high yielding varieties of castor and our analysis observed no variation in the protein sequence of *FAH12*. The cloned gene can be employed for production of industrially important ricinoleic acids in agriculturally amenable species through transgenic approach or other lipogenic microorganism for biotechnological intervention.

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