Industrial Crops & Products 140 (2019) 111621

Contents lists available at ScienceDirect

Industrial Crops & Products

journal homepage: www.elsevier.com/locate/indcrop

Dynamics of metabolites and key regulatory proteins in the developing seeds of *Pongamia pinnata*, a potential biofuel tree species

Kambam Tamna Singha[a], Rachapudi Venkata Sreeharsha[a], Sureshbabu Mariboina[a], Attipalli Ramachandra Reddy[a,b,*]

[a] Dept. of Plant Sciences, School of Life Sciences, University of Hyderabad, Hyderabad, 500046, India
[b] Yogi Vemana University, Kadapa, Andhra Pradesh, India

ARTICLE INFO

Keywords:
Pongamia pinnata
Seed development
Metabolome
Proteome
Triacylglycerols
Oil body

ABSTRACT

The present study analyzed the dynamic changes in metabolites and key proteins during the seed development of *Pongamia pinnata* L. (Family: Fabaceae) with a particular focus on lipid biosynthesis and oil accumulation. The developing seeds were collected at four different stages: 120 (stage 1), 180 (stage 2), 240 (stage 3) and 300 (stage 4) days after flowering (DAF), representing S1, S2, S3 and S4 respectively. The analysis of seed pigments and mRNA expression patterns of key photosynthetic genes confirmed the photo-autotrophic behavior of *P. pinnata* seed during the initial stages of development. The metabolite profiling of developing *P. pinnata* seeds also revealed differentially expressed sugars, amino acids, free fatty acids and organic acids. Proteins related to development, energy metabolism, lipid accumulation as well as stress responses were documented through MALDI-TOF-MS/MS analysis. The structure and pattern of oil body accumulation at each stage of seed development were determined by electron and confocal microscopy of the cotyledonary sections. The thin layer chromatogram of *P. pinnata* oil revealed higher amount of Triacylglycerides and the fatty acid profile of extracted triacylglycerides showed a rapid increase in oleic acid (C18:1) at S3 and S4. The outcomes reveal new insights into the complex oleogenic metabolism during *P. pinnata* seed development at macro level.

1. Introduction

Plant oils have a potential to replace petroleum based fuels by acting as feedstock for the oleochemical and biofuel industries. Understanding the regulatory networks of seed oil biosynthesis has been a target of agronomists and metabolic engineers for decades in order to enhance the oil production. The current knowledge on regulation of seed development and oil accumulation is limited to food crops and other model plants. While, very limited reports are available for biofuel plants including *Pongamia pinnata, Jaropha curcus, Camelina sativa* (Mudalkar et al., 2014; Chaitanya et al., 2015; Kumar et al., 2017). In general, the seed development bears a complex regulatory network of metabolic and developmental machinery which include signaling elements associated with accumulation of storage compounds (Baud et al., 2008; Atabani et al., 2013). Further, the seed storage-compound synthesis overlays the developmental progression of embryogenesis and is to an extent governed by the metabolite and hormonal signals inside the embryo (Borisjuk et al., 2003). The proportional distribution of seed storage products into carbohydrates, oils and proteins will depend on the influx of metabolic pathways during the development and can vary between species (Schiltz et al., 2005; Ekman et al., 2008; Chaitanya et al., 2015). For oil development, carbon is delivered to fatty acid synthesis in seed plastids via glycolysis with hexose and triose as the predominant carbohydrates entering the plastid. Previous reports signified the role of sucrose import into embryo and its metabolism in the cytosol and plastids in the formation of starch and oil (Luthra et al., 1991; Eastmond and Rawsthorne, 2000). It is well established in several model legumes that the dynamic changes of hexose/sucrose ratios play a key role in the commencement of storage product synthesis as the seed development progressed. On the

Abbreviations: ACP, acyl carrier proteins; BSA, bovine serum albumin; DAF, days after flowering; DAGs, diacylglycerides; DGAT, acyl-coA: diglyceride acyltransferase; DTT, dithiothreitol; DW, dry weight; EGTA, ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; FAMEs, fatty acid methyl esters; FFA, Free Fatty acids; FW, fresh weight; NADGA, N-acetyl- D-glucosamine; PBS, phosphate buffer saline; PCA, Principle component analysis; PDAT, phospholipid:diacylglycerol acyltransferase; PL, Phospholipids; PMSF, phenylmethylsulfonyl fluoride; TAGs, triacylglycerides; TCA, tricarboxylic acid; TLC, thin layer chromatography

* Corresponding author.
E-mail address: arrsl@uohyd.ernet.in (A.R. Reddy).

https://doi.org/10.1016/j.indcrop.2019.111621
Received 10 December 2018; Received in revised form 15 June 2019; Accepted 27 July 2019

other hand, the primary metabolism inside the seed which includes major pathways of glycolysis and TCA link the biosynthesis of carbohydrates, amino acids and fatty acids which also provide energy balance inside the developing seeds (Rolletschek et al., 2005; Schwender et al., 2015). Hence, the precise understanding of changes in these regulatory metabolites will provide insights into the seed development and oil composition in non-model legume tree species, specifically *P. pinnata*.

The oilseeds can be photo-autotrophic or heterotrophic depending on the presence of chloroplast and light plays an important role in regulating the green oil seed development. It was reported in certain crop plants that the seed carries out photosynthesis during embryogenesis which provides energy for FA biosynthesis and also helps in refixing the respiratory CO_2 release (Schwender et al., 2004). Though the absence of seed photosynthesis will not affect the overall FA biosynthesis, it will be considered as an important factor in promoting carbon storage, energy flux and lipid biosynthesis in oilseeds. The dynamic shift in the expression patterns of photosystem related proteins along with FA biosynthesis enzymes during the seed development of *Arabidopsis* and *Glycine max* support the active role of seed photosynthesis. Hence, studying the pigments and associated protein expression patterns in large seeds with long developmental cycles will provide more insights into the role of seed photosystems in synthesizing storage compounds.

The cultivation of *P. pinnata* is suitable in diverse tropical and subtropical environments. The oil content of *P. pinnata* seeds ranges from 35 to 40% of seed dry weight and 55–65 % of the total lipid has been reported as oleic acid which is the ideal fatty acid for biodiesel production (Sreeharsha et al., 2016; Xiong et al., 2018). Due to the accumulation of high levels of poly unsaturated fatty acids in the *P. pinnata* seed oil, it is considered as a potential biofuel feedstock with an optimized efficiency to use as a biodiesel for diesel engines (Singh et al., 2018; Jain et al., 2018). Moreover, not only the *P. pinnata* oil, but also the seed waste after oil extraction have recently been used to produce bio-ethanol (Muktham et al., 2016). The biochemical characteristics and protein profiling during seed development has been investigated in *P. pinnata* (Kesari and Rangan, 2011; Pavithra et al., 2014). The proximal chemical composition of mature *P. pinnata* seed along with fatty acid composition were also reported earlier (Bala et al., 2011; Sharma et al., 2011; Pavithra et al., 2012). However, in depth analysis of metabolite and proteomic profiles throughout the lengthy developmental period of *P. pinnata* seed have not yet been reported. It is crucial to study the metabolome and proteome of the developing seed to understand the complex association between various metabolites and FA biosynthesis to oil body formation. The objective of the current study is to decipher the metabolome and identification of key regulatory proteins in different developing stages of *P. pinnata* seed and their role in FA accumulation. In addition, the photosynthetic behavior of the *P. pinnata* seed also have been identified which will help in understanding the carbon influx to FA biosynthesis. Our results will deepen the knowledge on metabolic regulation of *P. pinnata* seed development and oil accumulation which is important for further metabolic engineering of this potential biofuel feedstock for different industrial and agricultural applications.

2. Materials and methods

2.1. Plant material and study site description

Ten year old *P. pinnata* plantation, established in the experimental farm of Tree Oils India Limited (TOIL) Zaheerabad, Medak district, Telangana state, India (latitude 17°36′; longitude 77°31′E; 622 m MSL) was selected for the present study. The study site has a tropical, hot-steppe agroclimate with the summer months between March to May having maximum temperature up to ~42 °C and an average temperature of 22–23 °C in the winter months of September to February, monsoon starts during June to October with average rainfall ranging from 700 to 1500 mm. Plants were maintained under natural photoperiod with uniform 2 m spacing between as well as within the rows. The recorded range of light intensity in the region on a normal sunny day ranged between 1200–2000 μmol/m^2/s during 10.00–14.00 h solar time. The plants were regularly watered during hot summer months with alternate watering during winter months and no watering during monsoon season. Nitrogen was provided by applying cow dung mixed with vermicompost as farmyard manure at the rate of 12 kg/year twice in equal splits during growth. Trees had a circular canopy with 15–20 tertiary branches. The flowering and fruiting seasonal events were recorded periodically for further experimental analyses. Flowering initiated in the month of March with visible inflorescence which continued until April. Natural pollination resulted in formation of pods with first visible appearance recorded at 80–90 days after the flowering started. Seed development and maturation was completed by nearly 300 days (~10 months) from the first visible appearance of flowers. The appearance of flower buds in each tertiary branches was considered as 0 days after flowering (DAF). Individual flowers were tagged randomly and the development of pod and seed was monitored regularly in the tagged trees. The tagged pods were collected at 120, 180, 240 and 300 DAF to study the seed morphology and stored at –80 °C for further biochemical and molecular analyses. The pod contained very tiny seeds with negligible dry mass during first initial stages of development (30 to 100 DAF). Therefore the experiments were carried out when the seeds reached 120 DAF till maturation. Seeds inside 120, 180, 240 and 300 DAF old pods were considered as stage1 (S1), stage2 (S2), stage3 (S3) and stage4 (S4) respectively. The study was performed for two successive flowering seasons of *P. pinnata* (for two consecutive years, March 2016 to February 2017 and March 2017 to February 2018).

