

Zygotic development and endosperm dynamics in *Hevea brasiliensis*: Progress, challenges and emerging opportunities of in vitro pollination and fertilisation

Rekha Karumamkandathil

rekha202052@gmail.com

Rubber Research Institute of India <https://orcid.org/0000-0002-2502-1583>

Jayasree Parukkuttiyamma Kumari

Rubber Research Institute of India

Vinoth Thomas

Rubber Research Institute of India

Gireesh T

Rubber Research Institute of India

Raheena K T

Rubber Research Institute of India

Athira P V

Rubber Research Institute of India

Jithu George

Rubber Research Institute of India

Alex Raju

Rubber Research Institute of India

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Abstract

Hevea brasiliensis is the primary source of natural rubber, a vital raw material with widespread applications across various industries. Owing to the consistent rise in demand for this strategic commodity, enhancing productivity remains as a key objective in rubber breeding. However, genetic recombination breeding in *Hevea* faces significant challenges, including seasonal flowering, low fertility, early fruit drop etc. These factors limit the production of sufficient full-sib families available for effective selection and thereby increase the cost and effort of artificial pollinations. Enhancing fruit/seed set is particularly important in a highly heterozygous perennial crop like *Hevea*. This study was aimed to investigate the fertilization and fruit set processes in *Hevea brasiliensis*, identify various factors contributing to low fruit set and explore the feasibility of an *in vitro* fertilization system to generate a higher number of desirable recombinants. The research was conducted using flowers and fruits from mature trees of clone RR II 105. Key factors such as age of the flower, methods of pollination, and culture media for fertilization and embryo formation were examined. Successful fertilization and embryo formation under *in vitro* conditions were demonstrated in *H. brasiliensis* for the first time. Additionally, the study provides novel insights into embryo and endosperm development under both *in vitro* and *in vivo* conditions, shedding light on potential causes of low fruit set. The findings will be beneficial for breeders, in the recovery of hybrid seeds especially from difficult crosses of breeding programs. The protocol developed in this study can be utilized for the consistent and sustainable development of hybrid embryos into seedlings from different cross combinations.

Key Message

The process of Pollination, fertilization, Zygotic embryogenesis, endosperm development and seed formation were studied under *both in vitro* and *in vivo* conditions in *Hevea brasiliensis*. Poor endosperm development was identified as a major factor contributing to the low seed set in this species. *In vitro* pollination experiments were successful, leading to fertilization and subsequent embryo formation. Under *in vitro* conditions, there is higher possibility of recovery of more number of hybrids, since the embryos without endosperm development can also be rescued.

Introduction

The Para rubber tree (*Hevea brasiliensis* Muell. Arg.) is a commercially important species known for producing high-quality natural rubber latex, a strategic raw material with diverse industrial application (Fig. 1). Among the 7500 species of rubber yielding plants, *H. brasiliensis* is the major contributor towards the global production of natural rubber. The demand for natural rubber always shows an escalating trend and improving the productivity through developing most suitable clones with enhanced yield coupled with the prevailing biotic and abiotic stress tolerance is the need of the hour. Tree requires at least 4–5 years to attain the ripeness to blooming and retains the capacity thereafter.

The recombination and selection cycles orienting towards enhanced growth and latex yield as well as the introgression of genes from wild populations to widen the genetic base through conventional crossing in rubber tree is cumbersome and laborious due to several factors like low fruit set, seasonal flowering, inaccessibility of flowers and disease infestations.

High yielding rubber clones are being selected from breeding populations consists of mainly full-sib progenies, through lengthy conventional crossing followed by selection cycles (Simmonds, 1989; Licy et al. 2003; Priyadarshan and Clemet-Demang, 2004). Low fruit set, seasonal flowering, non-synchrony and inaccessibility of flowers and disease infestations are the major constraints in *Hevea* breeding. Low fruit set prevalent in the female parents often results in the loss of numerous potentially good cross combinations in breeding programs. Consequent reduction in the family size and recombination frequency of full-sib families results in inefficient selection, thus limiting the progress of crop improvement (Mydin et al. 1989). Hand pollination program is labor intensive, and the number of crosses that can be done in a season is limited due to the inaccessibility of flowers and non-synchrony (Gireesh et al. 2008). Despite the efforts to circumvent these problems, the fruit set could not be raised beyond five percent (Mydin 1989). On the other hand fixing of elite F1 genotypic selections through vegetative means could capture promising gene combination, which is considered as the major advantage of *Hevea* breeding.

The presently cultivated rubber clones in Asia and Africa which contributes towards natural rubber production globally are successors of seedlings descended from Brazil to South-east Asia (Wickham collection) through Kew Garden and later undergone several cycles of breeding and selection and hence most of the present cultivars had a narrow genetic base. Reports suggests that the yield levels achievable through this currently popular Wickham gene pool is limited (James et al. 2021), warranting wide/interspecific hybridization and introgression of genes from wild populations to widen the genetic base through conventional breeding. Under such circumstances better recovery of hybrid seeds becomes inevitable and more imperative. The present study aims to examine the reasons for low fruit set and develop alternate strategies for increasing the recovery of recombinants.

b. A new flush with immature inflorescence developed from the axils

Flowering in *Hevea* is seasonal following the process of defoliation and refoliation during winter. Rubber tree is monoecious with both staminate and pistillate flowers in the same inflorescence which are developed in the axils of basal leaves of newly developed shoots (Fig. 2a &b). Extend of flowering and seed set largely depends on the geographical location and the prevailing climatic factors. Generally flowering occurs during March-April in the northern hemisphere and September to October in the southern hemisphere (Priyadharsan 2011). In Kerala flowering follows wintering during the period from January to March. Flowering is dichogamous with incomplete protandry. Male flowers mature earlier than female flowers. The emergence of few male flowers after the maturation of female flowers makes the protandry incomplete. Female flowers are comparatively fewer and larger than male flowers and are born at the end of main branches and central axis. They are pentamerous with tricarpeal ovary (Fig. 3).

Male flowers are numerous and smaller and seen in other parts of the panicle. The conjoined anthers often arranged in two rows over a staminal column. According to previous studies (Rao, 1961), *Hevea* is insect pollinated mainly by midges belonging to Ceratopogonidae and predominantly out crossed.

immature female flowers

1. b. Developing immature seeds separated from the ovule
2. c. T.S of *Hevea* fruit with three developing seeds

Mature *Hevea* fruit is a three lobbed capsule with a woody endocarp, a leathery mesocarp and contains three seeds (Fig. 4a,b&c). It will take 140 days from pollination to seed dispersal (Priyadarsan, 2011). Under open pollination, among the numerous female flowers developed, only 25% form fruits and among which only 25% will reaches maturity. In controlled pollinations pollinator ensures maximum pollination, the success rate varies from 5–10 percent. In the present scenario of unprecedented climatic vagaries the availability of flowers for hand pollination itself is less. Since the peak flowering time coincides with the hot summer in Kerala which makes hand pollination difficult. The inaccessibility of flowers is another problem. Practically it is possible to do only about 70–100 pollinations per person per day on the tree top. The maximum possible pollinations that can be achieved in a season are nearly 5000–6000. That will account for around 250–300 hybrid seeds per year. In order to carryout effective selection, the family size is important. In a heterozygous species, large family size ensures maximum combinations of alleles and the best combination can be identified and selected. Thus, it is of high importance to study the fertilization event under *in vitro* conditions with a view to explore the possibilities of *in vitro* fertilization and to discuss its implications for optimizing the recovery of hybrid plantlets.

