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Ontogenic development and structure of the embryo, seed, and fruit of *Jatropha curcas* L. (Euphorbiaceae)

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ABSTRACT

Jatropha curcas L. is a species from the Euphorbiaceae family, native to Mexico and Central America. This species has been a focus of recent research because its seeds can be used as a source for biodiesel production. In this study, we evaluated the morphological characteristics of development from ovary to fruit, and from ovule to seed, as well as early stages of embryo development. The results show that pollen grains have hexagonal and polygonal patterns. At 15 days post-anthesis (DPA), the early globular and cotyledonal embryo was observed; likewise, the caruncle around the micropyle exhibited an increase of body oils in the first 15 DPA. Ripening of fruits started at 40 DPA, but the seeds' testa became hard at 35 DPA. We show in this research new insights into fruit and seed development of *J. curcas*, which could be useful for genetic improvement of the species in order to produce more and better oil for biodiesel production.

1. Introduction

Jatropha curcas L. (Euphorbiaceae) is a native species from Mexico and Central America (Ovando-Medina et al., 2011); however, it is currently cultivated worldwide (Heller, 1996). The species has been a focus in the study of plants that can be used as a source for biodiesel production. Several studies suggest that the oil present in the *J. curcas* seeds can be used to produce biodiesel (Galaz-Avalos et al., 2012; Mukherjee et al., 2011; Parawira, 2010; Reddy and Pamidimarri, 2010) or biogas from seed cakes (Raheman and Mondal, 2012). Although many species have been suggested as an oil source, many of them, such as maize and sugar cane, among others, are important for food consumption as well (Loyola-Vargas et al., 2012). However, *J. curcas* can be used for oil extraction because this species does not compete with other plant crops (Galaz-Avalos et al., 2012).

J. curcas fruits present two or three seeds that contain between 20% to 40% oil and 22 to 35% protein, and the rest is mainly water and ash (Aminul Islam et al., 2012; de Oliveira et al., 2009; Gübitz et al., 1999; Hawash et al., 2009; Om Tapanes et al., 2008). The quality and quantity of the oil in the seeds of *J. curcas* depend on several factors, the most important of which is the full development of the fruits (Li et al., 2008; Xu et al., 2011). During the development of the seeds, environmental conditions can influence the biosynthesis and accumulation of the fatty acids' mixture present in the triacylglycerides (TAG) that form the oil storage inside the seeds (Canvin, 1965; Dombos and Mullen, 1992; Harris et al., 1978; Rotundo and Westgate, 2009).

Several investigations have been conducted around the floral biology of this species in order to understand its development (Bhattacharya et al., 2005; Rianti et al., 2010; Solomon Raju and Ezradanam, 2002; Wu et al., 2011). The only information available about the floral biology of this plant is that it is monoecious, with flowers consisting of five sepals and five petals. Male flowers are small and odorless, and they present ten stamens distributed in two series (Rianti et al., 2010; Solomon Raju and Ezradanam, 2002). On the other hand, female flowers are larger than their male counterpart, with a tricarpellary ovary and three apical spreading bifurcated stigmas (Solomon Raju and Ezradanam, 2002). Cultivated plants can bloom twice a year, with better water availability, and begin to bloom from June to May, with a second period from September to November around the tropical zones (Ghosh and Singh, 2008; Reddy and Pamidimarri, 2010). The male/female flower ratio reported in populations of this species ranges from 29:1 (Solomon Raju and Ezradanam, 2002) to 33:1 (Rianti et al., 2010). However, in a subhumid dry tropical environment (India), the reported ratio is 10:2 (Ghosh and Singh, 2008). New evidence demonstrates that the production of seeds can be increased, e.g., by the application of 1.42 μM 6-benzyladenine, which increases the ratio between female and male flowers in the inflorescences from 1:13.4 to 1:1.24 (Pan and Xu,

Abbreviations: DPA, days post-anthesis; FAA solution, formaldehyde, acetic acid, ethanol and water; PAS, periodic acid-Schiff; TAG, triacylglycerides.

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2011). Main pollinators for *J. curcas* include Hymenoptera (*Formicidae anoplolepis* and *Formicidae prenolepis*) and Apidea (*Xylocopa confusa*, *Apis cerana*, *Apis dorsata*). Others less frequently include members of Lepidoptera (*Graphium agamemnon*, *Ariadne ariadne*, *Junonia orithya*) and Diptera (*Eristalis tenax*) (Bhattacharya et al., 2005; Rianti et al., 2010; Solomon Raju and Ezradanam, 2002).

The first study on the structural features in early flower development was performed by Wu et al. (2011), who classified the development into 12 phases. The study was carried out before the flower anthesis, and the most significant discovery was the differences between the male tissues and the female flowers. According to the study, male flowers present unisexual tissues during floral development, while female flowers present bisexual tissues at early developmental stages that are degraded at late stages (Wu et al., 2011).

