SHOOT TIP CULTURE AS A METHOD OF MICROPROPAGATION OF *HEVEA*

By

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SUMMARY

Shoot tips of *Hevea* from asceptically grown seedlings were established in culture on Murashige and Skoog (MS) liquid medium with half strength salts, supplemented with BAP + IBA or MS solid medium with full strength salts with and without BAP + IBA. BAP was found to be better for axillary bud proliferation and growth than the other cytokinines. Proliferation of buds improved with successive subcultures. A multiplication rate of 30 + 2 shoots per explant was obtained with three subcultures in 165 days.

INTRODUCTION

Hevea is propagated vegetatively by grafting buds of selected clones on to unselected seedling rootstocks. Although the use of budgrafts made it possible to overcome the inherent variability of seedlings to some extent, yet, uniform growth and yield are not realised even under best management conditions (Combe, 1984). The variation can be due to many reasons, amongst which soil heterogeneity may be one, but much of it could be due to the heterogeneous rootstock. (Mc Indoe, 1958, Senanayake and Wijewantha, 1969). Propagation with rooted cuttings has failed due to the lack of a proper tap root that gives good anchorage (Tinley and Garner, 1960).

Tissue culture techniques have been used to overcome problems associated with conventional propagation in many crops. Shoot tip culture has been successfully used and the greatest success has been achieved with herbaceous plants (Murashige, 1974, Sri Skandarajah and Mullins, 1983). Among woody perennials that have been propagated successfully are fruit crops such as apple (Abott and Whitely, 1976, Jones et al. 1977, Zimmerman, 1983) Peach (Hammerschlag 1982) and Prunus (Jones and Hopgood, 1979) and forest trees Ulmus, Populus and Fagus (Chalupa, 1981). This method enables multiplication rates many times faster than conventional methods, and least likely to produce genetically deviant plants (Murashige, 1977).

Paranjothy and Gandhimathi (1975) reported the first attempt at shoot tip culture of *Hevea*. They were able to culture shoot tips of seedlings grown in aseptic culture on a modified MS medium and induce rooting, proliferation of buds and shoots was not achieved by them. Carron and others reported the production of plantlets from nodal cuttings of seedlings and from a few selected clones. Only a few plantlets could be obtained from one explant (Carron et al. 1982 and 1985). If this method is to be successfully used in propagation of *Hevea*, there should be rapid proliferation of shoots from an explant.

MATERIALS AND METHODS

Hevea seeds of clone PB 86 were collected during the seed fall in August – September, seed coats removed and surface sterilized with a solution of commercial bleach (20% Clorox) for 20 minutes followed by three rinses in sterile distilled water. Embroys with a piece of cotyledon were dissected under aseptic conditions and cultured in full strength Murashige and Skoog (1962) (MS) medium supplemented with 0.5 mg/l. Benzyl aminopurine (BAP), 0.5 mg/l Kinetine, 2.0 mg/l Giberellic Acid (GA_3) and 50 g/l Sucrose.

Cultured embryos developed into plantlets in 18 – 20 days. terminal shoots and axillary shootS that developed after removal of terminal shoots were used as explants.

Preparation of Explants for Culture

1.5 – 2.0 cm long shoots were excised from seedlings grown under aspetic conditions, expanded leaves dissected out leaving a part of the petiole attached to the stem and 2 – 3 unfolded leaves. These explant were used in culture without any surface sterilization.

Culture Conditions

50 ml. test tubes were used as culture vessels each culture tube contained 10 ml of the medium and they were closed with polypropylene closures and tied with rubber bands

All cultures were incubated at 26 $\pm$ 2 °C under 16 hours photo period at an intensity of 60 – 80 $Em^{-1} s^{-1}$ provided from day light fluorescent bulbs.

Culture Medium

Shoot tips were cultured on four different basal media viz. Murashige and Skoog (1962) medium (MS), a medium consisting of mineral salts in Murashige and Skoog medium and Vitamins of Skirvin and Chu (1980) medium (MS – 1), White's (1943) medium, (W), and Lloyd and Mc Cowan's (1980), Woody Plant Medium (WPM). Basal medium was supplemented with BAP, Kinetin, 6– – dimethyl allyl aminopurine (2ip), – NAA, IBA and GA3 at concentrations ranging from 0 – 10 mg/l. The pH of all media were adjusted to 5.3 before autoclaving. 5.3 – 5.4 g/l Phytagar (Gibco Chem. Co.) was used to solidify the media.