The technique of *in vitro* pollination and fertilization is a novel approach that has been used for overcoming pre-fertilization barriers (Rangaswamy and Shivanna, 1967; Valsala et al. 1996) and post fertilization barriers (Sahijram, 2013). *In vitro* pollination and fertilization system has been accomplished in more than 57 species belonging to 14 families including Brassicaceae, Caryophyllaceae, Liliaceae, Papaveraceae, Primulaceae and Solanaceae (Zentkler, 1992 and 1994). Various methods like developing interspecific and intergeneric hybrids (Zentkler, 1980; Chin et al. 1997), generation of haploids (Hess and Wagner, 1974; Musiał and Przywara, 1998), and pollen tube-female gametophyte interactions and the cellular events connected to fertilization and embryo development (Dupuis and Dumas 1989; Fernando et al. 1998) have been explored. Additionally, since the IVF system allows pollen and female gametophyte to be maintained in culture, for long periods, thus genetic transformations can be carried out on sperm or eggs coupled with hybridization (Fernando et al. 1998). Even though a vast literature is available on IVF systems in other plant species, *in vitro* fertilization studies in *Hevea* is meagre. A successful IVF system is relevant in a highly heterozygous perennial crop like *Hevea* where the breeding programmes are often delayed due to the seasonal flowering, low fruit set and early fruit drop. In this context, the present study was undertaken to elucidate the dynamics of zygote and endosperm development in *Hevea brasiliensis*, and to identify potential post-fertilization barriers inherent to the species. Based on insights gained from preliminary investigations (Rekha et al. 2002) further

experiments were conducted to standardize an *in vitro* pollination and fertilization system for *Hevea brasiliensis*.

Materials and Methods

1. In vitro pollination and fertilization

a. Pollen pistil interaction studies

Before going for *In vitro* fertilization experiments, we have studied the pollen pistil interaction after pollination under in vitro conditions. The fluorescent technique reported by Kho and Baer (1968) was adopted for pollen pistil interaction studies. The pollinated flowers were fixed in FAA (Johansen, 1940) at different intervals 12, 24 and 48 hours after pollination. After 24 hrs, the buds were transferred to 1N NaOH for eight hours at room temperature. After thorough washing with distilled water, the buds were stained in 0.1 per cent aniline blue in 0.1 N K_2HPO_4 for period of 18 hrs. The pistil was later macerated in 90 percent glycerol. *In vivo* pollen tube growth was then examined under the fluorescent microscope.

b. Standardization of pollination techniques in vitro

Age of the flower bud

The experiment was performed using the flowers of the Indian clone RR11 105. Female flowers at different developmental stages from one to three days before the anthesis (1DBA, 2 DBA, and 3 DBA), anthesis day (AD), and one day after anthesis (1 DAA) were used for *in vitro* pollination. Male flowers bearing pollen grains at the late uninucleate to early binucleate stage were selected for the experiment. Flower buds were collected in the early morning and washed in running tap water for three minutes. They were then sterilized using 0.1% $HgCl_2$ for five minutes and then thoroughly washed with sterile distilled water 4–5 times. The flowers were then blotted on a sterile filter paper to remove the moisture. Anthers were dissected from surface sterilized male flowers using fine forceps. The flowers were then blotted on a sterile filter paper to remove the moisture. Anthers were dissected from surface sterilized male flowers using fine forceps.

C. Method of pollination

In vitro self-pollination was done using different techniques described by Bhojwani and Razdan (1983) viz., (i) *In vitro* stigmatic pollination: the female flowers after sterilization were inoculated as such in the nutrient medium. Anthers dissected from the male flowers were placed on the stigma of the female flower; (ii) Intra ovarian pollination: After surface sterilization, the perianth was completely removed, and the stigma was excised to expose the ovary. Pollination was carried out directly on the cut surface of the ovary. (iii) *In vitro* placental pollination: Following the careful removal of the perianth, stigma, and ovary wall, the placenta bearing the ovules was exposed. Pollen was then applied directly onto the ovules under sterile conditions. (iv) *In vitro* ovular pollination/test tube fertilization: The perianth, stigma and the

ovary wall were removed carefully and the ovule supported by the funicle were isolated and inoculated in the medium. The anthers dissected from male flowers were placed close to the ovules. Test tube fertilization was performed as described below in four different ways. *Method 1:* The ovules were inoculated in the medium and the anthers were kept aside. *Method 2:* Ovules were inoculated in the medium and anthers were kept aside and few drops of sucrose solution were poured over them. *Method 3:* Ovules were inoculated in the medium and pollinated with anthers and then few drops of pollen germination medium were applied over them. *Method 4:* The ovules were immersed in 1% CaCl_2 for one day and pollinated with freshly isolated anthers. The pollinated flowers/ovaries/ovules were cultured on media in Petri dishes and sealed with Para film and incubated under dark conditions at $24 \pm 2^\circ\text{C}$. Unpollinated flowers/ovaries/ovules were also inoculated in the same culture medium as control. After one week, the swelled ovaries were counted as fertilized and they were transferred to new culture media in Petri dishes for embryo development and sealed with Para film and incubated under dark condition at $24 \pm 2^\circ\text{C}$. Observations were recorded at weekly intervals.

2. Culture media

The basal salts of MS, WPM (Lloyd, 1980) and N6 (Nitsch and Nitsch, 1969) were experimented initially, for the growth of fertilized ovules. Based on the swelling of ovules, the basal medium was fixed and varying concentrations of sucrose from 3–18% were experimented. After fixing the sucrose level, combinations of growth regulators such as BA, KIN, GA_3 , NAA and 2, 4-D were tried for the growth of fertilized ovules.

3. Histological studies

The ovules showing morphological similarities with *in vivo* seeds were recovered and fixed in FAA and processed for microtome sectioning. For general histology, sections were stained with Periodic Acid Schiff's reagent (Jensen, 1962). For detecting starch, I_2KI treatment and to localize lipids, Sudan IV dye (Johansen, 1940) was used.

4. Comparison of *in vitro* and *in vivo* fertilised ovules

a. Performance of in vitro and in vivo fertilised ovules in culture

In order to compare the growth of *in vivo* and *in vitro* fertilised ovules under *in vitro* culture conditions, *in vivo* pollination was performed as per standard procedure (Mydin and Saraswathiyamma, 2005) and the pollinated flowers were collected after three days and sterilized as described in the session 3.b. Ovules were isolated from these flowers and inoculated in the same medium and growth was observed.

b. Growth of the fruit, endosperm and embryo under field conditions

Fruits at different maturity were collected from the trees and, the developing ovules were dissected and observed for embryo/endosperm development, in order to study the endosperm growth under field

conditions. The developing seeds were collected from fruits of different maturity and cultured in nutrient medium and observed for the development of embryo and endosperm.

c. Growth and development of immature seeds collected from the field grown trees and cultured *in vitro*

Open pollinated fruits from the clone RR11 105 at different stages of maturity were collected and inoculated in media following *half ovulo* embryo culture (Rekha et al. 2015).