The aim of our study was to increase the knowledge about the ontogeny of fruits and seeds of *J. curcas* for a better understanding of triacylglyceride biosynthesis. In this study we analyzed the anatomical structure of both male and female flowers at different times (days) after anthesis, as well as the details of external pollen grain morphology, and the characterization of embryo development at early stage.

2. Materials and methods

2.1. Plant material

J. curcas is a perennial, deciduous, monoecious shrub or treelet. It grows up to three to five meters high and blooms during the summer season (July–August). Flowering and fruiting seasons may vary among tropical areas at the same latitude; also, variations can be observed at altitudinal levels even for the same species, as well as variations year to year (Giménez-Benavides et al., 2007; Lévesque et al., 1997). This is the main reason we specified that *J. curcas* individuals growing at CICY grounds bloomed from July to August in 2010 and 2011.

Normally, *J. curcas* produces racemes of mostly unisexual flowers with a central female flower surrounded by a group of male flowers (Abdelgadir et al., 2012; Solomon Raju and Ezradanam, 2002). The sampling of the biological material was accomplished during the flowering periods in the summers of 2010 and 2011. Three- to four-year-old plants from the Yucatan Scientific Research Center greenhouse were evaluated.

2.2. Floral bottoms labeling

Labeling of female flowers was carried out during the anthesis and development during 15 days post-anthesis (DPA). Three female flowers were collected every day, from three different plants; these were washed with a water dip and placed in either phosphate buffer or a fixing solution of formaldehyde, acetic acid, ethanol and water; 10:5:50:35 (FAA solution) (Ruzin, 1999). On the other hand, other female flower sets in anthesis were sampled during a period of 40 DPA. This experiment started at 15 DPA and ended at 40 DPA. Three fruits were collected every five days and the fresh and dry weight, length and width were recorded. The characteristics of the seeds were analyzed after they were separated from the fruits. The fresh and dry weights as well as the length were measured.

2.3. Histological analysis

To observe and document the changes in the internal structures of the female flowers during their development, i.e., from ovary into fruit and from ovules into seeds, samples of the ovaries after anthesis were collected every day, between 8 and 9 in the morning, during the first 15 days after the flowers opened. Samples were processed as previously reported, with some modifications (Arroyo-Herrera et al., 2008; Quiroz-Figueroa et al., 2002; Sánchez-Teyer et al., 2003). Every sample was fixed in FAA solution for 48 h and kept at 4 °C, rinsed with distilled water and dehydrated with ethanol (30, 50, 70, 85, 96 and 100%, v/v). At the beginning of each treatment, vacuum was applied for 15 min, and the samples continued in the solution for another 45 min. Before increasing the ethanol concentration, every treatment was repeated twice, with a new solution (Ruzin, 1999). The samples were embedded in resin (hydroxyethyl-methacrylate) according to the instructions of the manufacturer (Kit JB-A, Polyscience). Longitudinal sections (5 μm thickness) were cut with a microtome and acidified for 20 min with periodic acid (Sigma, 1% w/v), then rinsed with distilled water and stained for 8 min with the Schiff reagent [basic fuchsine 1% w/v (Sigma), in a solution of sodium metabisulfite 1.5% w/v (J. T. Baker), activated carbon 0.25% w/v (J. T. Baker) and HCl 15% v/v (Fermont)]. Then the samples were rinsed with distilled water twice (McManus and Mowry, 1960) and stained for 10 min with naphtol blue (Polysciences, 1% w/v in a 7% v/v acetic acid solution, Fermont) and, finally, rinsed twice with distilled water (Fisher, 1968). The observation and analysis of the structures was carried out with an Olympus (BX42) microscope provided with an UIS2 optical system, focus vertical stage movement, high color reproductivity LED light source and condenser Abbe (NA 1.1) for 4× –100× and photographed with a digital camera. The study was done in each of the three ovaries' collected samples.

2.4. Scanning electron microscopy (SEM)

For the SEM analysis, complete female flowers were collected at the same time as the samples for the histological analysis described earlier. The samples were processed according to Ruíz-May et al. (2011), with slight modifications. The samples were fixed for 48 h with glutaraldehyde (2.5% v/v) in a phosphate buffer (0.2 M; pH 7.3). The fixation was followed by a rinse with phosphate buffer (0.2 M; pH 7.3) (Ruzin, 1999) and the samples were gradually dehydrated with ethanol following the same protocol previously described for the histological analysis and dried with supercritical CO_2 (Balzers CPD 020 Critical Point Dryer; Bal-Tec, Schalksmuhle, Germany). The samples were mounted on metallic stubs with carbon conductive adhesive tape, sputter coated with colloidal gold and observed at 10–20 kV using a Zeiss DSM 940A scanning electron microscope (Zeiss, Oberkochen, Germany).

