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# THE PROBLEM OF SURFACE STERILIZATION OF SHOOT MATERIALS OF *HEVEA*

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## SUMMARY

The success rate at culture establishment of field-grown shoot materials of *Hevea* remained very low. The main difficulty was the surface sterilization of shoots as the high content of phenolic compounds made the problem more complex. Use of glass house-grown shoot materials was effective to increase the culture establishment rate. Regular spraying of benomyl reduced fungal contaminations further. Application of antibiotics either as a foliar spray or to incorporate into culture media was not effective. Two step sterilization procedure had an adverse effect on both disinfection and browning. Substituting NaOCl with $HgCl_2$ was the most effective treatment of all.

**Key words**: rubber, *in vitro*, culture establishment.

## INTRODUCTION

The establishment of *in vitro* cultures of woody plant species has frequently been hampered by low response to media, phase change, phenol productions and persistent microbial contaminations. Generally, the surfaces of the plants carry a microbial flora which need removing prior to establish in culture. When working with field grown plants, specially trees like *Hevea*, tissue like stems can be old and microbes may penetrate and survive within the plant tissues and are very difficult to remove by normal surface sterilization. This indigenous microbial contamination had been a serious problem with most of the forest trees (Fossard *et al*, 1977). So many techniques have been tested, some with success to overcome this type of contaminations.

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Soaking the explants overnight in running tap water, or in a weak solution of calcium hypochlorite, prior to normal surface sterilization has been tried by Cresswell & Nitsch (1975) for *Eucalyptus*. Zimmermann (1985) has suggested to grow shoots on low cytokinin containing medium under dark and then harvesting shoot tips from those proliferating cultures as they are normally free of bacterial contaminants.

Incorporating antibiotics into the culture medium has been reported to control bacterial contaminations effectively in a number of plant species including rubber (Carron *et al*, 1985; Enjalric *et al*, 1988; Phillips *et al*, 1981; Wilson & Power, 1989; Young *et al*, 1984). Another method which has been tried is protecting or covering the new growth of shoots even if the plants are grown in a glass house (Fossard *et al*, 1977). Treating the branches with fungicides or insecticides before pruning or covering with polythene bags has been found to destroy eggs and fungal spores (Cresswell & Nitsch, 1975). Spraying the buds with a mixture of antibiotics and harvesting newly sprouted shoots has been found to be very effective in reducing contamination as reported by Enjalric *et al* (1988), Hu & Wang (1983) and Litz & Conover (1978). The present report is mainly on the effectiveness of various techniques of surface sterilization of shoot materials of *Hevea*.

## MATERIALS AND METHODS

### Plant Material

Actively growing shoots were harvested from refoliating branches of about 5–6 year old RRIC 100 and RRIC 121 field grown trees, around March–April. Shoots were harvested from source bush nurseries of RRIC 100 and 121 also. When harvested from source bush plants, after pruning, the buds were covered with polythene bags to protect them from rain water and dust. Two–three year old glass house–grown plants of RRIC 100, RRIC 121 and PB 86 were also used to harvest shoot materials. These plants were regularly pruned to enhance lateral shoot development.

### Media

Murashige & Skoog medium supplied by Flow laboratories was used in preparation of culture media. All chemicals including sucrose, agar, activated charcoal, PVP *etc* were analytical grade supplied by BDH Chemical, England. Sucrose was supplied at 2% w/v, PVP at 0.01% w/v, agar at 0.6% w/v and the pH of the media was adjusted to 5.7 prior to autoclaving unless otherwise stated. Establishment medium was prepared free of growth regulators in 9 cm diameter petri dishes.

## Sterilization

All expanded leaves were cut off and shoots were cut into 6–8 cm pieces and washed thoroughly until all latex and rubber particles were removed. Explants were washed in 70% v/v ethanol for 1 min prior to sterilize with either NaOCl or $HgCl_2$ where a few drops of Tween–80 was added. They were shaken throughout and washed 5–6 times with sterilized water after sterilization treatment.

Two step sterilization procedure tested as suggested by Jones *et al* (1977) is given below.

