Coordinated changes in storage proteins during development and germination of elite seeds of *Pongamia pinnata*, a versatile biodiesel legume

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Abstract

Background and aims

The oleaginous legume *Pongamia pinnata* is a rapidly growing and economically important tree. The seeds are used increasingly as feedstock for biodiesel production, with the protein-rich residue providing valuable supplement to farm animal diets. However, little is known about seed development and the characteristics of germination. We therefore studied morphological, protein and ultrastructural changes during seed maturation and germination using seeds from a tree selected for superior morphological and reproductive characters (candidate plus tree).

Methodology

Phenology, sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), and scanning and transmission electron microscopy were used to investigate seed development from 90 to 350 days after flowering (DAF), and germination and seedling development from 0 to 45 days after the start of imbibition (DAI) (Stages 0–VII).

Principal results

Seven distinct developmental stages were identified during seed development. Fresh weight, length, breadth and thickness increased from Stage I (90 DAF) to V (270 DAF) and decreased at Stages VI (315 DAF) and VII (350 DAF), when the seeds were fully ripe. Marked changes in total soluble protein content and SDS–PAGE profile were observed in vegetative and reproductive tissues and in the cotyledons of germinating seedlings. Polypeptide fragments of 150–14 kDa were observed during seed maturation and germination. In SDS–PAGE the expression of three main polypeptide bands (50, 18 and 14 kDa) increased from Stage I to Stage V and then almost became the same until Stage VII during seed maturation. During germination the expression of 50 kDa polypeptide decreased and that of 18 and 14 kDa increased from Stage 0 (ungerminated seed) to Stage VI (30 DAI), respectively; however, all three polypeptides (50, 18 and 14 kDa) completely disappeared at Stage VII (45 DAI). Ultrastructural changes during four stages of seed maturation (early immature, 90–135 DAF; late immature, 180–225 DAF; early mature, 225–270 DAF; and late mature, 315–350 DAF) and three stages of germination and seedling development (early 10 DAI to late 45 DAI) localized marked gradients in protein storage reserves.

Conclusions

Increasing the knowledge base for *P. pinnata*, especially for its seeds, is an essential prerequisite for rapid and successful exploitation of this promising energy and animal feed crop. Our findings contribute to this by establishing key developmental features of the seeds as they form and germinate.

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Introduction

Oil-yielding crop plants are important for economic growth of the energy (biodiesel) and agricultural sectors of developing countries such as India. The legume tree *Pongamia pinnata* is such a species. It has several advantageous features: it is easy to grow, has a short generation time and produces large quantities of oil- and protein-rich seed. The trees also flower and crop twice a year. There is wide genetic variability in oil content (Kesari et al. 2008; Scott et al. 2008; Mukta and Sreevalli 2010), implying that rigorous selection and breeding will improve oil production and other useful characteristics. There is considerable potential for improving productivity by increasing our basic knowledge such as developmental studies especially by using seed from elite trees possessing desired characteristics, like high seed oil content (candidate plus trees). Here we used seeds from one individual tree possessing superior morphological, oil-bearing and reproductive characteristics (candidate plus tree—CPT) to study development in relation to seed protein content.

Protein is one of three major classes of storage products present in seeds, viz. storage lipids, storage proteins and oleosins. After extraction of oil (28–34 %) from the seeds of *P. pinnata*, a meal highly enriched in protein (crude protein ~ 40 %) remains. This is a valuable source of protein for animal nutrition (NOVOD 2009). To develop types with a higher added value due to improved protein content, knowledge of the seed proteins of *P. pinnata* is important. Seed proteins include storage proteins (40–70 % in legumes), housekeeping proteins and biologically active enzymes, and other proteins (protease inhibitors, lectins and allergens) classified as minor proteins. During germination and seedling development, seed storage proteins are typically degraded by proteases that convert the insoluble storage proteins into soluble peptides and then by hydrolases to generate free amino acids. These free amino acids are available for transport to the embryonic axis to support growth and as an energy source (Shutov and Vaintraub 1987; Bewley and Black 1994; Müntz et al. 2001). There are no reports available on the changes in protein profiling pattern during seed maturation, germination and subsequent seedling growth that contributes to major component of seed development in *P. pinnata*. The same is true of the other reproductive and vegetative tissues in *P. pinnata*. Much is already known about protein storage and fine structural changes during seed maturation and germination of other oil seed crops (Bhandari and Chitralekha 1984; Prego et al. 1998; Borisjuk et al. 2005; Neuberger et al. 2008). This literature helped guide our own studies with *P. pinnata*.

In previous work we identified the elite tree CPT, NGPP (North Guwahati *P. pinnata*) 46, based on morphometric traits from natural populations in North Guwahati, Assam, India (Kesari et al. 2008). This tree is helping to improve the agronomic value of *P. pinnata* through breeding and selection. The crop is cultivated for its seeds, but basic information on their development and germination is lacking. We wished to rectify this deficiency by (i) defining seed development in terms of changes in size and protein content in relation to biomass and seed yield; (ii) establishing the effect of seed maturation, germination and seedling development on changes in protein content and its spatial distribution using sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE); and (iii) identifying changes in the localization of storage reserves and related events using scanning (SEM) and transmission electron microscopy (TEM). The findings are intended to underpin the long-term objective of enhancing desirable traits such as heavier seed yield and greater oil and protein content of *P. pinnata*.

