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PROPAGATION OF CALLUS FROM *HEVEA* ANTHERS

BY

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ABSTRACT

This paper describes the first successful in vitro *culture of anthers of* Hevea brasiliensis (*Willd. ex Adr. de Juss.*) *Mull- Arg· Anthers of* H. brasiliensis, *cv. RRIC 52 and KH* 440, *were excised at a stage when pollen grains were individualized and cultured aseptically on a simple agar medium, containing modified Nitsch's medium with auxin,* 2, 4-D *and kinetin or coconut milk. Callus formation from tissues of the anther and pollen grains was observed after four to five weeks. Callus thus obtained has continued to maintain good growth on the same medium, through six sub-cultures over a period of six months. When auxin and kinetin were absent from the medium, the anthers and pollen grains became dark brown in colour and died off. The possibility is thus indicated for the development of haploid* Hevea *plants from callus tissue derived from pollen grains.*

INTRODUCTION

The recent discovery that pollen grains could be induced to develop into haploid plants either directly or *via* haploid callus formation has created considerable interest among plant breeders. The advantages of raising new homozygous lines in heterozygous populations are well known in plant breeding and have been theoretically discussed by Nei (1963). In the conventional method the plant breeder has to raise a considerable number of generations before obtaining a pure line in a heterozygous variety. If, however, haploid plants could be raised first, then chromosome doubling could be achieved through artificial means like colchicine treatment, to obtain homozygous lines in one or two generations.

The production of haploid plants directly from pollen grains has been reported in *Datura innoxia* (Guha & Maheswari, 1967) and in *Nicotiana tabacum* (Nakata & Tanaka, 1968; Nitsch & Nitsch, 1969; and Sunderland & Wicks, 1969). In *Oryza sativa,* Niizaki & 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 possible route to haploid plant production. Furthermore, the callus approach is preferable since callus cultures are more unstable and chromosome doubling frequently occurs through repeated sub-culturing. This could also be achieved through artificial means. From such diploid callus, diploid plants might be regenerated with less difficulty.

The observations reported in this paper were made during experiments carried out to establish and grow callus cultures from *Hevea* anthers.

MATERIALS AND METHODS

Male flower buds from *H. brasiliensis,* cv. RRIC 52 were used initially for the study during the flowering season—February/March 1971. During subsequent experiments, flower buds from cv. KH 440 were used; since it was possible to induce flowering in this clone right through the year and flower buds could be obtained when required.

Flower buds at various stages of development were sterilized in a filtered suspension of calcium hypochlorite (7% w/v) for five min and the group of anthers from each flower bud was asceptically removed under a dissecting microscope and placed on sterile nutrient media in culture tubes.

The basal medium comprised of Nitsch's inorganic and organic additives without myo-inositol (Nitsch & Nitsch, 1969) and 2% sucrose. This was supplemented with growth substances such as indole-3-acetic acid (IAA), 2, 4—dichlorophenoxyacetic acid (2, 4-D), gibberellic acid (GA), kinetin and coconut milk, either singly or in combinations for various treatments. The pH of the medium was adjusted to 5.5 with 0.1 N NaOH or HCl, before addition of 1% Difco Bacto Agar (DBA). Approximately 20 ml aliquots of the medium were dispensed into each culture tube and autoclaved at 15 lb pressure for 15 min. The cultures were incubated at room temperature 28 ± 2°C, under continuous fluorescent lighting. For microtome sectioning, the material was fixed in formalin-acetic-alcohol (5 : 5 : 90) washed in distilled water and dehydrated in a graded series of ethyl alcohol and tertiary butyl alcohol (Johansen, 1940). Paraffin sections 10 thick were cut and stained in Heidenhain's Iron Haematoxylin (Johansen, 1940).

RESULTS

Development of anthers in vitro

Young anthers inoculated at the microspore mother cell stage failed to grow on any of the media tested. Such anthers turned black in colour within two weeks and finally degenerated at the end of six weeks. Mature anthers, at a stage shortly before pollen is shed, also failed to show any response and degenerated by the end of six weeks. No embryoid formation from pollen grains was noticed.

Anthers in between these two stages, that is, when the pollen grains were fully individualized and at the uninucleate stage or immediately after the first mitotic division, showed response depending on the composition of the nutrient medium used. The response shown could be described as follows :

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

(2) On basal medium supplemented with IAA, 2, 4-D, GA, kinetin and coconut milk, separately at various concentrations, the anthers showed a slight increase in size but later turned dark brown in colour. They finally degenerated. No callus formation or embryoid formation from pollen grains were observed.

(3) On basal medium supplemented with auxins IAA, 2, 4-D and kinetin and 15% coconut milk in combination, the anthers grew to more than double the size in two weeks and showed callus proliferation.

Among the combinations of growth substances tested at different levels, good callus growth was obtained in the medium containing the following; IAA (1.0 mg/l) 2, 4-D (1.0 mg/l), kinetin (1.0 mg/l) and 15% coconut milk. In this medium vigorous pale yellow callus formation was observed in four to five weeks time (Table 1).