3. Results

3.1. Floral structure and morphology

J. curcas is a monoecious species, with both female and male flowers (Fig. 1a) in the same inflorescence; in addition to this fact, floral sex cannot be differentiated in floral buds (Fig. 1b). The elapsed time since the emergence of the floral buds until the beginning of anthesis was from six to seven weeks. On the other hand, the female as well as male flowers have five sepals and five petals (Fig. 1c, e). However, there is an evident difference between the flowers; first, in the female flowers, sepals and petals are spread out, while in the male flowers, sepals are shorter than those of the female flowers and the tips of the petals are rolled downward (Fig. 1c, e). Secondly, nectaries form a ring around the base of the ovary on female flowers, while in male flowers they are organized around the stamen filaments. Moreover, nectaries on female flowers are more conspicuous and have a heart-like shape; by contrast, in male flowers they are tiny and have an ellipsoidal shape. Thirdly, the base of the floral receptacle is two times larger on female flowers compared to those on male flowers (Fig. 1d, f). The stigmas present an intense green color during anthesis (Fig. 1d), which changes to yellow one day later and, by the third day, the stigmas are completely brown and senescent, probably due to the presence of phenols.

The analysis by SEM showed clearly the villose base of the petals in the female flower (Fig. 2a), as well as in the three pairs of bifurcated stigmas, characteristic of the female flower of *J. curcas* (Fig. 2b). The blue arrows show the bifurcation along the stigmas. Also, an abundance of pollen grains can be observed over the stigmas at the beginning of the

