Studies on Tissue Culture of Hevea brasiliensis I. Role of Osmotic Concentration, Carbohydrates and pH values in Induction of Callus Growth in Plumule Tissues from Hevea Seedlings

S. E. CHUA

An elaborate medium was devised for induction of callus formation in Hevea *plumule tissues under aseptic conditions, several earlier trials with conventional tissue culture media having failed. Different carbohydrates were compared and the optimal pH values and osmolar concentrations of media to induce callus growth were determined. Sucrose is the most suitable major source of carbon and energy. A pH below 6 and a total osmolar concentration of between 0.3 and 0.4 M were found to be optimal. Diverse minerals, amino acids, vitamins and growth regulators are essential. These callus tissues produced roots without subculturing.*

The general biological problems of cultivating cells in *in vitro* have been discussed by HABERLANDT (1902), though his methods were not successful. HARRISON (1907) and CARREL (1912) managed to culture animal cells, but it was not until 1934 when WHITE and GAUTHERET evolved an experimental technique for establishing plant tissue culture. More recently plant physiologists have adopted the method for studies of tissue metabolism, especially where the size of the plant presents difficulty for metabolic measurements *in vivo*. The method forms a valuable bridge between experiments on whole plants, which are physiologically ideal but physically difficult, and experiments on tissue homogenates which are physically easy but physiologically inept.

Previously cambial tissue of *Hevea* has been successfully cultured but the cultures could not be kept for an indefinite period because of heavy infection (RUBBER RESEARCH INSTITUTE OF MALAYA, 1954). This paper describes a successful method for the culture of plumule tissues of *Hevea* seedlings, to dissociate the plant into functional and organisational units for physiological studies. The main interest is to investigate the differentiation of latex vessels as distinct from the elements of xylem and phloem present in all vascular plants. The present account deals mainly with the role played by the osmolar concentration, carbohydrates and pH of media in induction of callus formation in plumule tissues.

MATERIALS AND METHODS

Culture Techniques

Hevea seeds were sown in pots in the nursery. Each seed took 2–3 weeks to germinate and the 3–4 days old seedlings (*Figure 1*) were washed in 1% Clorox (active ingredient, sodium hypochlorite). The middle portion of the plumules were cut and surface sterilised by immersion in 1% Clorox for 5 minutes. The plumules were then washed in sterile distilled water and sliced into pieces each an average of 1 mm thick. Two pieces of plumule tissues were implanted on 50 ml of sterile agar medium in 100 ml Erlenmeyer flasks plugged with non-absorbent cotton wool, with the mouth wrapped in polythene sheet to prevent evaporation. The whole operation was performed in a sterile

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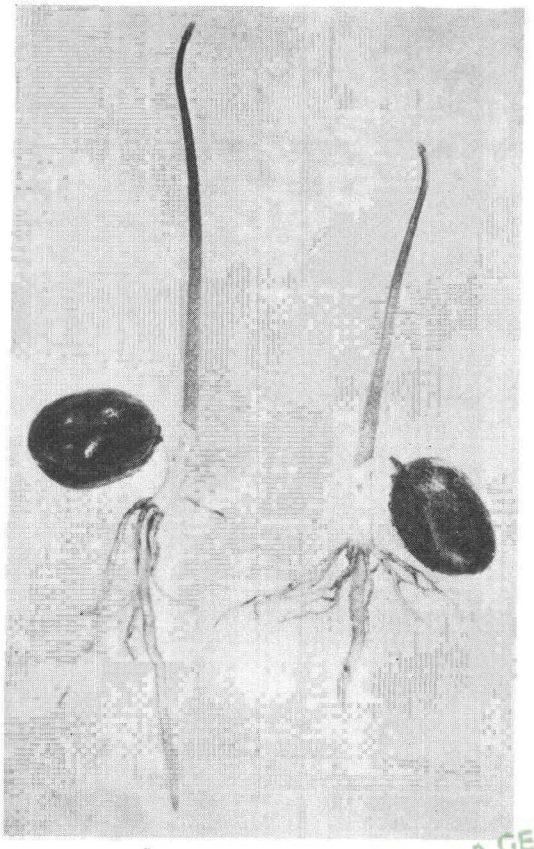


Figure 1. Young Hevea *seedlings.*

room fitted with ultraviolet germicidal lamps (2600 Å).