2.2. Seed morphology and biochemical analysis

Fresh seeds were initially weighed and dried for 12 h at 65–70 °C in a ventilated hot air oven. The weight of seed coat and cotyledon were measured gravimetrically and percentage was calculated using the formula: (weight of cotyledon / weight of total seed) ×100; (weight of seed coat / weight of total seed) ×100, respectively. The seed dry mass was recorded and seed moisture content (%) was calculated according to the formula: (FW–DW/FW) ×100.

Chlorophyll content in the seed samples was estimated according to the method reported by Hiscox and Israelstam (1979). Fresh seeds (100 mg) were ground and refluxed for 1 h in 10 mL of DMSO, extracted liquid was collected and absorbance was measured at 645 and 663 nm using a UV–vis spectrophotometer (Eppendorf, Germany) and chlorophyll contents were calculated according to Arnon (1949) using the formula: chlorophyll *a* (g/L) = 0.0127 × A_{663}– 0.00269 × A_{645}; chlorophyll *b* (g/L) = 0.0229 × A_{645} – 0.00468 × A_{663}; the results were expressed as mg/g FW (where A_{663}, A_{645} represents the absorbance measured at 663 and 645 nm respectively).

Total carbohydrates and starch content in the seeds were estimated according to Anthrone method as described by Hedge and Hofreiter (1962), where 100 mg of fresh seed tissue was refluxed with 5 mL of 2.5 N HCl followed by neutralizing with NaOH. The mixture was centrifuged for 10 min at 6000 × g. An aliquot of supernatant (1 mL) was taken and added with 4 mL of Anthrone reagent. After heating at 80–90 °C for 8 min in a water bath, the solution was allowed to cool rapidly and the absorbance was measured at 630 nm. Spectrometric readings were quantified using glucose standards and represented in mg/g FW of the seed.

For starch estimation, 100 mg of fresh seed tissue was first ground with hot 80% ethanol. The supernatant of this mixture was removed after centrifugation. The residue was washed repeatedly with 80% hot ethanol till the washings did not give green color with Anthrone reagent. The dried residue was mixed with 5 mL of water and 6.5 mL of 52% perchloric acid. The mixture was kept at 0 °C for 20 min and

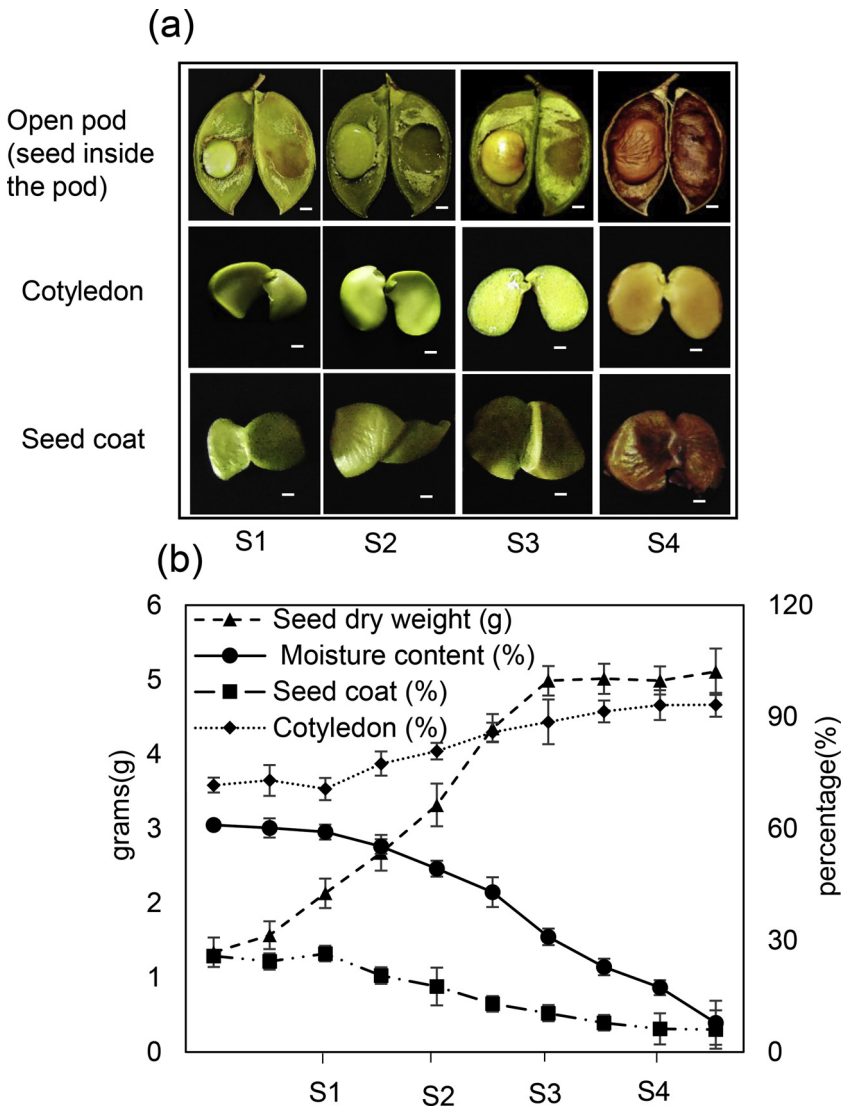


Fig. 1. Position of seed inside the pod and cotyledons separated from seed coat at each developmental stage **(a)**. Morphological and biochemical analysis of developing *P. pinnata* seeds. Changes in seed dry weight, moisture content, % cotyledon and seed coat throughout development **(b)**. Values are mean ± SD, (n = 40); the scale is presented by white bar with the length of 0.3 cm.

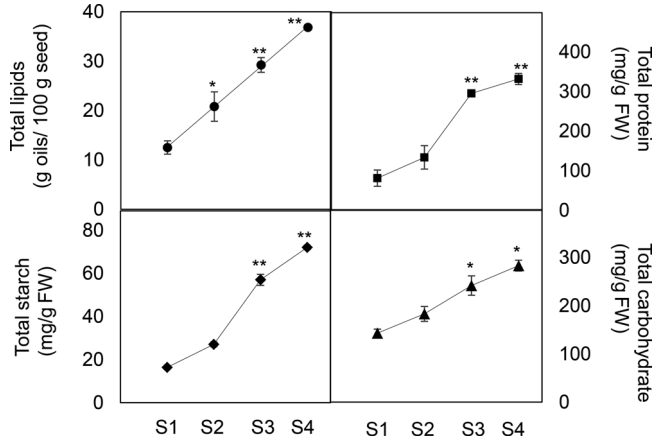


Fig. 2. Pattern of increasing storage products (lipid, protein, total carbohydrate and starch) with development. Values are mean ± SD (n = 3). Data are given as means ± SD; (n = 3). *, ** represents significant differences within the stages analyzed by one-way ANOVA, $P < 0.05$; $P < 0.01$ respectively.

centrifuged at 8000 × g for 5 min, supernatant was collected and the extraction was repeated twice. The collected supernatant (1 mL) was mixed with 4 mL of Anthrone reagent and starch was estimated by the method used in estimation of carbohydrates.

For total protein quantification, fresh tissue (100 mg) was ground with liquid nitrogen and mixed with 2 mL of protein extraction buffer consisting of 25 mM Tris − HCl (pH 7.2), 15 mM $MgCl_2$, 15 mM EGTA, 75 mM NaCl, 2 mM DTT, 1 mM NaF, 1 mM PMSF with 0.1% Nonidet, followed by centrifugation at 12,000 × g for 5 min at 4 °C. Supernatant was collected and protein was estimated according to Bradford (1976). Quantification of total protein was achieved by using BSA as the protein standard and represented as mg/g FW.

Total lipids were extracted by using the method described by Bligh and Dyer (1959) with slight modifications. Fresh seeds were ground with $CHCl_3$:MeOH:H_2O (2:2:1.8) v/v and the mixture was centrifuged at 10,000 × g for 15 min. The lower $CHCl_3$ layer was collected and the extraction was repeated three times. The chloroform was separated by a Rotary evaporator (Heidolph, Germany) and total lipid was estimated gravimetrically and represented as g oils/100 *g* seed. All of the chemical reagents used for biochemical analysis were purchased from Sigma Aldrich, USA.