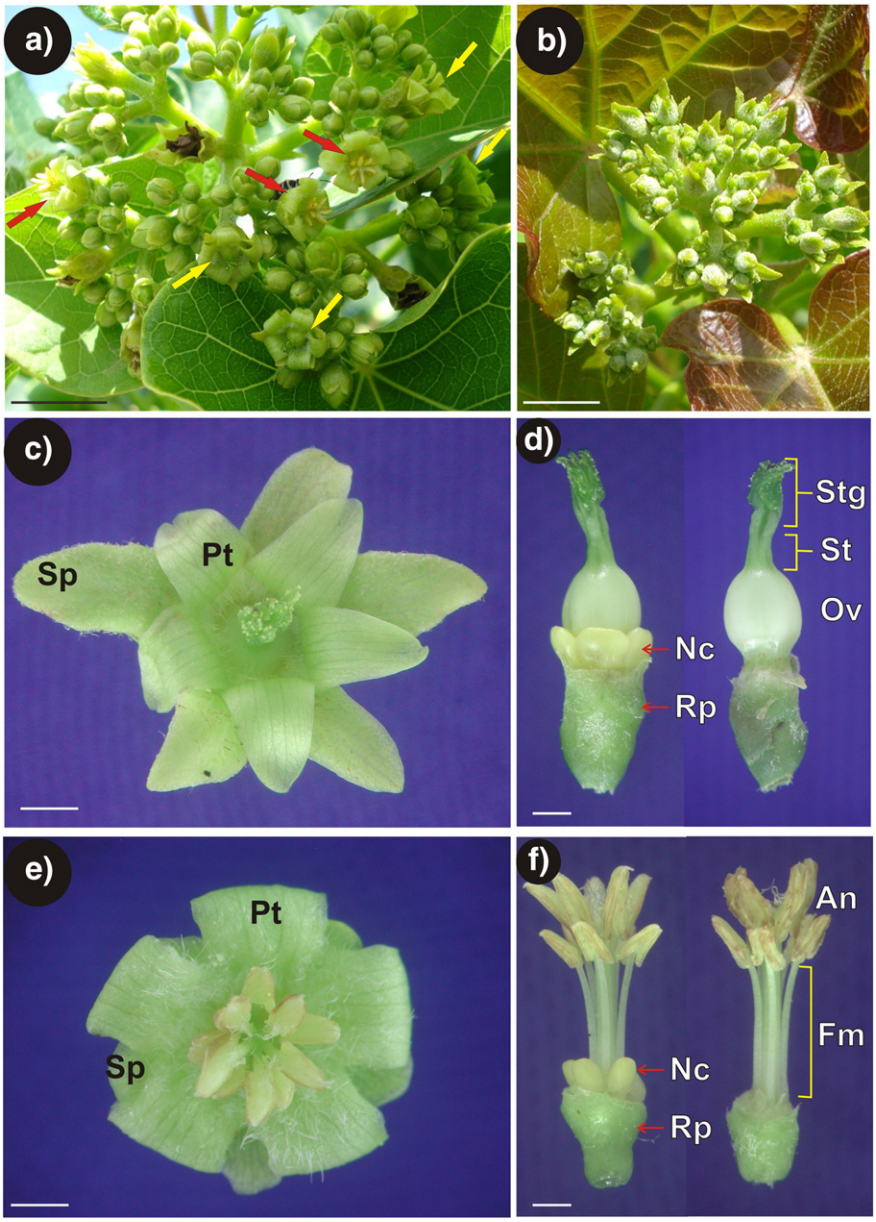

Fig. 1. Inflorescences and flowers of *Jatropha curcas*. a) Mature inflorescence (with male red arrows) and female (yellow arrows) flowers. b) Immature inflorescence; see the formation of different flower buds. At this stage, it is not possible to distinguish male from female flowers. c) Female flower. d) Structure of the gynoecium. e) Male flower. f) Structure of the androecium. Sp: sepal; Pt: petal; Nc: nectaries; Rp: receptacle; Stg; stigma; St: style; Ov: ovary; An: anthers; Fm: Filament.

anthesis (Fig. 2c). The stigmas form a fold at the base the ovary (Fig. 2d). The morphology of the pollen grains is as follows: the grain is non-aperturated and it has a large semi-spheroidal shape (diameter around 70 ± 5 μm). In addition, the pollen has a layer of exine with conspicuous verrucae (Fig. 2e); they are arranged in a regular polygonal or hexagonal pattern around a depression (Fig. 2f).

3.2. From ovary to fruit and embryo development

The fruit is a tricarpellary capsule with each carpel carrying one ovule. The morphological changes that take place in the ovary and the ovule lead to the formation of the capsule and seeds (Fig. 3). Five DPA, the petals and sepals start to age and the stigmas lose their shape and turgor (Fig. 3a, b). Ten DPA, the petals began to dry and the stigmas are totally closed and brown (Fig. 3e, f). At the end of the study (15 DPA), the flowers and stigmata were all fully dry (Fig. 3i, j). The histological studies showed that after the first five DPA the embryo is already in place (Fig. 3c, d, g, h, k, l). Inside the embryonic sac, it is possible to differentiate the caruncle, the funiculus and the internal and external integumental tissues (Fig. 3c). After the formation of the different tissues of the fruit, the maturation process begins and the formation of the early globular (Fig. 3h) and the cotyledonal embryo (Fig. 3l) can be recorded in the three carpels where each ovule was fertilized.

During the formation of the zygote in flowering plants, occur two fertilization events, one of them leading to the production of the zygote and the other to the formation of the endosperm, a triploid tissue. Following this initial occurrence, the ovule develops into a seed and the ovary differentiates into a fruit (Goldberg et al., 1994).

Early embryogenesis is the critical differentiation phase and the basic characteristics of the plantlet are established (Lau et al., 2012). The embryo contains the axis and the cotyledon. Both are composed of three primordial tissues, the protoderm, procambium and ground meristem, which, during the development of the seedling, will