Assessment of Growth

The extent of elongation of the terminal shoot, axillary bud break and extent of elongation of axillary buds, leaf growth, growth of callus and the general appearance of the cultures were assessed after incubation.

RESULTS

Establishment of Shoot Tip Cultures

Shoot tip explants from 3 weeks old seedlings could be established in culture on, 1/2 MS or FMS semi solid media with or without PGRs and 1/2 MS liquid medium with PGRs. Media were supplemented with 0.5 mg/l BAP and 0.005 mg/l IBA (Table 1). The cultures that remained fresh and green were considered as established. Most shoots on 1/2 MS liquid medium without PGRs started browning by about the tenth day. There was no visible change in colour of the medium such as darkening of the medium due to phenolic exudates.

Table 1. *Effect of different media on survival of explants 15 days after incubation*

Basal medium	PGRs mg/l	No. of explant browning, out of 10	No. of explant survival, out of 10
1/2 MS liquid	–	6	4
1/2 MS solid	–	0	10
1/2 FMS solid	–	0	10
1/2 MS liquid	+ BAP 0.5, IBA 0.005	1	9
1/2 MS solid	+ BAP 0.5, IBA 0.005	0	10
FMS solid	+ BAP 0.5, IBA 0.005	0	10

Orientation of the Explant

Shoot tips were placed on a 1/2 MS medium, supplemented with 4.0 mg/l BAP and 0.005 mg/l IBA. The shoot tips were placed vertically and horizontally on the medium.

Within 10 days of incubation, the shoot tips exhibited new growth of terminal buds, axillary buds and leaves. Growth of terminal buds was faster than axillary buds. Normal trifoliate leaves developed in some explants while on others simple. needle shaped, narrow leaves developed. Initially there was no difference in growth of shoots placed either vertically or horizontally. However, after 6 weeks, shoots placed vertically on the medium started losing the green colour and did not look fresh. Horizontally placed cultures remained fresh and green (Table 2).

Table 2. *Growth of shoot tips placed horizontally and vertically on the medium, 6 weeks after incubation.*

Orientation	Percentage of bud growth			Shoots with expanded leaves
	Terminal growth only	Axillary/bud growth only	Terminal + axillary bud growth	
Horizontal	50[a]	20[a]	30[a]	100
Vertical	20[b]	0	40[b]	80

a – terminal bud 5.0 – 0.7cm long
axillary bud 0.2 – 0.4cm long

b – terminal bud 0.3 – 0.5cm long
axillary bud 0.1 – 0.2cm long

Different replicates in the same treatment did not show similar type of growth. There was a variation in the extent of growth of terminal buds, axillary buds, leaves and callus. The percentage of explants with axillary budgrowth was greater with shoots placed horizontally compared with those placed vertically. Size and length of the buds was also greater.

As the horizontal orientation was found to be more suitable for axillary bud proliferation, the explants were placed flat on the medium in subsequent experiments.

Basal Media

Shoot tips were cultured on four basal media, MS, MS – 1, W and WPM, supplemented with 0.5 mg/l BAP and 0.1 mg/l IBA.

The sprouting of axillary buds was noticed in one week in all media and the terminal buds grew faster than the axillary buds. Leaves unfolded and turned green through copper brown. The response of replicates in a treatment was variable.

After four weeks, growth of axillary buds became slower in White's medium (Table 3). In 5 – 6 weeks, most cultures in MS – 1 medium lost their green colour and turned yellow in about 8 weeks and died. There was no noticeable difference between the growth of the shoots cultured on MS and WPM media upto 6 weeks.

The MS medium was selected as the basal medium. In subsequent experiments, shoot tips were cultured on a 1/2 MS semi solid medium supplemented with Plant Growth Regulators (PGRs) during the first two weeks and then transferred to FMS semi solid medium supplemented with PGRs. Cultures were transferred to fresh media every two weeks, unless stated otherwise.