Results and Discussion

1. In vitro pollination and fertilization

a. Pollen-Pistil interaction

Success of pollination depends on the sequential events starting from the acceptance of pollen by the stigma and a complex and cooperative interaction between viable pollen and a receptive pistil followed by pollen tube growth through the pistil to the ovule. The pollen pistil interaction studies indicated that there is profuse development of pollen tube after pollination and it enters the ovules 24–48 hours after pollination (Fig. 5) in all the samples tested. These results are in conformity with earlier reports by Sedgley and Attanayake (Sedgley and Attanayake, 1986). In flowering plants, the transport of non-motile sperm to distant egg cells is required for effecting double fertilization. The pollen tube penetration of the pistil and maintenance of structural integrity of the pollen tube while moving to the destination following the positional guidance cues is required for successful fertilization event. During the penetration of pistil, the pollen tube encounters numerous physical barriers preventing the ultimate fusion between male and female gametes. Studies on pollen- pistil interactions serve as a distinctive model for elucidating the mechanisms of plant cell to cell interaction and signaling, which are critical for successful fertilization. In the present study, the sufficient growth of pollen tube ensures the absence of any pre-fertilization inhibitors in the stigma/style leading to absence of seed set.

b. Age of the flower buds

Among the five developmental stages assessed for in vitro pollination, the highest percentage of ovule swelling, an indicator of successful pollination, was recorded in female flowers collected one day before anthesis (1 DBA) at 79.2%, followed closely by those collected on the day of anthesis (AD) with 75.2% success (Table 1, Fig. 6a & b). Flowers collected one day after anthesis (1 DAA) showed a moderate success rate of 58%. In contrast, significantly lower percentages were observed in flowers collected 2 and 3 days before anthesis, indicating these stages were less responsive to in vitro pollination. This indicates that the stage of the flower is an important factor in determining the success of fertilization and it is highly specific. Immature flowers are not suitable for pollination. Fertilization success is largely determined by stigma receptivity, with the timing and duration of receptivity playing a critical role in effective pollination and subsequent seed set. It may be assumed that lack of stigma receptivity may be

the reason for low fertilization success in immature flowers. In contrast, higher number of seeds was produced in *Lilium* when the flowers three days prior to anthesis were pollinated (Van Tuyl et al., 1991). Stigma receptivity is a species character and it varies among different species of same genus. In Eucalyptus, the stigma receptivity varies from 5 to 10 days among different species (Pauldasan et al 2023). Flowers on the day of anthesis or one or two days after anthesis improved seed set in crops like Ginger, Papaver, Nicotiana and Maize (Valsala et al. 1996; Kanta and Mahaswari 1963; Rangaswamy et al. 1967; Gengenbach, 1977). In legumes like *Swainsona formosa* also stigma receptivity started one day before anthesis (Zulkarnain et al. 2019). Even though no significant difference was noticed in the success rate of the male flowers on one day before anthesis (Fig. 6a&b) and on the day of anthesis in *H. brasiliensis*, the rate of contamination was high for the flowers taken on the day of anthesis. Similarly, a high fertilization success (58%) was noticed in female flowers one day after anthesis when compared to 2 DBA and 3 DBA but the rate of contamination was high. Based on these observations, the flowers one day prior to anthesis were utilized for further experiments.

Table 1
Effect of flower bud age on fertilization success after *in vitro* pollination in *Hevea brasiliensis*

Treatments	Age of the flower bud	Success (%)
1	3 DBA	39.2
2	2 DBA	46.0
3	1 DBA	79.2
4	Anthesis day	75.2
5	One DAA	58.0
	CD	9.2
DBA: days before anthesis; DAA: days after anthesis; values		
are means of multiple observations (p < 0.05)		

c. Methods of pollination

The effects of different methods of pollination on fertilization success as indicated by ovary/ovule/ development are presented in the Table 2. Based on the observations two weeks after pollination, the following results were derived. Among the four methods of pollination tried, *in vitro* stigmatic pollination and test tube fertilization showed some positive results, as indicated by the swelling of ovaries and ovules respectively after fertilization. The intra ovarian pollination and *in vitro* placental pollination failed to give any positive results and the ovules dried within a few days. Even though 40% of ovaries were enlarged under *in vitro* stigmatic pollination (Fig. 7), none of them contained seeds. In *Hevea*, the studies on pollen pistil interaction indicated that the pollen tubes enter the ovules 24–48 hours after pollination. This ensures the absence of any pre-fertilization inhibitions, for seed set. The fertilization may not have

taken place due to some incompatibility or the embryo might have aborted at an early stage due to some post fertilization inhibitions. This observation is comparable to the available reports under *in vivo* conditions (Sedgley and Attanayake, 1986). In *Hevea*, there are reports that the control mechanisms for fruit wall formation and embryo growth are different and independent (Priyadharsan 2011). Hence it is possible that fruit wall may grow even after embryo abortion. Lack of some essential nutritional factors can also be attributed to the absence of seeds in *in vitro* stigmatic pollination of *Hevea*. Development of fruits after stigmatic, stylar and intra ovarian pollination without seeds is reported in the *in vitro* fertilization of ginger (Valsala et al. 1996). In contrast, the application of pistil culture and *in vitro* stigmatic pollination techniques significantly improved seed set in *Petunia* and (Rangaswamy et al. 1967; Usha, 1965).

Table 2
Effect of pollination method on *in vitro* pollination of *Hevea brasiliensis*

Method of pollination	Cultures with ovary development (%)	Cultures with ovule development (%)
Stigmatic	40	0.0
<i>In vitro</i> placental	0.0	0.0
<i>Intra-ovarian</i>	0.0	0.0
Test tube	—	80

Among the different methods tried for test tube fertilisation, immersing the ovules in 1% CaCl₂ for 24 hrs followed by pollination and pouring pollen germination solution over the pollinated ovules was promising. 80% of the pollinated ovules swelled. Inoculating the ovules in the medium and keeping anthers over them didn't work. Pouring sucrose solution after pollination resulted in the swelling of 10–12 percent ovules. However these ovules dried after a few days.

d. Growth and development of ovules after test tube fertilization

In the current study, after *in vitro* ovular pollination (test tube fertilization), about 80% of the ovules enlarged two weeks after pollination. The fertilized ovules were ivory colored and opaque, whereas the unfertilized ovules were glassy in appearance, turned brown and dried after a few days (Fig. 8a). The unpollinated controls also turned brown within a few days. The swelled ovules were further cultured in different media combinations. Among the swelled ovules, further development was noticed in 40% of ovules. The rest of the ovules dried off after one month. Among the remaining swelled ovules, two types of developments were noticed wherein the first 10% ovules as a whole developed into small seeds. At 30 days post-fertilization, the *in vitro*-derived ovule attained a size similar to its *in vivo* counterpart, although its morphological characteristics closely resembled those of a mature seed. After 30 days, the size of the *in vitro* raised fertilized ovule was comparable with *in vivo* ovule but the morphology of the *in vitro*

developing ovule resembled a mature seed under field conditions. The seed coat is sclerified and characteristic mottling was observed in the surface of these ovules as in the case of mature seeds (Fig. 8.b). Since further embryo growth could not be observed in these cultures, they were fixed for anatomical studies.

The remaining 30% of the ovules showed a different pattern of development. The embryos were developed without endosperm and seed coat formation (Fig. 8c). By approximately 30 days after pollination, the integuments were observed to be displaced, exposing embryos at the globular stage. This is in contrast to the development of seeds under *in vivo* conditions. The lack of endosperm formation or its early degradation during the zygote or young embryo stage has been documented in several plant species, often posing a major constraint to successful embryo development (Kapoor, 1959; Töpfer and Steinbiss, 1985; Zenkteler and Nitsche, 1985). In the interspecific crosses of brassicas also absence of endosperm is observed after *in vitro* pollination. However, these embryos could be developed in to plantlets.