Materials and methods

Plant material

Leaves, flowers, buds and seeds were harvested from the CPT, NGPP 46, of *P. pinnata*. In our previous study for selection of CPTs, 10 locations (each representing one population) with 5 random trees (total of 50 trees named NGPP 1–50) from each location were scored for various morphological and reproductive characters using a combined analysis program of CROPSTAT (Kesari et al. 2008). Individuals performing above average for 75 % of characters were tagged as the CPT in the first-stage analysis. In the second-stage analysis, based on pod-seed traits and oil content, the best individual CPT, NGPP 46, was identified (Kesari et al. 2008). From August 2007 to April 2008, seeds were harvested between 90 and 350 days after flowering (DAF) at intervals of 40–45 days, transported to the laboratory in sealed polythene bags and stored at 4 °C. Matured seeds collected at the end of April were used for germination and seedling development studies. For this, seeds were rinsed in 25 % (v/v) ethanol for 10 min, washed with distilled water three times before placing in polythene bags containing sand and clay (ratio of 1:4), and hardened in a mist chamber at a temperature of 28 ± 2 °C and 70–80 % relative humidity. Germinated seedlings and young developing saplings were uprooted from the polybags at 5 days (Stage I), 10 days (Stage II), 15 days (Stage III), 20 days (Stage IV), 25 days (Stage V), 30 days (Stage VI) and 45 days (Stage VII) after the start of imbibition (DAI). Roots and cotyledons were harvested...
at each stage and analyses were repeated seven times overall.

For protein analysis, plant material (leaves, flowers, buds, developing seeds, roots and cotyledons of germinated and developing seedlings) was frozen in liquid nitrogen and stored in sealed containers at −20°C. Localization of storage reserves in the cotyledons was examined at four different stages of seed maturation, viz., early immature, 90–135 DAF; late immature, 180–225 DAF; early mature, 225–270 DAF; and late mature, 315–350 DAF. Similarly, localization of storage reserves in the cotyledons of germinated and young developing seedlings was performed from early germination (10 DAI) to late germination/seedling development (45 DAI).

**Phenotypic characterization of developing seeds**

Seeds of different developmental stages were scored for shape, colour, fresh weight, dry weight (DW), moisture content, length, breadth and thickness. Dry weights were taken after drying at 65°C for 4–7 days until the weight was constant. Seed moisture content (%) was calculated from (FW−DW)/FW × 100.

**Total soluble protein pattern during seed maturation, germination and seedling development**

**Total soluble protein extraction and quantification**

Approximately 0.5 g fresh weight of material was ground in a mortar and pestle using liquid nitrogen with 0.5 mL of extraction buffer (100 mM Tris-HCl pH 8.1, 10 mM EDTA pH 8.0, 10 mM β-mercaptoethanol and 0.2 mg mL⁻¹ phenylmethylsulphonyl fluoride). The homogenate was centrifuged twice at 13,000 g for 30 min; the supernatant was collected as the crude protein sample and mixed with an equal volume of 2× sample buffer (250 mM Tris-HCl pH 6.8, 4% SDS, 10% β-mercaptoethanol, 20% glycerol and 0.03% bromophenol blue), boiled for 5 min and stored at −80°C. Protein concentration was determined colorimetrically for the total crude extract of the protein sample according to Bradford (1976) using bovine serum albumin as standard. All the experiments were performed three times.

**SDS–PAGE analysis of total soluble seed proteins**

Denatured proteins were separated by SDS–PAGE by using a vertical slab gel (1.5 mm thick, Biorad Mini Protean II, Richmond, CA, USA). The concentrations of stacking gel (pH 6.8) and resolving gel (pH 8.8) were 5 and 12.5%, respectively. Tris-glycine buffer (pH 8.5) containing 0.1% SDS (w/v) was the electrode buffer. Protein samples dissolved in the sample buffer and the marker protein were denatured at 100°C for 5 min before being loaded onto the gel. Briefly, 50–100 μg protein samples, as quantified by the Coomassie Brilliant Blue G250 assay (Bradford 1976), were loaded onto each lane; electrophoresis was carried out at an initial voltage of 120 V and raised to 180 V when the tracking dye reached the gel mould. After electrophoresis, proteins were visualized by staining the gels with 0.05% (w/v) Coomassie Brilliant Blue-R 250 in 40% methanol and 10% acetic acid, and the band intensities were analysed by a gel documentation system (Molecular Imager Gel Doc XR system; Biorad, USA). The molecular weight of the polypeptide was determined by the mobility of the standard molecular weight markers for SDS–PAGE (Bangalore Genei, Bangalore, Karnataka, India). The protein profiles of various plant samples were confirmed by three separate extractions. Each experiment was repeated three times.

