

# Establishment of *in vitro* Culture of Rubber (*Hevea brasiliensis*) from Field-derived Explants: Effective Role of Silver Nanoparticles in Reducing Contamination and Browning

Moradpour M<sup>1\*</sup>, Aziz MA<sup>1,2</sup> and Abdullah SNA<sup>1,2</sup>

<sup>1</sup>Laboratory of Plantation Crops, Institute of Tropical Agriculture, Universiti Putra Malaysia, 43300 Serdang, Selangor Darulehasan, Malaysia

<sup>2</sup>Department of Agriculture Technology, Faculty of Agriculture, Universiti Putra Malaysia, 43300 Serdang, Selangor Darulehasan, Malaysia

## Abstract

The development of *in vitro* methods to produce high quality clonal planting materials of *c* for replanting and new planting is highly essential. The establishment of *in vitro* cultures of challenging woody plant species like *H. brasiliensis* has frequently been hampered by persistent microbial contamination, phenolic production, phase change and low response to media. Therefore, the overall goal of this study was to establish an efficient *in vitro* propagation method for *H. brasiliensis* through solving those persistent problems. In determining the most suitable method of reducing explant contamination, different concentrations of sodium hypochlorite, mercuric chloride and nano silver (NS) at different immersion times were assessed on explants derived from three physiological leaf stages (bronze, light green and stable green leaf stages) of preculture and non-preculture treated plants. The highest percentage of survival (94.44%) was from explants derived from the light green leaf stage of preculture treated plants immersed in 10 ppm NS for 20 min. Different types of adsorbents which were silver nitrate, activated charcoal and NS were assessed in controlling browning of *in vitro* cultures of *H. brasiliensis*. NS at 4 ppm significantly produced the highest percentage of explant survival (87.03%) with a low percentage of browning (10%). In determining the most suitable medium for *in vitro* culture of *H. brasiliensis* explants, Murashige and Skoog (MS) medium, Woody Plant medium (WPM) and Enjalric and Carron (MB) medium in combination with 3%, 6% and 9% sucrose concentrations were assessed. After 16 weeks of culture, the highest percentage of shoot formation (80%) was on MB medium supplemented with 6% sucrose.

**Keywords:** Micropropagation; *Hevea brasiliensis*; Sterilization; Phenolic compounds; *Hevea* leaf development

## Introduction

*Hevea brasiliensis* is commonly the rubber tree, is a tree belonging to the family Euphorbiaceae. It is the most economically important member of the genus *Hevea*. It is of major economic importance because the milky latex extracted from the tree is the primary source of natural rubber. *H. brasiliensis* is still traditionally propagated by grafting clonal axillary buds onto unselected seedlings as stock plant to maintain intraclonal heterogeneity for both vigour and productivity [1]. New methods of propagation are highly recommended in order to eliminate the possible incompatibility between rootstock and scion, as well as to reduce the cost and time required for plant production. Micropropagation is a promising technique for large scale multiplication of selected clones. It has the advantages of small space requirements and reduced costs when compared to conventional vegetative propagation techniques. Although gross experimentation was conducted in the past for standardizing *in vitro* technologies in rubber, there had been many setbacks in commercializing these procedures [2,3]. A number of aspects inherent in the explant tissue are responsible for the delay in commercialization. These include: (i) the release of phenols; (ii) contamination by bacteria and fungi; (iii) their recalcitrant status; (iv) reduced axillary branches; (v) lack of sufficient juvenility; and above all (vi) increased sensitivity of *in vitro*-raised plantlets towards environmental attributes. There are some methods and chemicals available to control *in vitro* contamination. The efficiency of some of these methods is low, and some of the chemicals are too toxic. The laboratory-based sterilization of explant cannot fulfil its potential to control *in vitro* contamination until it includes reference to the natural environment in which plants evolved. In fact, interaction with the environment is a key factor affecting the establishment of explants in plant tissue culture. Previous studies have shown that physiological aging negatively affects the micropropagation

capacity of *H. brasiliensis* from microcuttings [4,5]. This phase change phenomenon has been observed in many studies and is associated with a noticeable decline in the potential of micropropagation or somatic embryogenesis of most arborescent species [6-9]. The second difficulty in *in vitro* establishment of *Hevea* species is the rapid browning and/or necrosis of the explants. These problems are partly caused by oxidation of polyphenols which are abundant in *Hevea* species [10]. Also, inhibition of shoot organogenesis and necrosis of the explants are associated with considerable leaking of exudates into the culture medium [11]. There are no preliminary reports on reduction of browning in this plant material. To optimise an efficient tissue culture method for *Hevea* rubber, explant preparation is one of the important steps that need to be given attention in order to overcome the problem of microbial contamination and browning. Besides understanding the mother plant and its interaction with the environment it is also important that new types of sterilants are introduced to combat the problems faced during *in vitro* culture establishment. The antibacterial effects of Ag salts have been noticed since antiquity [12]. Nano silver is a new and non-toxic material which shows high capability in eliminating microorganisms, e.g., fungus, bacteria and viruses through the release of tiny particles of silver that could destroy the microorganisms [13].

