Q. Jl. Rubb. Res. Inst. Sri Lanka (Ceylon) (1973) 50, 91—97

HEVEA TISSUE CULTURE

By

R. Satchuthananthavale

Improvement of rubber as well as any other crop is based on variations which exist among individuals of a species. Such variations originate in differing hereditary characteristics. In the conventional methods of plant breeding two individual plants are selected for desirable traits such as high yield, disease resistance, growth vigour and other secondary characteristics and crossed with each other to produce an offspring possessing a combination of the desirable traits of the parents. In self-fertilized plants this method of producing new strains is quite straightforward and pure lines which breed true to type can be established in a reasonably short period of time and propagated by seed.

Cross-fertilized species do not breed true to type and the seedlings of selected plants with desirable traits are likely to be variable. In such heterozygous, perennials, mass selection is used, and the selected plant propagated vegetatively.

Hevea is a typically cross-pollinated plant and hence there is a high degree of variability in the species and even among individuals of the same mother tree. It is for this reason that rubber is propagated vegetatively. Grafting buds taken from a selected mother tree to a seedling stock has proved to be the only practicable method at present, of producing a fairly uniform stand of high yielding trees. This has helped the Plant Breeders' efforts towards increasing the yields of rubber to present day levels.

The grafting of buds of uniform genetic constitution on to seedling stocks of variable genetic make up imposes limitations on the performance of the scions. That the stock can influence the growth and yield of the scion and *vice versa* is generally accepted (Dijkman, 1951). Vollema (1937) suggested that the stock/scion interaction could be due to anatomical differences between the tissues of the stock and scion. It has also been shown that the stock can greatly influence the growth and yield of the scion (Murray, 1938). Schweizer (1938), showed that certain clonal scions do better on seedling rootstocks of certain other clones than they do on seedling stocks of their own.

Though the nature of the stock/scion interaction has not been fully resolved it could be inferred that propagation by bud-grafting limits yield potentials of clones.

Propagation by rooted cuttings has been tried as an alternative to overcome the stock/scion effect. Experiments indicated that cuttings grow better than buddings but the lack of a proper tap root system was a major drawback.

Plant tissue culture offers new scope in breeding and propagation of *Hevea*.

The recent discovery that pollen grains could be induced to develop into haploid plants either directly or *via* haploid callus formation has aroused considerable interest among plant breeders. The advantages of raising new homozygous lines in heterozygous populations are well known in plant breeding (Nei, 1963). In the conventional method of plant breeding a considerable number of generations have to be raised before obtaining

a pure line. If, however, haploid plants could be raised first, then chromosome doubling could be achieved through artificial means like colchicine treatment *etc*; to obtain homozygous plants in one or two generations.

The production of haploid plants directly from pollen grains has been reported in Datura (Guha & Maheswari, 1967) and in *Nicotiana tabacum* (Nakata & Tanaka, 1968; Nitsch & Nitsch, 1969; Sunderland & Wicks, 1969). In *Oryza sativa*, Niijaki & Oono (1968) reported the successful induction of haploid plants from haploid callus obtained from anther culture. The method of obtaining haploid plants directly from pollen grains as in the case of *Datura*,and *Nicotiana* may not be applicable to many other plant species. In such cases, the callus approach may be the only and preferable route to haploid plant production. It is preferable since callus cultures are more unstable and chromosome doubling frequently occurs through repeated sub-culturing. Chromosome doubling could also be achieved through artificial means. From such diploid callus, diploid plants might be regenerated with less difficulty.

Hevea tissue culture

From the published information available on *Hevea* tissue culture, it would appear that only sporadic attempts have been made up to now. Bouychou (1953) was able to obtain callus tissue from stem segments of young *Hevea* seedlings. Chua (1966) reported callus formation from plumule sections from 3 to 4 day old seedlings. Root differentiation was noticed in the callus after 5 to 6 months. The callus failed to grow on subculturing.

Differentiation of roots and plantlets from cotyledon cultured *in vitro* has also been reported recently. (Paranjothy, 1972).

The first successful attempt at continuous sub-culturing of *Hevea* callus tissue established from anthers was reported by Satchuthananthavale in 1971.

The experiments were initiated with a view to explore the possibilities of raising haploid plants. Male flower buds from *Hevea brasiliensis* Cv. RRIC 52 were used for the initial experiments. Since flowering is seasonal in this cultivar, experiments were restricted to the flowering season only. Subsequently Cv. KH 440 which flowers throughout the year was used to ensure a steady supply of flowers for continued experimentation.