**Localization and distribution of storage reserves in the cotyledonary tissues during seed maturation, germination and seedling development**

**Sample preparation for SEM**

For SEM, cotyledony tissues of different stages of seed development, germination and seedling development stages were fixed in 2.5% glutaraldehyde for 48 h and post-fixed in 1% osmium tetroxide (OsO₄) at 4°C. Sections 10–12 mm thick were dehydrated at room temperature at 15–30 min intervals in a graded series of aqueous ethanol solutions (10, 30, 50, 70, 90, 96 and 100%), critical point dried using CO₂, mounted directly on aluminium stubs using double-sided adhesive tape and sputter-coated with gold. Observations were made in a LEO 1430 VP instrument (Leo Electron Microscopy Limited, Cambridge, UK) at an accelerating voltage of 10 kV. For localization of lipids, cotyledonal tissues of matured ripened seeds were first defatted in acetone and n-hexane for 48 h, and then fixed in 2.5% glutaraldehyde. All experiments were performed twice. Two seeds were examined at each developmental stage.

**Sample preparation for TEM**

Cotyledons were cut with a sterile razor blade and fixed in Karnovsky’s fixative (Karnovsky 1965) for 48 h at 4°C. The material was post-fixed for 1 h in 1% (w/v) OsO₄ in 0.1 M sodium phosphate buffer (4°C, pH 7.0), rinsed three times for 10 min each in buffer, and dehydrated at room temperature for 15–30 min intervals in a graded series of aqueous ethanol (10, 30, 50, 70, 90, 96 and 100%). Dehydrated tissues were infiltrated and embedded in Spurr’s resin (Spurr 1969). Ultrathin sections (80 nm) were...
stained with 40 g L\(^{-1}\) uranyl acetate for 45 min, followed by 4 g L\(^{-1}\) lead citrate for 4 min, mounted on grids and observed in a Philips CM 10 instrument (Philips, Eindhoven, The Netherlands). All the experiments were performed twice. Two seeds were examined at each developmental stage.

**Results**

**Morphological parameters of developing seeds**

The dicotyledonous seed of *P. pinnata* has no endosperm (exendospermous) and is enclosed in a pericarp. It is thus strictly a fruit, but is conventionally considered a seed and is called such in this paper. Seed was collected from CPT, NGPP 46, at various stages of maturation from mid-August to the end of April. Seven stages were identified ranging from highly immature (90 DAF) to fully mature (350 DAF). Seed colour evolved from an initial dark green, through yellowish green, pale yellow and finally dark brown by Stage VII (350 DAF) (Fig. 1A). The moisture content of seeds ranged from 10.5 to 88.3 %: lowest levels in mature seeds (Stage VII) and highest levels at Stage I (Table 1). Fresh weight increased 57-fold between Stages I and V, but decreased in the subsequent stages when seeds ripened and turned yellow or brown. Similarly, length, breadth and thickness also increased from Stage I to Stage V before shrinking considerably during Stages VI and VII (Fig. 1B).

**Total soluble proteins during seed maturation, germination and seedling development**

Changes in total protein content during seed maturation, germination and seedling development

The total soluble protein concentration in fully mature seeds (300 DAF) was \(\sim 19 \text{ mg g}^{-1} \text{ FW}\) and over three times that of immature (90 DAF) seeds. Roots and flowers contained the least protein (\(\sim 4 \text{ mg g}^{-1} \text{ FW}\)) while leaves and buds contained about double this concentration (Fig. 2A). The high concentration of total soluble protein in mature seeds became dissipated...
during germination and seedling growth (Fig. 2B), and by 45 DAI (Stage VII) was almost undetectable. Protein loss was relatively slow for the first 20 days of germination (25% decrease), but the remaining protein was almost all degraded in the subsequent 25 days as seedlings developed an above-ground shoot system.

**SDS–PAGE analysis of total soluble seed proteins**

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis was used to characterize changes in seed storage proteins more precisely as seeds developed and matured. The electrophoretic spectra of total soluble seed proteins under denaturing conditions at different growing stages showed 15–20 distinct polypeptide bands (Fig. 3A). The highest-molecular-weight protein was ~150 kDa and evident in Stage I–III seeds. The smallest protein (14 kDa) was most evident at Stages III–VII. Between Stages I and VII, polypeptides of 14, 18, 21, 23, 25, 29, 35, 39, 50 and 95 kDa were observed (Fig. 3A). In summary, at Stage I, highly immature seeds (90 DAF) contained only trace amounts of detectable proteins. At Stage II (135 DAF), polypeptides of low intensity were the major soluble proteins. As development proceeded (Stages II–V), three main polypeptides of 50, 18 and 14 kDa became the major proteins and there was a clearly marked increase in their concentration upon maturation (Fig. 3A). It was also noted that 270 DAF (Stage V) and at later stages (Stage VII) SDS–PAGE profiles were substantially the same as those of the most mature seeds (Stage VII).