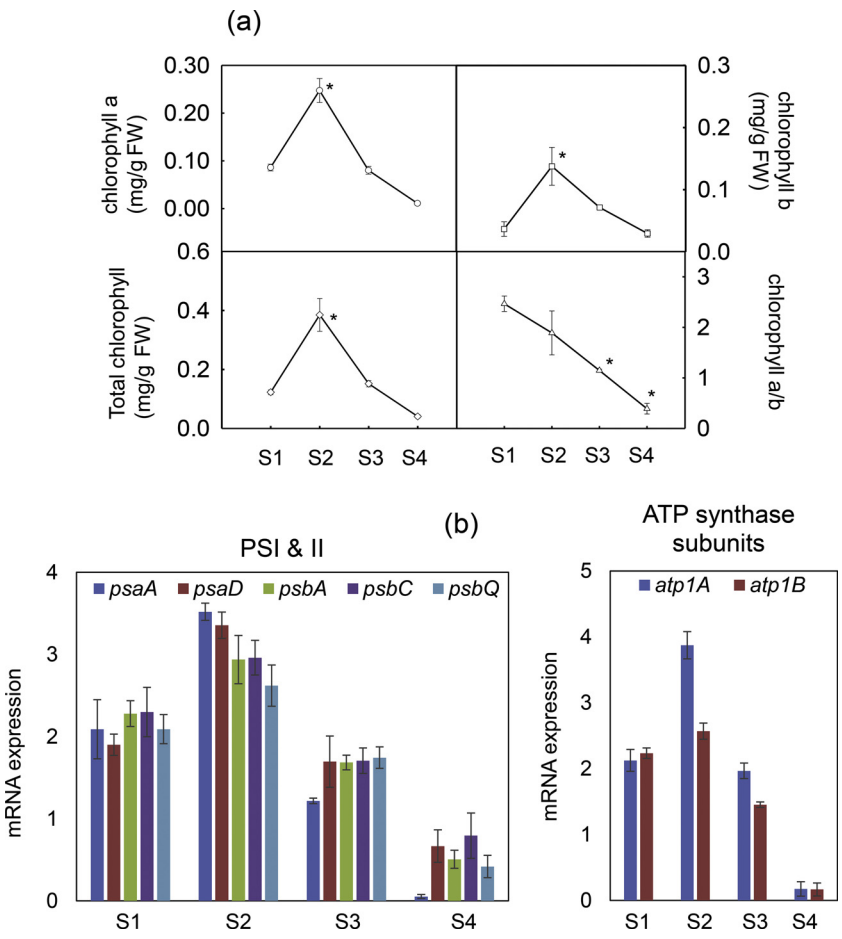


Fig. 3. Changes in the chlorophyll *a*, b, total chlorophyll and chlorophyll *a*/b **(a).** Expression patterns of key regulatory genes represented in fold change related to photosynthesis **(b).** Fold change > 2 is considered as upregulation. Values are mean ± SD (n = 3). Data are given as means ± SD; (n = 3). *, ** represents significant differences within the stages analyzed by one-way ANOVA, P < 0.05; P < 0.01 respectively.

2.3. Gene expression analysis by real time PCR

Total RNA was isolated from developing fresh seeds using Plant total RNA extraction kit by following manufacturer's instructions (Sigma Aldrich, USA). Primers for the required genes were designed from available *P. pinnata* transcriptome sequences (Sreeharsha et al., 2016). First strand cDNA was synthesized with 1 µg of RNA using Revert aid first strand cDNA synthesis kit (Thermo-Fischer Scientific, USA). Expression analysis of selected genes were carried out on Realplex thermal cycler (Eppendorf, Germany) using SYBR FAST qPCR universal master mix (2X) (KAPA Biosystems, USA) with 50 ng of cDNA as template and following the program: 2 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 30 s at 55 °C annealing temperature and 20 s at 72 °C, followed by the dissociation (melting) curve. The mRNA expression level was calculated according to the $2^{-\Delta\Delta Ct}$ formula (Livak and Schmittgen, 2001). For the internal control, 18 s ribosomal RNA gene was used.

2.4. Metabolite profiling

P. pinnata seeds at different developing stages were ground into fine powder in liquid nitrogen. 100 mg of the seed powder was homogenized with 1.4 mL precooled methanol by vortexing for 10 s and 60 µL of ribitol (0.2 mg/mL) was added to the mixture as internal standard followed by 10 s of vortexing. This solution was ultra-sonicated for 10 min, followed by centrifugation at 11,000 × g for 10 min. The supernatant was transferred to a new tube and mixed with 750 µL of precooled chloroform and 1.5 mL of precooled water. The mixture was then centrifuged at 2200 × g for 15 min. 150 µL of extraction solution from upper phase was dried under vacuum and stored at –80 °C until derivatization. The extract was methoxyaminated, silylated and the dried extract was dissolved in 20 µL of methoxy amine hydrochloride pyridine solution (40 mg/mL) which was incubated at 30 °C with vigorous shaking for 90 min. Then 80 µL of N-methyl-N-(trimethylsilyl) trifluoro-acetamide-solution was added to the sample followed by 30 min incubation at 37 °C with vigorous shaking. The derivatized sample was centrifuged at 20,000 × g for 8 min. The supernatant was then transferred to vials for measurement. Samples were measured with gas chromatography coupled with LECO Pegasus R 4D GC GC–TOF spectrometry (GC-TOF-MS) (Agilent 6890, USA). Each sample was injected under both split less and split 25 times mode for better quantification of candidates with a wide capacity range. Candidates were manually annotated by comparing their retention times (RTs) and mass spectra to those of standards in GMD database (Kopka et al., 2005) with a minimum match factor of 700. The peak areas of the same metabolite with different derived groups were merged and normalized to internal standard of ribitol. The concentration of the metabolites was calculated with respect to the known concentration of ribitol and represented as µg/g FW. The differential analysis at developing stages were analyzed using MetaboAnalyst Version 4.0 [Software] (available from http://www.metaboanalyst.ca/MetaboAnalyst/faces/home.xhtml).

2.5. 2-D protein profiling and identification using MALDI TOF MS/MS analysis

Gel based 2D proteome was carried out according to the method described by Sengupta et al. (2011). Protein extracted earlier (200 µL) was precipitated using 800 µL of 0.1% ammonium acetate in methanol and kept at –20 °C for 8–12 h. The mixture was centrifuged at 10,000 × g for 10 min at 4 °C and the precipitate was washed with fresh methanol for 3 times followed by 2 times wash with acetone. The

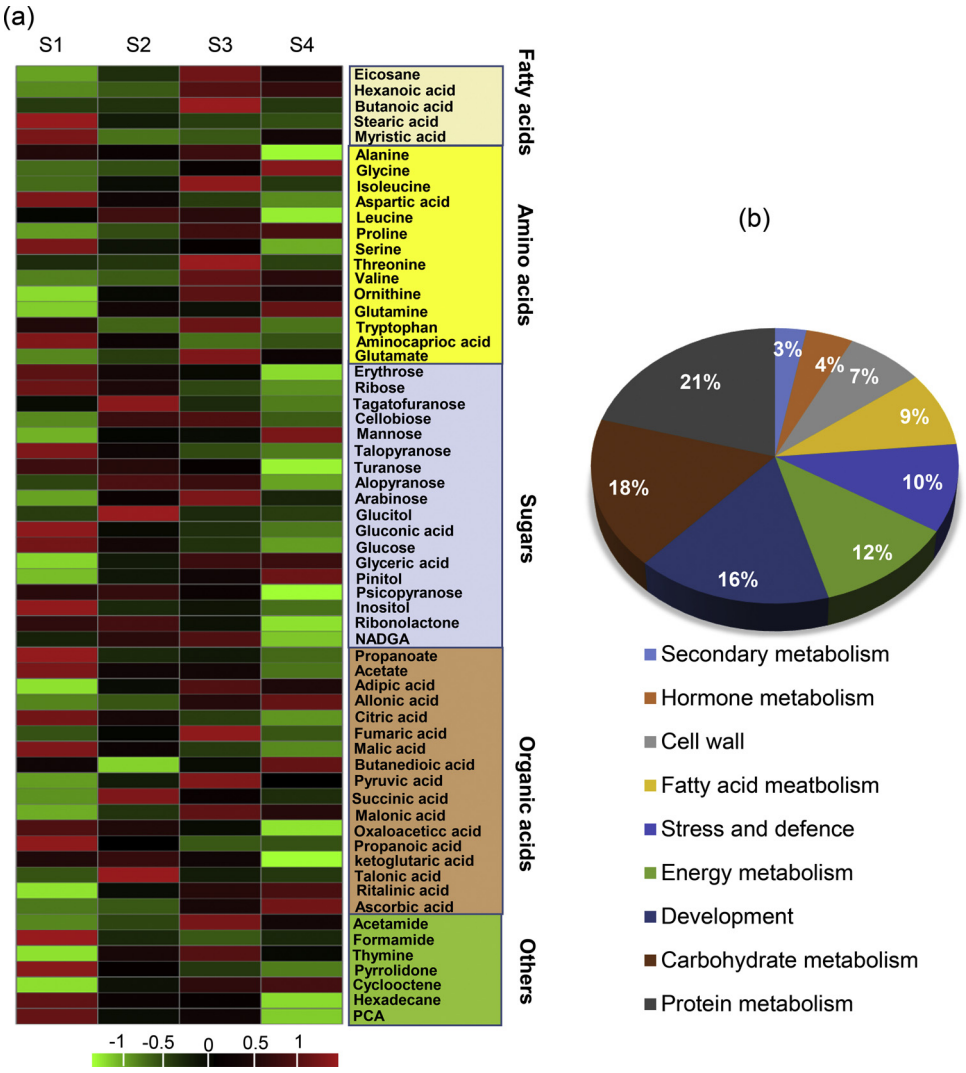


Fig. 4. Heat map based on the fold change in concentration of metabolites (μg/g FW) at S1, S2, S3 and S4 **(a)**. Pie chart representing functional classification of metabolites detected in all the developing stages **(b)**.

isolated protein sample (900 μg) was then solubilized in rehydration buffer followed by isoelectric focusing using pH 4–7 isoelectric focusing strips (18 cm, 4–7 pH linear gradient; GE Healthcare, USA) for 12 h at 50 V for first separation. Rehydration and focusing was carried out in Ettan IPGphor II (GE Healthcare, USA) at 20 °C, using the following program: 30 min at 500 V, 3 h to increase from 500 to 10,000 V and 6 h at 10,000 V (a total of 60,000 Vh). The IEF strips were then placed over 12% acrylamide gel for the second separation through SDS PAGE at 300–500 V using standard protocol. After staining, the gels were scanned and the gel image obtained was analyzed though Image Master 2-D Platinum version 6 image analysis software (GE Healthcare, USA).