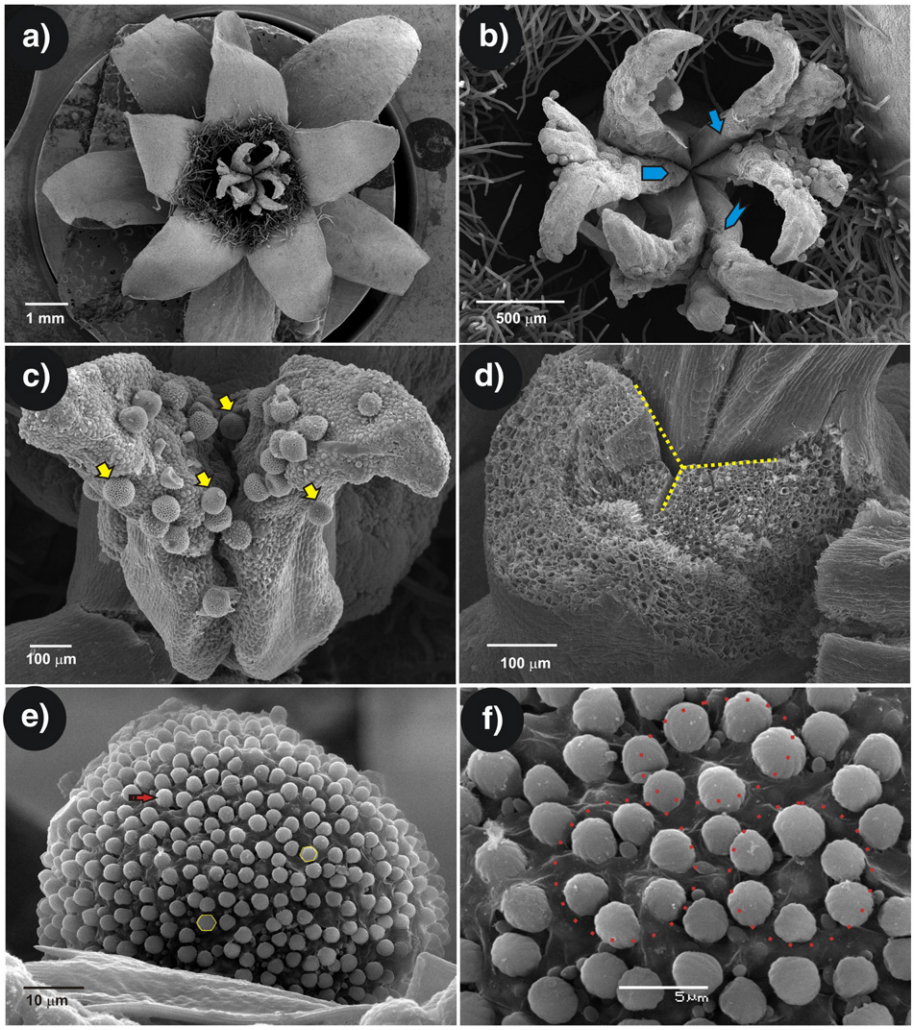

Fig. 2. Scanning electron microscopy of a female flower in anthesis of *Jatropha curcas*. a) Panoramic view of the female flower in anthesis; it can be observed that the petal base is full of trichomes. b) Pair bifurcate stigma. The blue arrows indicate a pair of stigma. c) Stigma with pollen grains. The yellow arrows indicate grains of pollen. d) Base of the style. The dotted yellow lines indicate convergence point of the stigma pair. e) Pollen grain; hexagonal or spherical shapes can be distinguished. The red arrow indicates verrucae structure and the yellow line the hexagonal shapes of these. f) Closeup of a grain of pollen; the dotted red circles indicate the distribution patterns.

become the epidermal, vascular, and parenchyma tissues, respectively (Goldberg et al., 1994).

The early polarization of the zygote is unknown. However, it has been established that the expression of WOX family transcription factors and the transport of auxins are factors in the healthy differentiation of the zygote into an embryonic axis (14,784). In our laboratory, we found that the indol-3-acetic acid biosynthesis and that of their conjugates play a fundamental role during the induction of somatic embryogenesis in *Coffea canephora* (Ayil-Gutiérrez et al., 2013).

One DPA (Fig. 4a), the ovule is visible inside the embryonic sac as well as the integument, which later will originate the seed coat. After five DPA, these structures were pale green and almost oval in shape (Fig. 4b). Five days later, seeds have already fully formed inside the ovary (Fig. 4c) and the capsule weighs on average 51.9 mg ± 0.28. The tissue that will later form the endosperm is completely visible at ten DPA (Fig. 4c).The carpels, with their seeds inside, have grown, and the embryo becomes visible inside the seeds (Fig. 4d). The seeds are now larger and completely white (data not shown). Fifteen DPA (Fig. 4d), the fruits are fully formed and the capsules have increased almost eleven times their weight (564 mg ± 0.20), and the color has changed from green to dark green. The carpels now contain seeds completely formed, and the structures of the seeds are visible and their color becomes yellow.

In addition, in the soft and fleshy structure known as the caruncle, the lipid content increases as the seeds develop (Fig. 4e–h). This structure will form the elaiosome (lipid-rich structure) in the seeds; this is a small, hard, brownish white and lobulated structure with a rich oily body center. Although we observed the embryo from a very early stage of development (Fig. 4i), it is not until 30 DPA (Fig. 4j) that the different seed components, such as the cotyledonary leaves, can be observed. The embryo is fully developed by 30 DPA (Fig. 4k, l).

3.3. Ontogeny development of fruits and seeds

The fruits and seed growth follows a typical S curve. For fruit and seed fresh weight, the growth exponential phase began 15 DPA (Fig. 5a, b), and dry weight at 25 DAP (Fig. 5b). The stationary phases were reached by 35 DAP in both cases. At the same time, a series of external morphological changes occurred in the fruits (Fig. 5c, upper row), with the skin fruit turning yellow, indicative of the beginning of the physiological ripening.

The seeds are soft and have high water content during the first 25 DPA. A few days later, the maturation process begins and the testa starts to gain a rigid consistency. After 35 DPA, the seeds turn brown and acquire a solid consistency (Fig. 5c, lower row).