The absence of endosperm may be either due to the failure of triple fusion during fertilization or due to endosperm abortion due to embryo endosperm incompatibility. Otherwise, after fusion, triploid nuclei may be degenerated due to some unknown reasons. The lack of proper nutrition can also contribute to the absence of endosperm. Another possible reason may be the suppression of the triploid endosperm by the faster growth of the diploid embryo. The development of embryo and germination of seedlings from the embryos without endosperm formation after IVF has been reported in Chicory (Castano, 2000). Similarly, in the test tube fertilization of *Eschscholzia californica* the embryo showed normal development during its early growth whereas the endosperm was purely developed and degenerated. This indicates the possibility of recovery of seedlings from such embryos in *Hevea* also. Optimization of nutritional requirements will be worth trying for saving these embryos. A few embryos could be recovered from these cultures and they were grown up to the cotyledonary stage. However, the size of the embryos was very small.

e. Histological observations

The fertilized ovules, with seed coat development were subjected to anatomical studies in order to understand the development pattern. The anatomical studies of the ovules 90 days after pollination revealed the presence of two layered outer walls (Fig. 9.a). Well-developed seed coat with a thin walled inner zone with radially elongated sclerified cells could be seen at this stage. Seed coat is single layered in the chalazal region. The abundance of starch and lipids were observed in the nucellar area (Fig. 9.b. & Fig. 9c). The zygote and primary endosperm nuclei at single cell stage were also visible. This indicates successful fertilization after *in vitro* pollination. When an ovule of the same age after *in vivo* pollination was observed, the seed coat was soft and ivory in color and not similar to *in vitro* in the case of seed coat. Under *in vitro* conditions, the seed coat formation seems to be faster and has been almost completed in about 90 days. The hard seed coat may be preventing the absorption of nutrients from the

medium which may in turn effect further development of the embryo. Subsequent experiments will focus on the removal of integuments at defined intervals following pollination.

The pollination success as indicated by the swelling of ovules under both *in vivo*/ *in vitro* conditions is presented in Table 3. In the *in vivo* pollination followed by *in vitro* culture, an initial success of 67.5% based on the swelling of ovules was achieved after 2 weeks of culture. Among these, only 19 percent of swollen ovules showed continued growth and rest of them dried off. In the case of *in vitro* fertilised ovules, among the 80% of swelled ovules 41 percent showed sustained growth after 1 month. Subsequently, they showed two types of growth pattern as described earlier. A few embryos in both *in vitro* and *in vivo* conditions developed up to cotyledonary stage (Fig. 10). Even though the initial success is comparable, more ovules showed sustained growth after IVF, compared to *in vivo* fertilization. After initial swelling of the ovules, the possibility of early embryo abortion cannot be ruled out in this situation. The sudden stress induced due to the detachment of pollinated flowers from the tree and the sterilization process might have triggered early embryo abortion. The *in vitro*-fertilized embryos were smaller in size compared to the *in vivo*-fertilized embryos.

Table 3
Pollination success under *in vivo* and *in vitro* pollination as indicated by the swelling of ovules

Methods of pollination	Percentage of ovules swelled	
	After 14 days	After 30 days
<i>In vivo</i> pollination	67.5	19
Test tube fertilization	79.9	41
T stat	20.6	34.79

e. Culture medium

The success of *in vitro* pollination depends on the formulation of appropriate medium, which will support pollen germination and tube growth leading to fertilization and zygote development. MS basal salts supplemented with 100 mg L⁻¹ boric acid and 20 gL⁻¹ sucrose promoted pollen germination and effective fertilization, as indicated by swelling of ovules. Among various basal media and concentrations of sucrose tried out, more number of swelled ovules were obtained in MS medium supplemented with 50 gL⁻¹ sucrose. The influence of sucrose on embryo development is well studied in other crops. In *Prunus*, *in vitro* pollination followed by culture on a medium containing 15% sucrose resulted in a high frequency of embryo development. (Dziedzic et al. 1999). In the media, for ovule growth, organic supplements viz., CW (20%), malt extract (200 mg/l), casein hydrolysate (400 mg/l) and banana powder (100 mg/l) was added as it was found to be good for the rescue of embryos from immature fruits in *Hevea* (Rekha et al. 2010). Among the five growth regulators tested, Kinetin (2mgL⁻¹) and GA₃ (2mgL⁻¹) were found to exhibit positive results when supplied individually. Other growth regulators such as 2, 4-D, NAA and BA supplemented in the medium, resulted in callus induction from the outer integument and prevented

further development of the ovule. However, when kinetin (2.0 mg L^{-1}) was combined with 2 mg L^{-1} GA3, the growth of the fertilized ovule was improved. A few embryos could be grown up to cotyledonary stage (Fig. 10). Further increase in concentrations of both had an adverse effect on ovule development. Addition of Kinetin promoted ovule development after *in vitro* pollination of two different species in Brassicas (Sosnowska and Teresa Cegielska-Taras, 2014). Kinetin had a positive effect on the growth of immature embryos rescued from field in *H. brasiliensis*. Experiments are underway to improve the nutritional status of the medium, for achieving further growth of the embryo. Standardization of the media requirements is very difficult using IVF embryos since tangible number of embryos could not be recovered with the present success rate. Hence experiments are being performed using open pollinated fruits. In *Hevea*, embryo culture was successful from 8 week old fruits onwards. Experiments are underway for the media optimization and culture requirements for very immature fruits (1–8 weeks old). Once this is achieved, the same can be used for IVF embryos as well.

f. Endosperm development under in vitro and in vivo conditions

When different stages of fruits (1–5 months) were dissected, it was observed that the embryos are visible only in later stages of fruit development (4–5 months). The different stages of fruit development (2–12 weeks) with their corresponding seeds are given in the Fig. 11. It is observed that, in *Hevea*, the growth of the fruits and seeds follow a specific pattern. The fruit wall grows faster and by 2–3 months it becomes thickened. After thickening of the fruit wall, the seeds will start developing and when it fills the locules, the endosperm development initiates. After the completion of endosperm development, the embryo starts developing. By this time, the fruits will attain maximum growth, seed coat will be thickened and the endosperm will be fully developed. Once the development is initiated, the embryos grow faster and a fully developed embryo with cotyledons intruding in to the endosperm will be formed within 10–15 days. Different stages of fruits (2–10 weeks old) with their corresponding ovules are given in Fig. 11a. No embryos are visible in any of these stages. Fruits of 15–18 weeks maturity are shown in Fig. 11b. In *H. brasiliensis*, it is reported that, under field conditions, the embryos will be small and microscopic till 14 weeks after fertilization (Muzick, 1954). Our results are in conformity with this. Only during the last few weeks of fruit maturity we could see the embryos (Fig. 12).