For the identification of proteins, MALDI TOF MS/MS was carried out through in-gel trypsin digestion and database searches (PMF and MS/MS) using MASCOT program [Software] (available from http://www.matrixscience.com). The similarity search for mass values was done with existing digests and sequence information from NCBInr and Swiss Prot database. The taxonomic category was set to Viridiplantae (green plants). The other search parameters were: fixed modification of carbamidomethyl (C), variable modification of oxidation (M), enzyme trypsin, peptide charge of 1+ and monoisotopic. Out of top ten most significantly identified proteins, results having highest score, peptide match, and similarity of molecular weights were considered.

2.6. Microscopic studies of P. Pinnata seeds

Fresh seeds were made into thin sections and fixed in 2.5% glutaraldehyde for 8–12 h followed by re-fixing again in 0.2% osmium tetra oxide for 2–3 h. After washing with 0.1% PBS and series of ethanol dehydration, thin sections were embedded in epoxy resin (Araldite 502). Ultrathin sections were made using ultra microtome (LEICA EM UC6, Germany) and observed under Scanning electron microscope (TESCAN S8000, Czech Republic). For TEM, ultrathin sections were fixed in grids and stained with uranyl acetate and lead citrate which was observed later in TEM (FEI Model, Tecnai G2S Twin, Spain) (200 kV). Nile red staining was performed by following the method reported by Greenspan et al. (1985) with slight modifications. Prefixed thin sections of the fresh tissues were infiltrated with the working concentration of 2 μg/mL Nile red solution in 0.1% HEPES buffer for 1–2 h under dark. After washing for 4–5 times with HEPES buffer, the sections were visualized under confocal microscope (LIECA TCS SP2 AOBS, Heidelberg, Germany) under excitation of 488 nm and emission range of 530–650 nm. All images were obtained with 10% laser power.

2.7. Thin layer chromatography for lipid profiles

Total lipid (5 mg) extracted from all the samples were dissolved in chloroform and spotted on the TLC Silica gel 60 F254 (Merck,

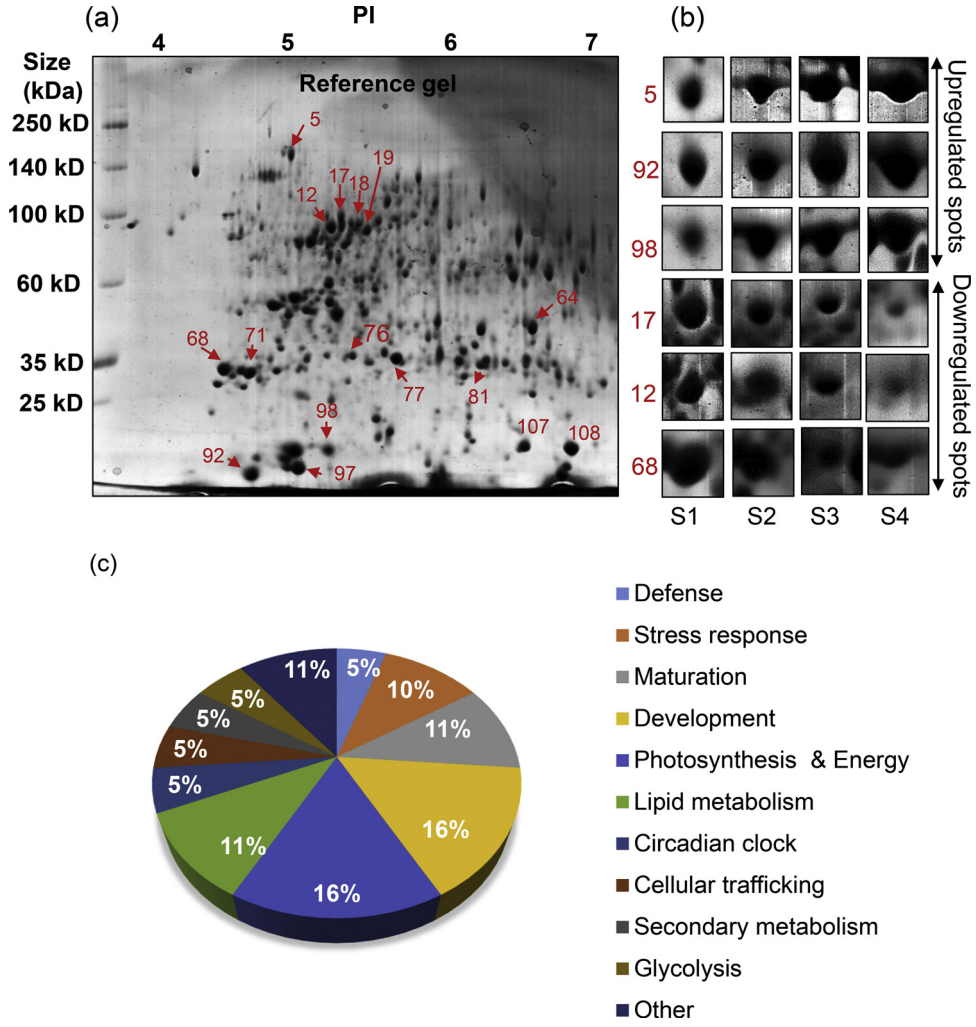


Fig. 5. 2D reference gel image where the identified spots were indicated by spot ids in red mark **(a)**, some of the upregulated and downregulated spots were presented as separated images at each stage **(b)**. Functional classification of identified proteins in pie chart **(c).**

Germany). The separation was carried out with hexane and ethyl acetate (9:1 v/v) as the mobile phase and silica gel bonded with aluminum sheet as the stationary phase. The TLC plates were air dried and exposed to iodine vapor. Iodine stained spots were compared with the standards of phospholipids, DAGs and TAGs separated in TLC plates with the same mobile phase.

The TAGs from the multiple TLC plates were scrapped carefully and extracted using chloroform. Further, chloroform was separated from the extracted TAGs with the help of rotary evaporator (Heidolph, Germany). The fatty acid profiling and quantification was carried by following the methods reported by Coetzee et al. (2008) and Sun et al. (2017) with slight modifications. FAMEs were prepared by refluxing 50 mg of the TAGs extracted with 5% H_2SO_4 in methanol (w/v) for 6 h on a hot plate. Prepared esters were analyzed through gas chromatography (GC-TOF-MS) (Agilent 6890, USA) with DB225 column (inner diameter = 0.25 mm, length = 37 m, thickness = 0.25 µm; Agilent, USA). The injector and flame ionization detectors were set at 250 °C and 270 °C, respectively. The oven temperature was set at 160 °C for 2 min and then increased to 230 °C at a rate of 5 °C/min. Nitrogen was used as the carrier gas at a flow rate of 1 mL/min. Fatty acids were confirmed by comparing with standard FAMEs mix (C14 – C22, Supelco, Sigma Aldrich, USA) analyzed at different concentrations. Quantification of fatty acids was carried out using area normalization method with percentage area of each peak corresponding to the identified fatty acid.

2.8. Statistical analysis

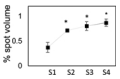
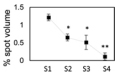
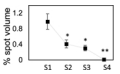
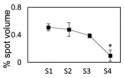
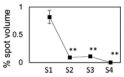
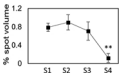
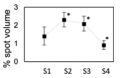
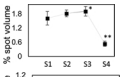
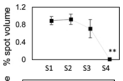
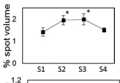
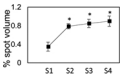
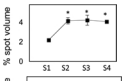
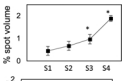
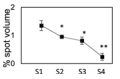
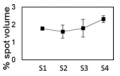
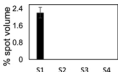
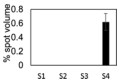
Each seed collected at various stages was considered as one unit (n = 40), where n is the number of seeds for the verification of difference in its morphological and physiological measurements. All the experiments were carried out in triplicates for the developing stages. PCA and heat map analysis was carried out for metabolome data using MetaboAnalyst online tool version 4.0 [Software] (available from http://www.metaboanalyst.ca/MetaboAnalyst/faces/home.xhtml). The mean values were compared and analysis of significance ($P < 0.05$) was determined by student's *t*-test and one way ANOVA using Sigma plot 11.0.

3. Results

3.1. Seed morphological characteristics and storage biochemistry during development

A single pod of *P. pinnata* generally houses either one or two seeds with 3–4 seeds occasionally/rarely observed. All the four different stages of *P. pinnata* seed with developmental progressing of pod, cotyledon and seed coat are shown in Fig. 1. The young seeds are green in color initially, which turned completely brown upon maturation (Fig. 1a). There was a gradual decrease in the seed moisture content from S1 to S4 whereas, the seed dry weight increased till S3 and got stabilized by maturation (Fig. 1b). Seed coat percentage decreased

Table 1
The list of protein spots identified with their % spot volume and MASCOT search results at four stages of development. Values are mean ± S.D, (n = 3), level of significant difference was analyzed by one-way-ANOVA with reference to S1. Note - *, P < 0.05; **, P < 0.01.

