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THE EFFECTS OF THE BASIC MEDIUM AND THE CARBOHYDRATE CONTENT ON SHOOT CULTURES OF *HEVEA BRASILIENSIS*

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ABSTRACT

Woody Plants Medium (WPM) was superior to Murashige and Skoog medium (M&S), which showed better axillary bud growth of both juvenile and mature shoot materials. The effect was more pronounced in later passages, in particular, on the survival rate of the cultures. Most of the cultures grown on M&S medium did not survive more than 24-28 weeks, while on WPM medium cultures survived for more than one and a half years, until the experiment was terminated.

The level of sucrose in the medium also had an effect on the growth of the primary axillary shoots, specially the length and the leaf growth. The length of primary axillary shoots increased with the increasing levels of sucrose in the medium up to 10% with clonal materials. Explants did not survive beyond 8 weeks when the medium was not supplied with sucrose.

Key words: *Hevea, in vitro*, M&S, rubber, sucrose, WPM

INTRODUCTION

Among the factors to be considered in *in vitro* culture of plants, medium composition receives possibly the second priority as the primary concern is the origin and the type of explants. However, the successful protocol is developed only after manipulating all these factors including the culture environment, subculture period *etc.*

As far as the literature is concerned, a number of basic mineral formulations, some with very high ionic strength while the others with low ionic strength have been used for tissue culture of woody plant species. Many plants will grow on a wide range of these formulations, but some species will show considerable difference in the quality of growth on different formulations. Among the reasons for this, inhibitory effects in high ionic strengths, effect of total nitrogen level, calcium deficiency, chloride sensitivity etc. are of importance. Generally, woody species are established in low salt formulations as low salt media are not inhibitory though, they may not be optimal and mean while, other factors such as contamination and browning *etc.* can be solved. Also woody species often contain phenolic compounds which are aggravated by high salt levels in the medium.

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Woody Plant Medium (Lloyd & McCown, 1980) which is a low salt formulation has been found to be better for many woody species including *Salix* (Garton *et al.*, 1981) *Alnus* (Read *et al.*, 1982), *Rhododendron* (Barns, 1985) & Poplar (McCown & Sellmer, 1987). However, there are exceptions too; Fink *et al.*, (1986) reported that the shoot tips of 'Pioneer' elm grew better on M&S medium producing multiple shoots.

For shoot culture of *Hevea*, Carron and Enjalric, (1982) have developed a medium (MB medium) which contains much higher levels of macro elements such as NH_4NO_3, KNO_3, $NaH_2PO_4.H_2O$ and $CaCl_2$ with compared to the composition of WPM.

Early studies on the requirement of a source of carbon in the medium has showed that number of carbohydrates could support the growth of the tissues in culture. But, over the years it has become apparent that sucrose is generally the best, in fact, the widely used energy source. In most species, sucrose is used at a concentration between two to four percent. However, it is important to note that though sucrose is widely used it may not necessarily be the most effective carbohydrate always. Other sugars such as fructose and glucose have been better than sucrose for mulberry bud culture (Oka & Ohyama, 1982). Similarly, it has been found that the growth in latter passages can only be supported by specific carbohydrates such as sorbitol in *Malus* culture (Pua and Chong, 1984). Further, there are reports to support that the carbohydrate requirement may be dependent on cultivar (Chong and Tapper ,1973), explant (Mathews *et al.*, 1973), culture passage (Pua and Chong, 1984) *etc.* and also it can influence on the type of organ differentiated by the primary explant (Kikuta and Okazawa, 1984). However, for most plant species it has been unnecessary to consider other sources of carbon other than sucrose as the growth is satisfactory with sucrose at a concentration of 2-3%. This has been proved by the fact that most of the tissue culture media contains sucrose within this range though, there are exceptions too. For *Hibiscus rosa sinensis* only 1% has been enough (Tian, 1984) whereas for crape myrtle 1.5% (Huang, 1984 a), for *Ginko biloba* 4% (Luo, 1985), for *Leucaena leucocephala* 5% (Huang, 1984 b) and for birch 10% (McCown & Amos, 1979) have been used. Enjalric & Carron (1982) have reported that 6% sucrose was better than 2% for *Hevea*.