**Stage I.** (a) wash in 0.01% teepol
(b) 10% chlorox for 3 min
(c) 3 rinses in sterile water
(d) culture on to hormone free medium

**Stage II.** (after 24 hours of incubation)

(a) 20 sec wash in 0.01% teepol
(b) soak in 0.1% benomyl for 15 min
(c) soak in 3% chlorox for 30 min
(d) rinse 3–4 times
(c) culture on to hormone free medium

All media, instruments and glass ware were sterilized in an autoclave at 121°C under 300 K Pas $m^{-2}$ for 20 min. Antibiotic mixtures were filter sterilized with millipore filters. The composition of different antibiotic mixtures tested in the study is as follows,

**Mixture I** – Vancomycin, tetracycline, cefatoxime, rifampicine, streptomycine and pimaracine each at 15 ppm.

**Mixture II** – Chloramphenicol, kenamycine, tetracycline and rifampicine at 60,20,20 and 20 pm respectively.

**Mixture III** – Gentamicin and tetracycline at 80 and 20 ppm respectively.

**Foliar spray** – A mixture of benomyl at 1% and streptomycine at 0.1%.

**Treatments with $HgCl_2$** – The following four treatments were tested with two concentrations of $HgCl_2$ and two time periods.

| | Concentration of $HgCl_2$ | |
|---|---|---|
| Time | 0.1% | 0.2% |
| 5 min | 1 | 2 |
| 10 min | 3 | 4 |

**Culture procedure and incubation**

After sterilization two 1 cm long pieces from either sides of the shoots were removed to make sure that any absorbed unsterilized water or sterilant is removed. A thin slice of about 1 mm removed from the base of the explant was cut in to two and cultured on to Nutrient agar (NA) and Potato Dextrose agar (PDA) media separately for easy detection of contaminations. In some experiments, the entire slice was cultured in nutrient broth and incubated in a roller drum in the growth room. Cultures were incubated at 25±2°C under 16 h photoperiod at irradiance of about 100 $\mu$mol $s^{-1}$ $m^{-2}$ supplied by coolwhite florescent tubes.

**Assessment and Statistics**

When cultures were examined for contaminations the respective NA and PDA or nutrient broth cultures were also checked. Contamination rate and phenolic browning was expressed as percentages.

## RESULTS

**Sterilization with NaOCl**

All possible combinations of 5%,10%,15% and 20% of NaOCl with 5,10,15 and 20 min durations were tested. Glass house–grown shoot materials were used for this and there were 15 replicates for each treatment. The percentage contamination rate and phenolic browning of cultures are given in Table 1.

Table 1. *Percentage of nodes that were contaminated and browned (n=15).*

| Time (min) | | Concentration of NaOCl 5% | 10% | 15% | 20% |
|---|---|---|---|---|---|
| 5 | C | 100 | 90 | 70 | 50 |
| | B | 50 | 60 | 90 | 100 |
| 10 | C | 100 | 75 | 50 | 25 |
| | B | 65 | 60 | 90 | 100 |
| 15 | C | 100 | 50 | 55 | 25 |
| | B | 75 | 80 | 100 | 100 |
| 20 | C | 100 | 50 | 50 | 25 |
| | B | 75 | 100 | 100 | 100 |

C – contaminations
B – browning

Both phenolic browning and contamination were observed in all treatments. Though 5% NaOCl was totally ineffective as a sterilant browning was observed at a higher rate. The higher concentrations of NaOCl were effective in cleaning shoots but at the same time explants were killed due to phenolic exudates. All these treatments were repeated at least twice individually or in groups, but the results were the same. 10% NaOCl for 10 min and 15 min were the only acceptable treatments which gave 25% and 50% clean cultures respectively, though phenolic browning too was as high as 60% and 80% respectively.

When the explants were treated with 70% v/v ethanol for 1 min prior to sterilize with 10% NaOCl for 2 min, about 50% of the cultures remained free of contaminations up to 2 weeks, but the browning remained around 60%. Use of water soluble PVP in the medium at 0.01% was very effective to control phenolic browning (Seneviratne & Wijesekara, 1994). However, the phenolic browning was 100% with both types of field-grown shoot materials, while contamination rate was about 90%. Incorporation of PVP at the same concentration was less effective with these shoot materials.

## Two stage sterilization procedure

The results of this experiment showed 100% contamination, with both bacterial and fungal and also 100% phenolic browning. Although the initial results were slightly different when tested at different days, the results after about 3 weeks were similar. Field-grown shoots too showed similar results.