Comparative profiling of leaves, root flowers, buds and seeds revealed considerable heterogeneity in terms of number and relative staining intensities of bands (Fig. 3B). Clearly, major proteins of mature seeds were very different from those of the roots and various shoot parts.

A further experiment examined the effect of germination and young seedling development on total soluble cotyledon proteins using SDS–PAGE during the first 45 DAI. Between 15 and 20 polypeptide bands ranging from 100 to 14 kDa (Fig. 4) were resolved in terms of changes in the number of protein bands and staining intensity. Notable features include the rapid loss of 50 kDa protein as germination proceeded. The decline continued during seedling development and was almost undetectable by 30 DAI. A second large polypeptide (29 kDa) disappeared completely within 20 DAI (Fig. 4). Simultaneously with these losses, new but smaller polypeptides appeared (25–14 kDa) after 10 DAI, and increased in abundance thereafter (Fig. 4). The developmental expression of 50, 18 and 14 kDa polypeptides during the seven stages of seed maturation exhibited an inverse relationship with seed germination and seedling developmental stages.

### Localization and distribution of the cotyledonary reserves during seed maturation, germination and seedling development

**Scanning electron microscopy** During early seed development (90–135 DAF), cotyledonous parenchyma formed large intercellular spaces and cells that were not well differentiated (Fig. 5A). At later stages (180–225 DAF), these spaces increased in number (Fig. 5B). As the seeds became more mature (225–270 DAF), well-defined vascular bundles appeared (Fig. 5C). These are shown more clearly at a higher magnification in the inset to Fig. 5C. At this stage, elongated palisade parenchyma cells just beneath the epidermal cells became distinguishable from the more rounded spongy parenchymatous cells of the central regions (Fig. 5C). The parenchymatous cells of highly mature seeds (315–350 DAF) were more compact, highly differentiated with smaller but still numerous intercellular spaces (Fig. 5D). Higher resolution SEMs revealed a considerable increase in cell wall thickness during seed maturation. Thickness varied from ~60 μm (early immature seeds) to 100 μm (late mature seeds) [see Additional Information Fig. A–D]. The epidermal cells of the flat inner cotyledon surface of highly

**Table 1 Fresh weight, DW and moisture content (%) in different developmental stages of seed maturation in CPT, NGPP 46, of *P. pinnata*.

| Seed developmental stages | Fresh weight | Dry weight | Moisture |
|---------------------------|-------------|------------|----------|
| I (mid-August; 90 DAF)    | 0.09 ± 0.01 | 0.01 ± 0.00 | 88.3     |
| II (end of September; 135 DAF) | 0.33 ± 0.02 | 0.08 ± 0.00 | 74.7     |
| III (early November; 180 DAF) | 1.14 ± 0.08 | 0.34 ± 0.04 | 70.3     |
| IV (mid-December; 225 DAF) | 2.77 ± 0.18 | 1.30 ± 0.11 | 53.2     |
| V (early February; 270 DAF) | 4.92 ± 0.08 | 2.77 ± 0.44 | 43.6     |
| VI (mid-March; 315 DAF) | 4.39 ± 0.23 | 2.84 ± 0.54 | 35.3     |
| VII (end of April; 350 DAF) | 3.38 ± 0.03 | 3.02 ± 0.12 | 10.5     |

Values are mean ± SE of triplicates.
immature seeds were of irregular shape with no distinct stomata [see Additional Information Fig. A]. In contrast, the epidermal cells of mature seeds were very closely packed and displayed angular-shaped stomata [see Additional Information Fig. D].

The homogeneous parenchymatous cells of young cotyledons at an early immature stage (90–135 DAF) contained few obvious reserve deposits (Fig. 5E).

Young mesophyll or spongy parenchyma cells commenced accumulating protein reserves in their central area (Fig. 5E) at a time when intercellular spaces were developing. The protein bodies (PBs) were deposited centrally within the cell whereas lipid droplets accumulated adjacent to the cell walls (Fig. 5F). In addition, thin cytoplasmic strands or networks were found interspersed with lipid and protein droplets. Later stages of

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**Fig. 2** Total soluble protein content of *P. pinnata* seeds (NGPP 46). (A) Vegetative and reproductive tissues and (B) during seven stages (0–VII) of seed germination and seedling development from 0 to 45 DAI (days after the start of imbibition). SG, seed germination; SD, seedling development.
seed development were characterized by the accumulation of large pools of storage reserves (early mature stage, 225–270 DAF) (Fig. 5G). Reserve material continued to accumulate in all parenchyma cells of the cotyledons during early and late maturation (Fig. 5G and H). Individual parenchyma cells became packed with protein, lipid and other storage reserves. To confirm the localization of lipid bodies within cotyledon cells, mature ripened seeds (within 350 DAF) were treated with acetone and \( n \)-hexane for 48 h prior to fixation and it was observed that large intracellular spaces were formed within parenchyma cells of treated seeds (Fig. 5I and J). Organic solvents (hexane and acetone) have been used for extracting or solubilizing lipids from the tissues as they can overcome the strong forces of association between tissue lipids and other cellular constituents, such as proteins and polysaccharides. However, even polar complex lipids, which do not normally dissolve easily in non-polar solvents, can sometimes be extracted with slightly polar organic solvents such as acetone or in a mixture of solvents when the tissues are in the presence of large amounts of simple lipids such as triacylglycerols.