Table 3. *Growth of terminal and axillary buds on 4 basal media*

Basal media	No. of explants with terminal growth, out of 9	No. of explants with axillary bud growth, out of 9	Appearance
MS	9[a]	8	Healthy, green colour
WPM	9[a]	7	Healthy, green colour
MS – 1	9[b]	7	Yellowing of leaves after 8 weeks
W	9[b]	2	Healthy, green colour

a – terminal bud, 0.5 – 0.7cm long
b – terminal bud, 0.3 – 0.49cm long

Effect of Different Growth Regulators on Growth and Proliferation of Axillary buds

Shoot tip explants were cultured on media supplemented with different concentrations of IBA and BAP to select the most suitable combination of BAP and IBA for establishment of shoot tips in culture and to induce proliferation of buds. Growth of shoot tips was found to be slow on all combinations of BAP and IBA tested, (Table 4). There was sprouting of axillary buds in about 7 – 9 days in almost all the cultures. Without any PGRs in the medium, terminal and axillary bud growth was very slow. There was no callus formation at the basal end, and the leaves developed into narrow, needle like structures.

In media supplemented with only IBA there was slow terminal and axillary bud growth and in 7 – 10 days, the explant got covered with callus in media with 0.1 mg/l IBA. By about the 8th week leaves turned yellow and later most of the cultures died. With BAP concentration of 7 – 10 mg/l, cultures showed a rosette type of growth with a large number of buds growing close together. When these were separated and subcultured, they failed to develop into normal shoots, but formed more clusters of buds.

With only 0.5 mg and 4.0 mg/l BAP and 0.5 and 0.5 mg/l BAP in combination with 0.1 mg/l IBA, (treatments 2,4 and 14) there was elongation of axillary buds. In a few cultures, each shoot developed two axillary buds just below the terminal shoot, 0.5 – 0.7 cm in length and the terminal bud elongated to 0.7 – 1.0 cm in length. In others where the axillary buds elongated to more than 1.0 cm, the other buds remained suppressed. There were still others where 2 – 4 axillary buds developed more than 0.4 cm in length.

Except for cultures in media 2, 4 and 14 others did not show further growth, their leaves turned yellow and most of them did not survive after about 20 weeks.

Table 4. *Effect of different combination of BAP and IBA on shoot growth after 4 weeks of incubation.*

Medium No.	IBA mg/l	BAP mg/l	Mean length of apical shoot + 0.1 cm	Mean number of axillary buds/culture	
				Actual	Transformed
01	0.0	0.0	0.2	1.5	1.4^{ab}
02		0.5	0.4	0.4	0.9^{abc}
03		0.2	0.2	0.6	1.9^{abc}
04	4.0	4.0	0.2	0.2	0.8^{bc}
05		7.0	0.1	0.0	0.7^{c}
06		10.0	*0.2*	*0.0*	*0.7*
07	0.05	0.0	0.4	0.5	0.9^{abc}
08		0.5	0.2	0.2	0.8^{bc}
09		2.0	0.3	1.5	1.3^{abc}
11		7.0	*0.2*	*1.5*	*1.4*
12		10.0	0.2	2.0	1.5
13	0.1	0.0	0.2	1.7	1.5^{a}
14		0.5	0.3	0.2	0.8^{bc}
15		2.0	0.2	0.4	0.9^{abc}
16		4.0	0.2	0.3	0.8^{bc}
17		7.0	0.2	0.3	0.8^{bc}
18		10.0	*0.2*	*1.0*	*1.0*

Any pair with different letter or letter combination is significantly different at 5% level.

Underlined values are not included in the comparison.

Fresh shoot tip explants were cultured on media supplemented with 0.5 mg and 4.0 mg/l BAP and 0.005, 0.1, 0.5, 0.1 and 0.1 mg/l IBA (Table 5). Axillary bud growth was found to be better on 0.5 mg/l BAP + 0.005mg/l, IBA 0.5 mg/l BAP + .1 mg/l IBA and 4.0 mg/l BAP + 0.005 mg/l IBA. After six weeks of incubation, cultures in these three media were separated and subcultured on the same medium. Cultures failed to grow on 0.5 mg/BAP + .005 mg/l IBA and 4.0 mg/l BAP + 0.005 mg/l IBA on subculture, there was yellowing of leaves and most of the cultures failed to survive after 5 – 6 weeks. When subcultured on 0.5 mg/l BAP + 0.1 mg/l IBA 54% of the cultures produced 2 – 4 axillary buds per culture which were longer than 0.5 cm. 46 of the cultures developed into single shoots (Table 5). All the cultures looked fresh and healthy.