In the work reported here, only WPM and MS media were compared as the MB medium suggested by Carron and Enjalric (1982) and the medium of Anderson (1975) were found similar to M&S in previous studies. The effect of different levels of sucrose was also looked at, alone and in combination with the basic medium.

MATERIALS AND METHODS

Both juvenile (seedling) and mature (clonal) origin shoot materials were used. Stock plants were grown in glass house and were pruned regularly in order to improve axillary shoot growth. Shoots about 15-20 cm long were harvested from stock plants, all expanded leaves were removed and washed thoroughly until all latex and rubber particles were washed off. They were then cut into 9-10 cm pieces and surface sterilized with 70% ethanol for 1 min followed by 0.2% w/v $HgCl_2$ to which a few drops of Tween-80 were added, for 10 min. Explants were shaken throughout and washed 5-6 times with sterilized water after sterilization.

Two 1 cm long pieces were removed from either sides of the explant before they were cultured on to media.

All chemicals including sucrose and agar were analytical grade supplied by BDH Chemicals, England. Stock solutions to prepare the media were stored at 4^0C in a refrigerator except vitamins which was stored frozen.

The culture establishment medium was prepared in 9 cm diameter glass petri dishes and after 8 weeks cultures were transferred to media prepared in 100 ml jars. Media, instruments, and glassware were sterilized in an autoclave at 121^0C and under 300 k pas min^{-1} for 20 min. Cultures were incubated at 26 ± 2^0C and 12 hr photoperiod under 100 umol $s^{-1}m^{-2}$ supplied by cool white fluorescent bulbs. Cultures were transferred to fresh media every 4 weeks and observations were recorded at transferring. Axillary bud number, length and leaf growth were assessed and the means of the treatments were compared. SEM values were calculated for treatment means.

For both types of explants, sucrose at 2%, 4% and 8% were tested in combination with WPM and M&S (Murashige & Skoog,1962) basic media. Plant growth regulators used were kinetin 2 mg/l, BA 1 mg/l, NA 0.2 mg/l for juvenile origin materials and kinetin 7.5 mg/l, BAP 3.75 mg/l and NAA 0.2 mg/l for mature origin materials. These two combinations of growth regulators were found to be better than all the other combinations and cytokinin types tested at concentration up to 15 mg/l (Data not present here).
No growth regulators were used on the establishment medium during the first 4 weeks. The effect of sucrose alone was also tested with clonal materials using WPM medium and sucrose at 0,2,4,6,8 & 10%. Replication was 20-30.

RESULTS

Juvenile origin explants

Results to compare the basic medium was affected by the insufficient number of replicates on M&S media at all 3 levels of sucrose after 12 weeks of culture. Contrary to this more than 80% survived in WPM media up to 52 weeks, showing a marked difference between M&S and WPM media for the survival of explants. Also, the quality of the axillary shoots produced on WPM media was better than those produced on M&S media. They were thick, dark green and contained better leaves. Mean length of axillary shoots produced on six media for a period of 20 weeks are shown in Fig.1.

The primary axillary shoots were not subdivided into nodes until 20 weeks of culture as the axillary shoots were elongating until then. As shown in Fig.1, there was no effect of basic medium on the length of axillary shoots. But, the amount of sucrose in the medium was positively effective on the elongation of primary axillary shoots. No interaction between the sucrose levels and the medium was found. After the first subdivision at 20 weeks, a higher number of nodes were produced from those grown at 8% sucrose. The growth of the secondary nodes was very poor in M&S medium at all 3 levels of sucrose; both the leaves and the stems turned yellow. The quality of the axillary shoots was good in those grown on WPM

media irrespective to the sugar level.

Although the cultures were better grown and maintained on WPM media, the growth rate of secondary and tertiary shoots was low and therefore, the differences observed in the growth of primary cultures was not observed in latter passages. However, the growth at 2% was comparatively poor, and the growth at 4% and 8% was similar. Only 5-6 cultures remained an M&S media at each sugar level by the end of 36 weeks.

Clonal origin shoots

The results of a similar experiment done with clonal shoots are shown in Fig.2.

