Natural Rubber Research, **23** (1&2): 47-54, 2010

EMBRYO RESCUE AND PLANT REGENERATION IN *HEVEA BRASILIENSIS*

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Genetic improvement of *Hevea brasiliensis* by conventional breeding is difficult due to its perennial nature, long breeding cycle, seasonal flowering and asynchronous flowering among clones. The major bottleneck in *Hevea* breeding through conventional methods is the low recovery of fruits after artificial pollination and fruit loss due to fungal diseases. The average fruit set after hand pollination is usually less than 5%. In this context, the present work was undertaken with an objective to develop a protocol for the rescue of immature embryos of *H. brasiliensis*, thereby improving the recovery of hybrid seeds. Open-pollinated fruits of different maturity (1-8 weeks) were collected from the field-grown trees and inoculated in nutrient medium. Different basal media, various combinations and concentrations of growth regulators and two sterilization techniques were tried. Embryos could be rescued from 5-week-old immature fruits in Nitsch basal medium supplemented with the growth regulators kinetin, zeatin and GA_3. The highest frequency of embryo recovery (42%) was obtained with 3.0 mg/L zeatin coupled with 1.0 mg/L GA_3. Plantlets were developed, hardened and established in the field.

Keywords: Embryo rescue, GA_3, *Hevea brasiliensis*, Kinetin, Zeatin

INTRODUCTION

The Para rubber tree, [*Hevea brasiliensis* (Willd. ex Adr. de Juss.) Muell.Arg.)] belonging to the family *Euphorbiaceae*, is cultivated extensively in South East Asia as a source of natural rubber. The tree is monoecious with a strong out-breeding tendency. The long life span and lack of sufficient fruit set render crop improvement laborious and time consuming. Heterozygosity of parents necessitates screening of large families to achieve tangible results. Production of *Hevea* hybrids of desired genetic constitution through breeding involves controlled artificial cross-pollination which is labour intensive. Eventhough *Hevea* tree produces flowers in abundance, hardly 3% of the female flowers develop into fruits (Maas, 1919). Under artificial pollination also, the fruit set and recovery of hybrid fruits are equally low. Several investigations have already been made to address the problem of low fruit set in *Hevea* (Warmke, 1951; Rao, 1961; Gandhimathi and Yeang, 1984). The low rate of recovery of fruits at the mature stage poses a serious hindrance to *Hevea* breeding efforts, necessitating considerably large number of hand pollinations every year (Mydin *et al.*, 1989). According to Leconte *et al.* (1984), use

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of growth regulators like NAA and GA_3 promoted fruit set to some extent. Changing the hand pollination method (Mydin *et al.*,1989) and addition of boric acid to the pollen germination medium were suggested to improve fruit set (Majumder,1964). However, the fruit set could not be raised to more than 5% (Mydin *et al.*, 1989). Chandrashekar *et al.* (2004) reported 19% fruit set in *Hevea* in a cross combination of PB 330 x RRII 414 during 2002 and that is the highest rate ever recorded following hand pollination. Normally in *Hevea*, the fruits attain maturation within 22-24 weeks after pollination. Extensive withering of pollinated flowers and loss of developing fruits occur during the first 10-12 weeks (Chandrahekar *et al.*, 2004). The exact reason for the fruit drop is unknown except for fungal attacks. In this context, any attempt to increase the fruit set will be of great use in *Hevea* breeding to increase recombination frequency and efficient selection. Hence, the present work was initiated with a view to develop a protocol for the rescue of very young embryos of *Hevea* and subsequent plant regeneration.

MATERIALS AND METHODS

Explant sterilization

Open-pollinated fruits from the clone RRII 105 at different stages of maturity (1-8 weeks after fertilization) were collected from the field and sterilized. Two sterilization methods experimented were as follows. (1) Fruits were washed thoroughly in distilled water and sterilized using 0.1% $HgCl_2$ for 5 min. After washing in sterile distilled water, they were allowed to dry on a sterile filter paper. (2) Fruits were dipped in 80% alcohol for 5 min and allowed to dry on a filter paper.