Table 5. *Axillary bud proliferation and elongation on MS medium + 0.5 mg/l BAP and 1 mg/l IBA*

Axillary bud proliferation			Elongation of shoots		
% of cultures[a] with 2,3 or 4 axillary buds per culture	Mean no. of axillary buds per culture	Shoot length (cm)	1.5 – 3.0	0.5 – 1.5	0.5
			% shoots 41	44	15
42	2.8		Mean length[a] (cm) 1.4 + 0.1		

a – only shoots longer than 0.5cm were considered

With three successive subcultures on the same medium one shoot tip explant produced 49 buds and shoots, (Table 6 and Table 7) in 165 days.

During the first subculture, the growth of buds was slow, with subsequent subcultures, growth of buds into shoots was rapid and there was development of more buds which on subculture developed into shoots. When very small buds (> 0.4 cm) were separated individually, and subcultured, they failed to grow any further. They had to be separated so that each segment had at least one well developed shoot, for them to survive and develop into shoots.

Table 6. *Number of buds longer than 0.3 cm produced in the subcultures from a single explant*

	No. of cultures	Total No. of buds
1st subculture	3	10
2nd subculture	9	25
3rd subculture	12	49

Each initial shoot tip explant on MS medium with 0.5 mg/l BAP + 1.0 mg/l IBA produced, 30 + 2.0 shoots longer than 0.5 cm after 3 subcultures. The total number of shoots longer than or equal to 0.5 cm and buds smaller than 0.5 cm, were 45 + 4.0.

Table 7. *Effect of 0.5 mg/l BAP + 0.1 mg/l IBA on axillary bud proliferation : Assessments made 6 weeks after each subculture*

Subculture	1st	2nd	3rd
Mean no. of shoots (> 0.5cm) per culture + SEM	3.0 + 0.0	2.4 + 0.6	2.4 + 0.4
Mean no. of shoots (> 0.5cm) + buds (< 0.5cm) per culture + SEM	3.0 + 0.0	2.8 + 0.7	4.4 + 0.4
Mean length of shoots (> 0.5 cm) + SEM	0.91 + 0.01	0.86 + 0.01	0.85 + 0.06

2ip and kinetin were not as effective as BAP and IBA was found to be better than NAA.

Effect of GA_3 on elongation of proliferated buds

GA_3 at 0.5 and 2.0 mg/l were not effective in improving the growth of proliferated axillary buds (Table 8). 0.5 mg/l GA_3 did not have any significant effect and 2.0 mg/l GA3 was found to suppress elongation of buds.

Table 8. *Effect of GA_3 on elongation of shoots*

Concentration of GA_3	Mean length of shoots + SEM
0.0	0.71 + 0.1a
0.5	0.70 + 0.1a
2.0	0.30 + 0.1b

Means with same letters are not significantly different at 5% level.

Rooting

Rooting of proliferated shoots was not attempted in this study. However, shoot. tip explants formed roots after 4 – 5 weeks of incubation on a solid WPM medium supplemented with 0.1 mg/l IBA, 0.5 mg/l BAP and 0.5% activated charcoal, and on 1/2 MS liquid medium supplemented with 1.0 04 2.0 mg/l IBA and 0.5% activated charcoal.

DISCUSSION

The shoot tips excised from aseptically grown seedlings were used in this study and the problems met with in cleaning the explants of the microbial contaminants was overcome by this method. The microbial contaminants have been a serious limiting factor in studies with *Hevea* (Enjarlic, 1986). There was no exudation of phenolic substances that interfered with the growth of cultures as reported by Carron et al. (1985).