**\*Corresponding authors:** Mahdi Moradpour, Laboratory of Plantation Crops, Institute of Tropical Agriculture, Universiti Putra Malaysia, 43300 Serdang, Selangor Darulehasan, Malaysia, Tel: 0389471043; ; E-mail: [maherandmr@gmail.com](mailto:maherandmr@gmail.com)

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The detrimental effects of nano silver have been shown on more than six hundred microorganisms [14]. There are no preliminary reports on reduction of contamination and browning using silver nanoparticles in *in vitro* culture of *Hevea*. This study attempts to establish an efficient *in vitro* culture of *H. brasiliensis* from field-derived explants through determining the most suitable method of reducing contamination and browning, as well as determining a suitable medium for promoting shoot formation (Figure 1).

## Materials and Methods

### Plant materials

One to two year-old grafted plants of *H. brasiliensis* clone RRIM 2025, grown in polybags were obtained from Rubber Industry Smallholder Development Authority (RISDA) nursery in Jelebu, Negri Sembilan, Malaysia. The plants were maintained in the Department of Agriculture Technology, Faculty of Agriculture, Universiti Putra Malaysia. The plants were regularly pruned to enhance lateral shoot development for use in this study. In order to assess the effects of environmental conditions on the degree of microbial infection of the *Hevea* tissues, the grafted plants were divided into two groups, with preculture treatment and without preculture treatment (control). The preculture treatment was applied by spraying the plants with a mixture consisting of 0.1% white oil, 0.2% benomyl and 90 mg L<sup>-1</sup> Welgro fertilizer. Then, explants consisting of shoot tips and axillary buds were taken from branches at different leaf developmental (physiological) stages (Figure 2) indicated by the leaf colour: (1) bronze leaf stage, (2) light green leaf stage and (3) stable green leaf stage of the preculture and non-preculture treated plants.

### Effects of sterilant type and concentration, preculture treatment and leaf developmental stage on overcoming contamination of *H. brasiliensis* explants

Sodium hypochlorite (NaOCl) at 10%, 20%, 30% and 40%, mercuric chloride (HgCl<sub>2</sub>) at 0.1% and 0.2% and NS at 2.5, 5, 10, 50 and 200 ppm were freshly prepared prior to use and dispensed in jam jars. *Hevea* explants were harvested from the bronze, light green and stable green leaf stage of both preculture treated plants and the control. The explants were harvested at 10-12 days after pruning for the bronze coloured leaf stage, 23-25 days after pruning for the light green leaf stage and 40-45 day after pruning for the stable green leaf stage. All expanded leaves were removed and the explants were excised into 1 cm pieces and washed thoroughly until all latex and rubber particles were removed. The explants were washed in 70% v/v ethanol for 30 seconds. Then, the explants were immersed in the different concentrations of NaOCl for 10, 20 and 30 min, in HgCl<sub>2</sub> for 5 and 10 min and in NS for 5, 20 and 60 min, added with a few drops of Tween-20. They were shaken throughout in the sterilant and washed 5-6 times with sterile distilled water. Explants without exposure to the sterilants were considered as the control. Finally, the cut ends of the explants were trimmed and the explants were cultured on 40 mL of MS medium containing 3% sucrose, 0.3% Gelrite without hormone supplementation. Each treatment combination was repeated three times and each replication per treatment consisted of 6 explants. At the end of 4 weeks of culture, the percentage of explant survival was recorded.

### Control of browning of *in vitro* cultures of *H. brasiliensis*

To reduce the occurrence of browning of cultures, different adsorbents which were silver nitrate (AgNO<sub>3</sub>), activated charcoal (AC) and NS at various concentrations were evaluated. The explants were derived from the light green leaf stage of preculture treated plants and

sterilized with 10 ppm NS for 20 min (best sterilization procedure determined above). MS basal medium was supplemented with 2, 4 and 6 ppm of AgNO<sub>3</sub>; 1, 3 and 5 ppm of AC and 4, 8, 12 and 16 ppm of NS. The explants were cultured on 10 mL aliquots of the respective media contained in 25 x 100 mm vials. Explants placed on hormone-free MS medium were considered as the control. Each treatment was replicated three times and each replication per treatment consisted of 6 explants. The percentage of browning and percentage of explant survival were recorded at the end of 5 weeks of culture.

### Effects of basal medium and sucrose concentration on shoot induction

Low response to media is another hindrance in the tissue culture of *H. brasiliensis* explants. In this experiment, full strength of WPM, MS and MB media were prepared in combination with 3%, 6% and 9% sucrose. The explants were derived from the light green leaf stage of preculture treated plants and sterilized with 10 ppm NS for 20 min (best sterilization procedure determined above). To overcome browning, the media were incorporated with 0.5% AC (best adsorbent treatment determined above). The explants were cultured on 40 mL of solid medium contained in 100 mL Pyrex Erlenmeyer flasks. Each treatment



Figure 1: *Hevea brasiliensis*.