Male flower buds of various stages of development were surface sterilised in a filtered suspension of 7% (W/V) calcium hypochlorite for 5 min. After washing in sterile distilled water the anthers were aseptically removed and placed on sterile nutrient media in culture tubes. The basal medium comprised of Nitsch's inorganic and organic additives (Nitsch & Nitsch, 1969) and 2% sucrose. This was supplemented with growth substances such as IAA, 2, 4-D, GA, Kinetin, and coconut milk either singly or in combinations, for different treatments. The pH of the medium was adjusted to 5.5 with 0.1 N, NaOH or HCl. The medium was solidified with 1% Difco Bacto Agar. Approximately 20 ml aliquots of the medium were dispensed into each culture tube and sterilised. The anthers were incubated at 28 ± 2 °C, under continuous fluorescent lighting.

It was observed that the developmental stage of the anther was critical for callus formation. Fig. 1 shows male flowers at different stages of development, before anthesis, that were used for the study. Anthers removed from flowers at stage 3 from right to left, were found to be the ones that developed callus.

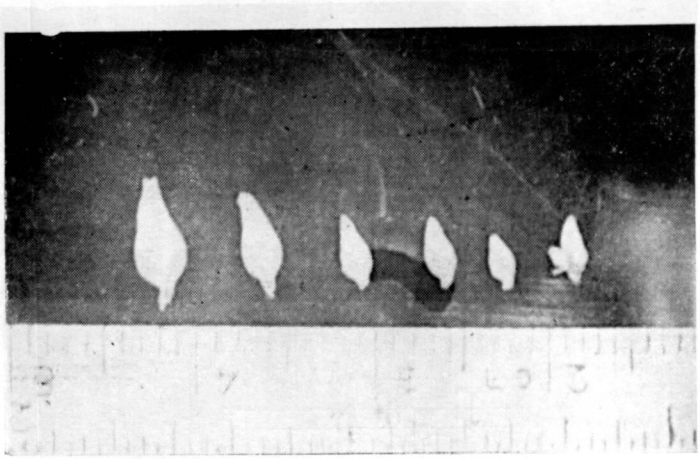

Fig. 1 Male flower buds of *Hevea* at different stages of development

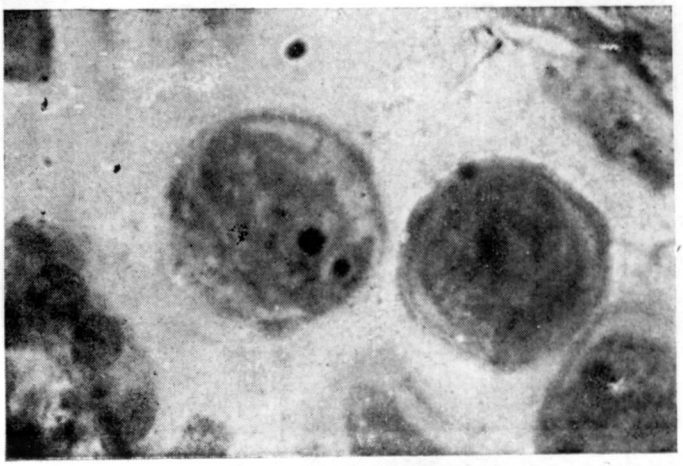

Fig. 2 Fully individualised pollen grains with vegetative and tube nuclei

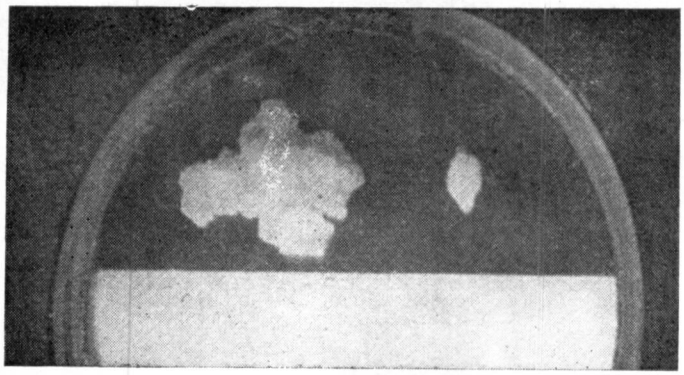

Fig. 3 A six week old callus established from an anther (left) compared with a group of anthers removed from a male flower (right).