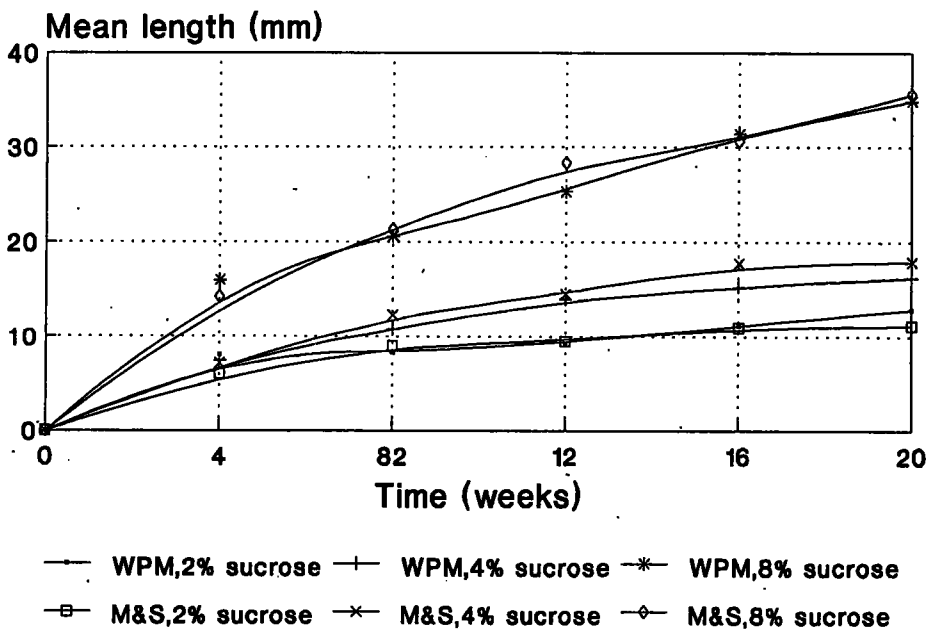

Fig.1 The mean axillary shoot length at 3 levels of sucrose and two basic media (n=20).

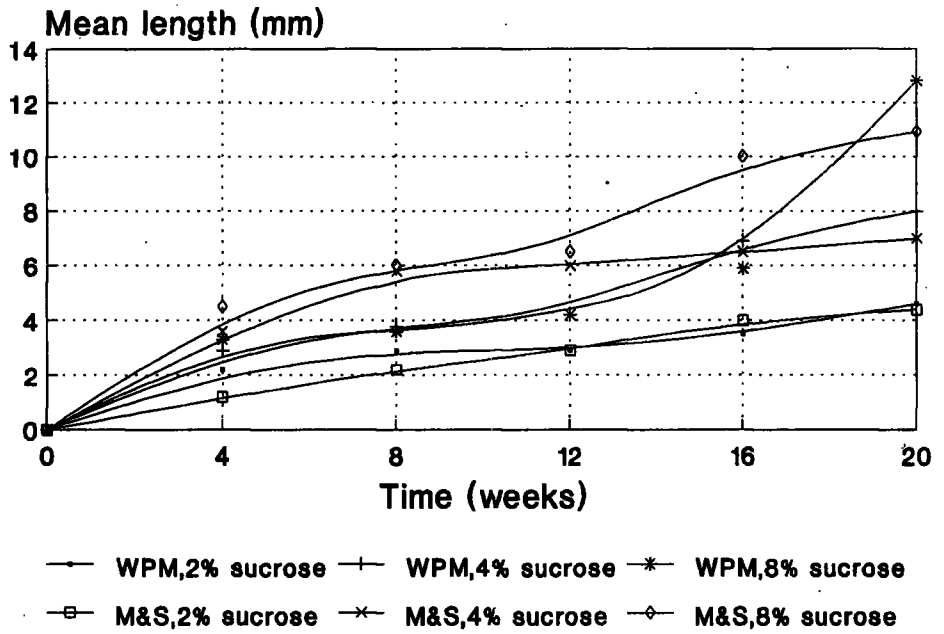

Fig.2. The mean axillary shoot length at 3 levels of sucrose and two basic media (n=20).