When mature seeds developed *in vivo* are observed, many seeds are without endosperm development (Fig. 12a). In this case, embryos also seemed to be degenerated. Partially developed endosperm was also observed in some seeds where the embryos are live and fully developed (Fig. 12b & c). In immature fruit culture, it was observed that endosperm development is partial or completely absent in many of the ovules. However, embryos will grow normally in culture even in the absence of endosperm (Fig. 12.d) and plantlets could be recovered from these embryos. These observations reveal that embryo- endosperm communication is apparent under field conditions and may be attributed to the low seed set. After double fertilization, seed development initiates with the endosperm formation from the primary endosperm nucleus and development of embryo from the zygote. It is assumed that the development of endosperm and embryo is happening in coordination under *in vivo* condition. Although embryo and endosperm development are distinct processes, proper seed formation depends on elaborate and

synchronized communication between the two. There are accumulating evidences which demonstrates the requirement of certain protein regulators originating from the endosperm for proper development of the embryo (Ingram et al. 2020). The ratio of maternal to paternal gene products in the endosperm plays a critical role in determining the success of crosses between individuals of differing ploidy levels within a species, as well as interspecific crosses. Maternally expressed small-interfering RNAs (siRNAs) are believed to regulate this balance by targeting growth-promoting genes, thereby influencing endosperm development and hybrid viability (David Haig, 2013). The endosperm acts as a major metabolic sink for absorbing nutrients from the maternal tissues and sequestering them in the central vacuole. Nutrients are then re-exported to the embryo from the endosperm (Ingram et al. 2020). In *Hevea*, early fruit drop is common under natural conditions as well as after hand pollination. These observations reveal that embryo-endosperm communication is apparent under field conditions and may be attributed to the low seed set. In the present experiment, after *in vitro* fertilization a few embryos developed without endosperm development. When mature fruits from the field are observed, there also endosperm is absent/partially developed. These observations lead to the assumption that failure of endosperm may be one of the main reasons for low fruit set. Hence future investigations focusing on these aspects need to be carried out. In immature fruit culture, the embryos developed with /without endosperm but plant regeneration was successful from both cases. Embryo rescue and culture is most successful in the recovery of plants where fruit drop is caused due to endosperm degradation. It is generally presumed that the tissue culture medium compensates for the absence of endosperm by supplying the essential nutrients required for embryo development. Continued refinements in culture media have permitted younger and smaller embryos to be rescued and grown to maturity in many crops (Stewart, 1981). In our experiments also, embryos could be rescued and successfully regenerated in to plantlets.

Under *in vitro* conditions, the embryo/endosperm dependency is not visible and many of the embryos develop into a seedling surpassing the endosperm influence. This opens up the possibility of recovering more number of hybrid seedlings by *in vitro* pollination. In *Hevea*, early fruit drop is a common phenomenon under natural conditions as well as during artificial pollination. Comparable embryo development was observed when fertilized ovules derived from *in vivo* pollination were subsequently cultured under identical conditions. In Spite of high initial success (67%), the value was substantially reduced (17%) (Table.3). This may be attributed to Lack of proper communication between endosperm and embryo leading to the degeneration of either of them. However, further research is needed to confirm the hypothesis. Deriving accurate nutrient composition for promoting the independent growth of the zygote is a major challenge in the success of *in vitro* fertilization and plant recovery.

There is a possibility of inducing calli/secondary embryos from the zygotic embryo and uniform seedlings can be generated. Induction of zygotic polyembryoni and development of uniform seedlings are already reported in *Hevea*, using open pollinated immature seeds (Rekha et al.2015). This possibility can be applied to IVF embryos as well and multiple seedlings of desirable crosses can be generated. They enables replicated evaluation in the nursery stage itself and selection will be more effective. This is especially important in the case of perennial tree crop like *Hevea*, where long term field trials are inevitable for a meaningful selection of elite individuals. Callus induction and subsequent

embryogenesis enables development of elite, uniform rootstocks. Embryogenic calli derived from the zygote is an ideal explants for gene transfer experiments, since the regeneration and hardening will be more efficient. There is also a possibility of developing triploids of desired combinations. In a triploid, 2 sets of maternal chromosomes and one set of paternal chromosomes will be there. Only for compatible crosses, endosperm develops *in vitro*. Hence there is always a possibility of regenerating viable triploid plants through somatic embryogenesis.

Conclusions

The present study explores the feasibility of achieving *in vitro* pollination and fertilization in *Hevea brasiliensis*, followed by normal embryo development under controlled conditions. The results confirm successful fertilization and embryo formation, with ovules cultured up to 90 days post-fertilization, leading to embryo development up to the cotyledonary stage. Further refinements in pollination techniques, along with optimization of culture media composition and environmental conditions, are likely to enhance embryo maturation and facilitate subsequent seedling development. This approach could significantly improve the recovery rate of hybrid seedlings, thereby increasing the efficiency of selection for elite traits in breeding programs. The study also highlights that defective endosperm development or impaired endosperm-embryo communication may contribute to low fruit set under natural field conditions a limitation that is circumvented under *in vitro* conditions, allowing for higher plant recovery. Beyond enhancing hybrid production, a successful *in vitro* fertilization system offers a platform for advanced crop improvement strategies, including gene transfer, gene editing, and the development of uniform elite rootstocks.

Abbreviations

RRII

Rubber Research Institute of India

IVF

In vitro fertilization

DBA

Days Before Anthesis

DAA

Days After Anthesis

AD

Anthesis Day

FAA

Formalin Acetic acid

MS

Murashigae and Skoog

WPM

Woody Plant Medium

SiRNAs
Small-interfering RNAs
BA
Benzyl adenine
GA
Gibberellic acid
NAA
Naphthalene Acetic Acid
2, 4, D
2, 4, Dichlorophenoxy acetic acid
FAA
Formaline Acetic Acid
CW
Coconut Water
CH
Casein Hydrolysate

Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Conflicts of interest

The authors declare no conflict of interest.

Author contributions

All the authors contributed equally to the work. *The study was conceptualised and designed by the senior authors Rekha K and Kumari Jayasree. Material preparation was done by Raheena KT and Athira.P.V, data collection and analysis were performed by Vinoth Thomas, GireeshT ,Jithu George and*

Alex Raju. The first draft of the manuscript was written by Rekha K and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data availability statement

No additional data is associated with this manuscript

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Figures



Figure 1

A mature rubber plantation where plants are being tapped for the collection of latex, which is the main source of natural rubber



Figure 2

a. A newly emerging shoot with initiation of flowering

b. A new flush with immature inflorescence developed from the axils



Figure 3

A protandrous inflorescence of rubber tree with matured male flowers and immature female flowers