The orientation of the explant on the medium was found to have a significant effect on growth and proliferation of axillary buds (Table 2). Horizontally placed shoot tips have more contact with the medium than those placed vertically, and absorption of nutrients may have been enhanced through a bigger surface area. *Hevea* shows strong apical dominance and the presence of BAP in the medium and placing shoots on a horizontal position may have helped to release axillary buds from apical dominance. Similar effect has been reported for *Trifolium pratense* (Campbell and Tomes, 1984)

The response of *Hevea* shoot tips to various growth regulators in the medium was found to be slow. The growth of axillary buds into shoots started only after 12 weeks of incubation. Shoot tip cultures of woody plants such as apple (Dunstan et al., 1985, Jones et al., 1977) peach (Hammerschlag, 1982) cherry (Cerovica and Ruzic, 1987) respond quickly in the culture medium, producing a large number of axillary buds. Slow responses in cultures has been reported in plants such as *Rhododendron* (Economou and Read, 1984).

BAP in combination with IBA was found to be the most effective for proliferaton and growth of axillary buds compared to 2ip, Kineting, BAP and IBA have been found to be more suitable than the other cytokinines and auxins in many other crops (Hammerschlag, 1982, Welander, 1985, Bargahchi and Alderson 1982).

With one explant 30 + 2 new shoot could be obtained on three successive subcultures. Initially the growth was found to be slow, but with successive subculture the rate of proliferation of buds and growth into shoots improved. The slow establishment followed by rapid proliferation indicates that conditioning of the explant has occurred in culture (Hasegawa, 1980). Rooting was not attempted with proliferated shoots in this study. However, shoot tip explants rooted on media supplemented with either high IBA levels or in the presence of activated charcoal. Rooting has not been found to be difficult in shoot tip cultures of Hevea by other workers. (Paranjothy and Gandimathi, 1975, Carron et al, 1985).

GA_3 at 0.5 mg/l had no effect on elongation of buds; at 2.0 mg/l there was inhibition of shoot growth (Table 8). 0.5 mg/l GA_3 may have been suboptimal whereas 2.0 mg/l may have been supra optimal for shoot growth. A level of GA_3, between these two may be effective in improving shoot elongation (Economou and Spanoudaki, 1986).

The reports to date on Hevea tissue culture shows that none of the methods developed so far could be used in rapid clonal propagation of Hevea. In this study, the possibility of axillary bud proliferation in culture has been demonstrated. With one explant 49 shoots could be produced in 165 days. By repeated subculture, rate of proliferation may be further improved as in other plants (Barghchi and Alderson, 1983). However, the rate of proliferation and growth is still too low compared to other plants (Jones et al., 1977, Economou and Read, 1984, Bhojwani et at., 1984) and further studies will have to be made to improve rate of proliferation.

The possibility of usuing shoot tip culture for the development of a micropropagation system for Hevea is demonstrated in this study. The procedure developed with seedling shoot tips may not be directly applicable to clonal shoot tips, although this has been possible with some plants (Barghchi and Alderson, 1983). However, it has been reported that cultures of *Hevea* clonal material is much more difficult than those of seedlings and re-juvenation of mature plant material is necessary for clonal explants (Carron et al., 1984). Therefore, the method of shoot tip culture that has been developed here, may be successfully adopted after inducing reversion of juvenility of the mature clonal material.