Scanning electron microscopy was also used to examine the utilization pattern of seed storage reserves in cotyledons during germination and seedling development (10–45 DAI). Within 10 DAI, lipids, proteins and other unidentified cytoplasmic networks were seen (Fig. 6A) but their appearance was dissimilar to those seen in matured seeds. It was observed that the SEM images of the cotyledon sections of initially imbibed/germinated seeds showing the localization of storage reserves had a very similar appearance to the acetone-treated cotyledons of the study material. Within 30 DAI, PBs increased in size and became rounded (Fig. 6B and C) while smaller PBs fused to form two or four larger ones. Cells were completely devoid of storage reserves by 45 DAI or during seedling development (Fig. 6D). In addition, cotyledonary tissues of 30-day-old imbibed seeds or of developing seedlings showed highly differentiated vascular bundles with distinct xylem and phloem cells (Fig. 6E and F). Cell wall shrinkage and distortion were observed after 45 DAI (Fig. 6G–I).

**Transmission electron microscopy** The high moisture content of immature seeds (90–135 DAF) was associated with the highly vacuolated cells (Fig. 7A). Cells were 13–16 \( \mu \)m in diameter with a well-defined wall and a large central vacuole. These vacuoles were dissected by sheets of cytoplasm that extended to the periphery. The cytoplasmic regions contained well-differentiated mitochondria and abundant granular...
material possibly of a proteinaceous nature (Fig. 7B and C). Rudimentary plastids were seen in the cytoplasm of cotyledonal cells of mature but still green seeds (Fig. 7D). All cells of mature seed contained abundant protein and lipids in the form of protein and lipid bodies (Fig. 7D and E). Lipids and proteins are apparently the most abundant reserves in developing seeds (Fig. 7F). Lipid globules, with a diameter of 1–3 µm, were found in a spatial association with protein reserves (Fig. 7F).

Further lipid and protein accumulation was always associated with dense cytoplasm with a full range of organelles, viz., nucleus, mitochondria, numerous ribosomes, polyribosomes, and smooth and rough endoplasmic reticulum (Fig. 7G). Both the smaller and larger PBs contain globoid inclusions/crystals in the proteinaceous matrix (Fig. 7H and I). These varied in diameter from 0.5 to 3 µm. The cotyledons also contained large amounts of phenolics in cells.
interspersed with other cell constituents as revealed by TEM section that were not visible in the SEM images. Cotyledonary cells of late maturing seeds (315–350 DAF) contained numerous electron-dense vesicles while other normal cells remained present. Endoplasmic reticulum occurred as closely packed sheets of cisternae. Transmission electron microscopy revealed the presence of a small number of starch grains in the cotyledonary cells of mature seeds.

Ultrastructural changes in cotyledonary tissues of germinated and young developing seedlings are shown in Fig. 7J–R. The structure of the PBs was different from those of maturing seeds. Although the PBs retained a limiting membrane, they expanded to create a much

Fig. 6 Scanning electron micrographs of cotyledon parenchyma at three stages of seed germination and seedling development in P. pinnata seeds (NGPP 46). (A–D) Single cells, (E) and (F) vascular bundles and (G–I) transverse section of outer cell wall of the cotyledon of germinating seeds. (A) and (G) are early germination stage (within 10 DAI); (B), (C) and (H) are middle germination stage (within 30 DAI); (D) and (I) are late germination stage (45 DAI); (E) well-developed vascular bundle connecting a number of neighbouring cells in the cross-section of cotyledon of 30-day-old seedlings; (F) highly differentiated conducting elements (xylem and phloem cells) at an advanced stage of seed germination (30 DAI). P, protein; L, lipid; CW, cell wall; IS, intracellular space.
Fig. 7 Transmission electron micrographs of cross-sections of cotyledons of P. pinnata (NGPP 46) showing ultrastructural changes in cells of maturing seeds, germinating seeds and during seedling development. (A–C) are late immature stages. Large central vacuole with few oil bodies and non-membrane-bound protein granules were found associated with regions of active cytoplasm; mitochondria, oil bodies, starch, cell wall and vacuoles are evident. Starch grains were often found in amyloplasts with a vacuole-like structure. Cell walls displayed an irregular thickening thought to be the deposition of reserve material. (D–I) are late mature stages. They illustrate dense cytoplasm with rudimentary plastids, oil bodies, vacuoles, protein storage vacuoles containing globoid crystals, a nucleus and endoplasmic reticulum forming sheets of cisternae, numerous ribosomes and amyloplasts. (J–M) are initial germination stages (within 10 DAI). (N–P) are middle germination stages (within 30 DAI). (Q) and (R) are late germination stages (45 DAI). CW, cell wall; ER, endoplasmic reticulum; Nu, nucleus; OB, oil bodies; P, protein granules; PB, protein bodies; Pl, rudimentary plastids; PSV, protein storage vacuole; St, starch; V, vacuole.
larger space between the included storage protein aggregation and the membrane (Fig. 7N). Transmission electron microscopy images show that as germination proceeded, PBs fused together, resulting in protein masses that occupied the centre of the cell, pushing other cell contents to the periphery (Fig. 7N and O). At this stage, PBs were devoid of all globoidal inclusions. Subsequently, networks of proteinaceous particles replaced the masses, which ultimately disappeared leaving large vacuoles in the centre of the cells (Fig. 7P–R).