Explant inoculation

The ovules were isolated from the fruits using a sterile knife and inoculated in the nutrient media. Since embryos were not visible at early stages and were difficult to dissect, the ovules were cut into two halves and placed in the medium with the micropylar end touching the medium.

Culture media

Preliminary experiments were carried out to identify the most suitable basal media and growth regulators for the culture of immature embryos. The basal media tried were MS (Murashige and Skoog, 1962), Woody Plant Medium (Lloyd and McCown,1980) and Nitsch medium (Nitsch and Nitsch, 1969). Since GA_3 has earlier proved to be beneficial for the development of *in vitro* fertilised embryos of *H. brasiliensis* (Rekha *et al.*, 2002), all the basal media were supplemented with 1mg/L GA_3. Out of the three basal media tried, Nitsch basal medium was found to be the most suitable one for the growth of the embryos and hence this medium was used in experiments for optimizing the growth regulator levels other than GA_3. Different growth regulators like NAA (1.0 - 2.0 mg/L), 2,4-D (1.0 - 2.0 mg/L), BA (1.0 - 3.0 mg/L, kinetin (1.0 - 5.0 mg/L) and zeatin (1.0 - 5.0 mg/L) were tried in combination with 1mg/L GA_3. The basal medium was also supplemented with 10% coconut water, 500 mg/L casein hydrolysate and 50 g/L sucrose. The pH of the medium was adjusted to 5.7. Autoclaving was done at 121 ^{0}C and 15 lb pressure for 10 min. Cultures were incubated in both dark and light (40 mE/m^2/s) at 25±2 ^{0}C.

Observations and statistical analysis of data

The experiments were arranged in a completely randomized design with five replications. Observations on the percentage recovery of embryos were recorded. The values were subjected to square root transformation and analysed.

RESULTS AND DISCUSSION

Explant sterilization and culture initiation

For the excision of embryos and their transfer to nutrient medium, aseptic procedures are necessary. Normally in *Hevea*, the embryo development occurs inside the ovules which in turn are lodged in the sterile environment of the fruits. Therefore, disinfection of the embryo as such is not necessary. Instead, the fruits were sterilized and ovule/embryos were dissected aseptically. When ovules were dissected out from unsterilized fruits, recovery of contamination-free cultures was as low as 20%. Among the two sterilization methods tried, both $HgCl_2$ and alcohol sterilization were on par when the contamination rate was considered. After sterilization, contamination-free cultures could be recovered at a frequency of 80%. Considering easy and safe handling, the alcohol sterilization method was used for further experiments.

During explant inoculation, the embryos were not visible until 14 weeks of age. Hence, *in ovulo* embryo culture was tried. In the preliminary experiments, ovules were isolated from the sterilized fruits and intact ovules were inoculated in the medium. However, they failed to grow and dried after a few days. Therefore, for further experiments, the ovules were cut into two halves and inoculated in the medium with the micropylar end touching the medium. By this method, after one month in culture, embryos became visible (Fig. 1) and bipolar differentiation occurred within two months (Fig. 2). This type of *in ovulo* embryo culture has been successfully carried out in several crops including cotton and *Alstroemeria* (Stewart and Hsu, 1977; Buitendijk *et al.*, 1995).

Incubation conditions

During embryo culture, the incubation conditions were found to influence the embryo development. Dark incubation was found to be ideal for embryo maturation. Light inhibited the development of embryos and no embryo under light incubation could be regenerated into plantlet. The influence of exposure to dark/light on shoot bud formation was reported in *Hevea* by Das *et al.* (2003). According to them dark incubation of cultures for two weeks was optimum for multiple shoot induction from zygotic embryos of *Hevea*. Similar observations were

Table 1. **Recovery of embryos from fruits of different maturity (2-8-week-old) in Nitsch basal medium supplemented with 1 mg/L GA_3**