Sp. ID	Spot intensities	Protein identified	MW observed in gel	MW from literature	Known Function	Organism related to	Accession no.	MS/MS Score	Peptide sequences matched
5		Acetyl coA carboxylase	200kD	226 kD	Fatty acid biosynthesis	*Sesamum indicum*	XP011083400.1	98	DEGRGPMR EDAFFQAVTEVACAQK ASQLLEQTK(Total matched peptides-29)
12		Midasin	95kD	116kD	Female gamete and seed development	*Arabidopsis thaliana*	MDN1_ARANTH	94	WMYLESIFVGSDDIRHQLPAEAK KSFEMVSLAVSQK (Total matched peptides - 34)
17		AP2 Complex subunit alpha 1	91kD	112kD	Vesicular transport and development	*Arabidopsis thaliana*	AP2A1_ARATH	94	FAPDLSWYVDVILQLIDK LVLFMGWK (Total matched peptides - 12)
18		Phosphoenol pyruvate carboxykinase	95kD	73.7kD	Photosynthesis carbon fixation and energy metabolism	*Zea maize*	PCKA_MAIZE	64	GLFGVMHYLMPK AQTIDELHSL QR(Total matched peptides - 12)
19		Probable disease resistance protein	100kD	104.5kD	Defence and disease resistance	*Arabidopsis thaliana*	PX24L_ARATH	108	VLGGLLAAKYTLHDWKR ERKDEIQNMK(Total matched peptides - 31)
64		Glyceraldehyde-3-phosphate dehydrogenase	45kD	36.5kD	Glycolysis and energy metabolism	*Oryza sativa*	G3PC1_ORYSJ	278	AASFNIIPSSTGAAK LKGIIGYVEEDLVSTOFVGD SR(Total matched peptides - 7)
68		ATP synthase subunit beta	35kD	53.3kD	Photosynthesis electron transport and energy	*Brimeura amethystina*	ATPB_BRIAM	68	AVAMSATDGLTR EGNDLYMEKESGVINEK(Total matched peptides - 8)
71		Ent-copalyl diphosphate synthase 1	35kD	99kD	Secondary metabolism	*Oryza sativa*	CPS1_ORYSJ	88	ARNFSYEFLR EIEQNMDYVNR(Total matched peptides - 8)
76		1-cys peroxiredoxin B	35kD	24.4kD	Vitamins	*Oryza sativa*	REHYB_ORYSI	67	DTAGGELPNR VVIPPGVSDEEAK(Total matched peptides - 8)
77		GTP Binding protein SARB1	30 kD	22.02kD	Vesicular transport of storage bodies	*Arabidopsis thaliana*	SAR1B_ARATH	74	VWKDYYAK YHLGLTNFTTGKG (Total matched peptides - 9)
81		Heat stress transcription factor c-1b	30kD	27.4 kD	Abiotic stress regulator	*Oryza sativa*	HFC1B_ORYSJ	97	NFASFVR QLNTYGFR(Total matched peptides - 17)
92		Lipid transfer like protein VAS	10kD	17kD	Lipid transfer metabolism	*Arabidopsis thaliana*	VAS_ARATH	90	WSSQAER EVPQVCCNPLK(Total matched peptides - 8)
98		Integrin linked protein kinase 1	25kD	52.4kD	Signal transduction (Plant defence response)	*Cucurbita maxima*	XP_022970695.1	101	TNPGSRSFSK WGSTPLADAIYYK(Total matched peptides - 6)
107		Class I heat shock protein	10.5kD	17.7kD	Heat shock and stress	*Solanum pennelli*	XP_015078018.1	88	FRLPENAK VEVEEDRVLQISGER(Total matched peptides - 7)
108		Class I heat shock protein	10.3kD	17.8kD	Heat shock and stress	*Solanum lycopersicum*	HSP11_SOLLC	66	FRLPENAK VEVEEDRVLQISGER(Total matched peptides - 6)
97		Protein ELF 4 Like	15kD	14.07kD	Circadian clock	*Arabidopsis thaliana*	EF4LT_ARATH	58	DGDTTTTTTGSS NVGLINEINISQVMEIYSDLSLNFAK (Total matched peptides - 2)
N17		Allergen LEN	90kD	47.7kD	Allergen protein in non-edible plants	*Lens culineris*	CAD87730.1	112	IFENLQNYR KSVSSESESEPFNLR(Total matched peptides - 8)

gradually while the percentage of cotyledon increased as the seed matured (Fig. 1b). Accumulation of all the storage products including starch, protein and lipids increased gradually, reaching the highest levels during S4 (Fig. 2). Protein and lipid contents were similar at younger stages (S1–S2), while the lipid content reached up to 37% of total 100 g of seeds (~36.8 g oil/100 g seed), whereas protein content reached to 33% (~331 mg/g FW) by the end of the development. Similarly, the total carbohydrate content in the mature seeds (S4) was ~298 mg/g FW (1.9 folds higher when compared to S1), out of which the content of starch was only ~80 mg/g FW (4.3 folds higher when compared to S1) (Fig. 2). Chlorophyll *a*, chlorophyll b and total chlorophyll concentrations showed a distinct pattern when the seeds were green where their contents increased from S1 to S2 with a further decrease thereon at S3 and S4, during which the seeds started to develop brown color (Fig. 3a). Similarly, chlorophyll *a*/b ratio also consistently decreased with the progression of seed development (Fig. 3a). Relative gene expression of some of the key photosynthetic genes were analyzed at different stages of development by considering S1 as reference (Fig. 3b). All genes related to Photosystem I and II major subunits (*psaA, psaD psbA, psbC, psbQ*) and ATP synthase subunit (*atp1A* and *atp1B*) were upregulated at S2 which subsequently decreased from S3 to S4 (Fig. 3b).

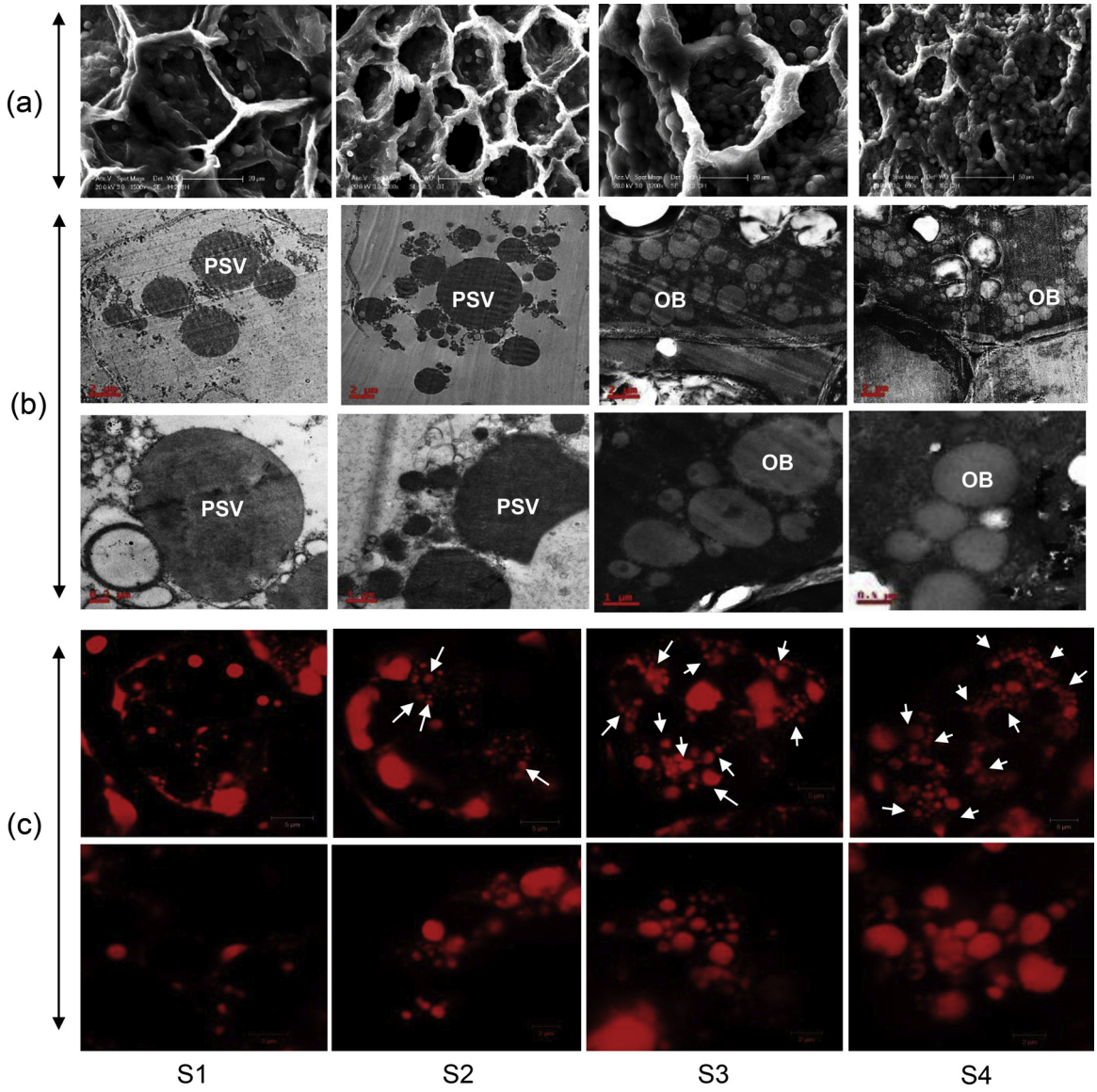


Fig. 6. *P. pinnata* seed cross section under SEM **(a)** (20–50 μ) and TEM **(b)** (2–0.2 μ). Nile red staining of *P. pinnata* seed sections observed under confocal microscope, oil bodies are marked by white arrows **(c)** (5–2 μ). PSV- protein storage vacuole; OB- oil body.