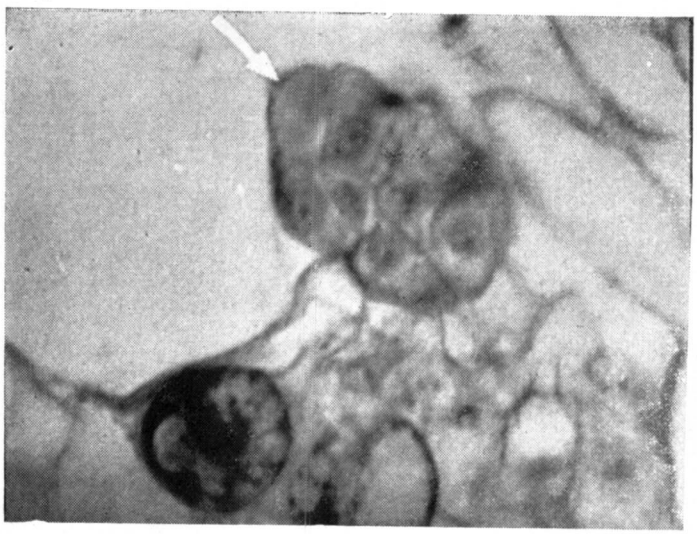

Fig. 4 Section through callus showing a dividing pollen grain (arrow).

An examination of the anthers at this stage showed that they had fully individualised pollen grains as shown in Fig. 2. The first mitotic division appeared to have occured in some of the pollen grains. From the nature of the exine of the pollen grains it is evident that they are not fully mature. It is at this stage that the anthers showed response depending on the composition of the nutrient medium used. Sunderland & Wicks (1969) demonstrated that in *Nicotiana tabacum* the haploids obtained were derived from the tube nucleus of the pollen grain. We are not certain whether this is the critical stage for callus formation in *Hevea* anthers.

Very young anthers at the microspore mother cell stage or fully mature anthers failed to show any response on any of the media tried. These anthers turned black in colour within two weeks of inoculation and degenerated by the end of six weeks. The anthers which showed response remained fresh. Within two weeks of inoculation they enlarged, and further callus development was determined by the composition of the nutrient medium on which they were cultured. The response shown could be categorised as follows :—

(1) On basal medium alone with 2% sucrose and without any added growth substances, the anthers remained fresh for about two to three weeks, but later turned brown and then black and finally degenerated.

(2) On basal medium supplemented with IAA, 2, 4-D, GA, Kinetin and coconut milk singly at various concentrations, they were seen to enlarge only. No callus formation was observed.

(3) On basal medium supplemented with auxin IAA, 2, 4-D, Kinetin, and coconut milk in combination at the various concentrations tested anthers enlarged and showed callus proliferation.

The optimum levels of growth substances for good callus development were found to be IAA (1.0 mg/l), 2,4-D (1.0 mg/l), Kinetin (1.0 mg/l) and coconut milk 15%. Incorporation of GA into the medium had an adverse effect on callus formation whether it was used singly or in combination with other growth substances.

In the above medium, vigorous, pale yellow callus was seen to develop in 4 to 5 weeks. The callus had a nodular appearance as shown in Fig. 3. On sub-culturing the callus grew satisfactorily on the same medium. No diminution in growth vigour was evident through repeated culturing.

Plantlet development from pollen grains as has been reported for *Datura*, or *Nicotiana* was not observed. Organ differentiation too did not occur on prolonged culturing on the same medium.

Histological studies were carried out to determine the mode of origin of the callus. Microtome sections of anthers during the early stages of callus development revealed that cell proliferation occurred from the anther wall and connective tissue but not from the central column. Cell division was also noticed in a few pollen grains as seen in Fig. 4 which is of interest. We have reasons to believe that these multicellular bodies, are in fact dividing pollen grains. The cell wall is distinctive and exine like, resembling the exine of the undivided pollen grain. These multicellular bodies were also seen scattered among groups of aborted pollen grains, which is again evident from Fig. 4.

Microtome sections of much older callus (about eight weeks or more) did not show the presence of such multicellular bodies. It is very probable that with subsequent cell divisions they are lost among the tissue of the callus originating from the anther wall, which is very fast growing. Attempts to culture the pollen grains in isolation on nutrient media have failed.