Age of the fruits (Weeks)	Embryo recovery (%)
2	2 (1.39)
3	3.8 (1.94)
4	13.6 (3.69)
5	21.8 (4.67)
6	22.4 (4.73)
7	22 (4.69)
8	24 (4.90)
CV	4.89
CD (P = 0.05)	0.24

Transformed values are given in parentheses

also made in *Pinus radiata* by Biondi and Thorpe (1982) and Villalobos *et al.* (1984). A higher rate of germination was observed in cultures incubated two weeks in dark and subsequently transferred to light at 16h photoperiod for two weeks in floribunda rose (Mohapatra and Rout, 2004).

The fruit age also significantly influenced plant regeneration (Table 1). It was observed that the percentage of rescued embryos increased with the age of the fruits. The highest embryo recovery was recorded for eight-week-old fruits (24%) whereas the lowest recovery was for two-week-old fruits (2%). Eventhough, a few embryos were obtained from two-week-old fruits, plants could not be regenerated. It was reported that the successful production of plants from the cultured embryos largely depends upon the maturation stage and the composition of the medium (Sharma, *et al.*, 1996). The probable reason for the low recovery of embryos during very early stages of development could be the dissection and transfer shock or the unsatisfied nutritional requirements of the embryo. A complex culture medium may be required for the embryo during its early developmental stages. At a later stage, the embryo is capable of synthesizing substances required for its growth. The critical stage at which the embryo passes from the heterotrophic to autotrophic stage varies with species (Razdan, 1983). Similar results were reported in chickpea, where a study on the response of immature ovules following rescue *in vitro* demonstrated a positive correlation between germination of the embryo and age of the rescued ovule. Germination ranged from zero in five-day-old ovule to 43% in twelve-day-old ovules (Clarke *et al.*, 2006).

Healthy plantlets could be regenerated from embryos obtained from five-week-old fruits. No significant differences were observed between five-week-old (21.8%) and eight-week-old (24%) fruits in terms of embryo recovery. Since withering and fruit drop occur in *Hevea* during the first 10-12 weeks after pollination (Chandrashekar *et al.*, 2004), culture of embryos at the youngest stage possible is preferred. Hence, five-week-old fruits were used for further studies.

Culture media

In the present study, the nutrient medium played a significant role for normal embryo development and plant regeneration. Among the three basal media tried, Nitsch medium supplemented with 1mg/L GA_3 was found to be the most suitable for culture establishment and embryo development. Nitsch basal medium has been extensively used in *in vitro* fertilisation experiments of *Papaver, Nicotiana, Melandrium, Argimone, Petunia, etc.* to promote the growth of the immature embryo. When auxins like NAA and 2,4-D were used along with GA_3, callusing of the outer integument was observed and as a result embryos could not germinate. Since its discovery in 1954, GA_3 has been extensively used in inter-generic and inter-specific crosses and the influence of GA_3 on ovule and embryo development is well established (Larter and Enns, 1960; Larter and Chaubey, 1965). In the present experiment, Nitsch basal medium containing 1.0 mg/L GA_3 helped embryo formation from 2-8-week-old fruits. The beneficial effect of GA_3 for the development of fertilized ovule after *in vitro* pollination in *Hevea* has been reported earlier (Rekha *et al.*, 2002).

Experiments on the effect of three different cytokinins along with GA_3 on embryo development revealed a positive

Table 2. **Effect of kinetin in presence of GA_3 (1 mg/L) on embryo development in Nitsch basal medium.**

Kinetin (mg/L)	Embryo recovery (%)
1	18 (4.24)
2	23 (4.79)
3	24 (4.90)
4	26.4 (5.14)
5	32.4 (5.69)
CV	2.81
CD (P = 0.05)	0.18

