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Recent Advances in Anther Culture of *Hevea brasiliensis* (Muell.-Arg.)

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Summary. The yield of pollen embryoids from cultured *Hevea* anthers was increased 4 fold by optimizing the proportion of ammonium nitrate to potassium nitrate in the dedifferentiation medium. For optimal differentiation of pollen embryoids, kinetin, 2,4-D and α-naphtalene acetic acid are required. Anther culture for 50 days on the dedifferentiation medium is a prerequisite for the selective development of calli and embryoids from microspores.

The determination of chromosome numbers in embryoids, plantlets and regenerated trees reveals that they originate from (poly)haploid pollen grains ($n=2x=18$). Aneuploid, triploid ($3x=27$) and tetraploid ($4x=36$) cells were encountered in increasing frequencies as the embryoids and plants developed. A few haploid cells with 9 chromosomes were consistently observed. Buds from shoots with mixoploid chromosome numbers can be grafted and the change in the chromosome constitution of the developing new shoots followed.

Key words: Pollen plants – Chromosome counts – Callus formation – Embryoid formation – Plant regeneration – Rubber-tree

Introduction

It is unrealistic to produce pure lines of perennial cross-pollinating trees such as *Hevea* by inbreeding over many generations. Homozygous diploid plants, however, can be obtained by regenerating haploid plants from pollen by anther culture and doubling of their chromosomes. By this short-cut procedure, pure lines of different genotypes should be obtainable and provide the basis for producing hybrids of *Hevea* exhibiting heterosis.

After the first pollen plantlets of the rubber tree, *Hevea brasiliensis*, had been obtained by anther culture in 1977 (Anonymous 1977), optimal conditions for the development of microspores into embryoids have been worked out. The frequency of embryoids obtained was initially an average 25 per 100 inoculated anthers (a maximum of 80 was occasionally achieved). With the present techniques 100 embryoids are formed per 100 inoculated anthers (with a maximum of 140). The frequency of viable pollen plantlets has been enhanced from 0.05% to 3%. Several hundred pollen plantlets have been obtained from three genotypes of rubber tree. Some of them have been transplanted into the field and grow normally (Fig. 1). In the present paper we detail the optimal culture conditions and the analyses of chromosome numbers on calli, embryoids, pollen plantlets and transplanted plants.

Materials and Methods

The parent plants, the inoculation techniques and the histological as well as cytological techniques have been described previously (Chen Chêng-hua et al. 1978a, b). The production of pollen plants is carried out in three steps:

Step 1. The anthers are inoculated on a dedifferentiation medium consisting of modified basal medium (Chen Chêng-hua et al. 1978a, b) supplemented with 1 mg/l kinetin, 1 mg/l 2,4-D, 1 mg/l α-naphtalene acetic acid, 5% coconut milk and 7% sucrose. On this medium the somatic tissues in the anthers form calli at the same time or somewhat earlier than the pollen grains.

Step 2. After 50 days the anthers showing calli are transferred to the differentiation medium consisting of Murashige-Skoog medium (1962) supplemented with 0.5–1 mg/l kinetin, 0.2 mg/l naphtalene acetic acid, 0.5 mg/l gibberellin and 7–8% sucrose. On this medium the pollen calli differentiate embryoids and the preexisting microscopic embryoids grow to become visible to the naked eye.

Step 3. The embryoids are transferred to a Murashige-Skoog medium supplemented with 1–2 mg/l gibberellin (GA_3), 1 mg/l indole acetic acid, 1 mg/l 5-bromo-uracil and 5% sucrose.

In all three types of media 0.7–0.8% agar-agar was used and the initial pH was 5.8.

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Results

Effects of Nitrate and Ammonium Ions and Hormones on the Induction of Pollen Embryoids

The amount and chemical form of the nitrogen appears to be of special significance for the development of the microspores. From the experiments summarized in Table 1 it emerges that treatment 1 with a relative high proportion of ammonium nitrate to potassium nitrate is optimal for embryoid formation. A decrease in the total amount of nitrogen in the medium favours callus formation from the anther while an increase in total amount of nitrogen is inhibitory altogether. A high proportion of KNO_3 relative to NH_4NO_3 (treatment 5) permits the formation of embryoids but decreases their frequencies.

The presence and concentrations of plant growth substances also notably affected the dedifferentiation and development of the embryoids. The supplement of the dedifferentiation medium with kinetin and 2,4-D induced a considerable frequency of callus formation by the anthers. But for the development of embryoids the addition of all three growth hormones (kinetin, 2,4-D and naphtalene acetic acid) was necessary. Naphtalene acetic acid had a pronounced effect on the formation of multicellular masses from pollen grains. After 20 days of culture from anthers inoculated on a single day and from a single plant, it was observed that in the presence of naphtalene acetic acid (1 mg/l) 149 out of 730 studied pollen grains had developed into multicellular masses (20.4%) whereas in its absence 84 out of 795 had done so (10.6%). Reduction of the amount of naphtalene acetic acid from 1 mg/l to 0.5 mg/l decreased the number of embryoids formed. Thus, in an experiment employing 1 mg/l in the medium, 245 embryoids were obtained from 1073 anthers inoculated (22.8%) whereas in an experiment with half this concentration 160 embryoids formed on 1166 inoculated anthers (14.2%).

