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IN VITRO FERTILISATION IN *HEVEA BRASILIENSIS* : A PRELIMINARY INVESTIGATION

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ABSTRACT

A preliminary investigation to optimize the conditions for successful *in vitro* fertilization and subsequent embryo development in *Hevea brasiliensis* has been conducted. The pollination method, stage of the flower and culture medium for effective fertilization have been optimised. *In vitro* ovular pollination has been identified as the suitable method of *in vitro* pollination in *Hevea brasiliensis*. The flower one day before anthesis was identified as the optimum stage for *in vitro* pollination. The fertilized ovules could be grown up to three months and embryos were grown up to the heart shaped stage, which is the first report in *Hevea brasiliensis*. MS medium supplemented with complex organic nutrients viz. coconut water, casein hydrolysate, malt extract, banana powder and extract prepared from *Hevea* endosperm promoted ovule/embryo development. The growth regulators GA_3 (1.0mg/l) and KIN (2.0 mg/l) also improved the ovule/embryo development. Anatomical studies of the developing ovules revealed the abundance of starch and lipids in the nucellar area 30 days after pollination. A well developed seed coat was observed at 90 DAP. The morphology of 90 day old developing seed after *in vitro* fertilization resembled a mature seed *in vivo*.

INTRODUCTION

In a broad sense, the terms *in vitro* pollination and fertilization include any manipulation of the maternal tissue that accomplishes pollen tube penetration of the embryosac by means other than the normal *in situ* process (Stewart 1981). Kanta *et al.* (1962) were the first to achieve artificial fertilization of a flowering plant, *Papaver somniferum*, by the pollination of excised ovules with mature pollen. The *in vitro* fertilisation (IVF) system has been accomplished in 57 species representing 14 families including *Brassicacea, Caryophyllaceae, Liliaceae, Papaveraceae, Primulaceae* and *Solanaceae* (Zentkler 1992, 1994). The technique of *in vitro* pollination and fertilization is a novel breeding tool and have been used for overcoming pre-fertilization barriers (Rangaswamy and Shivanna, 1967); Valsala *et al.* 1996; Lech *et al.* 1994), production of interspecific and intergeneric hybrids (Zenktler 1980, Chin *et al.* 1997), haploid production (Hess and Wagner 1974) and for studying the pollen tube - female gametophyte interactions and the cellular events surrounding fertilization (Dupuis and Dumas 1989; Fernando *et.al*, 1998).

Recently, the potential of IVF system in genetic transformation has also been identified. Since the IVF system allows pollen and female gametophyte to be maintained in culture, genetic transformations can be carried out on sperm or eggs (Fernando *et al.* 1998). Coupled with hybridization and genetic transformation, IVF could result in the development of stable novel genotype of economically superior crop plants. In conifers, work on this line has been started earlier (Fernando *et.al.* 1998).

In *Hevea*, the major constraints in conventional breeding are low fruit set, seasonal flowering, lack of synchrony in flowering among clones and the inaccessibility of the flower. The brief and periodic nature of flowering impedes the progress of pollination programme. Very often, it becomes impossible to attempt sufficient number of artificial pollinations to obtain families large enough to conduct effective seedling selection in the nursery. In hybridization programmes, the low fruit set results in the loss of numerous potentially good cross combinations, thus limiting the progress of genetic improvement. Low fruit set also reduces the size of legitimate families on which selection is to be applied for the evolution of new clones with the desired attributes. The inaccessibility of flowers also makes the hand pollination programme labour intensive and limits the number of crosses that can be performed in a season. Efforts to overcome the problem have been in vain and fruit set could not be raised to more than 5% at RRII (Kavitha *et al.* 1989). So it is necessary to conduct intensified hand pollination programme bringing about heavy input involvement.

At this context, the present work on *in vitro* pollination and *in vitro* fertilization are being highly significant. With the objective of establishing an *in vitro* system for seed production to compliment the conventional breeding, an attempt was initiated to standardize the conditions for effective *in vitro* pollination and fertilization in *Hevea brasiliensis*. The system could be further utilized for interspecific and intergeneric hybridization, genetic transformation and haploid induction.