TABLE 1

EFFECTS OF GROWTH SUBSTANCES ON CALLUS FORMATION FROM ANTHERS OF *HEVEA*

Medium*	Growth substances					Response**
	IAA	2,4-D	GA	KIN	CM	
BM	—	—	—	—	—	—
BM	—	—	—	—	15%	—
BM	1 mg/l	—	—	—	—	—
BM	—	1 mg/l	—	—	—	—
BM	—	—	—	1 mg/l	—	—
BM	2 mg/l	—	—	—	15%	—
BM	—	1 mg/l	—	—	15%	+
BM	—	2 mg/l	—	—	15%	+
BM	—	—	2.5 mg/l	—	15%	—
BM	—	—	5 mg/l	—	15%	—
BM	2 mg/l	—	—	1 mg/l	15%	—
BM	1 mg/l	1 mg/l	—	1 mg/l	—	+++
BM	1 mg/l	2 mg/l	—	1 mg/l	—	+
BM	1 mg/l	1 mg/l	—	—	15%	+++
BM	1 mg/l	1 mg/l	—	1 mg/l	15%	++++
BM	2 mg/l	1 mg/l	—	1 mg/l	15%	+
BM	1 mg/l	2 mg/l	—	1 mg/l	15%	++
BM	2 mg/l	2 mg/l	—	—	15%	+
BM	2 mg/l	2 mg/l	—	1 mg/l	—	—

* Nitsch's medium + 2% sucrose—BM (basal medium)
** — represents no response; + anthers showing enlargement;
++ discernible callus formation; +++ good callus formation
++++ very good callus formation.

The callus proliferation was observed from cells forming the wall of the anther as well as from inside the anther. The central column on which the anthers are situated did not show any callus formation. These callus masses had a warty appearance (Fig. 1). Some of these callus nodules turned green in colour while the others remained pale yellow. On sub-culturing, these callus masses grew satisfactorily on a medium containing IAA, 2, 4-D, kinetin and coconut milk at the same levels as for anther callus initiation. The oldest such sub-cultures have been maintained for approximately one year now, without any loss of vigour through continued sub-culturing every six to eight weeks.

Microtome sections of anthers, during callus formation, revealed that cell proliferation occurred from cells of the anther wall as well as from pollen grains. A section through a six week old anther in culture, is shown in Fig. 2. where a pollen grain in the early stage of callus formation is clearly seen.

No differentiation has so far been noticed in these callus cultures. But further experiments are being carried out to induce shoot and root differentiation and plantlet development.

DISCUSSION

Organ differentiation and plantlet formation has been reported from callus cultures obtained from various plant organs such as root, stem, leaf, floral primodium, ovary and anther in different species of plants. This indicates that somatic cells have the potential to develop into plantlets. It is also evident from published work that different growth substances either singly or in combinations as well as

different cultural conditions are required to trigger differentiation and plantlet formation from callus cultures of various plant species. For example, in these experiments with *Hevea* anthers, callus formation was obtained when IAA + 2, 4-D were supplied together, although kinetin and coconut milk could be substituted for each other in the medium. Earlier attempts by many workers to establish callus cultures of *Hevea* from apical meristem and cambium were not successful. Chua (1966) reported callus formation from plumule sections of three to four day old *Hevea* seedlings, which after five to six months produced roots, without sub-culturing. Whether this callus could be sub-cultured and maintained in an undifferentiated state has not been reported. This study has shown that *Hevea* callus cultures can be established from anthers and such callus could be sub-cultured continuously. It is possible that given the required conditions, these cultures could be induced to differentiate organs and plantlets. Studies are being continued to establish *Hevea* plantlets by this method.

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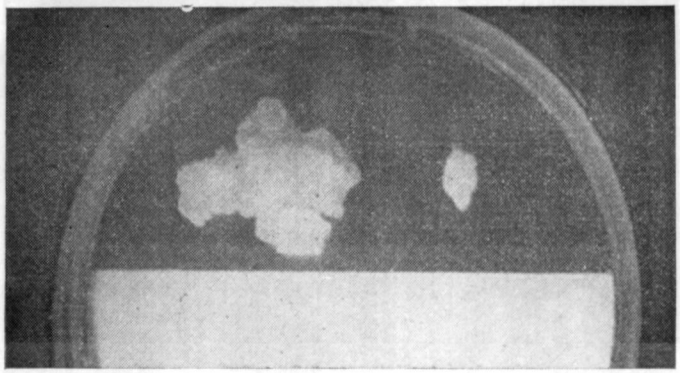

Fig. 1

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

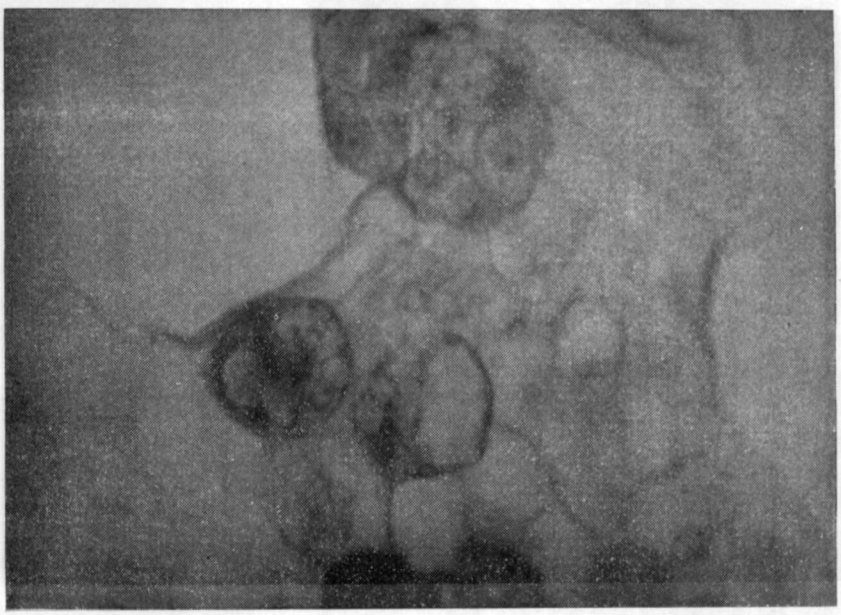

Fig. 2

Section through anther six weeks after inoculation, with pollen grain showing cell divisions (top mid centre). Few pollen grains not showing cell division are seen below (mid centre).