Suppression of growth of the diploid callus originating from the wall of the anther and the simultaneous enhancement of growth of the haploid callus arising from the pollen grains, could be tried. It has been reported that parafluorophenylalanine stimulates the growth of haploid tissues at the same time inhibiting that of diploid tissue (Navendra Gupta & carlson, 1972). This growth promoting chemical could be tried to isolate the haploid callus from a mixture of haploid and diploid callus, and grow it separately.

CONCLUSION

Haploid plantlet formation directly from pollen grains may not be possible in *Hevea*. The haploid callus approach could be tried as a possible route to haploid plant production.

The aphorism 'all cells from pre-existing cells' and 'living cells are 'totipotent', that is, they have the ability to develop into entire plants provided that the correct stimulus is given to trigger growth and development, is becoming more and more evident. Organ differentiation and plantlet formation has been reported from proliferating pieces of stem, root, leaf, floral primordium, embryo, anther, fruit and even endosperm tissue. In most cases some growth hormone or some other complex substance like coconut milk for example, has to be present for differentiation.

In *Hevea* too, induction of callus from stem sections of young seedlings; root differentiation from callus obtained from plumule sections; root and plantlet formation from cotyledons have been reported. There is no doubt that *Hevea* tissue is amenable to tissue culture.

We believe that the utilization of the recent technique of anther culture in the improvement of *Hevea*, rests on devising suitable techniques to culture haploid callus and providing the precise nutritional conditions required for plantlet development.

ACKNOWLEDGEMENT

The author wishes to thank Mr. G.de Mel and Mr. Z.E. Irugalbandara for technical assistance.

REFERENCES

BOUYCHOU, J. G. (1953) La culture *in vitro* Des Tissues *d'Hevea*. *Arch. Rubb. cult.* Extra No. 2 Paper A5, 50-53.

CHUA, S. E. (1966) Studies on tissue culture of *Hevea brasiliensis*. I. Role of osmotic concentration, carbohydrates and pH values in induction of callus growth in plumule tissues of *Hevea* seedlings. *J. Rubb. Res. Inst. Malaya*, **19**, 272-276.

DIJKMAN, M. J. (1951) Hevea, *thirty years of research in the Far East*. Florida: Univ. of Miami Press.

GUHA, S. AND MAHESWARI, S. C. (1967) Development of embryoids from pollen grains of *Datura in vitro*. *Phytomorphology*, **17**, 454-461.

MURRAY, R. K. S. (1938). The budgraft as a complete plant. *Quart. Circ. Rubb. Res. Scheme Ceylon* **15**, 166-174.

NAKATA, K. AND TANAKA, M. (1968) Differentiation of embryoids from developing germ cells in anther culture of tobacco. *Jap. Jour. Genet*, **43**, 67-71.

NARENDRA GUPTA AND CARLSON, P. S. (1972) Preferential growth of haploid plant cells *in vitro*. *Nature New Biology*, **239**, 86.

NEI, M. (1963). The efficiency of haploid method of plant breeding. *Heredity*, **18**, 95-100.

NIIJAKI, H. AND OONO, K. (1968) Induction of haploid rice plants from anther culture. *Proc. Japan Acad.* **44**, 554-557.

NITSCH, J. P. AND NITSCH, C. (1969) Haploid plants from pollen grains. *Science*, **163**, 85-87.

PARANJOTHY, K. (1972) Personal communication.

SATCHUTHANANTHAVALE, R. (1971). *A. Rev. Rubb. Res. Inst. Ceylon* 1971, p. 82.

SCHWEIZER (1938) Over den wederzijdschen invloed van boven-en onderstam bij *Hevea brasiliensis*. *Bergcultures* **12**, 773-778. (Abstract Bibliography of *Hevea* Rubber Vol. I. Library Information Bulletin No: 7/69 *Rubber Research Centre Hatyai, Thailand*).

SUNDERLAND, M. AND WICKS, F. M. (1969) Cultivation of haploid plants from tobacco pollen. *Nature*, **224**, 1227-1229.

VOLLEMA, J. S. (1937) Veredeling van *Hevea*. *Landbouw* **13**, 482-456. (Abstract Bibliography of *Hevea* Rubber Vol. I. Library Information Bulletin No. 7/69. *Rubber Research Centre. Hatyai, Thailand.*)