Three growth substances, namely kinetin, naphtalene acetic acid and gibberellin (GA_3) were required in the differentiation medium. In the absence of kinetin the induction frequency of embryoids decreased and bud formation was prevented. GA_3 favoured the growth of the embryoids and the formation of cotyledons. In its absence the embryoids remained small in size and developed anomalous cotyledons.

Table 1. Effect of nitrogen concentration and ammonium ions relative to nitrate on the frequency of embryoid development

Treatment	KNO_3 mg/l	NH_4NO_3 mg/l	Total nitrogen mg/l	Anthers inoculated	Callusing anthers %	Embryoids %
1	950	1,650	708	204	52	45
2	1,900	825	552	200	81	3
3	1,900	2,800	1,229	126	13	1
4	950	825	420	244	80	1
5	1,900	1,650	841	240	54	25

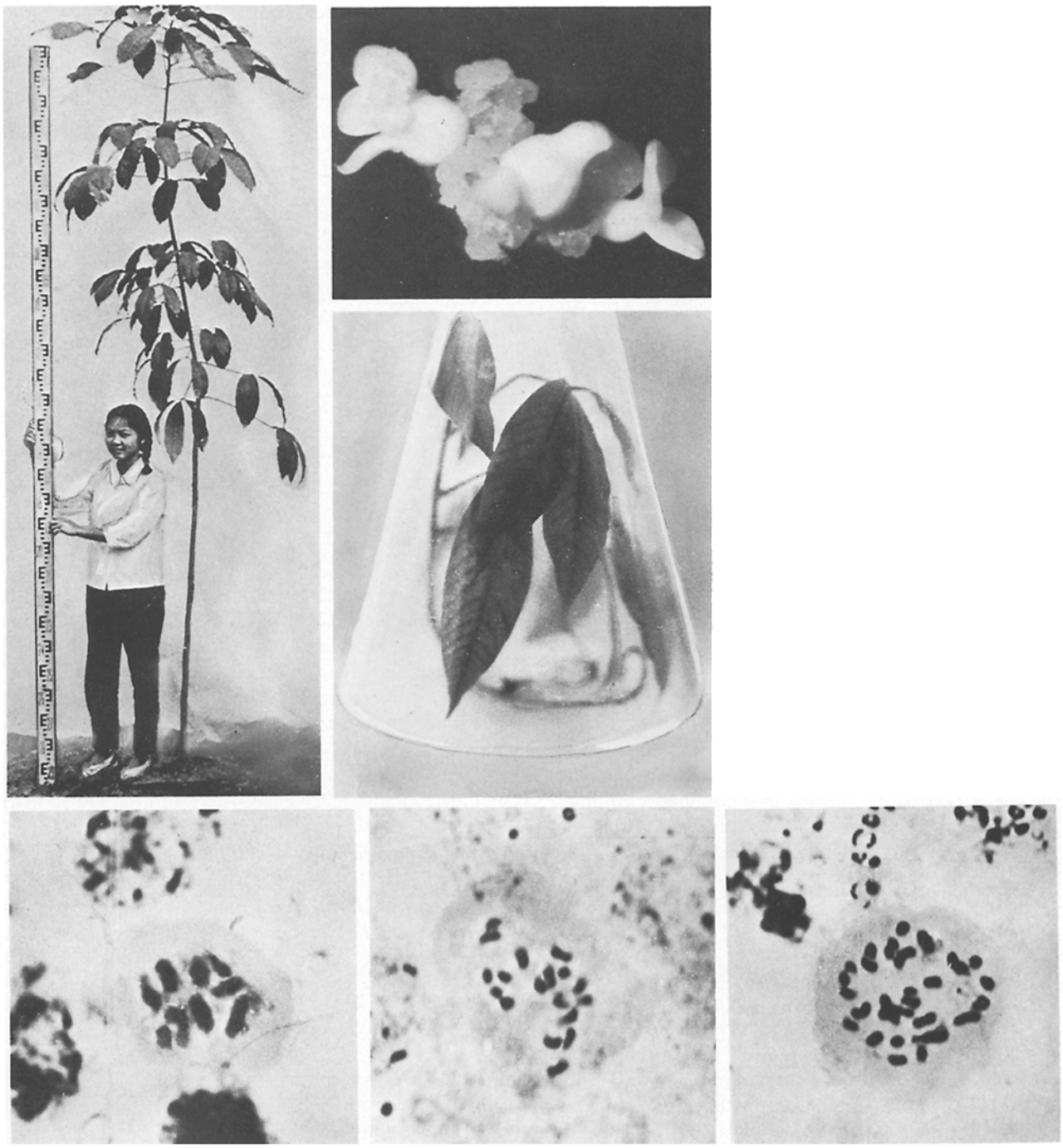
The Chromosome Numbers of the Anther Cultures and Transplanted Pollen Plants