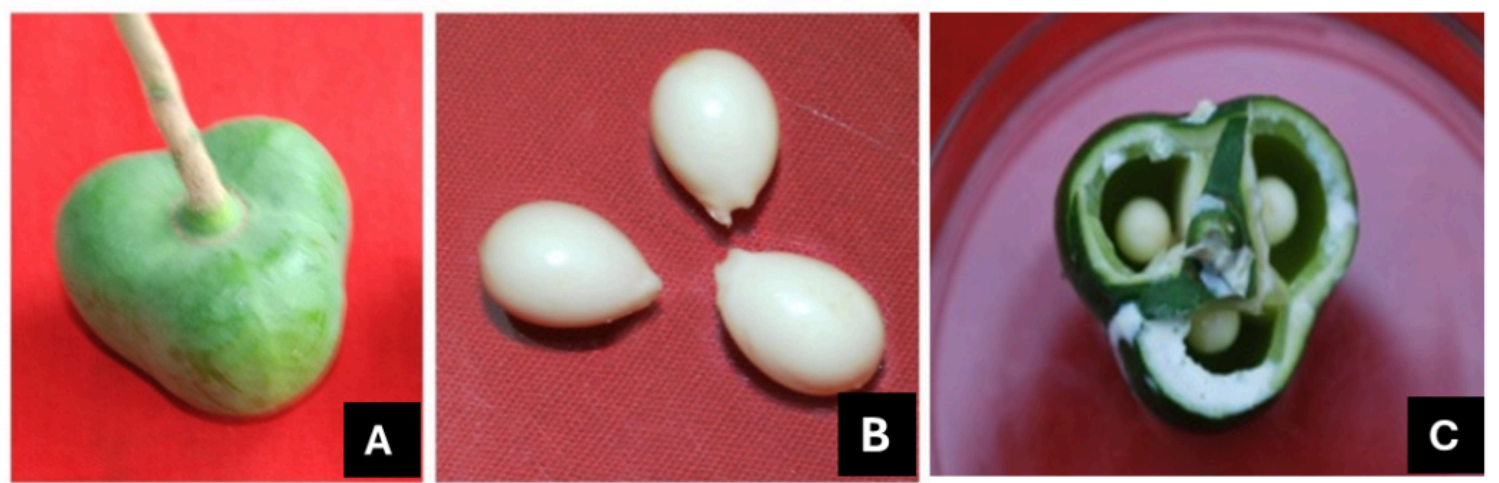


Figure 4

- a. Fruit of *H.brasiliensis* with 3 lobes
- b. Developing immature seeds separated from the ovule
- c. T.S of *Hevea* fruit with three developing seeds

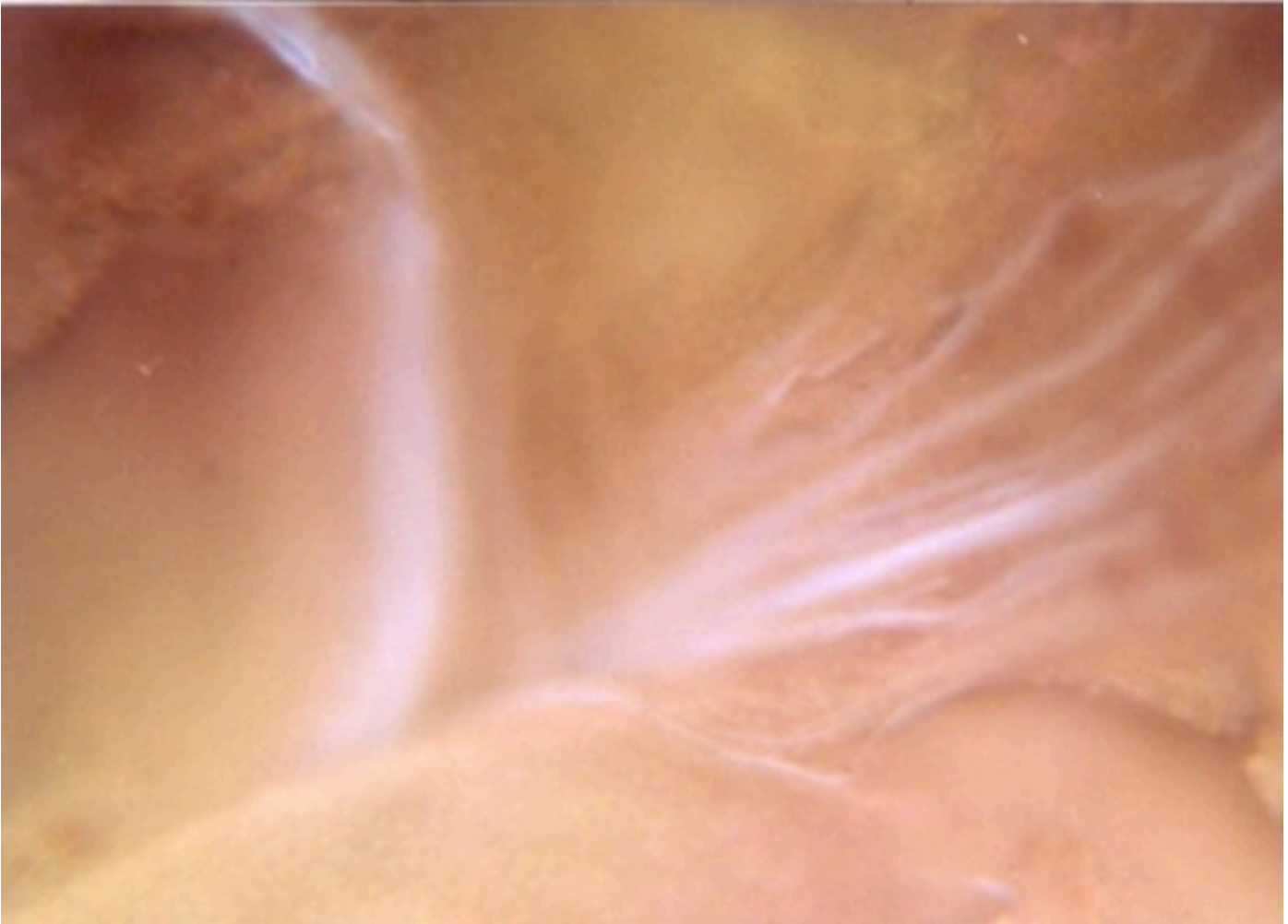


Figure 5

Profuse growth of the pollen tube and penetration of the ovules (x100)