3.2. Complete metabolome analysis of developmental stages of P. Pinnata seed

In all the four stages, 61 metabolites were identified with the help of GCMS analysis (Supplementary data 1). Metabolites were classified based on their chemical structure (Fig. 4a) and biological functions (Fig. 4b). The variation among distinctive profile of metabolites was observed through PCA analysis where there was overall 71.7% separation among the developing stages (Supplementary data 3). Separation or variation of seed metabolites among S2 and S3 was less when compared to S1 and S4. Majority of the metabolites detected were functionally related to protein metabolism (21%), carbohydrate metabolism (18%) and development (16%). Rest of the metabolites belonged to energy (12%), stress and defense (10%), fatty acids (9%), cell wall formation (7%), hormonal responses (4%) and secondary metabolism (3%) (Fig. 4b). The dynamics of the metabolites were determined by their concentration during the 4 developing stages analyzed through heat map using Pearson's test in MetaboAnalyst online software. Metabolites were classified as fatty acids, amino acids, sugars and organic acids depending on their respective chemical characteristics (Fig. 4a). Among the sugars, ribose, glucose, erythrose, gluconic acid, inositol and ribonolactone had higher concentrations at S1 which gradually decreased with the maturation. Glyceric acid, pinitol and mannose were abundant at S4 whereas sugars like cellobiose, arabinose and NADGA were higher during S1 to S2. In contrast to the sugars, most of the amino acids and organic acids were abundant during S3 and S4 (Fig. 4a). Leucine, threonine, alanine, tryptophan, valine, glutamate and some organic acids including keto-glutaric acid, fumaric acid, succinic acid, pyruvic acid and malonic acid were abundant in S3 and S4. The concentrations of glycine, proline and glutamine were higher in S4. Similarly, adipic acid and allonic acids were higher during S3 and S4 respectively (Fig. 4a). In contrast, few organic acids including propionic acid, acetate, malic acid, citric acid, oxalic acid and certain amino acid such as aspartic acid were more at S1, which gradually decreased to the lowest levels at S4. Fatty acids which were abundant in S1 were stearic acid and myristic acid, hexanoic acid was abundant in S3, while others including butanoic acid, and eicosane were more in S4 (Fig. 4a).

3.3. 2D- proteomic profile of developing P. Pinnata seed

The seed protein expression patterns were quantified at different

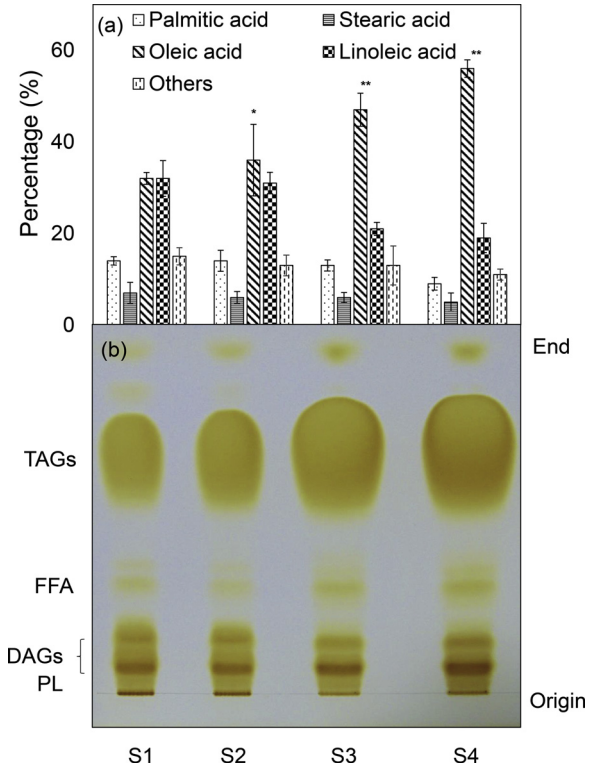


Fig. 7. Percentage of fatty acids accumulated in the TAGs at different developing stages **(a)**. Total lipid profiling through TLC **(b)**. Values are mean ± SD (n = 3). *, ** represents significance difference (P < 0.05, P < 0.01) between the fatty acid content with respect to 120 DAA, analyzed by *t*-test.

developing stages of *P. pinnata* seed. More than 300 spots were reproducibly detected in all the gels with Image Master 2D Platinum software and 125 spots matched in all the four stages (Fig. 5a). Based on the percent spot volume in the S1 stage, we have determined the significantly upregulated, unchanged or downregulated spots throughout developing stages. Some of the spots were selected and successfully identified using MALDI MSMS (Table 1). Rest of the spots which did not give any significant match in the MASCOT search were not considered. All of the differentially regulated spots were represented as gel picture in the supplementary data 2, and the changes in some of the spots among the different stages were represented along with the reference gel in Fig. 5a,b. The spot distribution and intensity at each stage were analyzed through PCA analysis which illustrated the variation in the form of percentage separation (Supplementary data 4). There was overall 64.4% separation among the stages and the graph clearly showed the protein profile of S4 was different compared to the younger stages (120–240 DAF). The profiles of S2 and S3 were more similar with least separation (Supplementary data 4). The functions of the identified spots were classified based on the available literature (Fig. 5c). The upregulated spots from S1 to S4 were identified as Accase, lipid transfer like protein VAS, ATP synthase subunit β and GTP binding protein SAR1b. The unchanged spots were identified as PEP carboxykinase and G3PD. Two of the downregulated spots included Midasin and ap2 complex subunit alpha1 (Table 1). The protein spot identified as ELF 4 was observed only in S1 whereas, Allergen Len was observed only in S4 (Table 1).

3.4. Visualization of storage granules and oil bodies in developing P. Pinnata seeds

The surface morphology of *P. pinnata* cotyledonary cross sections analyzed by SEM at various stages showed an increase in storage granules from S1 to S4 (Fig. 6a). Further the biochemical nature of these storage granules were studied through TEM analysis and identified large protein vacuoles at S1 which further increased during S2. The appearance of a large amount of oil body accumulation was observed in S3 and S4, where the lipid droplets were located at cellular periphery (Fig. 6b). For more clarification of the accumulation of oil bodies in developing stages, Nile red fluorescent staining was performed which specifically stains only the lipid containing substances. Oil body accumulation was very low at S1 and S2, but showed rapid increase at S3 to S4 (Fig. 6b, c). Moreover, the oil bodies were localized near the periphery of the cell with sizes varying from 1 to 2 μ while the protein storage vacuoles have unusually large diameter (~2–5 μ) and situated mostly in the central region (Fig. 6).

3.5. Fatty acid profiling of the TAGs

The separation of phospholipids, DAGs and TAGs during developing stages were compared by the intensities on the TLC plates which have been loaded with equal quantity and separated with the same mobile phase. There was a visible change in the TAGs accumulation where it progressively increased as the seeds reached maturity (Fig. 7b). The fatty acid profile of the accumulated TAGs was examined with the help of GCMS analysis and compared with the FAMEs standards, based on which the percentage was also determined. The major fatty acids during initial stages of development (S1 and S2) were oleic and linoleic acid which were present in equal amounts (Fig. 7a). In later stages of development, oleic acid gradually increased with a significant ($P < 0.05$) decrease in linoleic acid (Fig. 7a). Furthermore, palmitic acid also decreased gradually from S1 to S4 (Fig. 7a) while there was no change in stearic acid content in all the stages.

4. Discussion

The basic seed morphology and biochemistry of developing *P. pinnata* seed share certain similar developmental regulatory processes with other legumes where, the pods were nearly flat in younger stages with a tiny developing seed inside and the pod thickness increased as the seed developed, finally filling the pod (Wright and Lenssen, 2013). The duration of seed development in *P. pinnata* is a prolonged process making it complex to understand the seed filling and oil accumulation patterns in this imminent biofuel tree species. Thus, knowledge on physiological and molecular dynamics of this lengthy seed development will boost the attempts to improve the seed productivity towards potential biofuel feedstock. The current study thoroughly examined the seed developmental processes of *P. pinnata* and deduced the physiological, biochemical and molecular changes through metabolomics and proteomic approaches. Our present studies also provide insights into the photo-autotrophic nature of *P. pinnata* seed.