MATERIALS AND METHODS

Plant material

Flower buds of *Hevea* clone RRII 105 were selected for this study. Female flower buds at different developmental stages from three days before anthesis to one day after anthesis (3 DBA, 2 DBA, IDBA, anthesis day and 1 DAA) were used for *in vitro* pollination. Male flowers at late uninucleate stage to early binucleate stage were selected.

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 5 minutes and then rinsed with sterile distilled water 4-5 times. The flowers were then blotted on a sterile filter paper to remove the moisture.

Pollination

Anthers were dissected from male flowers after surface sterilization using fine forceps. *In vitro* self pollination was done using different techniques described by Bhojwani and Razdan (1983).

(1) *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.

(2) Intra ovarian pollination - After sterilization the perianth was completely removed and the stigma was cut back and the pollination was done on the cut surface.

(3) *In vitro* placental pollination - In this case, the perianth, stigma and the ovary wall were removed carefully and the ovules attached to the placenta was pollinated.

(4) *In vitro* ovular pollination/test tube fertilization - The perianth and stigma were removed. The ovary wall was carefully pealed with sterile tweezers and blade, leaving the ovules supported by the funicle. The isolated ovules were inoculated in nutrient medium. The anthers dissected from male flowers were placed close to the ovules.

The pollinated flowers/ovaries/ovules were cultured on media in petridishes and sealed with parafilm and incubated under dark conditions at 24±2°C. Unpollinated flowers/ovaries/ovules were also inoculated in the same culture medium as control. After one week, the ovules were subcultured into the second medium and observations were recorded at weekly intervals.

Histological observations

The pollinated pistils after *in vitro* stigmatic pollination were fixed in FAA (Johansen 1940) at different intervals after pollination and the pollen-pistil interaction was studied using the staining technique given by Kho and Baer, (1968).

Developing ovules were fixed at different intervals in FAA and processed for microtome sectioning. Sections were stained with periodic acid Schiff's reagent (Jensen 1962) for general histology, I_2KI (Johansen 1940) for starch and Sudan IV (Jensen 1962) for lipid.

Culture media

For the initial one week, MS media (Murashige and Skoog 1962) supplemented with 100 PPM boric acid and 20 g/l sucrose were used. After one week, the enlarged ovules were subcultured into different combinations of nutrient media. The basal salts of MS, WPM (Lloyd, 1980) and N6 (Nitsch and Nitsch, 1969) and varying concentrations of sucrose from 3-6% were tried. The basal medium was supplemented with varying concentrations and combinations of growth regulators such as BA, KIN, GA, NAA and 2, 4-D. The effect of complex organic nutrients viz. coconut water, casein hydrolysate, malt extract, banana powder and *Hevea* endosperm extract on IVF were also studied.

RESULTS AND DISCUSSION

Methods of pollination

The effects of different methods of pollination on fertilization success as indicated by ovary/ovule development are presented in Table 1. The observations were recorded 2 weeks after pollination. Among the 4 methods of pollination tried, 40% ovary development was noticed in *in vitro* stigmatic pollination and 80% ovule development was noticed in *in vitro* ovular pollination/test-tube fertilization. The intra ovarian pollination and *in vitro* placental pollination failed to give any positive results. The ovules dried within a few days in these two methods. In test tube fertilization, about 80% of the ovules enlarged two weeks after pollination. The fertilized ovules were ivory coloured and opaque, where as the unfertilized ovules were glassy in appearance, turned brown and dried after a few days (fig. 2). The unpollinated controls were also turned brown within a few days.