1 Chromosome Numbers of Calli

Chromosomes were counted on calli from anthers on the dedifferentiation medium in order to determine the growth rate of somatic calli and those originating from pollen cells. In calli from 10 anthers cultured for 25 days, 80% of the mitotic metaphase plates revealed a diploid chromosome number of $2n=36$. In a sample of 142 metaphases from 10 anthers cultured for 50 days only 18 (12.7%) had 36 chromosomes, while the majority namely 96 metaphases (67.5%) had 18 chromosomes. Of the rest, 22 metaphases (15.5%) had 19 to 27 chromosomes and 6 metaphases (4.3%) contained 28 to 35 chromosomes. During the first 25 days of inoculation calli were thus primarily proliferating from the somatic tissue, but after 50 days of culture the calli and small embryoids originating from the microspores began to divide vigorously. This then was considered the right moment to transfer the callus forming anthers to the differentiation medium.

2 Chromosome Numbers of Embryoids (Fig. 2) and Root-tip Cells of the Plantlets (Fig. 3)

Chromosomes were counted in metaphases of embryoids and root-tip cells of plantlets developed from the anthers cultured in a single flowering season (Table 2). Among 66 metaphase plates analyzed from 13 embryoids, half of them (51.5%) had 18 chromosomes and 39.4% had 27 chromosomes. A few cells had 9 chromosomes (Fig. 4) and a few more than 27 chromosomes. Since the mother tree has 36 chromosomes we can conclude that with the employed culture technique the embryoids arose from pollen cells. From 5 plantlets root tips were studied and 38 mitotic metaphases analyzed. 39.5% of the cells contained 18 chromosomes (Fig. 5) and 52.6% of the cells had 27 chromosomes (Fig. 6). Two of the plates revealed aneuploid numbers of 20 and 24 chromosomes respectively and one cell with 9 chromosomes was identified. The origin of the cells with 27 chromosomes is unclear but probably due to an unbalanced increase in the number of chromosomes during growth of the pollen calli.



Figs. 1 – 6. 1 A transplanted pollen tree; **2** the pollen embryoids (the cotyledon type embryoids 5 mm in length); **3** a pollen plantlet; **4** a cell of embryoid with 9 chromosomes. Magnification 600 ×; **5 and 6.** A root cell of plantlet with 18 chromosomes **(5)** and with 27 chromosomes **(6)**. Magnification 600 ×

3 Chromosome Numbers of Leaves from Transplanted Trees

Metaphases of leaf cells were studied after transplantation into the field. In plants less than 50 cm in height the leaf cells revealed a considerable variation in chromosome number (Table 3 A), most of the cells having counts between 18 and 27 chromosomes. A larger tree with a height of 160 cm had many leaf cells with chromosome numbers between 28 and 36 chromosomes and

Table 2. Chromosome numbers observed in mitotic metaphases of embryoids and root-tips of plantlets

Tissue	Number of specimens	Number of metaphases	Chromosome counts							
			9	18	20	24	27	32	26	45
Embryoids	13	66	4	34	0	0	26	1	0	1
Root-tips	5	38	1	15	1	1	20	0	0	0

Table 3. Chromosome counts in metaphases of young leaf cells from transplanted pollen plants (A) and one grafted shoot of a pollen plant (B)

Number of plants resp. shoots	Height of plant (cm)	Number of leaves	Number of metaphases	Chromosome counts			
				9 – 17	18 – 27	28 – 36	> 36
A 8	50	8	221	38	172	6	5
1	160	10	69	1	26	41	1
B 1	50	2	11	3	8	0	0
1	170	8	67	0	48	19	0

relatively few cells with 18 to 27 chromosomes. This may indicate a successive increase in chromosome numbers, whereby cells with euploid numbers are favoured.

Two buds of a pollen plant were grafted and grew into new plants. In a plant of 50 cm in height cells with 9 to 27 chromosomes were found (Table 3 B), whereas the same plant had chromosome counts ranging from 18 to 36 when it reached 170 cm in height. This fact also reveals a successive increase in chromosome numbers during the growing period of the bud grafted stocks of pollen.

4 Possible Origin of Plants from Somatic Tissues

Embryoids and plantlets with 36 chromosomes could arise from the somatic tissue of the anthers rather than from the generative pollen grains. We observed that 85.3% of the cells from embryoids induced on media without naphtalene acetic acid had chromosome counts of 28 to 36 (Table 4) whereas in the presence of the growth hormone 93.7% of the cells had chromosome numbers close to 18.