Discussion

Seeds of *P. pinnata* are potentially important for the biodiesel industry and as a food source for farm animals. Basic research into the physiology and development of the plant and its seeds is still at an early stage. The present study addresses this deficiency by analysing seed development with respect to phenotypic traits, phenology, protein profile, tissue-specific localization and gradients in storage reserve deposition during maturation, germination and seedling development. A similar but less wide-ranging study of seed development phenology has previously been documented for *Jatropha curcas*, another potential biodiesel crop (Annarao et al. 2008).

Our results show that seed maturation in *P. pinnata* takes ≏1 year from a May flowering. In general, seed development and acquisition of the ability to germinate are associated with an overall loss of moisture (Adams and Rinne 1980; Li et al. 1999) and, as expected, this also applied to *P. pinnata* seeds. Noticeable changes in mass occurred in cotyledons with the progress of seed maturation. Evaluation of changes in fresh weight, dry weight, moisture content, colour, length, breadth and thickness revealed seven stages of the seed maturation process with a clear separation between immature and mature stages. The present study identified large differences in the protein content between the different stages of seed development and between different tissues. According to Sagwan et al. (2010), the protein content obtained was greatest in root (24.0 mg g⁻¹ DW) and least in leaf (4.2 mg g⁻¹ DW). However, in the present study, the protein content observed was greatest in mature ripened seeds and least in roots. *Pongamia* seed development could be usefully divided into an initial period of protein synthesis (before 135 DAF, Stage I), a second period of rapid synthesis (135–270 DAF, Stages II–V) and a final period of slow synthesis (270–350 DAF, Stages V–VII). The protein contents we report are comparable with those of other legume species (Nagaraj et al. 2007). During germination and early seedling development, the overall loss of total soluble protein in cotyledons was 81.5 % by 45 DAI, indicating that the PBs of seeds are degraded and subsequently utilized to support the growth of germinating seedlings. This is as expected because many authors have reported a similar or earlier decrease in the protein content of germinating cotyledons [e.g. *Macrotyloma uniflorum* (Karanagaran and Rao 1990; Rajeswari and Ramakrishna 2002), *Lupinus albus* (Nandi et al. 1995) and *Lathyrus sativus* (Chandna et al. 1995)]. Storage protein mobilization was also observed in *Pisum sativum*, *Glycine max*, *Vicia sativa* and *Phaseolus vulgaris* (Schlereth et al. 2000; Tiedemann et al. 2001).