Eventhough 40% of ovaries were enlarged in *in vitro* stigmatic pollination, none of them contained seeds. The fertilization may not have taken place due to some pre fertilization barriers or the embryo might have aborted at an early stage due to some post fertilization inhibitions. In *Hevea*, the studies on pollen pistil interaction indicated that the pollen tubes enters the ovules 24-48 hours after pollination (Fig.1). This observation is comparable to the available reports under *in vivo* conditions (Sedgley 1986). This ensures the absence of any pre-fertilization barrier. Some post fertilization inhibitions or nutritional factors can be attributed to the absence of seeds in *in vitro* stigmatic pollination of *Hevea*. Similar results were also reported in the *in vitro* fertilization of ginger (Valsala *et al.* 1996) where ovaries developed into fruits after stigmatic, styler and intra ovarian pollination but none of them contained seeds. However, the pistil culture and *in vitro* stigmatic pollination was found to improve seed set in *Petunia* and *Antirrhinum* (Rangaswamy *et.al.* 1967, Usha 1965). In the present study, since all the other methods failed to give seeds, the method of test tube fertilization has been adopted for further investigations.

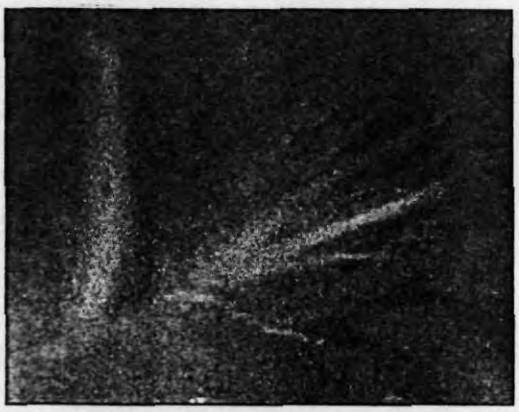

Fig. 1. Pollen tube penetration of the ovule

Fig. 2. A fertilized ovule and an unfertilized ovule - 2 weeks after polination

Table 1. Effect of pollination method on *in vitro* pollination of *Hevea brasiliensis*

Sl No.	Methods of pollination	Cultures with ovary development (%)	Cultures with ovule development (%)
1	Stigmatic pollination	40	0.0
2	Intra ovarian pollination	0.0	0.0
3.	*In vitro* placental pollination	0.0	0.0
4	*In vitro* ovular pollination	NA	80

Observations were recorded 2 weeks after pollination.

Age of the flower bud

Among the five developmental stages used for *in vitro* pollination, the maximum percentage of ovule development was obtained for the flowers one day before anthesis (79.2) followed by flowers on the day of anthesis (75.2) (Table 2). For the flowers 2 and 3 days before anthesis, the values were significantly lower and 58% success was obtained for flowers one day after anthesis. The stage of the flower is an important factor in determining the success of fertilization and it is highly specific for different species. 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 Maheswari 1963; Rangaswamy *et al*; 1967, Balaktova *et al.* 1977; Gengenbach 1977). In contrast to this, the flowers pollinated 3 days prior to anthesis resulted in more number of seeds in *Lilium* (Van Tuyl *et al.* 1991). Our results are similar to that of *Gossypium* where the flower buds one day prior to anthesis were suitable for IVF (Refant *et al.*, 1984). Eventhough no significant difference was noticed in the success rate of the flowers on one day before anthesis and on the day of anthesis in *Hevea*, the contamination was high for the flowers on the day of anthesis. Similarly, a high fertilization success (58%) was noticed in flowers one day after anthesis compared to 2DBA and 3DBA, however the rate of contamination was high. Based on these observations, the flowers one day prior to anthesis were utilized for further experiments.

Culture medium

The success of *in vitro* pollination depends on the formulation of an appropriate medium, which will support pollen germination and tube growth leading to fertilization and zygote development. In *Hevea*, the MS basal medium supplemented with 100mg/l boric acid and 2% sucrose promoted the pollen germination and effected fertilization. Among various basal media tried for the growth of the fertilized ovule, the macro and micro elements of MS medium was found to be ideal. Sucrose concentration of 50g/l was found to be suitable for the ovule development. The survival of the fertilized embryos could be raised from 15 to 40 days by the addition of organic supplements viz. CW (20%), malt extract, (200 mg/l), casein hydrolysate (400 mg/l) and banana powder (100mg/l). The extract prepared from *Hevea* endosperm, when added to the medium could further increase the survival up to 90 days.