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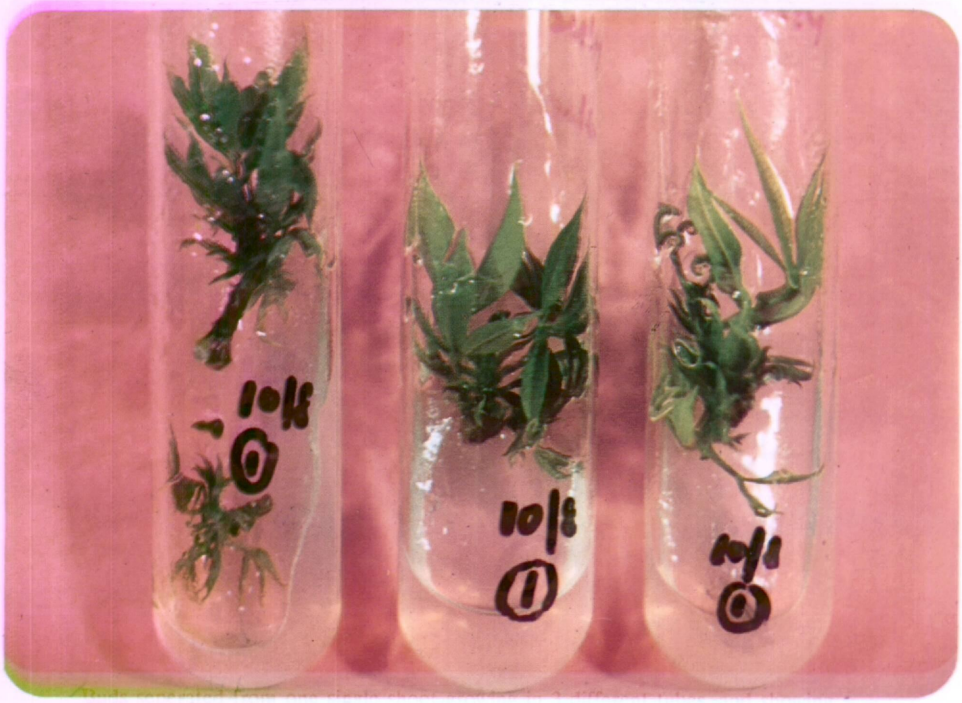
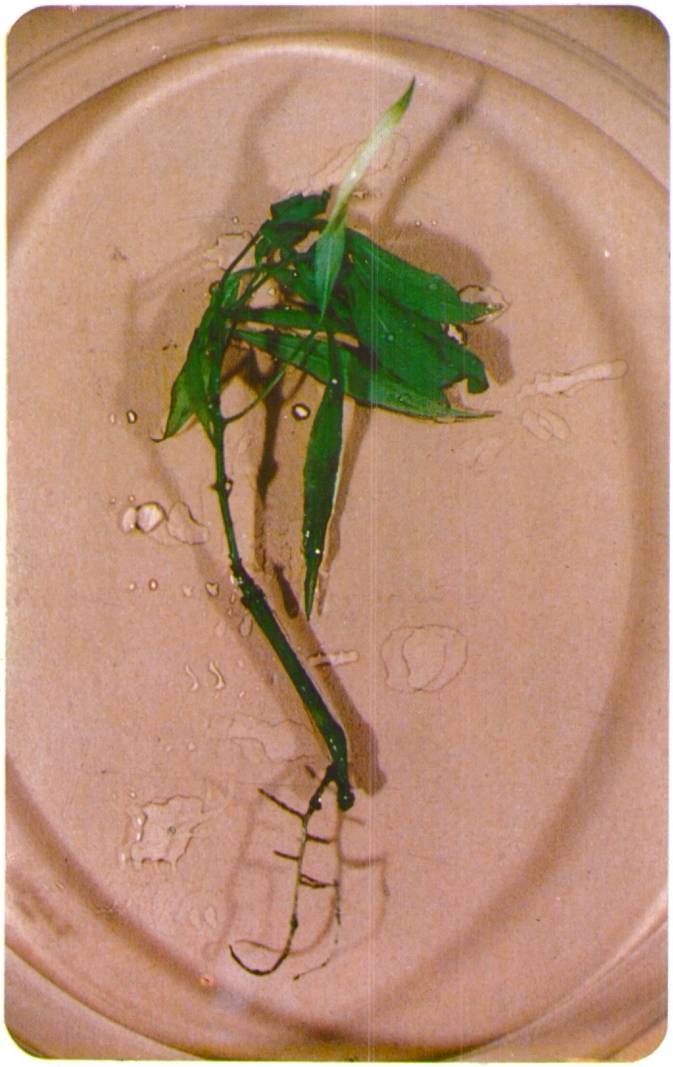


Buds seperated from one single shoot growing in 3 different tubes, and showing proliferation.