Legume seed development and differentiation is characterized by three rapid growth phases separated by two lag phases (Weber et al., 2005). Majorly, the growth phases are marked by the development of endosperm, seed coat and embryo associated with maturation and cell expansion. The differentiation of processes during seed development is a sequential process which involves active mitotic cell division, sucrose uptake, cell expansion, greening and gaining of photosynthetic activity as well as accumulation of storage products (Baud et al., 2002). For oil seeds, the differentiation phase is of special interest because during this stage a regulatory network initiates the accumulation of storage products. The developing seeds of *P. pinnata* are green in color at younger stages (S1-S2) which turned brown during maturation with a gradual increase in seed weight, storage products and lipid content but decrease in moisture content as well as the weight of seed coat. The importance of maternal seed coat in providing the necessary sources of filial cotyledons for development and controlling germination is well known in legumes (Weber et al., 2005). Seed coat percentage in terms of its structural weight is high during younger stages of development which indicated its active role in maintaining seed development. During

natural seed maturation process, there is a gradual increase in seed desiccation, wherein the water content declined significantly, (Angelovici et al., 2010). In general, chlorophyll content and the ratio of chlorophyll *a*/b ratio play a significant role in developing green seeds and are directly related to the photosynthetic efficiency of the green seeds (Eastmond and Kolacna, 1996). In the current study, initial stages of *P. pinnata* seed showed higher chlorophyll *a*/b ratio (> 1), which declined gradually with maturation. This clearly indicated the active photosynthesis in *P. pinnata* developing seeds during S1 and S2. To support our results, we have also analyzed the gene expression of some of the key photosynthetic electron transport genes. PS I and PS II related gene expression was reported in many green and oil yielding seeds and it is also considered to be an important factor for embryo development and fatty acid biosynthesis (Niu et al., 2009; Allorent et al., 2015). The expression of photosynthetic genes in *P. pinnata* was higher till S2 after which there was a steady decline in expression levels indicating very active photosynthetic process during the initial stages of seed development. The temporal induction of ATP synthase subunit gene (atp1A) could have also contributed for substantial production of ATP which is needed for the energy productions and fatty acid biosynthesis. The seed photosynthesis may provide energy for the fatty acid biosynthesis during subsequent stages and also helps in re-fixing the respiratory CO_2 release. The release of O_2 through the photosynthesis can aid in reducing NO:O_2 ratio, thereby preventing anoxia in seeds during development (Borisjuk and Rolletschek, 2009). Active photosynthesis might also contribute for significant accumulation of storage products including protein, starch and other carbohydrates which was evident in this study with *P. pinnata* seeds.

Metabolites are considered as very robust and sensitive as they maintain the seed physiology and storage biochemistry (Weselake et al., 2009). Understanding the dynamics of metabolic profile among various stages of development might give new insights into the mechanism of seed oil biosynthesis with respect to quantity and quality of lipids in this potential biofuel crop. The stage dependent metabolite changes of various crops including *Oryza sativa*, *Glycine max* and *Brassica napus* were recently reported which had enlightened the role of metabolites in regulating oil biosynthesis, cellular morphology, seed filling and maturity (Tan et al., 2015; Hu et al., 2016; Gupta et al., 2017). We have detected sugars, organic acids, free fatty acids and amino acids through our metabolite analysis in developing *P. pinnata* seed. Sugars and organic acids take part in energy metabolism thus providing reducing compounds as well as precursors for fatty acid biosynthesis, cell division and differentiation. Among the detected sugars, glucose is utilized as a signal for cellular multiplication and differentiation while other sugars like ribose and erythrose take part in nucleotide formation and energy metabolism such as Calvin cycle. Interestingly, sugars related to cell wall formation and cell division were higher in S2-S3, for example NADGA, cellobiose, pyranose and arabinose indicating that the active stages of cellular division and differentiation are from S1 to S3. Sugars such as mannose were most abundant in the mature stage S4 and it may contribute to the production of bioethanol from remaining seed residue after the extraction of oil. The major organic acid components of TCA cycle were also detected in the metabolites where, the stage wise quantitative changes were observed in *P. pinnata* seeds. The presence of a flux mode for respiratory energy synthesis through TCA may be the reason of expression of these metabolites in higher concentration during initial stages (S1-S3), which in turn can aid the fatty acid biosynthesis. Amino acids in the developing seeds contribute in various metabolic activities including storage protein accumulation, germination, stress responses and in respiratory catabolic processes to maintain optimum energy status inside the developing seeds (Miranda et al., 2001; Galili et al., 2014). Most of the amino acids were abundant in S2-S3, while glycine and proline which are having stress responsive characteristics were abundant during S4 providing clues for stress tolerance nature of *P. pinnata* seed as the development progressed (Hayat et al., 2012; Czolpinska and Rurek, 2018). The other important amino acids such as glutamate, aspartate, glutamine were also detected which play a central role in the synthesis of other amino acids during seed development (Brian et al., 2007; Jander and Joshi, 2009).

Total seed storage protein profile analyzed through 2D gel electrophoresis gave the overall picture of stage wise variation in protein expression and regulation, where some key regulatory proteins have been successfully identified. The proteins, related specifically to seed development are Midasin and ap2 complex subunit alpha which take part in the development of female gametophyte as well as the cotyledonary development (Chantha et al., 2010). On the other hand, glyceraldehyde-3-phosphate dehydrogenase, a glycolytic enzyme showed higher expression during younger stages which decreased significantly at S4 indicating an active cellular mechanism such as glycolysis at younger stages S1-S2 which gradually decreased with maturation. ATPase subunit beta of photosynthetic electron transport and PEP carboxykinase of carbon assimilation metabolism were upregulated at S2-S3. Our data infers that photosynthesis in *P. pinnata* seed plays a critical role in carbon recycling and energy metabolism which is crucial for fatty acid biosynthesis. Significantly upregulated spots from S1 to S4 included acetyl CoA carboxylase (Accase), the first catalyzing enzyme of fatty acid biosynthesis and lipid transfer like proteins (LTPs). The LTPs are low molecular weight (9–10 kD) proteins and have a significant role in plant development such as transporting lipids for membrane biosynthesis, defense responses and vesicular transports (Liu et al., 2015). A subcellular localization study in seeds also showed the involvement of LTPs with protein as well as lipid bodies (De O. Carvalho et al., 2004). In addition, GTP binding protein SAR1b, which is well known for taking part in vesicular protein traffic from Endoplasmic reticulum to Golgi leading to storage body formation was detected in *P. pinnata* seeds (Memon, 2004). Moreover, recently in liver cells, phosphorylation of SAR1b had been proven to be an important step for release of lipid chylomicrons (Siddiqi and Mansbach., 2012). However, in plants particularly the high oil yielding species like *P. pinnata*, it still remains a question to whether this protein carries a role in the synthesis of lipid bodies.

The accelerating accumulation of oil bodies in developing seeds could help in determining the best harvest time of *P. pinnata* seeds. However, the size and pattern of oil body accumulation during seed development is important for understanding the dynamics of molecular regulation behind this process. It was evident from our data that TAG levels were increased gradually as the seed development progressed and the fate of most of the TAGs accumulated is to ultimately form oil bodies (Hills, 2004). The increasing number of oil bodies with maturation suggest that majority of storage product observed were lipids in *P. pinnata* seed. The FAMEs composition of biofuel and its blend can determine its efficiency for fuel capacity (Sbihi et al., 2018; Xiong et al., 2018). In *P. pinnata*, the fatty acid composition of accumulated TAGs at each stage had shown that oleic acid was predominantly incorporated only after S3 making the seed oil of *P. pinnata* rich with unsaturated fatty acids and making it a valuable source of biodiesel which meets all the standard criteria for biodiesel production (Karmee and Chadha, 2005).

5. Conclusion

Present study highlights the active role of metabolites and some key proteins in the stage specific regulation of *P. pinnata* seed development. The pattern of gene expressions along with metabolites and the proteome data can contribute to possible regulatory networks of cellular metabolism responsible for synthesis and accumulation of oil in Pongamia seeds during different developmental stages. Major metabolites and proteins of each stage belonging to various pathways are identified and their profiles reveal that energy metabolism and cell division are major processes in S1 and S2 which is followed by rapid increase in storage products along with oil bodies from S3 to S4. The TAGs are the major form of lipids in Pongamia oil and we confirmed

that in mature seeds they contribute to the highest content of oleic acid, which also concur with high content of unsaturated fatty acid in *P. pinnata* making it desirable for biofuel production. These results will possibly lay the foundation for metabolic engineering of oilseed crops for improving the quality and quantity of seed oil as a potential feedstock in the biofuel industry.

Declaration of Competing Interest

Authors declare to have no conflict of interest.

Acknowledgements

The present study was supported by a grant from Department of Biotechnology (DBT), Govt. of India (BT/PR12024/BCE/8/1097/2014). Authors like to thank Tree Oils India Limited for providing access to the farm for sample collection and experimental setup. KTS thanks DBT, New Delhi and RVS, SM thanks UGC, New Delhi for fellowship.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.indcrop.2019.111621.