Here also, the effect of sucrose was more prominent than that of basic medium for axillary shoot length. The cultures grown on WPM media were more greener than those grown on M&S media at all 3 levels of sucrose. As for juvenile origin explants, the most important factor was the higher survival rate of the cultures grown on WPM media, irrespective to the content of sucrose. No interaction was observed between the basic medium and the sucrose level.

After the primary axillary shoots were cut into nodes at 20 weeks, as for juvenile materials, the differences between the different sucrose levels became small. Any how, the growth at 4% and 8% was better than 2% sucrose.

The effect of sucrose alone was tested using WPM basic medium and 5 levels of sucrose. In this experiment, media contained thidiazuron, at 0.002, BAP at 0.2 and NAA at 0.2 mg/l. Results up to 16 weeks of culture are shown in Plate 1 and Fig.3.

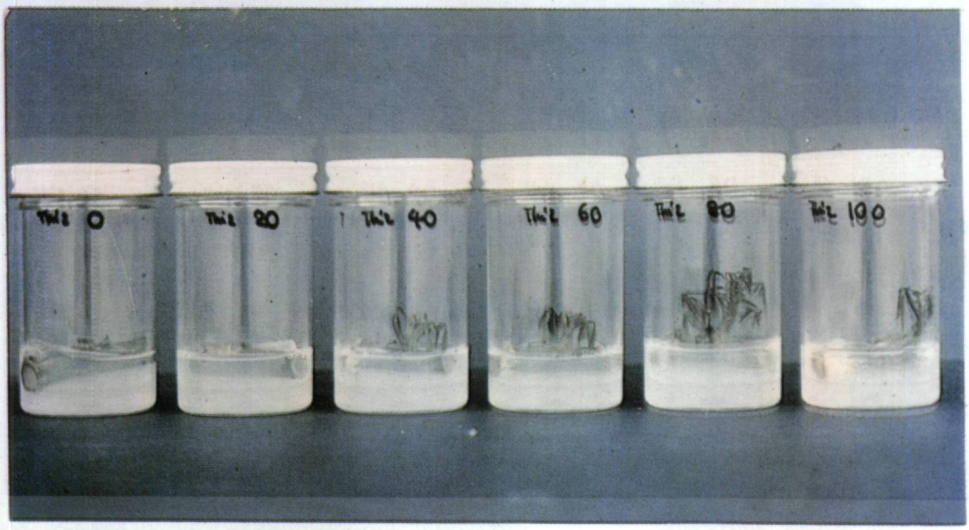

Plate 1. Axillary shoot growth of nodal explants grown at 5 levels of sucrose

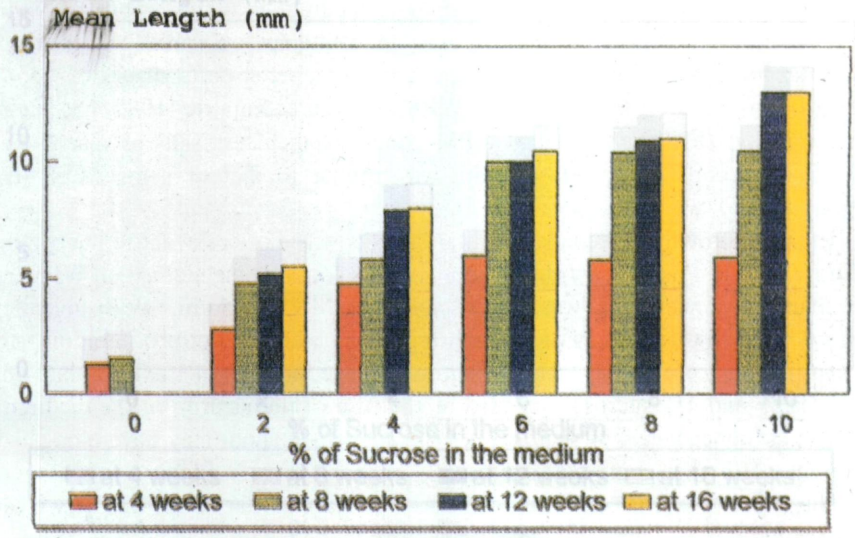

Fig.3. Mean length of axillary shoots produced at five levels of sucrose (n=15)

Effects of the medium on *in vitro* growth

As expected cultures grown on sucrose free medium did not survive beyond 8 weeks of culture. The length of the axillary buds increased with the increasing amount of sucrose in the medium from 2% to 10% (Plate 1). Considering the shoot quality and the leaf growth of the axillary shoots produced, both 6% and the 8% sucrose were equally good. They were all single axillary shoots with leaves on them. About 20% of the axillary shoots produced on 2% and 4% media showed more than one axillary shoot.