The number of polypeptides revealed by SDS–PAGE analysis in the seeds of *P. pinnata* coincides with that reported by Ghafoor and Arshad (2008) for *P. sativum* and by Ghafoor and Ahmad (2005) for *Vigna mungo* seeds. Also, the size range of polypeptides (150–14 kDa) we detected is similar to those in groundnut (*Arachis hypogaea*; Schlereth et al. 2000), sesame seed (*P. sativum*; Prakash and Narasinga 1986), mustard seed (*Brassica bavarica*; Ghafoor and Arshad 2008) and mustard seed (Aluko and McIntosh 2004). There are previously published SDS–PAGE profiles of *Pongamia* seed proteins from Australia and the Chitradurga and Kolar districts of Karnataka (India) showing bands of 90–10 kDa (Scott et al. 2008) and 9.4–48.9 kDa and 21.8–53.5 kDa (Pavithra et al. 2010), respectively. In the current analysis, the difference between polypeptides was in the relative intensity of the stained bands and number. No high-molecular-weight components were observed in the electrophoretic spectra in the present study, and this might be because of the presence of β-mercaptoethanol, which breaks the disulphide bonds present in the polypeptides. Globulins may be the main seed storage proteins in *Pongamia*. This group includes legumins (11S) and vicilins (7S) represented by band numbers 2–8 in SDS–PAGE gels. However, this needs confirmation by mass spectrometry and immunoblotting. There are many reports demonstrating that legume globulins comprise two main fractions: the legumins (350–450 kDa) and the vicilins (150–250 kDa). Legumins are mixtures of trimers and hexamers comprising subunits of 50–60 kDa bound by non-covalent interactions (Hayashi et al. 1988; Shotwell et al. 1988; Wang et al. 2001). A polypeptide with molecular masses of 40–80, 37, 29, 20 and 17 kDa has been reported in legumes (Ghafoor and Arshad 2008; Wang et al. 2001). The most representative bands of soluble proteins are 43.3 and 14.4 kDa (Navari-Izzo et al. 1992). These results concur with the banding pattern that we observed. There were three high-intensity bands: one of ≏50 kDa and two of ≏18 and ≏14 kDa, respectively. In the present study, a polypeptide of
14–18 kDa first appeared about 180 DAF, became prominent by Stage IV (225 DAF) and Stage V (270 DAF), and remained so thereafter. Virtually all the minor polypeptides are present at very low levels in protein fractions prepared from young seed tissues. Altogether, the data reflect that proteins increased in seeds of *P. pinnata* as they developed and matured, as shown by sequential increases in the number and intensity of polypeptide bands. *Pongamia pinnata* seeds were found to accumulate large quantities of protein at maturity (39 % of DW; NOVOD 2009) and possibly represent a major metabolic event during development of this legume, presumably the outcome of tightly regulated changes in gene expression. In contrast to seeds, storage proteins were absent from practically all vegetative parts and flowers. Two prominent polypeptide bands of 55 and 16 kDa were visible in leaf tissues. This may represent Rubisco large and small subunits, respectively.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis is widely used for monitoring protein mobilization in early stages of seed germination and seedling development (Hussain *et al.* 1988; Ahmed *et al.* 1995; Krochko and Bewley 2000). The SDS–PAGE profiles of germinated seeds and developing seedlings that we report for *P. pinnata* are in line with those of other legume crops (Bhatty 1982; Ahmed *et al.* 1995; Ramakrishna 2007). Studies with seeds of *L. sativus*, *Dolichos lablab*, *Cicer arietinum*, *Vicia faba* and also *Gossypium hirsutum* revealed various protein fractions ranging from 92 to 12 kDa with a faster degradation of high-molecular-weight proteins (Chandna *et al.* 1995; Vigil and Fang 1995; Müntz *et al.* 2001; Ramakrishna 2007). Savelkoul *et al.* (1992) suggested that the high-molecular-weight proteins that disappeared during germination were legumins and vicilins. It is our belief that the fast-degrading high-molecular-weight polypeptides during the early stages of germination from cotyledons of *P. pinnata* seeds may be the globulins. The reason behind this speculation is that there are reports which revealed that globulins are the main seed storage proteins in legume seeds (Lqari *et al.* 2004) and it is a well-known fact that storage proteins are degraded during germination and seedling growth. The results of the present study are supported by other reports showing selective protein band disappearance and appearance (Mitsuhashi and Oaks 1994; Ahmed *et al.* 1995). Ahmed *et al.* (1995) suggested that protein disappearance represents degradation of reserve proteins, while new proteins appearing at specific times during germination and seedling development have stage-specific developmental functions. The constant proteins are presumably housekeeping proteins or structural components, while the declining proteins are precursors for other proteins or enzymes.

Protein breakdown is brought about by a variety of proteases that convert insoluble storage proteins into soluble peptides, which are further hydrolysed to free amino acids. These free amino acids are mobilized to the embryonic axis to support its growth and also to provide energy (Shutov and Vaintraub 1987; Bewley and Black 1994; Müntz *et al.* 2001). The mobilization or degradation of polypeptide of 50 kDa protein during germination confirmed its storage role. The SDS–PAGE gel images revealed that the seed storage proteins start to accumulate from 180 DAF and continue until 270 DAF (mid-maturation stage) of seed development. Thereafter, concentrations become constant and are later utilized during germination and seedling development. The SDS–PAGE gel images also showed that they are synthesized only in the seed (in cotyledons) and not in other tissues. These proteins probably lack any other functional activity. The proteins synthesized during seed development are packaged in PBs via one or more steps of the secretory pathway. The seed storage proteins of plants are a group of evolutionary conserved proteins that serve as a source of carbon, nitrogen and sulphur for the germinating seedling (Shotwell and Larkins 1989). Comparison of the electrophoretic patterns would allow the effects of maturity on protein content to be identified and the complex effect of both factors on the composition of seed proteins.