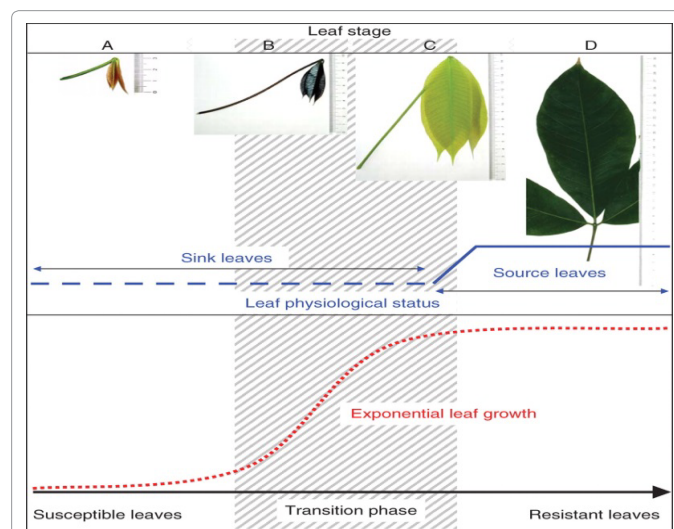


Figure 2: Relationship between exponential leaf growth, transition phase from completely susceptible leaf stage to completely resistant leaf stage, and the short physiological step from sink to source leaves. This qualitative comparison attempts to visualize the different developmental processes that turn a susceptible rubber tree leaf into a completely age-resistant organ (Lieberei, 2007).

was replicated three times and each replication per treatment consisted of 18 explants. The explants were subcultured on fresh medium of the same constituents at 4-week intervals. The percentage of shoot formation was recorded at the end of 16 weeks of culture.

### Culture conditions

Cultures were incubated on racks in an air-conditioned culture room with the temperature maintained at  $25 \pm 2^\circ\text{C}$  and a relative humidity of 50 to 70%. Illumination was provided by 80 watt fluorescent lamps (Philips TL 80 W/55T) at an intensity of  $45 \mu\text{mol m}^{-2} \text{s}^{-1}$  for 16 hours photoperiod.

### Statistical analysis

Statistical analyses were performed using the statistical analysis system (SAS) program (Version 9.2). Data were subjected to one-way analysis of variance (ANOVA) for mean comparison and significant differences were calculated according to Duncan's multiple range test (DMRT). Differences at  $P=0.05$  were considered statistically significant.

## Results

### Effects of NaOCl in combination with preculture treatment and leaf developmental stage

Table 1 shows the effects of different treatments of NaOCl on survival of explants derived from the three leaf developmental stages of preculture and non-preculture treated plants. It is obvious that explants derived from the three leaf developmental stages of preculture treated plants generally showed moderate to high percentage of explant survival. Increase in the concentration of NaOCl and duration of immersion decreased the percentage of survival of explants taken from all leaf developmental stages. Explants from the bronze leaf stage of preculture treated plants showed up to 79.62% survival after sterilization in 20% NaOCl for 20 min, while explants from the bronze leaf stage of non-preculture treated plants exhibited a maximum of 57.40% survival after sterilization in 20% NaOCl for 20 min. Meanwhile, explants from the stable green leaf stage of preculture treated plants exhibited a maximum of 62.96% explant survival after sterilization in 20% NaOCl for 20 min, while explants from the stable green leaf stage of non-preculture treated plants attained the highest survival of 51.85% also after sterilization in 20% NaOCl for 20 min as well. Overall, NaOCl was effective to some extent the increase in immersion time resulted in loss of explants due to browning followed by leaching of chlorophyll from the explants. In order to use NaOCl as a sterilizing agent for *H. brasiliensis* explants, it is therefore recommended to take explants from the bronze leaf stage of preculture treated plants and sterilized with 20% of NaOCl for 20 min.

### Effects of HgCl<sub>2</sub> in combination with preculture treatment and leaf developmental stage

Table 2 shows the effects of different treatments of HgCl<sub>2</sub> on survival of explants derived from the three leaf developmental stages of preculture and non-preculture treated plants. The survival of explants was noticeably high (42.59%) for explants derived from the stable green leaf stage of preculture treated plants whereas the highest survival was only 27.77% for explants derived from the stable green leaf stage of non-preculture treated plants, both after sterilization in 0.1% of HgCl<sub>2</sub> for 10 min. Meanwhile the highest survival recorded for explant derived from the bronze leaf stage of preculture treated plants was 38.88% after sterilization in 0.1% of HgCl<sub>2</sub> for 10 min, while the highest from the bronze leaf stage of non-preculture treated plants was 37.03% survival after immersion in 0.1% of HgCl<sub>2</sub> for 5 min. In the case of explants

derived from the light green leaf stage of preculture treated plants the highest survival was 31.47%, while the highest survival of explants taken from the light green leaf stage of non-preculture treated plants reached only 18.51%, both after exposure to 0.2% of HgCl<sub>2</sub> for 5 min.