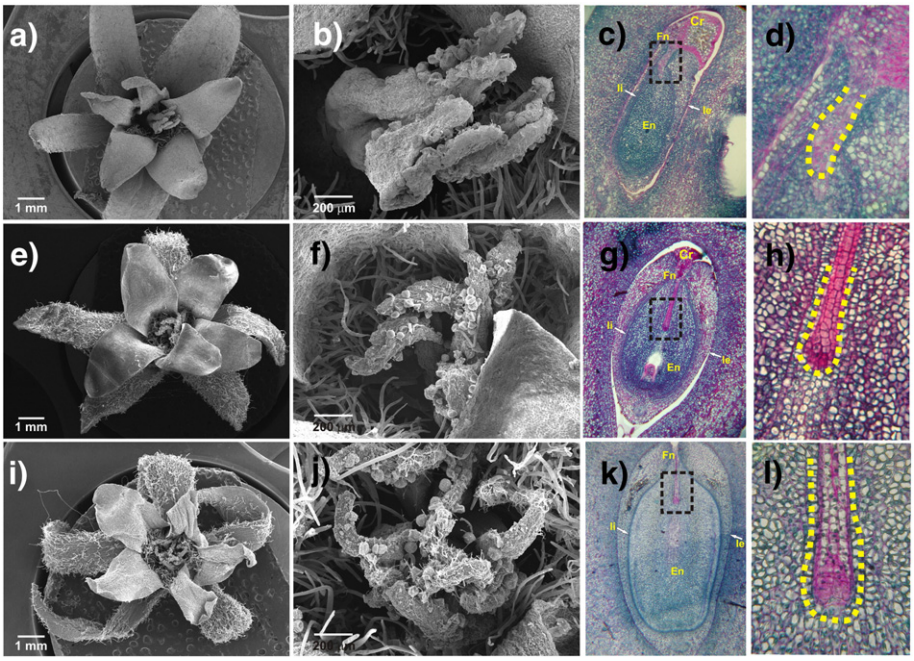

Fig. 3. Morphological changes and development of the zygotic embryo. a–d) Five DPA; e–h) 10 DPA; i–l) 15 DPA; a, b, e, f, i, j) scanning electronic microscopy. c, d, g, h, k, l) histological sections. d, h, l) close up from c, g, k, respectively. The dotted lines show the development of the embryo inside the ovule. Cr: caruncle; Fn: funicule; Ii: inner integument; Ie: outer integument; En: endosperm.

4. Discussion

Several reports on floral morphology describe the general structure of the flowers of *J. curcas* (Bhattacharya et al., 2005; Rianti et al., 2010; Solomon Raju and Ezradanam, 2002; Wu et al., 2011). There are important differences between female and male flowers, such as the nectaries; in the female flower they are larger than those on male flowers, containing 4.54 ± 0.82 µL and 1.92 ± 0.44 µL of nectar, respectively