After 16 weeks of culture, axillary shoots were divided into nodes and cultured onto the same medium. The internodal lengths were very short and each newly formed node contained about 1-5 axillary buds on them. The maximum mean number of shoots produced was 2, at 8% sucrose. The growth after wards, in all five media, was low compared to primary axillary shoot growth. Also, the differences observed in the lengths of primary axillary shoots was not seen in the secondary axillary shoots, though the cultures grown at levels above 4% sucrose, were better than those grown at 2%.

DISCUSSION

Woody Plant Medium was better than M&S medium for both juvenile and mature materials of *Hevea*. The survival rate of the cultures grown on WPM medium showed better results at all three levels of sucrose. Several woody plants have been reported to grow better on low salt concentrations than those described by Murashige & Skoog. Economou & Read (1984) reported sustained growth of shoot tips of azalea in a medium containing reduced levels of NH_4NO_3 and KNO_3. M&S medium at 1/4 strength has been better for red raspberry clones (Pyott and Converse, 1981). The main feature of WPM is its low levels of chloride, for which the woody species are known to be sensitive. As suggested by McCown & Sellmer (1987), the response of plant tissues to the basic medium is highly genotype dependant. According to him one genotype of poplar could be maintained on WPM indefinitely though the growth has been poor. There are numerous reports on better performance of woody plant species grown on low salt media including WPM. Carron & Enjalric (1982) has suggested a medium for *Hevea* (MB medium) but WPM contains much less salts as macro elements. Personal experience of using MB medium was similar to that of using M&S (data not presented).

The poor growth in secondary axillary shoots was more relevant to the origin of the explant in *Hevea*. But however, *Hevea* shoot cultures could be maintained on WPM medium for more than 1 year while this was not possible with M&S medium. With juvenile origin explants, by using thidiazuron as the growth regulator, a high shoot proliferation rate could be achieved (data not presented). The reason to produce more than 1 axillary bud on clonal shoot tips at 2% and 4% sucrose may also be the use of thidiazuron in the medium. This was not seen at higher levels of sucrose, perhaps the single shoot may have elongated faster in the presence of higher content of sucrose.

The effect of sucrose on the elongation of primary axillary buds was very clear. The length increased with the increasing level of sucrose in the medium, in both M&S and WPM media. Enjalric and Carron (1982) have also reported that 6% sucrose was better than 2%

for axillary shoot growth of juvenile materials of *Hevea*. In the present study, the effect of the basic medium and the level of sucrose on the survival rate and the primary axillary shoot elongation respectively were similar for both juvenile and mature origin materials.

The quality of the shoots was equally good at both 6% and 8% sucrose. At 100%, leaves looked vitrified though the shoots had the highest mean elongation.

As the growth of the shoots and the leaf growth was similar at 6% and 8%, and also as the effect of sucrose was not very prominent in latter passages, 6% sucrose was chosen to use. It was observed that the growth of primary axillary shoots is somewhat independent of the exogenous growth regulators supplied in the medium suggesting that the primary axillary buds generally, grow out and elongate as a result of releasing them from apical dominance together with their endogenous content of hormones.

At the primary axillary shoot state, as the explants consist of the required amounts of growth regulators, the optimum concentration of sucrose is expressed. But, as they reach the secondary or tertiary stages, they depend on the external supply of plant growth regulators.

Apart from the medium composition , there may be other factors which influence the growth of *in vitro* cultures. For clonal materials of *Hevea*, the poor growth or the low response in the later passages has been identified as a problem related to the phase change of the mother plant which may not simply be improved by manipulating culture medium or conditions. However, on the other hand, it is important to identify the optimum levels of medium composition and culture conditions as the growth in poor.

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