### Effects of Nano silver in combination with preculture treatment and leaf developmental stage

Table 3 shows the effects of different treatments of NS on survival of explants from the three leaf developmental stages of preculture and non-preculture treated plants. It is very clear that explants derived from the light green and bronze leaf stages of preculture and non-preculture treated plants generally showed high survival. Increase in the concentration of NS and duration of immersion decreased the percentage of survival of explants from all three leaf developmental stages of preculture and non-preculture treated plants. The survival of explants from the light green leaf stage reached 94.44% for preculture treated plants and 88.88% for non-preculture treated plants after sterilization in 10 ppm of NS for 20 min. Explants taken from the bronze leaf stage of preculture treated plants attained the highest survival of 85.18% following immersion in 10 ppm of NS for 5 min, while explants from the bronze leaf stage of non-preculture treated plants reached 77.77% of survival after sterilization in 10 ppm NS for 20 min. Explants from the stable green leaf stage of preculture treated plants could also attain 77.77% survival after sterilization in 50 ppm of NS for 20 min. This study revealed that NS was more potent than NaOCl and HgCl<sub>2</sub> for effective disinfection of field-derived shoot tip and axillary bud explants of *H. brasiliensis*.

### Control of browning of *in vitro* cultures of *H. brasiliensis*

Figure 3 shows that the effect of AgNO<sub>3</sub>, AC and NS in controlling the browning of *in vitro* cultures of *H. brasiliensis* after 5 weeks of culture. NS at 4 ppm significantly produced the highest percentage of explant survival (87.03%) with a low percentage of browning (10%). In contrast, explants cultured on solid MS medium supplemented with 2 ppm AgNO<sub>3</sub> showed the highest percentage of browning (70%) compared to the rest of the treatments. The present study also showed that NS at 12 ppm and 16 ppm could totally prevent the incidence of *in vitro* browning of *Hevea* explants but with significant reduction in the survival of explants compared to NS at 4 ppm. Figure 4A shows shoot of *H. brasiliensis* free of contamination and browning

### Effects of basal medium and sucrose concentration on shoot induction

The results showed poor shoot formation (18%) on WPM medium with 6% sucrose. Meanwhile, there was no shoot formation on WPM medium with 3% and 9% sucrose and MS medium at all concentrations of sucrose tested (Figure 5). On the contrary, there was 80% shoot formation on MB medium supplemented with 6% sucrose, which differed significantly compared to the other treatments. This was followed by 34% shoot induction on MB medium with 9% sucrose and 28% on MB medium with 3% sucrose. It can clearly be seen that MB medium was most effective on induction of shoots from the field-derived *H. brasiliensis* explants compared to WPM and MS medium, with the percentage of shoot formation being most enhanced in the presence of 6% sucrose Figure 4B shows healthy and well- formed regenerants on MB medium supplemented with 6% sucrose.

## Discussion

A successful tissue culture initiation step often begins with an effective explant sterilization technique and in the micropropagation of