## Use of antibiotics

All three mixtures were tested separately to soak explants for ½h after sterilization. When incorporated the same mixtures into solid medium, the antibiotic solution was filter sterilized and then incorporated into medium when the agar was about to set. For liquid media, antibiotics were incorporated just before culturing.

Soaking treatment showed higher content of browning and also higher rate of fungal contaminations. Incorporation treatment showed no difference from the control, as measured by the amount of browning or the rate of fungal contaminations after about 1 month of culture. Antibiotics could delay the appearance of contaminations. Field-grown shoots were not used in this experiment as the results were expected to be worse.

## Sterilization with $HgCl_2$

In all experiments reported so far, the explants were sterilized with NaOCl. With NaOCl at 10% for 10 min along with 1 min dip in 70% ethanol, the maximum number of clean cultures obtained was about 50% and the browning was observed at 60% as stated earlier. In this experiment, a solution of $HgCl_2$ was used as the sterilant. Initially four treatments were tested and the results are shown in Fig.1.

Use of $HgCl_2$ as the sterilant, not only increased the number of clean cultures, but also reduced the extent of browning. From the four treatments tested, 0.2% $HgCl_2$ for 10 min showed the best results giving about 80% clean cultures with browning at about 20%.

## The effect of foliar spray

The reason to use foliar sprays was in fact, to control the leaf diseases in glass-house grown plants. However as benomyl is a systemic fungicide it was expected that the effect would extend to *in vitro* cultures also to reduce contamination.

Obviously the diseases were controlled in the glass house but the effect of that in culture was difficult to detect. The result obtained at different times were different but it was evident that at least fungal contaminations were reduced to a certain degree.

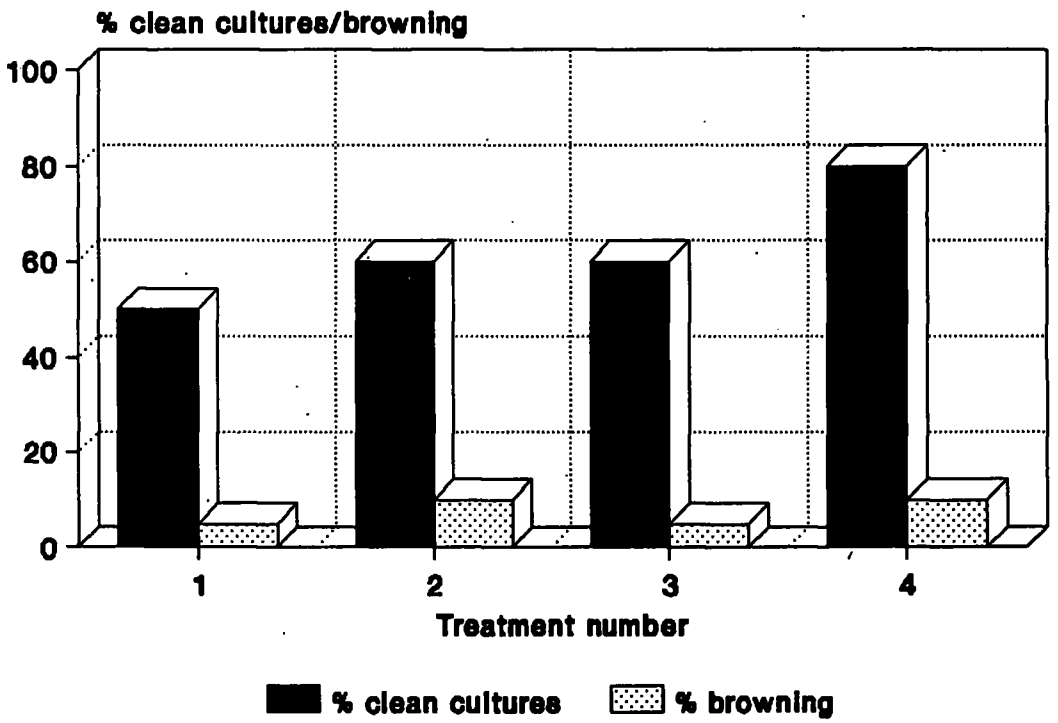


Fig. 1. Percentage of clean cultures and phenolic browning of shoot explants sterilized with $HgCl_2$ (n=15).