Table 4. Chromosome counts in metaphases of embryoids obtained on media with and without α-naphthalene acetic acid

Naphthalene acetic acid mg/l	Number of embryoids	Number of metaphases	Chromosome counts	
			9 – 18	28 – 36
0	17	34	5 (14.7%)	29 (85.3%)
1	11	112	105 (93.7%)	7 (6.3%)

A low temperature treatment of the inflorescences prior to inoculation affected the development of callus from pollen grains but not that from somatic tissues. We inoculated anthers from inflorescences stored at 3–5 °C for 20 h, others at 11 °C for 24 h and as control anthers without storage. We followed callus and embryoid formation in the anthers by microscopy. The anthers stored at a low temperature prior to inoculation had fewer multicellular masses derived from pollen than the controls. In the latter no embryoids with a majority of cells containing 28 to 36 chromosomes were observed. Among five embryoids derived from anthers stored at 11 °C for 24 h three had a majority of cells with 36 chromosomes. Among ten embryoids derived from anthers stored at 3–5 °C for 20 h there were nine in which most cells had 36 chromosomes.

Discussion

The microscopical analysis of callus formation from anthers is helpful to determine the best genotypes for culture, the optimal developmental stage of the microspores anthers prior to inoculation and the optimal composition of the medium. In some dicotyledonous crop plants, such as cotton (Hu Shao-an 1977; Pan Chingli et al. 1977) and tea-oil tree, callusing anthers had been obtained several years ago, but embryoids from microspores have so far not been obtained. Experimental approaches to induce the development of microspores in culture might be successfully explored in these species by following their differentiation micro-

scopically. In cereals microscopic monitoring of the development of microspores (Sun Chingsan et al. 1977; Wang Chingchu et al. 1973; Wang Chingchu et al. 1974; Chu Zhihching et al. 1975) has been helpful in optimizing culture conditions. For instance the N_6 medium was developed by screening for frequencies of multicellular masses and calli originating from pollen grains (Chu Zhihching et al. 1975).

We have previously found that high concentrations of sucrose (7%) and coconut milk (5%) favoured the development of microspores, while low levels of sucrose (3%) and absence of coconut milk appeared to be adequate for development of somatic tissue (Chen Zhenghua et al. 1979). In the present paper it is shown that the balance of nitrate versus ammonium and the level of plant hormones can regulate the relative frequency of calli and embryoids arising from the microspores and the somatic tissue of the anthers. Also the length of time required on the different media can be optimized. Thus, the calli from the somatic tissue of the anthers proliferating during the first 25 days of culture underwent senescence during another 25 days of culture permitting the calli and embryoids of the microspores to divide vigorously at the later stages. This time then was the right moment to transfer the callusing anthers to the differentiation medium promoting the development of the haploid embryoids.

The chromosome number of *Hevea brasiliensis* was established unequivocally as $2n=36$ by Perry (1943) and subsequently confirmed by others (Mendes 1946; Majumder 1964; Ong 1975). From the occurrence of multivalents in metaphase I of meiosis in species hybrids, Ramaer (1935) deduced *Hevea brasiliensis* to be an amphidiploid with a basic number of $x=9$. Occasional occurrence of cells of the rubber tree with 9 chromosomes has been reported earlier (cf. Perry 1943) but was attributed to poor cytological techniques. The consistent occurrence of a few dividing cells with 9 chromosomes in our preparations of calli, embryoids and root-tips lend support to the amphidiploid nature of the rubber tree. The deregulation of the chromosome number of the polyhaploid to the true haploid chromosome number is an intriguing problem requiring further study.

In previous studies on cereals such as wheat and rice a successive increase in chromosome number in the developing pollen calli and plantlets has been observed, whereby multiples of the basic chromosome numbers were favoured (Chen Ying et al. 1974; Hu Han et al. 1978; 1980). In certain in vitro cultures of calli, endomitosis, fusion of nuclei, endoreduplication and multipolar mitosis of cells are frequently observed. Such anomalies of mitotic division might be the cause of chromosome doubling, mixoploidy and aneuploidy (McComb 1978; Hu Han et al. 1980). The mechanism of the increase in chromosome number leading to aneuploid, triploid and tetraploid cells in embryoids of *Hevea* arising from microspores needs further elucidation. The frequencies of cells with different chromosome numbers appeared to continue to change in the transplanted trees, whereby again balanced chromsome numbers, i.e. multiples of 9 seem to be favoured. As buds from such trees can be propagated it will be possible to analyse the chromosomal variation in shoots originating from buds with various euploid and aneuploid constitutions. This chromosomal variation might be useful in the breeding work with the rubber tree.

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