Time (min)	NaOCl concentration (%)	Mean percentage of explant survival					
		Bronze Leaf Stage		Light Green Leaf Stage		Stable Green Leaf Stage	
		Control	Preculture	Control	Preculture	Control	Preculture
Control	Control	0.0 ± 0.0 <sup>f</sup>	1.85 ± 3.2 <sup>h</sup>	0.0 ± 0.0 <sup>d</sup>	5.55 ± 5.55 <sup>g</sup>	0.0 ± 0.0 <sup>e</sup>	3.7 ± 3.2 <sup>e</sup>
10	10	33.32 ± 5.55 <sup>bcd</sup>	44.44 ± 5.56 <sup>cde</sup>	1.85 ± 3.2 <sup>d</sup>	11.10 ± 5.55 <sup>fg</sup>	9.25 ± 6.41 <sup>de</sup>	5.55 ± 5.55 <sup>e</sup>
	20	40.73 ± 3.21 <sup>b</sup>	49.99 ± 5.55 <sup>bcd</sup>	11.10 ± 5.55 <sup>c</sup>	16.66 ± 5.55 <sup>ef</sup>	9.25 ± 6.41 <sup>de</sup>	18.51 ± 3.2 <sup>cd</sup>
	30	31.47 ± 8.48 <sup>bcd</sup>	53.7 ± 3.2 <sup>bc</sup>	16.66 ± 5.55 <sup>c</sup>	24.07 ± 3.2 <sup>de</sup>	12.96 ± 13.97 <sup>cd</sup>	22.22 ± 11.11 <sup>c</sup>
	40	16.66 ± 5.55 <sup>e</sup>	33.32 ± 5.55 <sup>f</sup>	12.96 ± 3.2 <sup>c</sup>	33.32 ± 5.55 <sup>bc</sup>	22.21 ± 5.55 <sup>bc</sup>	24.07 ± 3.2 <sup>c</sup>
20	10	37.03 ± 3.2 <sup>bcd</sup>	55.55 ± 5.55 <sup>b</sup>	29.62 ± 3.2 <sup>b</sup>	38.88 ± 5.55 <sup>b</sup>	29.62 ± 3.21 <sup>b</sup>	38.88 ± 5.55 <sup>b</sup>
	20	57.40 ± 3.21 <sup>a</sup>	79.62 ± 3.21 <sup>a</sup>	51.85 ± 3.2 <sup>a</sup>	55.55 ± 5.55 <sup>a</sup>	51.85 ± 3.2 <sup>a</sup>	62.96 ± 3.2 <sup>a</sup>
	30	38.88 ± 5.55 <sup>bc</sup>	46.29 ± 3.21 <sup>bode</sup>	27.77 ± 5.55 <sup>b</sup>	49.99 ± 5.55 <sup>a</sup>	27.77 ± 5.55 <sup>b</sup>	44.44 ± 5.56 <sup>b</sup>
	40	27.77 ± 5.55 <sup>d</sup>	33.32 ± 5.55 <sup>f</sup>	11.10 ± 5.55 <sup>c</sup>	16.66 ± 5.55	16.66 ± 5.55 <sup>cd</sup>	22.21 ± 5.55 <sup>c</sup>
30	10	29.62 ± 8.48 <sup>cd</sup>	38.88 ± 5.55 <sup>ef</sup>	24.07 ± 3.2 <sup>b</sup>	37.03 ± 3.2 <sup>b</sup>	12.96 ± 3.2 <sup>cd</sup>	38.88 ± 0.0 <sup>b</sup>
	20	33.32 ± 5.55 <sup>bcd</sup>	42.59 ± 8.48 <sup>def</sup>	12.96 ± 3.2 <sup>d</sup>	25.92 ± 3.2 <sup>cd</sup>	20.36 ± 3.21 <sup>bcd</sup>	27.77 ± 5.55 <sup>c</sup>
	30	27.77 ± 5.55 <sup>d</sup>	33.32 ± 5.55 <sup>f</sup>	0.0 ± 0.0 <sup>d</sup>	12.96 ± 3.2 <sup>fg</sup>	11.10 ± 5.55 <sup>cd</sup>	5.55 ± 5.55 <sup>e</sup>
	40	11.10 ± 5.55 <sup>e</sup>	22.21 ± 5.55 <sup>g</sup>	1.85 ± 3.2 <sup>d</sup>	5.55 ± 5.5 <sup>g</sup>	12.96 ± 3.2 <sup>cd</sup>	11.10 ± 5.55 <sup>de</sup>

Table 1: Effects of NaOCl, preculture treatment and leaf developmental stage on percentage of survival of *H. brasiliensis* explants after 4 weeks of culture. Means followed by the same letter in the same column are not significantly different based on DMRT (p=0.05).

Times (min)	HgCl <sub>2</sub> Concentration (%)	Mean percentage of explant survival					
		Bronze Leaf Stage		Light Green Leaf Stage		Stable Green Leaf Stage	
		Control	Preculture	Control	Preculture	Control	Preculture
Control	Control	0.0 ± 0.0 <sup>d</sup>	5.55 ± 5.55 <sup>b</sup>	0.0 ± 0.0 <sup>c</sup>	1.85 ± 3.2 <sup>c</sup>	0.0 ± 0.0 <sup>c</sup>	0.0 ± 0.0 <sup>c</sup>
5	0.1	37.03 ± 3.2 <sup>a</sup>	24.07 ± 23.13 <sup>ab</sup>	14.81 ± 3.2 <sup>ab</sup>	27.77 ± 0.0 <sup>a</sup>	11.10 ± 5.55 <sup>b</sup>	25.92 ± 3.20 <sup>b</sup>
	0.2	24.07 ± 3.2 <sup>b</sup>	18.52 ± 6.41 <sup>ab</sup>	18.51 ± 3.21 <sup>a</sup>	31.47 ± 8.48 <sup>a</sup>	22.21 ± 5.55 <sup>a</sup>	20.36 ± 3.21 <sup>b</sup>
10	0.1	12.96 ± 3.2 <sup>c</sup>	38.88 ± 24.21 <sup>a</sup>	11.10 ± 5.55 <sup>b</sup>	27.77 ± 5.55 <sup>a</sup>	27.77 ± 5.55 <sup>a</sup>	42.59 ± 8.48 <sup>a</sup>
	0.2	0.0 ± 0.0 <sup>d</sup>	1.85 ± 3.2 <sup>b</sup>	1.85 ± 3.2 <sup>c</sup>	14.81 ± 6.41 <sup>b</sup>	5.55 ± 5.55 <sup>bc</sup>	5.55 ± 5.55 <sup>c</sup>

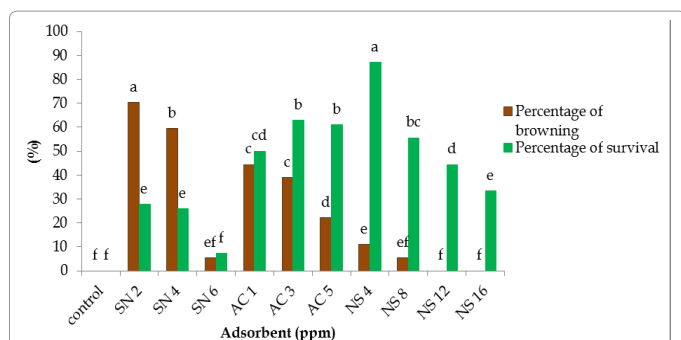
Table 2: Effects of HgCl<sub>2</sub>, preculture treatment and leaf developmental stage on percentage of survival of *H. brasiliensis* explants after 4 weeks of culture. Means followed by the same letter in the same column are not significantly different based on DMRT (p=0.05).