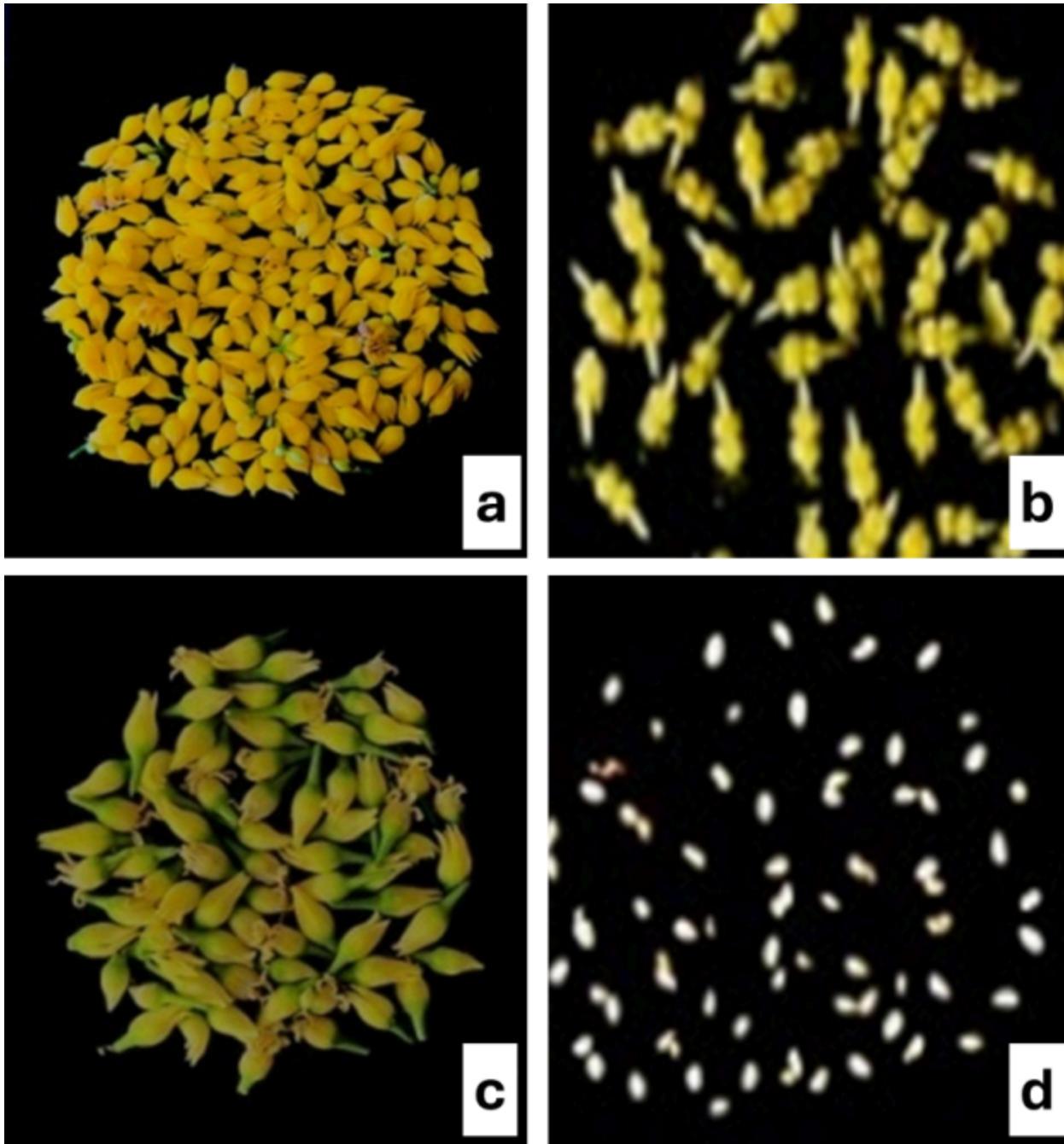


Figure 6

a. Male flowers; **b.** Anther column with pollen at late uninucleate to binucleate stage

c. Female flowers **d.** Excised ovules

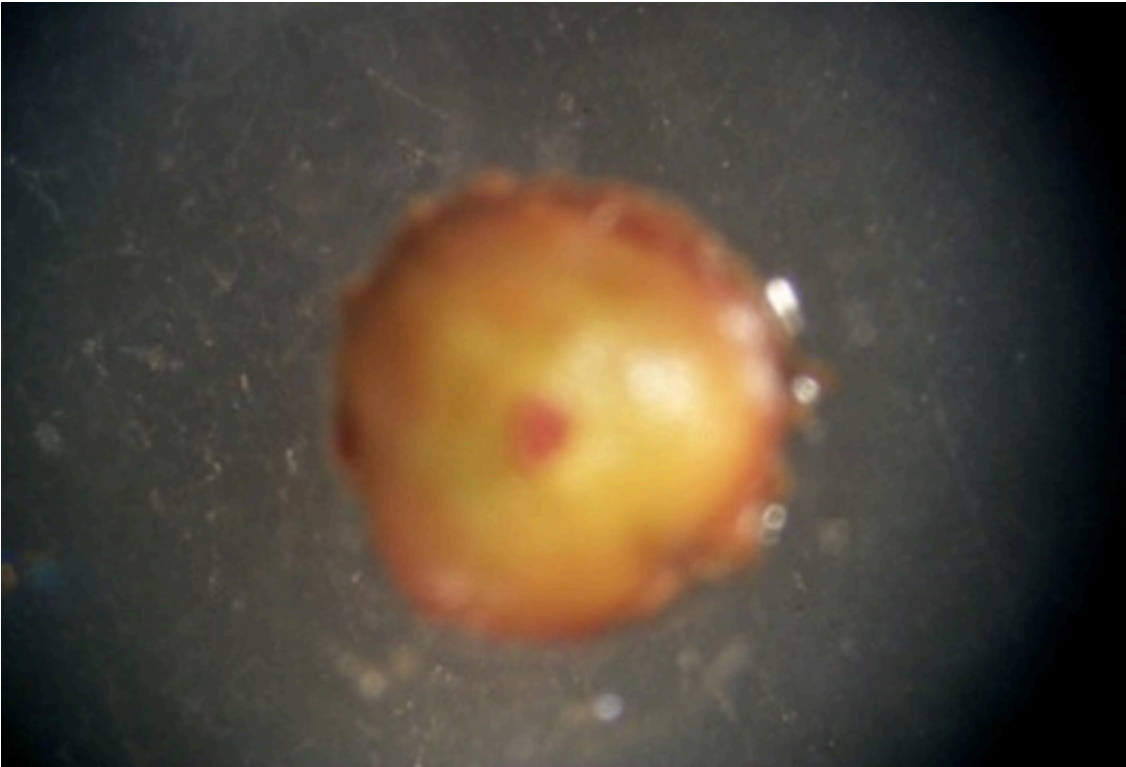


Figure 7

A swelled ovary 2 weeks after performing *in vitro* stigmatic pollination



Figure 8

- a. A fertilized and unfertilized ovule after *in vitro*ovular pollination
- b. A *in vitro* fertilized seed showing the characterized mottling as seen in *vivo* fertilized mature seeds
- c. Embryo development without seed coat and endosperm formation

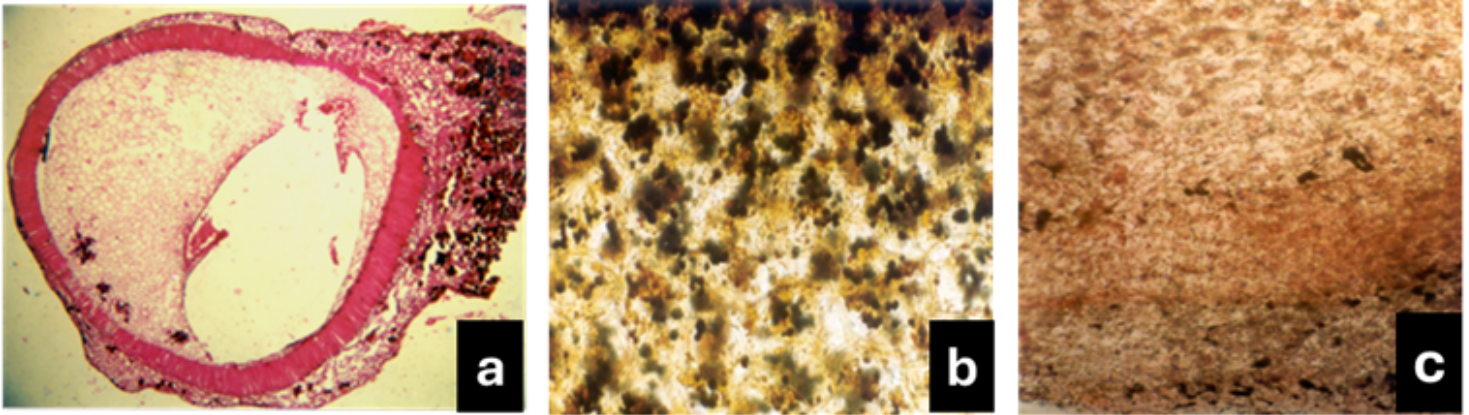


Figure 9

- a.** Cross section of fertilised seeds showing well developed seed coat, single celled zygote and primary endosperm nuclei
- b.** Abundance of starch grains in the nucellar area
- c.** Abundance of lipids in the nucellar area