The cultures were maintained in a chamber fitted with fluorescent lamps supplemented with tungsten lamps to give an intensity of 100 ft candles. The temperature was kept at $(26 \pm 1)°C$ when the lights were on and at $(25 \pm 1)°C$ at night when the lights were switched off. The duration of artificial light given to the cultures was 8 hours per day.

Media

Analytical grades of chemicals and distilled rain water were used in making up the media. All vitamins were filter-sterilised whereas the other compounds together with 1% agar were autoclaved for 15 min at 15 lb in.^{2}

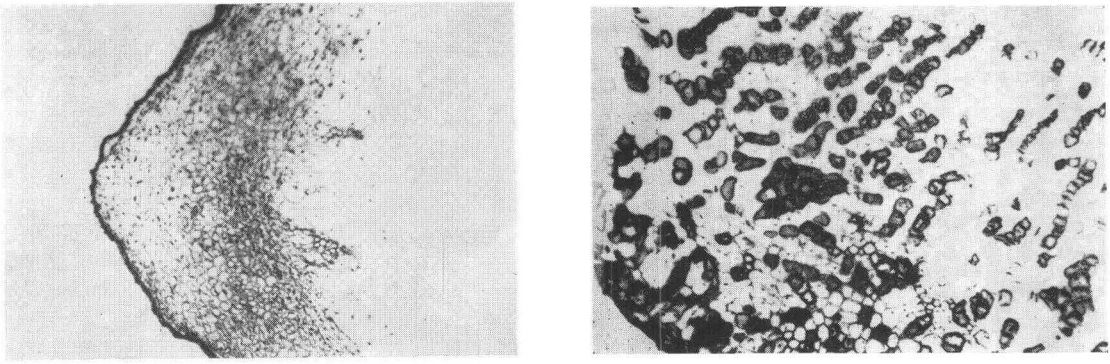
EXPERIMENTAL RESULTS

Callus Induction

Attempts to induce callus formation using the media of GAUTHERET (1942, 1950) or WHITE (1943a, and b) were unsuccessful. Both were modified and additional growth factors added, but still no satisfactory growth was achieved. It was then decided to develop a new medium by altering the concentration of each compound used and by increasing the sucrose concentration from 2 to 10% (*Table 1*). On this medium, with the pH maintained at 6.8, the plumule tissues began to proliferate in

TABLE 1. COMPOSITION OF EFFECTIVE CULTURE MEDIUM—AMOUNT OF NUTRIENTS IN 1 LITRE OF DISTILLED WATER

Nutrient	*Amount*	
Sodium sulphate	800	mg
Calcium nitrate (hydrated)	400	mg
Magnesium sulphate	180	mg
Potassium nitrate	80	mg
Potassium chloride	65	mg
Sodium dihydrogen orthophosphate (hydrated)	33	mg
Sequestrene sodium iron	40	mg
Manganese sulphate (hydrated)	4.5	mg
Zinc sulphate (hydrated)	6.0	mg
Boric acid	0.375	mg
Potassium iodide	3.0	mg
Glycine	12.0	mg
Aneurine hydrochloride	2.0	mg
Pyridoxine	2.0	mg
Cysteine—HCl	20	mg
Calcium panthothenate	2.0	mg
Biotin	2.0	mg
Vitamin B 12	2.0	mg
Nicotinic acid	20	mg
Ascorbic acid	20	mg
Citric acid	20	mg
Tryptophan	10	mg
Phenylalanine	10	mg
Tyrosine	10	mg
Leucine	20	mg
Caseine hydrolysate	5	mg
Coconut milk (by vol.)	10	%
Sucrose	100	g
Inositol (Meso)	100	mg
2,4-dichlorophenoxyacetic acid (2,4-D)	1	mg
Indoleacetic acid (IAA)	1	mg
Kinetin (6-furfuryl-amino-purine)	1	mg
Yeast extract (Yeastrel)	1	mg
Agar (Oxoid No. 3)	10	g