Transformed values are given in parenthesis

influence of kinetin and zeatin. BA had no positive influence in the embryo development, which was indicated by the gradual drying of the ovules in the medium supplemented with BA. Table 2 shows the influence of kinetin on embryo recovery. It was observed that when kinetin and GA_3 were given in equal concentrations (1 mg/L), a slight reduction in the percentage of embryo recovery was observed compared to the medium supplemented with GA_3 alone (Table 1). However, the frequency of embryo recovery increased gradually with increase in kinetin concentration. The frequency of embryo recovery could be raised to 32.4% when 5.0 mg/L kinetin along with 1.0 mg/L GA_3 was used. Further experiments will be required to understand the effect of kinetin at higher levels (>5.0 mg/L). In a previous study, a combination of 2.0 mg/L kinetin along with 1.0 mg/L GA_3 improved the growth of the fertilized ovules after *in vitro* fertilization of *Hevea* (Rekha *et al.*, 2002). The promotive effect of kinetin during the initial stages of growth was reported earlier in many crops including cowpea (Pellegrineschi *et al.*,1997).

More promising results were obtained when kinetin was replaced with zeatin in combination with GA_3. A steady increase in the embryo recovery was observed with increase in concentration of zeatin up to 3.0 mg/L. Further increase in zeatin concentration reduced the embryo recovery. Maximum embryo recovery (42.2%) was achieved with a hormonal combination of 3.0 mg/L zeatin and 1.0 mg/L GA_3 (Table 3). Zeatin being the natural cytokinin, triggers

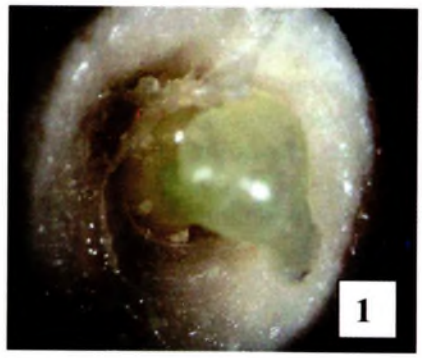
Table 3. **Effect of zeatin in presence of GA_3 (1.0 mg/L) on embryo development in Nitsch basal medium**

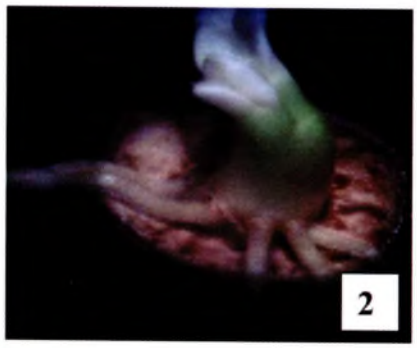
Zeatin (mg/L)	Embryo recovery (%)
1	16.2 (4.02)
2	22.4 (4.73)
3	42.2 (6.4)
4	25.6 (5.06)
5	20.4 (4.64)
CV	3.3
CD (P = 0.05)	0.22

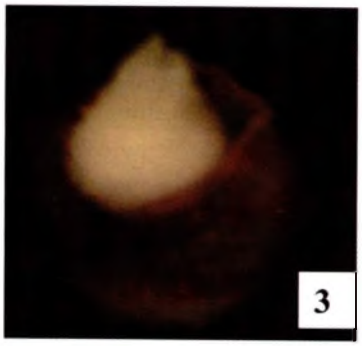
Transformed values are given in parenthesis

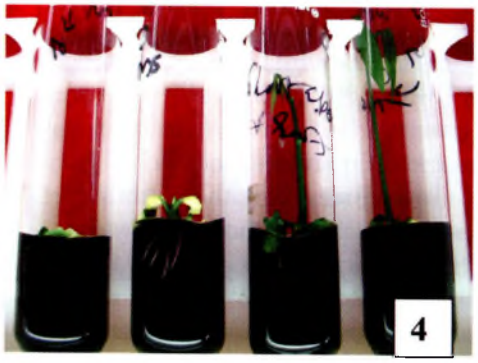
the development of the zygote and subsequent embryo formation. In cowpea embryo rescue experiments, it was reported that 0.1 mg/L zeatin promoted embryo development and maturation (Pellegrineschi, 1997). Similarly, 1.0 mg/L zeatin promoted *in ovulo* embryo development in chickpea (Clarke *et al.*, 2006).