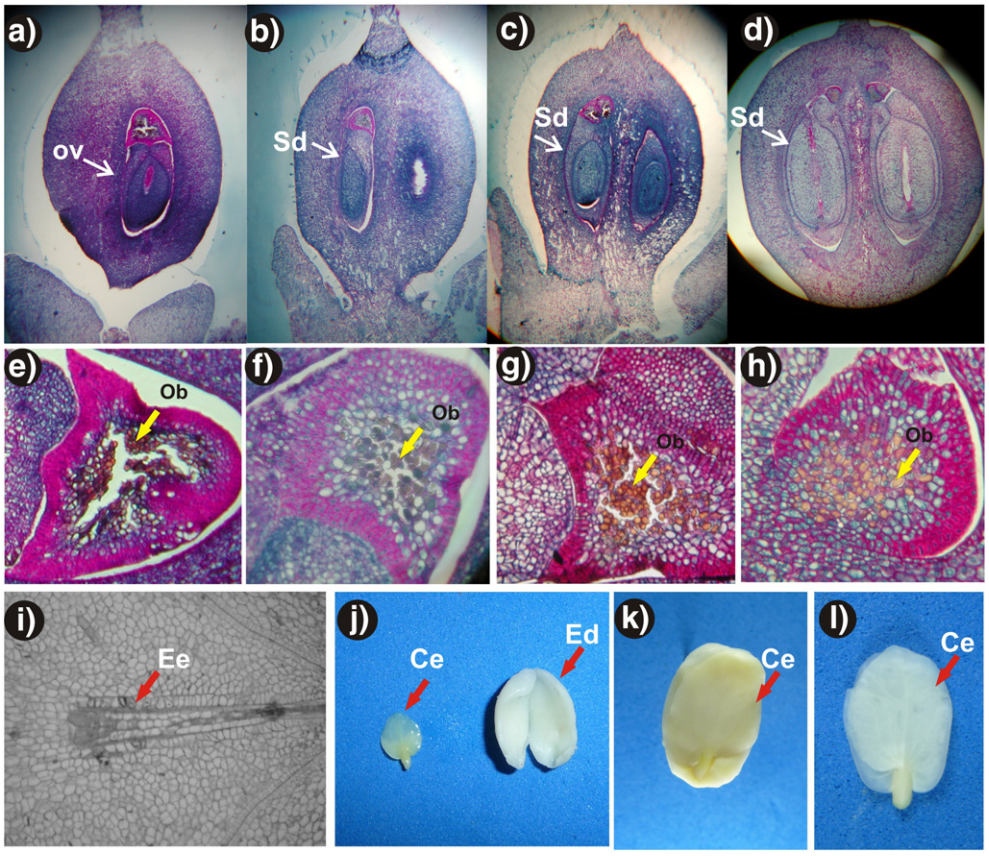

Fig. 4. Seed and embryo development. a, e) 1 DPA; b, f) 5 DPA; c, g) 10 DPA; d, h) 15 DPA; e–h) Lipid accumulation in the caruncle of the seeds. i) Embryo in early stage of development. j, k, l) Embryo in mature cotyledonal stage. Ov: Ovary; Sd: Seed development; OB: Oil body, yellow arrows. Ee: Early embryo stage; Ce: Cotyledonal embryo; Ed: Endosperm.

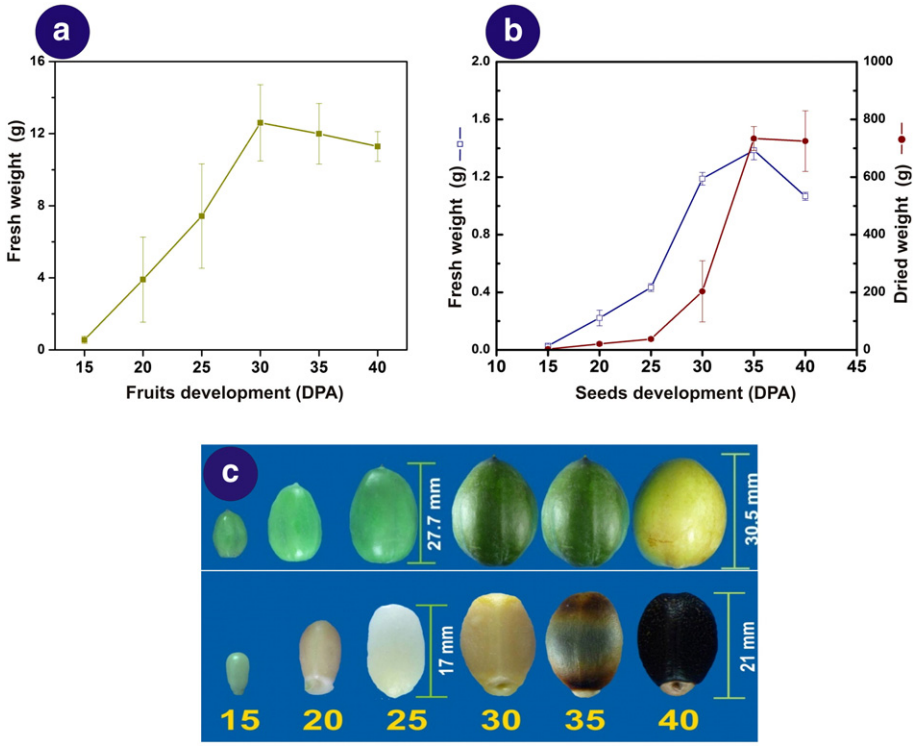

Fig. 5. Development and morphology of fruits and seeds of *Jatropha curcas*. a) Fruit growth. b) Seed growth. c) Upper row, fruits at different stage of ripening.; lower row, the ontogeny of seed changes. For graph A, $n = 3$ at each day post-anthesis. For graph B, $n = 9$ for each day post-anthesis.

(Bhattacharya et al., 2005). Nectar, as well as pollen, is the main pollinator reward (Proctor et al., 1996).

The fate of the female flower, as well as of the pollen, has a profound effect on fruit and seed production. We observed that the stigmas are viable for two or three days, and drop off mostly on the third day, as has been previously reported (Solomon Raju and Ezradanam, 2002). On the other hand, the high pollen/ovule ratio (6332:1) is common on xenogamous species (Cruden, 1977), ensuring enough pollen to fertilize the ovules (Solomon Raju and Ezradanam, 2002). In this study, we observed that pollen grains of *J. curcas* shared some characteristics with other Euphorbiaceae members, such as pollen shape and external verrucose ornamentation (Miller and Webster, 1962; Park, 1997).

Fruit and seed development depend on the pollen viability, levels of self-compatibility and its capacity to germinate. For successful fertilization, the pollen must germinate on the stigma, grow through the style and find and penetrate the micropyle of the ovule (Wang et al., 2002). Abdelgadir et al. (2012) have demonstrated that the pollen tube enters the ovary during the first 8 h post-pollination. In the present study, it is shown that there is an abundance of grains of pollen on the female flowers and the evidence suggests that effective fertilization could be carried out earlier than previous reports, within the first three DPA.