(a) (b)

Figure 2. Anatomical sections of (a) cells of plumule tissue before cell division and (b) cells of callus tissue after cell division has taken place. Magnification × 100.

7–10 days. Cell division took place as seen in anatomical section (*Figure 2*). At the end of 3 months, the 1 mm blocks had grown to an average size of 15 mm and callus growth occurred in large clumps also appearing to be hard and compact (*Figure 3*). Green-brown colourations appeared on callus structure grown in artificial light at 100 ft candles intensity at later stages. Some callus tissues produced roots at the end of 5–6 months without sub-culturing (*Figure 4*).

Effect of Osmolar Concentration and different Carbohydrates

The increase in sucrose concentration raised the effective osmolar concentration (as measured by a vapour pressure osmometer) from 0.1 to 0.3 M. To test whether growth was induced by the increase in sucrose concentration or by the increase in osmotic pressure of the medium, media were made up by substituting mannitol for sucrose for osmotic values ranging from 0.1 to 0.8 M, but maintaining constant the concentration of other compounds. The pH was maintained at 6.8. The results (*Table 2*) showed that sucrose played a very important role in callus induction. At low osmolar concentration (0.1 M) in the presence of sucrose, cell division occurred only after 21 days. By increasing the sucrose concentration to raise the osmolar concentration

TABLE 2. EFFECT OF OSMOLAR CONCENTRATION, SUCROSE AND MANNITOL ON CALLUS INDUCTION

Treatment	Average number of days taken to induce growth at different osmolar concentrations					
	0.1 M	0.2 M	0.3 M	0.4 M	0.6 M	0.8 M
Medium containing sucrose	21	20	8	9	No growth	No growth
Medium containing mannitol instead of sucrose	No growth	No growth	No growth	No growth	No growth	No growth
Medium containing both sucrose and mannitol	No growth	No growth	21	21	No growth	No growth

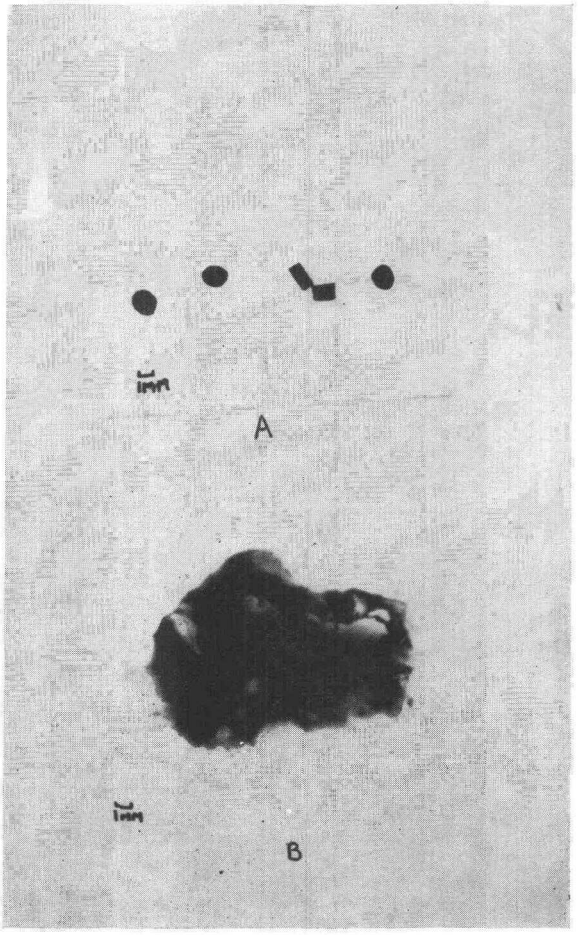


Figure 3. Plumule tissues: (A) before cell division; (B) after cell division (callus formation).