Among the 5 growth regulators tested, KIN and GA_3 were found to exhibit positive results when supplied individually. However, when KIN (2.0 mg/l) was combined with GA3, the growth of the fertilized ovule was improved. Further increase in concentrations of both had an adverse effect on ovule development. When the medium was supplemented with 2, 4-D, callus was induced from the outer integument and prevented the ovule growth. Addition of BA also showed a similar callusing response and incorporation of NAA alone in the medium had detrimental effect and the ovules turned brown. However, the synergic effect with BA (1.0 mg/l) leads to callus induction.

Table 2. Effect of flower bud age on fertilization success after *in vitro* pollination in *Hevea brasiliensis*

Age of the flower bud	Success (%)
3 DBA	39.2
2 DBA	46.0
1 DBA	79.2
Anthers day	75.2
One DAA	58.0
CD (.05)	9.2

Fertilization

Preliminary attempts on test tube fertilization resulted in successful fertilization and embryo formation (Fig.3). Among the 50 ovules pollinated, 80% enlarged after two weeks, indicating a high percentage of fertilization success. The unfertilized ovules as well as the control were shrunken and dried after a few days. However, further development was noticed in 40% of the ovules. Out of these, embryos were developed without endosperm and seed coat formation in about 30%. The remaining 10% ovules showed normal development comparable to *in vivo* fertilization. After 90 days, the size of the *in vitro* raised ovule was comparable with *in vivo* ovule but the morphology of the *in vitro* developing ovule resembled a mature seed. The seed coat is

sclearified and characteristic mottling was observed in the surface of these ovules as in the case of mature seeds under field conditions (Fig.5). Since further growth was not observed, they were fixed for anatomical studies.

Fig. 3. Embryo development without endosperm and seed coat

Another important phenomenon noticed in *in vitro* fertilization of *Hevea* was the development of embryos without endosperm and seed coat formation. The two integuments were found to be pushed back and the embryos become visible by about 30 days after pollination (Fig.3). This is in contrast to the development of seeds under *in vivo* conditions. Under field conditions, the embryos will be small and microscopic till seven weeks after fertilization in *Hevea brasiliensis*, (Muzick 1954). The failure of triple fusion during fertilization may be the reason for the absence of endosperm. Otherwise, after fusion the 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.

However, 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 where as the endosperm was purely developed and degenerated. This indicates the possibility of recovery of seedlings from such embryos in *Hevea* also. In *Hevea*, the embryos survived up to globular stage. By improving the nutritional status of the medium, the survival could be raised up to the heart shape stage. This also indicates the possibility of obtaining seedlings from these embryos by optimizing the culture medium.

Fig. 4. Heart shaped embryo along with suspenser

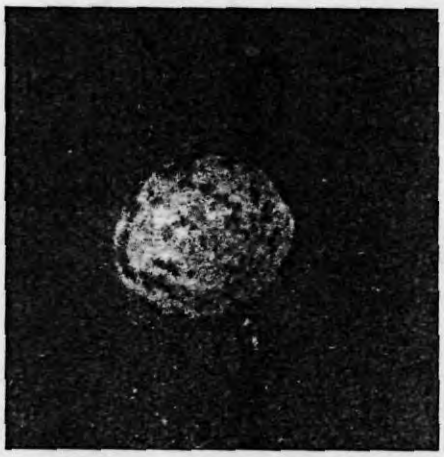

Fig. 5. Developing seed after IVF showing the characteristic mottling

Histological observations

The anatomical studies of the ovules 30 days after pollination revealed the presence of 2 layered outer wall. The abundance of starch and lipids were observed in the nucellar area (Fig. 6&7). The longitudinal section of a developing ovule 90 days

after pollination is presented in Fig.8. A well developed seed coat with a thin walled outer zone and thick walled inner zone with radically elongated sclearified cells could be seen at this stage. Seed coat is single layered in the chalazal region. The embryos were not visible at this stage. When a 90 days old ovule was dissected after *in vivo* pollination, similar results were observed except in the case of seed coat. Under *in vitro* conditions, the seed coat formation was faster and has been almost completed by 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. Further experiments are to be conducted by removing the integuments few days after pollination.