It has been reported in some Euphorbiaceae that the ovule is anatropous, bitegmic and crassinucellate (Bertechine Gagliardi et al., 2012; Khasim et al., 2013; Krishnamurthy, 2013). In *J. curcas*, seeds originate from an anatropous, bitegmic and crassinucellate ovule, and contain a short funicle. The micropyle is formed by both inner and outer integument. After the fertilization, the ovule expands rapidly due to the proliferation and growth of the maternal tissue cells. The maternal tissues grow very fast and lead to an early increase in the length of the endosperm. The endosperm is essentially nucleus-free from five to ten DPA (Greenwood et al., 2005), and it becomes primarily cellular by 15 DPA. The cotyledons grow through the central space of the successively expanding cellular endosperm from 10 to 15 DPA. During the development of the seeds, the cells of the nucellus die, while the cellular endosperm, the major seed storage organ, expands and begins to accumulate reserves. Greenwood et al. (2005) have proposed that the destruction of the nucellus and the integument of the seeds is a developmentally programmed cell death.

Krishnamurthy (2013) recently described the microsporogenesis, microgametogenesis and megasporogenesis of *Jatropha* species, including *J. gossypiifolia* and *J. curcas*. The results in the present study showed that in *J. curcas* the formation of the embryo in early globular and cotyledonal stage occurs between five and 15 DPA. In *Brassica napus* L. (Brassicaeae), it has been observed that the zygotic embryos reach the heart-shape stage 15 DPA and after 24 DPA the embryo is completely formed (Crouch and Sussex, 1981). The development of the closely related *Ricinus communis* L. (Euphorbiaceae) zygotic embryos is very similar to that of *J. curcas*. Globular and cotyledonal-shaped embryos can be observed after 10 and 25 DPA, respectively (Greenwood et al., 2005). Soon after fertilization in *J. curcas*, the seeds have a yellow color, in contrast with those seeds on *R. communis*, which are pale green (Botega Baldoni et al., 2010), as well as in *Brassicanapus* (Yu-Qing and Yan, 2009).

Members of the Euphorbiaceae family display an explosive seed dispersal mechanism (Narbona et al., 2005) based on the presence of the caruncle. The differences in explosive dispersal distance between species seem to depend on both seed mass and caruncle retention. In addition to this mechanism, some members of the family, like species of *J. curcas*, have a secondary method to disperse the seeds. This secondary mechanism is in general insect-dependent (Bhattacharya et al., 2005; Rianti et al., 2010; Solomon Raju and Ezradanam, 2002), mainly including members from the order Hymenoptera. The seeds of *J. curcas* have a lipid-rich caruncle that functions as an elaiosome, attracting ants and initiating a myrmecochorous secondary dispersal mechanism (Berg, 1975). We observed that oily bodies develop very early (one DPA) in the caruncle of *J. curcas*. The presence of these structures increases sharply, until they completely fill the elaiosome.

Recently, some characteristics of the *J. curcas* seed structure have been described. The cells of the testa are walled and unlignified, and the outer epidermis is made of narrow columnar cells with slightly

thickened and pitted radial walls and dark brown content (Fig. 5c). The inner epidermis generally is formed with short, thin-walled, columnar cells (Khasim et al., 2013). These observations are consistent with the observations made in this study (Fig. 5c); 40 DPA the testa of the seeds is dark brown, suggesting that the seeds have reached their total maturity as it has been also reported by Rao et al. (2008). By contrast, in *R. communis*, also a member of the Euphorbiaceae family, the fruits reach their maturity 60 DPA (Botega Baldoni et al., 2010).

We observed three main stages in the seed development of *J. curcas*. The first stage is characterized by high cell division, with the development of zygotic embryogenesis. The second stage consists on cell expansion, when proteins and lipids are accumulated and water loss initiates. This result is consistent with previous observations (Xu et al., 2011) that during first developing stages, seed growth is gradually slow with a relative high increase of fresh weight towards more advanced stages. During the last and third stage, seeds stop growing and begin the process of desiccation, with a simultaneous increase of lipid accumulation in the storage tissues achieving a maximum value (data not shown).

In conclusion, this study provides new insights into fruit and seed development of *J. curcas*, opening the possibility to study the proteome and genomic interactions of the pollen and stigmas, as well as the biosynthesis of fatty acids that are used later in the TAG synthesis during the ontogeny of the seeds. This knowledge could lead to a better understanding of the reproduction of *J. curcas*, which must lead to its breeding improvement in order to produce new varieties with potential resistance to diseases and a better oil production.

Author contribution

CNCY carried out the morphological, histological, and electron microscopy studies and drafted the manuscript. FAB helped to perform the electron microscopy studies. IMRM helped to draft the manuscript. VMLV conceived of the study, participated in the design of the experiments and drafted the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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