Times (min)	NS concentration (ppm)	Mean percentage of explant survival (%)					
		Bronze Leaf Stage		Light Green Leaf Stage		Stable Green Leaf Stage	
		Control	Preculture	Control	Preculture	Control	Preculture
Control	Control	0.0 ± 0.0 <sup>f</sup>	0.0 ± 0.0 <sup>i</sup>	0.0 ± 0.0 <sup>h</sup>	0.0 ± 0.0 <sup>h</sup>	0.0 ± 0.0 <sup>h</sup>	0.0 ± 0.0 <sup>h</sup>
5	2.5	49.99 ± 11.11 <sup>cd</sup>	55.55 ± 5.55 <sup>f</sup>	33.32 ± 5.55 <sup>ef</sup>	46.29 ± 3.21 <sup>e</sup>	16.66 ± 5.55 <sup>g</sup>	22.21 ± 5.55 <sup>g</sup>
	5	61.11 ± 11.11 <sup>bc</sup>	66.66 ± 5.55 <sup>de</sup>	40.73 ± 3.21 <sup>de</sup>	66.66 ± 5.55 <sup>cd</sup>	22.21 ± 5.55 <sup>fg</sup>	35.18 ± 3.2 <sup>ef</sup>
	10	66.66 ± 11.11 <sup>ab</sup>	85.18 ± 8.48 <sup>a</sup>	44.44 ± 5.5 <sup>d</sup>	90.73 ± 8.48 <sup>a</sup>	33.32 ± 5.55 <sup>cd</sup>	46.29 ± 3.21 <sup>cd</sup>
	50	75.92 ± 13.97 <sup>ab</sup>	81.47 ± 6.41 <sup>ab</sup>	62.96 ± 3.2 <sup>bc</sup>	77.77 ± 5.55 <sup>b</sup>	38.88 ± 5.55 <sup>c</sup>	49.99 ± 5.55 <sup>c</sup>
	200	61.10 ± 5.55 <sup>bc</sup>	72.21 ± 5.55 <sup>bcd</sup>	38.88 ± 5.55 <sup>de</sup>	49.99 ± 5.55 <sup>e</sup>	38.88 ± 5.55 <sup>c</sup>	51.85 ± 3.2 <sup>c</sup>
20	2.5	62.96 ± 3.20 <sup>abc</sup>	64.81 ± 3.2 <sup>def</sup>	55.55 ± 5.55 <sup>c</sup>	61.10 ± 5.55 <sup>d</sup>	29.62 ± 3.21 <sup>def</sup>	33.32 ± 5.55 <sup>ef</sup>
	5	68.51 ± 8.48 <sup>ab</sup>	72.21 ± 5.55 <sup>bcd</sup>	61.10 ± 5.55 <sup>bc</sup>	66.66 ± 5.55 <sup>cd</sup>	31.47 ± 3.21 <sup>cde</sup>	38.88 ± 5.55 <sup>de</sup>
	10	77.77 ± 5.55 <sup>a</sup>	77.77 ± 5.55 <sup>abc</sup>	88.88 ± 5.55 <sup>a</sup>	94.44 ± 5.56 <sup>a</sup>	51.85 ± 3.2 <sup>b</sup>	70.36 ± 3.21 <sup>a</sup>
	50	64.81 ± 8.48 <sup>abc</sup>	61.10 ± 5.55 <sup>ef</sup>	44.44 ± 5.56 <sup>d</sup>	66.66 ± 5.55 <sup>cd</sup>	53.7 ± 3.2 <sup>b</sup>	77.77 ± 5.55 <sup>a</sup>
60	2.5	18.51 ± 3.21 <sup>e</sup>	18.51 ± 3.21 <sup>h</sup>	27.77 ± 5.55 <sup>fg</sup>	44.44 ± 5.56 <sup>ef</sup>	66.66 ± 5.55 <sup>a</sup>	61.10 ± 5.55 <sup>b</sup>
	5	64.81 ± 3.20 <sup>abc</sup>	66.66 ± 5.55 <sup>de</sup>	44.44 ± 5.56 <sup>d</sup>	66.66 ± 5.55 <sup>cd</sup>	24.07 ± 3.2 <sup>efg</sup>	27.77 ± 5.55 <sup>g</sup>
	10	68.51 ± 6.41 <sup>ab</sup>	68.51 ± 6.41 <sup>cde</sup>	57.40 ± 3.21 <sup>c</sup>	72.21 ± 5.55 <sup>bc</sup>	24.06 ± 6.41 <sup>efg</sup>	38.88 ± 5.55 <sup>de</sup>
	50	61.11 ± 11.11 <sup>bc</sup>	66.66 ± 5.55 <sup>de</sup>	66.66 ± 5.55 <sup>b</sup>	75.92 ± 3.2 <sup>bc</sup>	49.99 ± 5.55 <sup>b</sup>	44.44 ± 5.56 <sup>cd</sup>
	200	38.88 ± 11.11 <sup>d</sup>	44.44 ± 5.56 <sup>a</sup>	22.21 ± 5.55 <sup>g</sup>	37.03 ± 3.2 <sup>f</sup>	29.62 ± 3.21 <sup>def</sup>	44.44 ± 5.56 <sup>cd</sup>
		0.0 ± 0.0 <sup>f</sup>	0.0 ± 0.0 <sup>i</sup>	0.0 ± 0.0 <sup>h</sup>	16.66 ± 5.55 <sup>g</sup>	5.55 ± 5.55 <sup>h</sup>	3.7 ± 6.41 <sup>h</sup>