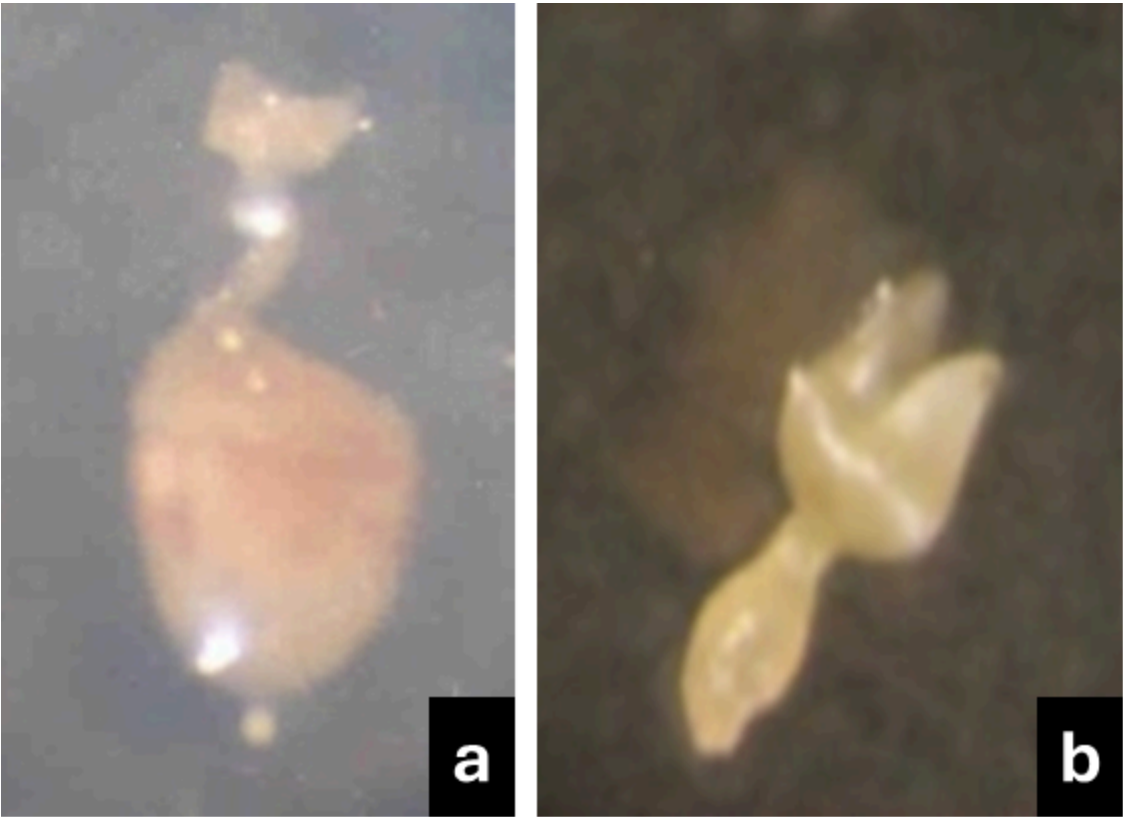


Figure 10

- a.** *in vitro* fertilised embryo after 2 months in culture

b. An *in vivo* fertilised embryo after 2 months in culture (x 100)



a



b

Figure 11

a Stages of fruit development with corresponding ovules from 2-12 weeks

b Developmental stages of fruits (15-18 weeks old) with corresponding seeds and embryos

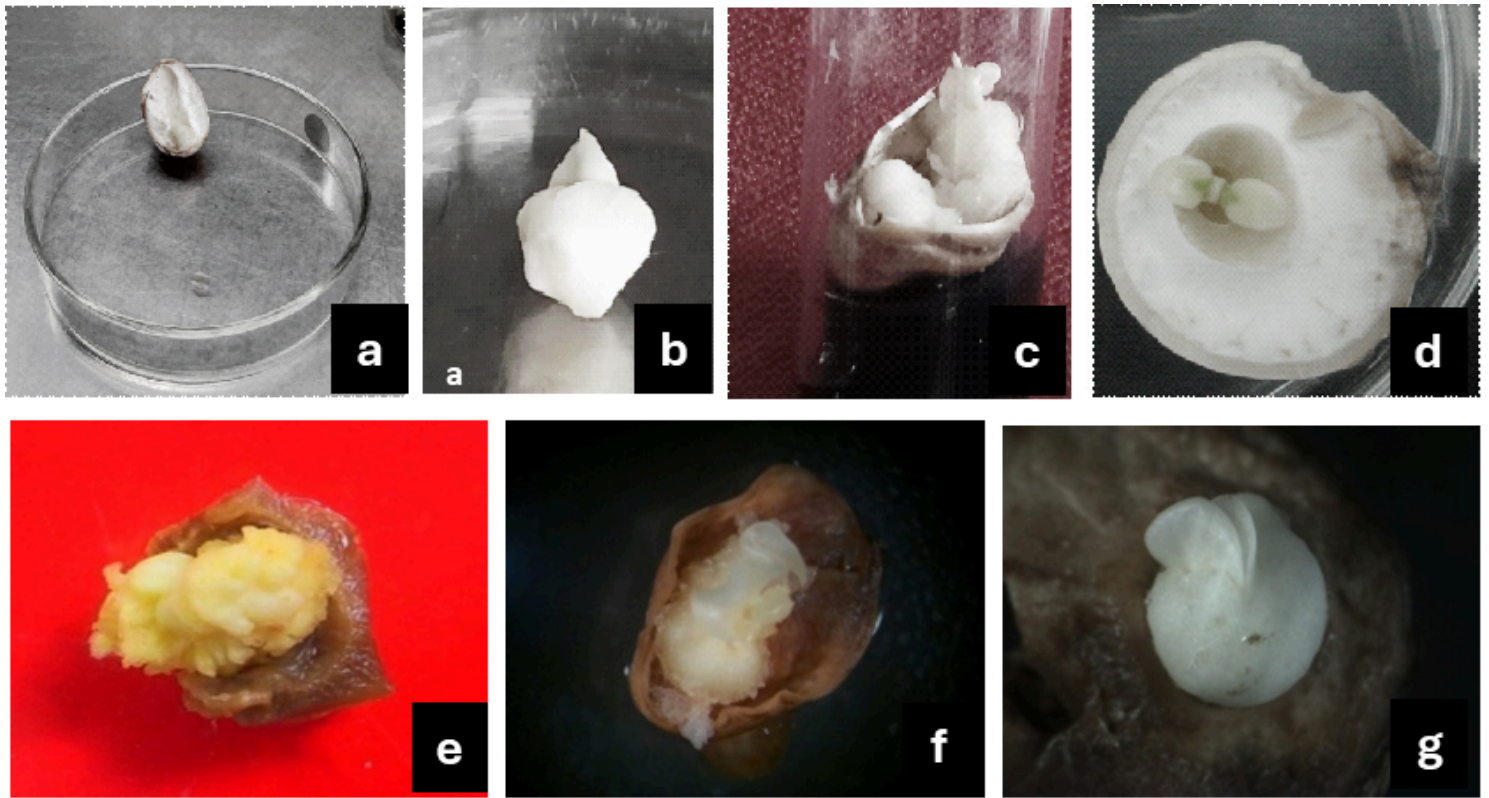


Figure 12

Types of endosperm development observed in *Hevea*

a. Absence of endosperm and embryo in mature seeds of *H.brasiliensis*

b& c. A fully developed embryo with partially developed endosperm

d. Development of seedling from a fruit without endosperm development

e. Profuse development of endosperm from immature fruits

f &g. Embryos with partially developed endosperm from immature fruit culture

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