References

Allorent, G., Osorio, S., et al., 2015. Adjustments of embryonic photosynthetic activity modulate seed fitness in Arabidopsis thaliana. New Phytol. 205, 707–719.
Angelovici, R., Galili, G., et al., 2010. Seed desiccation: a bridge between maturation and germination. Trends Plant Sci. 15, 211–218.
Arnon, D., 1949. Copper enzymes isolated chloroplasts, polyphenoloxidase in Beta vulgaris. Plant Physiol. 24, 1–15.
Atabani, A.E., Silitonga, A.S., et al., 2013. Non-edible vegetable oils: a critical evaluation of oil extraction, fatty acid compositions, biodiesel production, characteristics, engine performance and emissions. Renew Sust Energ Rev. 18, 211–245.
Bala, M., Nag, T.N., et al., 2011. Proximate composition and fatty acid profile of *Pongamia pinnata*, a potential biodiesel crop. J. Am. Oil Chem. Soc. 88, 559–562.
Baud, S., Boutin, J., et al., 2002. An integrated overview of seed development in *Arabidopsis thaliana* ecotype WS. Plant Physiol. Biochem. 40 (2), 151–160.
Baud, S., Dubreucq, B., et al., 2008. Storage reserve accumulation in *Arabidopsis*: metabolic and developmental control of seed filling. Arabidopsis. Book. 6, e0113.
Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37, 911–917.
Borisjuk, L., Rolletschek, H., 2009. The oxygen status of the developing seed. New Phytol. 182, 17–30.
Borisjuk, L., Rolletschek, H., et al., 2003. Differentiation of legume cotyledons as related to metabolic gradients and assimilate transport into seeds. J. Exp. Bot. 54 (382), 503–512.
Bradford, M.M., 1976. A Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.
Chaitanya, B.S.K., Kumar, S., et al., 2015. Pivotal role of sugar fluxes between the inner integument and endosperm in lipid synthesis during seed ontogeny in *Jatropha curcas* L. Ind. Crops Prod. 76, 1106–1113.
Chantha, S.C., Gray-Mitsumune, M., et al., 2010. The MIDASIN and NOTCHLESS genes are essential for female gametophyte development in Arabidopsis thaliana. Physiol. Mol. Biol. Plants 16 (1), 3–18.
Coetzee, R., Labuschagne, M.T., et al., 2008. Fatty acid and oil variation in seed from kenaf (Hibiscus cannabinus L.). Ind. Crops Prod. 27 (1), 104–109.
Czolpinska, M., Rurek, M., 2018. Plant glycine-rich proteins in stress response: an emerging, still prospective story. Front. Plant Sci. 9, 302.
De O. Carvalho, A., De S. Teodoro, C.E., et al., 2004. Intracellular localization of a lipid transfer protein in *Vigna unguiculata* seeds. Physiol. Plant. 122, 328–336.
Eastmond, P.J., Kolacna, L., 1996. Photosynthesis by developing embryos of oilseed rape Brassica napus. J. Exp. Bot. 47, 1763–1769.
Eastmond, P.J., Rawsthorne, S., 2000. Coordinate changes in carbon partitioning and plastidial metabolism during the development of oilseed rape embryos. Plant Physiol. 122 (3), 767–774.
Ekman, A., Hayden, D.M., et al., 2008. Carbon partitioning between oil and carbohydrates in developing oat (*Avena sativa* L.) seeds. J. Exp. Bot. 59 (15), 4247–4257.
Galili, G., Avin-Wittenberg, T., et al., 2014. The role of photosynthesis and amino acid metabolism in the energy status during seed development. Front. Plant Sci. 5, 447.
Greenspan, P., Mayer, E.P., et al., 1985. Nile red: a selective fluorescent stain for intracellular lipid droplets. J. Cell Biol. 100 (3), 965–973.
Gupta, M., Bhaskar, P.B., et al., 2017. Integration of omics approaches to understand oil/protein content during seed development in oilseed crops. Plant Cell Rep. 36 (5), 637–652.
Hayat, S., Hayat, Q., et al., 2012. Role of proline under changing environments: a review. Plant Signal. Behav. 7 (11), 1456–1466.
Hedge, J.E., Hofreiter, B.T., 1962. In: Whistler, R.L., Be Miller, J.N. (Eds.), Methods in Carbohydrate Chemistry 17. Academic Press, New York.
Hills, M.J., 2004. Control of storage-product synthesis in seeds. Curr. Opin. Plant Biol. 7, 302–308.
Hiscox, J.D., Israelstam, G.F., 1979. A method for the extraction of chlorophyll from leaf tissue without maceration. Can. J. Bot. 57, 1332–1334.
Hu, C., Tohge, T., et al., 2016. Identification of conserved and diverse metabolic shifts during rice grain development. Sci. Rep. 6, 20942. https://doi.org/10.1038/srep20942.
Jander, G., Joshi, V., 2009. Aspartate-derived amino acid biosynthesis in Arabidopsis thaliana. Arabidopsis Book 7, e0121.
Karmee, S.K., Chadha, A., 2005. Preparation of biodiesel from crude oil of Pongamia pinnata. Bioresour. Technol. 13, 1425–1429.
Kesari, V., Rangan, L., 2011. Coordinated changes in storage proteins during development and germination of elite seeds of *Pongamia pinnata* a, a versatile biodiesel legume. AoB Plants 2011 (2011), plr026.
Kopka, J., Schauer, N., et al., 2005. GMD @ CSB. DB: the golm metabolome database. Bioinformatics 21, 1635–1638.
Kumar, S., Sreeharsha, R.V., et al., 2017. Molecular insights into photosynthesis and carbohydrate metabolism in Jatropha curcas grown under elevated CO2 using transcriptome sequencing and assembly. Sci. Rep. 7, 11066.
Liu, F., Zhang, X., et al., 2015. Non-specific lipid transfer proteins in plants: presenting new advances and an integrated functional analysis, J. Exp. Bot. 66 (19), 5663–5681.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real time quantitative PCR and the $2-\Delta\Delta CT$ method. Methods 25, 402–408.
Luthra, R., Munshi, S.K., et al., 1991. Elationship of carbohydrate metabolism with lipid biosynthesis in developing sunflower (*Helianthus annuus* L.) seeds. J. Plant Physiol. 137 (3), 312–318.
Memon, A.R., 2004. The role of ADP-ribosylation factor and SAR1 in vesicular trafficking in plants. Biochim. Biophys. Acta 1664 (1), 9–30.
Miranda, M., Borisjuk, L., et al., 2001. Amino acid permeases in developing seeds of *Vicia faba* L.: expression precedes storage protein synthesis and is regulated by amino acid supply. Plant J. 28, 61–71.
Mudalkar, S., Golla, R., et al., 2014. De novo transcriptome analysis of an imminent biofuel crop, Camelina sativa L. Using Illumina GAIIX sequencing platform and identification of SSR markers. Plant Mol. Biol. 84, 159–171.
Muktham, R., Ball, A.S., et al., 2016. Bioethanol production from non-edible de-oiled *Pongamia pinnata* seed residue-optimization of acid hydrolysis followed by fermentation. Ind. Crops Prod. 94, 490–497.
Niu, Y., Wu, G.Z., et al., 2009. Global analysis of gene expression profiles in *Brassica napus* developing seeds reveals a conserved lipid metabolism regulation with *Arabidopsis thaliana*. Mol. Plant 2, 1107–1122.
Pavithra, H.R., Gowda, B., et al., 2012. Oil, fatty acid profile and karanjin contentin developing *Pongamia pinnata* (L.) Pierre seeds. J. Am. Oil Chem. Soc. 89, 2237–2244.
Pavithra, H.R., Gowda, B., et al., 2014. Biochemical changes in the composition of developing seeds of *Pongamia pinnata* (L.) Pierre. Ind Crops Pro6d 53, 199–208.
Rolletschek, H., Radchuk, R., et al., 2005. Evidence of a key role for photosynthetic oxygen release in oil storage in developing soybean seeds. New Phytol. 167, 777–786.
Sbihi, H.M., Nehdi, I.A., et al., 2018. Study of oxidative stability and cold flow properties of Citrillus colocynthis oil and *Camelus dromedaries* fat biodiesel blends. Ind. Crops Prod. 122, 133–141.
Schiltz, S., Munier-Jolain, N., et al., 2005. Dynamics of exogenous nitrogen partitioning and nitrogen remobilization from vegetative organs in pea revealed by 15N in Vivo labeling throughout seed filling. Plant Physiol. 137 (4), 1463–1473.
Schwender, J., Goffman, F., et al., 2004. Rubisco without the Calvin cycle improves the carbon efficiency of developing green seeds. Nature 432, 779–782.
Schwender, J., Hebbelmann, I., et al., 2015. Quantitative multilevel analysis of central metabolism in developing oilseeds of oilseed rape during in vitro culture. Plant Physiol. 168 (3), 828–848.
Sengupta, D., Kannan, M., et al., 2011. A root proteomics-based insight reveals dynamic regulation of root proteins under progressive drought stress and recovery in *Vigna radiata* (L.) Wilczek. Planta 233 (6), 1111–1127.
Sharma, S., Guleria, S., et al., 2011. Lipid accumulation in developing soybean: influence of seed position on stem axis. Genet. Plant Physiol. 1, 56–67.
Siddiqi, S., Mansbach, C.M., 2012. Phosphorylation of Sar1b protein releases liver fatty acid-binding protein from multiprotein complex in intestinal cytosol enabling it to bind to endoplasmic reticulum (er) and bud the pre-chylomicron transport vesicle. J. Biol. Chem. 287 (13), 10178–10188.
Singh, Y., Sharma, A., et al., 2018. Optimization of performance and emission parameters of direct injection diesel engine fuelled with Pongamia methyl esters-response surface methodology approach. Ind. Crops Prod. 126, 218–226.
Sreeharsha, R.V., Mudalkar, S., et al., 2016. Unravelling molecular mechanisms from floral initiation to lipid biosynthesis in a promising biofuel tree species, *Pongamia pinnata* using transcriptome analysis. Sci. Rep. 6, 34315.
Sun, C., Jia, L., et al., 2017. Natural variation in fatty acid composition of *Sapindus spp*. Seed oils. Ind. Crops Prod. 102, 97–104.
Tan, H., Xie, Q., et al., 2015. Dynamic metabolic profiles and tissue-specific source effects on the metabolome of developing seeds of Brassica napus. PLoS One 10 (4), e0124794.
Weber, H., Borisjuk, L., et al., 2005. Molecular physiology of legume seed development. Annu. Rev. Plant Biol. 56, 253–279.
Weselake, R.J., Taylor, D.C., et al., 2009. Increasing the flow of carbon into seed oil. Biotechnol. Adv. 27 (6), 866–878.
Wright, D., Lenssen, A.W., 2013. Staging soybean development. Agri.Environ.Exten. Pub. 191.
Xiong, B., Zhang, Z., et al., 2018. Biodiesel from *Lindera glauca* oil, a potential non-food feedstock in Southern China. Ind. Crops Prod. 122, 107–113.