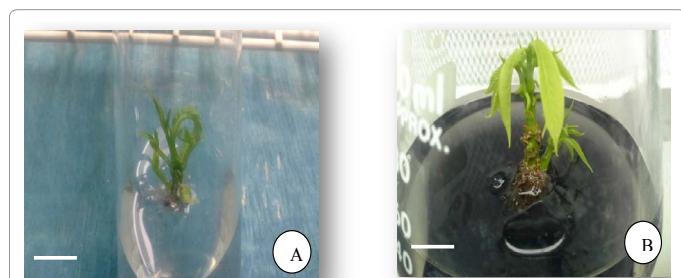
Table 3: Effects of NS, preculture treatment and leaf developmental stage on percentage of survival of *H. brasiliensis* explants after 4 weeks of culture. Means followed by the same letter in the same column are not significantly different based on DMRT (p=0.05).

*H. brasiliensis* the establishment of an axenic culture is one of the most challenging steps. In this study, NaOCl, HgCl<sub>2</sub> and NS were assessed as sterilants on explants derived from different leaf developmental stages of preculture and non-preculture treated plants of *H. brasiliensis*. The use of increasing strength of NaOCl and HgCl<sub>2</sub> or extending the time of immersion in the sterilants resulted in the loss of explants due to

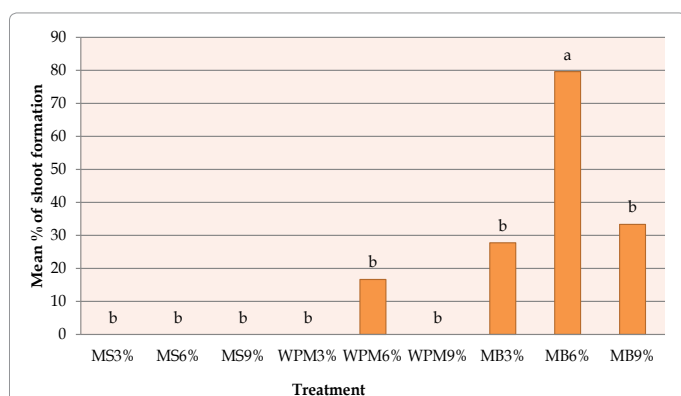
browning followed by leaching of chlorophyll from the explants. The results indicated the necessity of using the correct concentration and immersion time in NaOCl and HgCl<sub>2</sub> as well as a suitable leaf developmental stage and with preculture treatment in order to overcome the contamination of *H. brasiliensis* explants *in vitro*. Debergh and Maene [15] proposed a scheme focusing on the preculture phase to obtain



**Figure 3:** Effects of different adsorbents on control of browning of in vitro culture of *H. brasiliensis* after 5 weeks of culture. Values represent means for 18 explants per treatment per replication. The same letters represent no significant difference between treatments according to one-way analysis of variance ( $P=0.05$ ).



**Figure 4:** *H. brasiliensis* shoots after NS treatment (bar=0.75 cm), (A) shoot without contamination and browning (bar=0.28 cm), (B) healthy and well-formed regenerants (bar=0.75 cm).