Medium – MS – BAP 0.5 IBA 0.1 mgle

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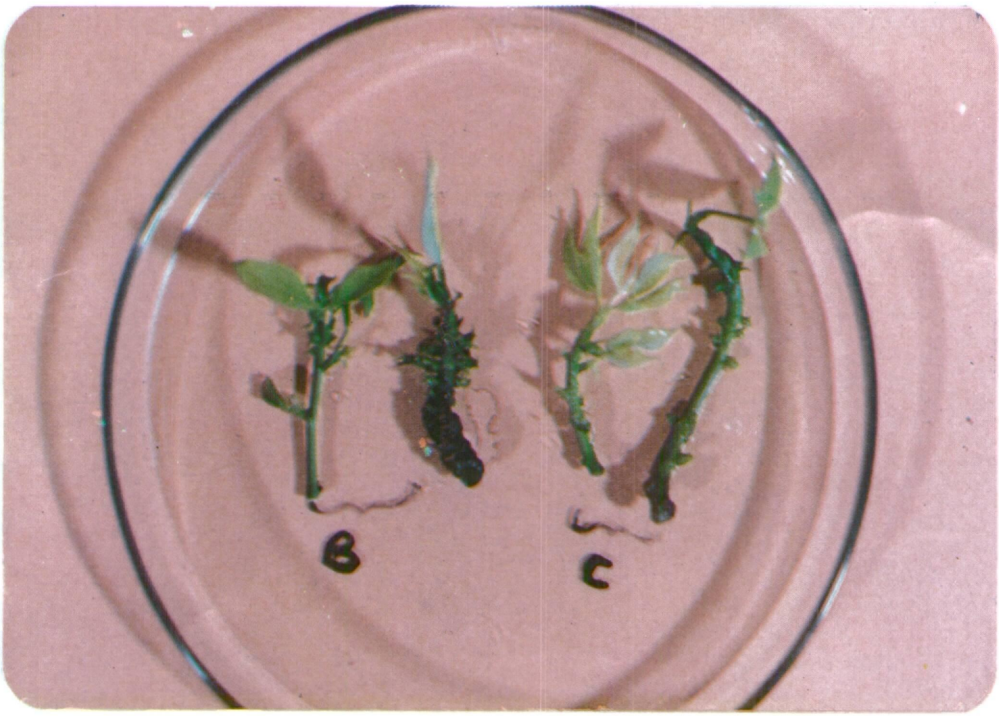
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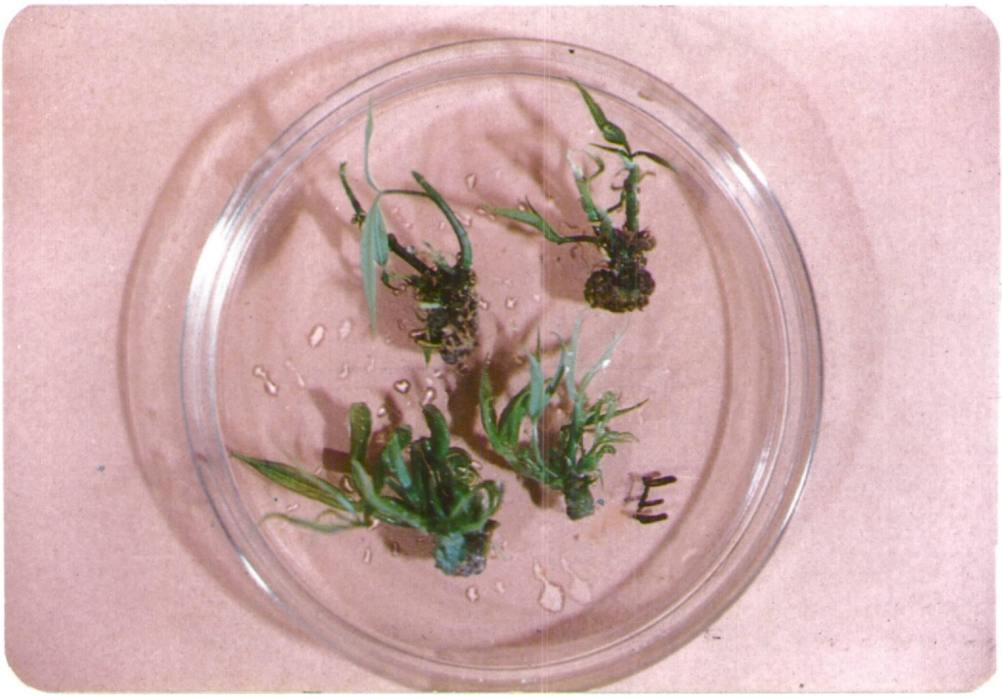
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