## DISCUSSION

The higher contamination rate observed in field grown shoot materials may be due to their exposure to rain water and dust. Also one of the reasons that they cannot be sterilized by increasing the strength of the sterilant or by lengthening the time period is that they contain a very high amount of phenolic compounds compared to those grown in the glass house. But however, the behaviour of seedling and clonal plant materials grown in the same glass house was different in the sense of percentage clean cultures obtained after the same sterilization treatment.

The high content of phenolic compounds in clonal shoots could be controlled by incorporating PVP in to culture medium. This was further reduced by substituting NaOCl with $HgCl_2$ as the sterilant. Always a higher number of clean cultures was obtained with seedling explants compared to clonal shoots grown in the same glass house under the same conditions. Therefore, the reason may be the bark texture of clonal materials, for instance rough surfaces are difficult to sterilize. Though the efficiency of the sterilant is increased by using a prewash with 70% ethanol and also by incorporating Tween–80 in to the sterilant solution, it does not seem to clean the surface properly or there may be some contaminants located within the tissues which cannot be removed by normal surface sterilization. Enjalric *et al* (1988) has cleaned the surfaces by brushing in ethanol in addition to the normal sterilization treatment with NaOCl for 15–30 min with a prewash in 95% ethanol for 3–4 min. The personal experience in brushing with even a damped piece of cotton wool resulted higher levels of phenolic exudates that could not be controlled by PVP in the medium. Enjalric *et al* (1988) have reported of lower success rates in surface sterilization with $HgCl_2$. But they have used a very low concentration, *ie* 0.5 g/l for 15–20 min where as in the present study 2 g/l solution was used for 10 min. Perhaps this was possible in the present study as the PVP was incorporated in the medium to control phenolic browning. Enjalric *et al* (1988) has not mentioned about phenolic exudates in clonal cultures. $H_2O_2$ has also been effective in their trials but NaOCl has given more consistent results. When the same sterilization treatment was carried out in two steps, no improvement has been observed. Our experience on two stage sterilizing procedure was also similar to theirs, adverse effects were observed with any type of plant material.

Studies done by Enjalric *et al* (1988) on the microorganisms present in the explants has revealed a large number of bacteria and fungi in association with the plant tissues, either at the surfaces or at the lower end of the explant. It has been reported of some 20 different germs, with bacteria being predominant and responsible for about 73% of infections. Use of antibiotics was not successful in the present study. The failure in soaking treatment may also be due to possible spread of remaining bacteria cells and fungal spores and also due to aggravated phenolic browning. As reported by Enjalric *et al* (1988) use of a mixture of antibiotics mixed with a fungicide either incorporated in to the medium or to soak explants for 20 h has not improved the number of aseptic cultures. Further, when this mixture was incorporated in to the medium a negative effect on bud development has been noticed. Spraying the same mixture to the plants *in vivo*, every two days for a period of 15 days prior to harvesting explants has been better as the negative effect on the bud development was not present.

As reported by Wilson and Power (1989) eight bacterial species have routinely been found in stem tissues of rubber. Further, both streptomycine and tetracycline have controlled seven of them. Inhibitory and stimulatory effects of antibiotics in culture have also been discussed.

However, the personal experience of the author confirms that most of the contaminants found in the surfaces of the shoot explants are a result of growing the plants in tropical climatic conditions. Use of 0.2% $HgCl_2$ for 10 min with a prewash in 70% ethanol for 1 min often gave more than 90% clean cultures when the stock plants were grown in a glass house in the UK. A similar situation has been experienced by Enjalric *et al* (1988) also for *Hevea* shoot cultures.

Use of the apical part of the shoot which is believed to contain less contaminants was not successful for *Hevea* as they contained more phenolics and also due to poor axillary bud expansion in shoot tip explants relating to high apical dominance. When working with field-grown shoot materials, in almost all the cases, the major problem was phenolic exudates. Contaminations appeared after a certain period of time. The maximum period that they could be retained in culture was 1-2 months. However, when the explants were obtained from refoliating top branches, the cultures remained for about 3 months. But the success rate was very low and non of them showed any axillary bud expansion until they were discarded due to browning.

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