to 0.3 and 0.4 M, cell division took place in 7–10 days. At higher concentrations growth was inhibited. When mannitol was substituted no growth occurred even at 0.3 or 0.4 M. Further, although growth occurred at 0.3 and 0.4 M in the presence of both sucrose and mannitol, it took 21 days for growth to begin. This showed clearly the role of sucrose in callus induction of plumule tissues in addition to its regulation of osmolar concentration.

At the optimal osmolar concentration of 0.3 M, sucrose gave callus growth within 8 days. When mannose, glucose or fructose were used at the same osmolar concentration in place of sucrose, in all cases callus formation was delayed until 21 days, and even after three months, growth was very small compared with that on the sucrose medium.

Effect of pH

Media were prepared as in *Table 1* with an osmolarity of 0.3 M and the pH was adjusted to levels ranging from 5.4 to 6.8 with 0.1 N HCl. No buffering was used. Within this range the commencement of proliferation of plumule tissues was unaffected, but at pH 5.4 to 6.0 soft spongy callus masses of 13–15 mm diam. were produced during 3 months' growth, in contrast to the firmer growths of only 6–7 mm diam. at pH of 6.2 to 6.8.

DISCUSSION

No one medium will be completely satisfactory for all plant species, and it is clear that callus formation by *Hevea* plumule tissues is enhanced by increasing sucrose concentration from 2 to 10%. Sucrose is required as a source of carbon or energy, rather than to increase osmolarity, because there was no growth when mannitol was substituted for suc-

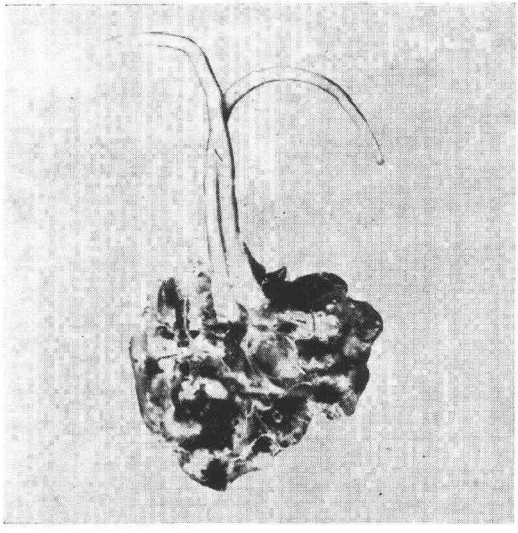


Figure 4. Callus with roots at end of 5 months.

rose. WHITE (1934), who first achieved the continuous culture of excised tomato roots, used a culture medium which contained sucrose as the source of carbon and energy in place of glucose in earlier, unsuccessful, attempts. While hexoses such as glucose, fructose and mannose can induce growth of *Hevea* callus, the period of induction is much longer than with a medium containing 10% sucrose. WHITE (1940) and later DORMER AND STREET (1949) produced strong evidence that the superiority of sucrose over the sugars was not due to its containing growth-active impurities.

The optimum osmolar concentration seems to be in the region of 0.3 to 0.4 M, which approximates to the osmolar concentration of latex obtained by tapping *Hevea* trees. The cells are thus in an isotonic medium and cell division takes place at a rapid rate because there is no need for adaptation to a new environment. Thus the maintenance of isoosmolar conditions is important in facilitating cell division. The pH does not influence the period for callus induction markedly.

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Botanical Division
Rubber Research Institute of Malaya
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