One important observation in the present experiment was the absence of endosperm development in many of the fertilized ovules cultured (Fig. 3). Either the failure of triple fusion during fertilization or the degeneration of triploid nuclei after triple fusion due to unknown factors may be the reason for the absence of endosperm









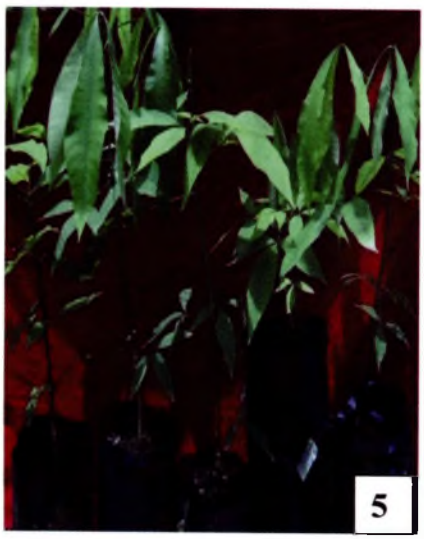


Fig. 1. A developing embryo inside the ovule
Fig. 2. A cotyledonary embryo emerging from the ovule
Fig. 3. Embryo development without endosperm formation
Fig. 4. Different stages of plant regeneration
Fig. 5. Plants after hardening
Fig. 6. A rescued plant in the field

development. It was reported that in many instances barriers to hybrid embryo survival occur due to lack of endosperm or abnormal endosperm development, which causes embryo starvation and abortion of developing fruit (Stewart and Hsu, 1981). Similar results were also obtained from *in vitro*-fertilized ovules of *Hevea*. Out of the growing embryos after fertilization, only 10% showed endosperm development (Rekha *et al.*, 2002). The absence of endosperm or poor development of endosperm may be contributing towards poor seed set in *Hevea*. Embryo rescue and culture is most successful in the recovery of plants where endosperm breakdown is the cause of fruit drop. It is presumed that tissue culture medium provides the necessary nutrients normally supplied by the endosperm tissue. Continued refinements in culture media have permitted younger and smaller embryos to be rescued and grown to maturity in many crops (Stewart and Hsu, 1981). In the present experiments, even though embryos developed without endosperm in many of the cultured ovules, plants could be successfully regenerated from those embryos too.

After one month in embryo formation medium, embryos from each ovule were separated and transferred to embryo maturation medium. For maturation of the embryos ½ MS basal medium supplemented with 5% sucrose, 0.2% charcoal and a growth regulator combination of GA_3 (0.3 mg/L) and BA (0.3 mg/L) were used. After bipolar differentiation, embryos were transferred to germination medium containing the same components with low (2%) sucrose (Fig. 4). Fully developed plantlets were planted out in big polybags with potting mixture. The plants were covered with thin transparent plastic bags to maintain 90-95% relative humidity for the initial few days. After 3-5 days, the relative humidity was gradually reduced by making holes in the plastic covers. After two weeks, the covers were removed and the plants were maintained in the net house until field planting (Fig. 5) and the plants were well established in the field (Fig. 6).

CONCLUSION

In the present study, a protocol could be developed for the rescue of immature embryos in *H. brasiliensis* by adopting *in ovulo* embryo culture with successful plant regeneration. By optimizing suitable culture media, considerably high frequency (42.2%) of embryos could be recovered. This technique can be used for increasing the percentage recovery of hybrids following hand pollination. It can be employed for the production of inter-specific and inter-generic hybrids in *Hevea*. By refining the technique, it is possible to enhance the percentage of recovery further and to reduce the time taken from pollination to seedlings. There is also a possibility of inducing polyembryony *via* this technique, thus facilitating replicated evaluation at the nursery stage itself.

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