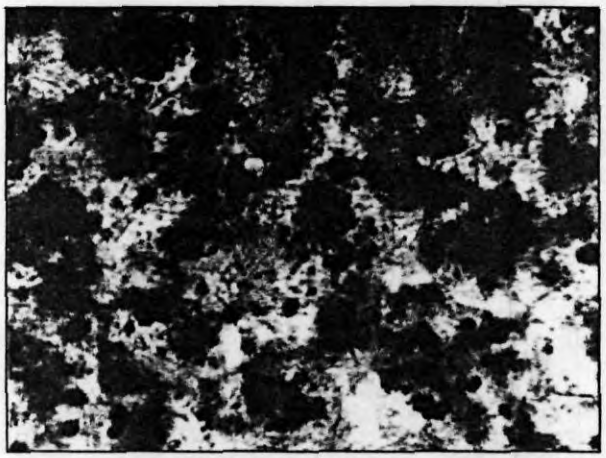

Fig. 6. CS of a developing ovule showing the abundance of starch (30 DAP)

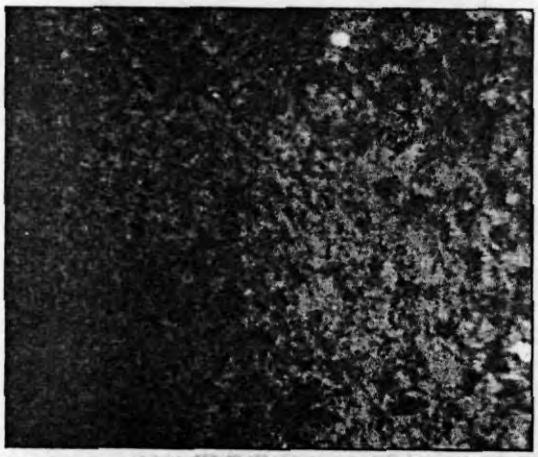

Fig. 7. CS of a developing ovule showing the abundance of lipids (30 DAP)

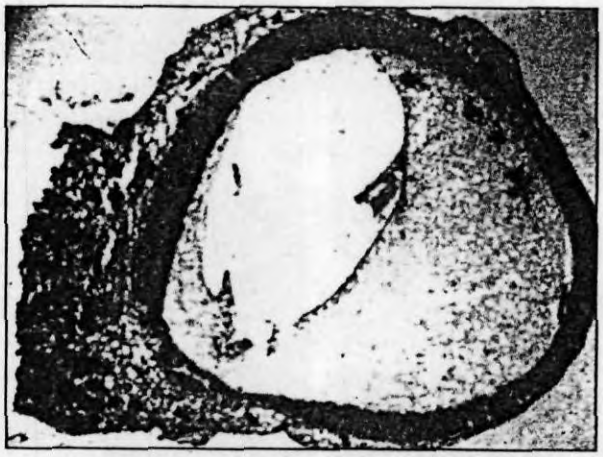

Fig. 8. LS of a developing seed (90 DAP)

CONCLUSION

The preliminary attempt on IVF of *Hevea* has resulted in successful fertilization and embryo formation. The fertilized ovules were grown up to 90 days after fertilization and embryo growth up to the heart shaped stage was achieved. This is the first report in *Hevea*. Eventhough, in this preliminary attempt the embryos could grow only up to heart shaped stage, the results throw light towards the possibility of recovering seedling after IVF by improving the nutritional status and culture conditions. However, further research has to be conducted to refine the technique in all stages so as to recover considerable number of progenies and to utilise the system in *Hevea* breeding.

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