The seed genetics of *P. pinnata* have been studied with respect to total lipid profile, total seed protein profile and molecular markers (Scott *et al.* 2008; Kesari *et al.* 2009, 2010; Pavithra *et al.* 2010). The ultrastructural changes in the oleaginous seed of *P. pinnata* with maturation are not only of interest from the physiological point of view but are also a challenge for anatomists studying a structure–function relationship. We successfully identified ultrastructural changes in the seeds of *P. pinnata* during maturation (90–350 DAF), germination and seedling development (0–45 DAI). Scanning electron microscopy showed seed maturation to be characterized by cell wall thickening, more compact and highly differentiated cells, and the appearance of stomatal grooves. Dodd *et al.* (1989) showed that seed development in *Podocarpus henkelii* occurred with increases in wall thickness that were irregular and frequently associated with numerous organellar bodies. During seed development, deposition of storage reserves (proteins, lipids and carbohydrates) begins at the late immature stage and increases until the late maturation stage. The SEM images of the cotyledon parenchyma cells of developing *P. pinnata* seeds revealed that the synthesis of storage components (mainly lipids and proteins) followed a maturation gradient inside the fruit that corresponds to the high-intensity protein deposition from 270 DAF.
in the seeds as dissected by SDS–PAGE. Seed storage proteins are synthesized from the expression of large gene families that are highly conserved among diverse species (Kinney et al. 2001). The increase in DW and total soluble protein content as observed based on the study of seed phenotypic traits and SDS–PAGE was reflected especially by the accumulation of lipids and protein detected by SEM. In our previous study, determination of the fatty acid composition of individual CPT, NGPP 46, from the same geographical location demonstrated the predominance of polyunsaturated fatty acid, viz. oleic acid (Kesari et al. 2009). The origin of lipid bodies was not clear but for other lipid-rich plants such as Brassica napus and Cucumis sativus lipid bodies were reported to arise from the swelling of endoplasmic reticulum (Wanner et al. 1981). Ultrastructural studies concerning the deposition of storage protein in legume seeds are available for V. faba (Neumann and Weber 1978; Alder and Müntz 1983; Johansson and Walles 1994), Pisum (Craig et al. 1979) and Glycine (Yoo and Chrispeels 1980). It was reported that the storage protein is initially deposited in the central vacuole, which later buds off as small vacuoles, i.e. future PBs. In the present study, due to the interaction of lipid globules with protein reserves, it was not possible to define the oleosin membrane. Guilloteau et al. (2003) reported the existence of two classes of oleosins in Theobroma cacao. We suggest that the rapid coalescence among the lipid bodies occurs when they are exposed to temperatures >28 °C. Fresh histological sections were unable to confirm the existence of the oleosin membrane enclosure in our current study. The functional significance of plastid type in the seeds of P. pinnata is unclear, although proplastids with phytoferritin in seed tissues have been reported in the cotyledons of some members of Fabaceae: P. sativum (Lobreaux and Briat 1991), V. faba (Johansson and Walles 1994) and Cercis siliquastrum (Baldan et al. 1995). According to Greenwood (1976), the grain shape of starch depends on amylose content: the less angular, rounded grains have relatively higher amylose levels. The grain shape of P. pinnata starch granules, like that of Quinoa and Amaranthus hypocondriacus seeds, indicates that they contain mainly amylopectin (Prego et al. 1998). Our TEM observations suggested that reserve deposition started at the immature stage of seed development.

Fresh brown seeds of P. pinnata are not dormant. They germinate quickly and would be expected to have an ‘opportunist’ germination strategy. During germination and seedling development, lipids and PBs of the seed break down to provide monomeric units to the growing seedling. The digestion of PBs may be of the more common internal type, the entire protein matrix breaking down uniformly. The digestion of PBs may also be of the external type, digestion beginning at the surface of the matrix in the form of numerous peripheral vacuoles that increase in size and fuse with each other, resulting in a large vacuole (Bhandari and Chitralekha 1984). The breakdown of PBs during germination and seedling development has been ascribed simply to the amount of water in the cells (Bain and Mercer 1966) or to the influence of the embryonic axis (Varner et al. 1963; Opik 1966; Penner and Ashton 1967; Guardiola and Sutcliffe 1971; Yomo and Srivivasan 1973; Halmer et al. 1978; Kern and Chrispeels 1978). Davies and Chapman (1979) have suggested that the seed coat might also have a role in controlling protein hydrolysis during seed germination in Cucumis. Thus, views concerning the source of the factor initiating PB degradation are controversial. Our ultrastructural study in P. pinnata seeds during germination and subsequent seedling growth indicates that mature seed constituents such as protein and oil are degraded and utilized to support the growth of the embryonic axis.

Conclusions and forward look

This study is a prelude to more detailed physiological studies on the production, storage and subsequent breakdown of seed reserves in P. pinnata. Here we focused on the synthesis of reserves from the beginning of their deposition to seed maturity. The pattern and type of reserves accumulated suggest continuous ongoing development without the intervention of drying or developmental arrest. The SEM and TEM micrographs gave no evidence of oleosin membrane cotyledonary storage cells. The findings provide a solid foundation for future identification and characterization of seed storage lipids and protein and, ultimately, of genetic engineering to introduce desirable characteristics: for example, to improve the protein nutrient value of seed residue after lipid is extracted for biodiesel production. There is still much to learn about the identity and function of storage proteins in biochemistry and plant development. A thorough understanding of the regulatory mechanisms controlling oil seed reserve deposition, stability and breakdown is now in prospect.

Additional information

The following additional information is available in the online version of this article:

Figure. Scanning electron micrographs of transverse section of outer cell wall of the cotyledons at four developmental stages of seed maturation: (A) early immature stages (90–135 DAF); (B) late immature stages (180–
225 DAF; (C) early mature stages (225–270 DAF); (D) late mature stages (315–350 DAF). CW, cell wall.

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All the authors contributed to a similar extent overall and each author has seen and agreed to the submitted manuscript.

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Conflict of interest statement
None declared.

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