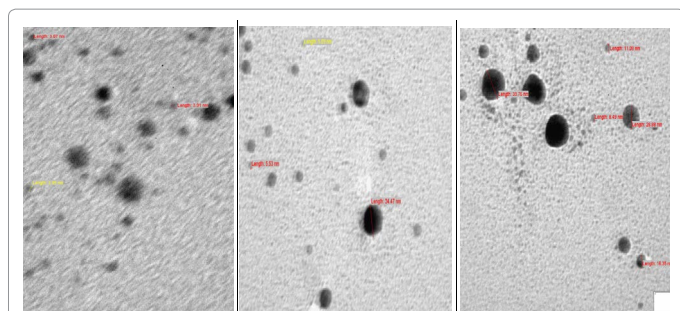


**Figure 5:** Effects of basal medium and sucrose concentration on shoot induction of *H. brasiliensis* explants after 16 weeks of culture. Medium formulations: MS, WPM and MB; Sucrose concentrations: 3%, 6% and 9%. Values represent means for 18 explants per treatment per replication. The same letters represent no significant difference between treatments according to one-way analysis of variance ( $P=0.05$ ).

healthier explants and uniform responses. Growth, morphogenesis and rates of propagation *in vitro* can be improved by applying appropriate environmental and chemical pretreatments to the stock plants. To our knowledge, there are no reports so far investigating on the effects of NaOCl and HgCl<sub>2</sub> in combination with leaf developmental stage and preculture treatment on overcoming contamination of field-derived explants of *H. brasiliensis*. In the assessment of NS as a sterilant, the results revealed that it was more effective than NaOCl and HgCl<sub>2</sub> in reducing contamination of *H. brasiliensis* explants. Increasing the strength of NS or extending the time of immersion in the sterilant did

not cause browning and leaching of chlorophyll from the explants. The highest percentage of survival (94.44%) was attained when explants derived from the light green leaf stage of preculture treated plants were sterilized in 10 ppm of NS for 20 min. Chen and Schluesener [16], Mahna et al. [17] and Razali et al. [18] also suggested the use of NS for disinfection of *in vitro* plant species they worked on. Abdi et al. [14] and Abdi [19] reported on the application of 100 ppm NS for 60 min as the most effective decontamination procedure for *Valeriana officinalis* explants. However in this study, a low concentration of 10 ppm NS at an immersion time of 20 min was highly effective in reducing microbial contamination and increasing the survival of *H. brasiliensis* explants. The mechanism of action of NS in terminating the microorganisms is not known clearly. However, it has been shown that NS releases silver ions (Ag<sup>+</sup>) slowly which can destroy the cell structure of microorganisms [20]. Dibrov et al. [21] stated that the effects of Ag<sup>++</sup> on microorganisms may be due to their chemosmotic activity. These researchers showed that Ag<sup>+</sup> affects the phospholipids and destroy the cell membrane of microorganisms. Ag<sup>+</sup> also may substitute the sulphur in the -SH groups of cell membrane of microorganisms and destroy them. The destructive effects of Ag<sup>+</sup> are related to the production of active silver containing organic compounds. These compounds can attract the microorganisms and destroy their structure. The use of nano-sized silver particles as antimicrobial agent has become more common as technological advances make their production more economical. One of the potential applications of silver is in the management of plant diseases. Since silver displays multiple modes of inhibitory action on microorganisms, it may be used for controlling various plant pathogens in a relatively safer way compared to synthetic fungicides [22]. Results obtained in this study including the overall low percentage of microbial contamination of explants derived from the different phenological stages after sterilization in NS reconfirmed the antifungal activity of NS as reported by Jo et al. [22].

Another most frequently encountered problem during micropropagation of *H. brasiliensis* is the occurrence of browning. Several authors have reported on methods of controlling phenolic compounds exudation from the species [23-25]. This study revealed the effectiveness of nano silver particles as an antioxidant and polyphenol adsorbent compared to silver nitrate and activated charcoal on explants of *H. brasiliensis*. Earlier investigations revealed that the dark exudate was very difficult to eradicate by pretreatment washes [26]. Ziv and Halvey [27] found that phenolic compounds inhibited shoot formation in *Strelitzia reginae* and *Geranium* species and also caused browning and necrosis of *Kangaroo paws*. Reports have shown that in other woody species when activated charcoal was added to the medium, with or without dark periods at the beginning of the experiment, it eliminated browning and was beneficial to shoot formation [28,29]. However, in our study on rubber though the explant survival reached 65% in the presence of 3 ppm activated charcoal the percentage of browning was still high at 40%. Meanwhile, evaluation on effects of AgNO<sub>3</sub> showed even higher percentage of browning (70%) with an explant survival of less than 30% upon application of 2 ppm AgNO<sub>3</sub>. Earlier, Housti [10] reported that AgNO<sub>3</sub> showed only a slight positive effect on organogenesis of *H. brasiliensis* and a very slight antibrowning effect on callus of this species. It was also evident from our study that shoots of *H. brasiliensis* produced in the presence of activated charcoal and silver nitrate were not vigorous and showed symptoms of chlorosis. Interestingly, our study shows that nano silver is highly effective on the explant survival, behaviour and response to organogenesis. Transmission electron microscopy (TEM) images (Figure 6) of the silver nanoparticles used in this study indicated the



**Figure 6:** Transmission electron microscopy (TEM) image of silver nanoparticles. Scale bars: 50, 100 and 200 nm.

extremely minute size of the particles. A study by Zhang et al. [30] reported that Ag<sup>+</sup> release rates were found to depend on the primary particle size and concentration of NS, when other environmental factors (e.g. dissolved oxygen and protons) were kept constant. To our knowledge, there are no reports so far investigating the effects of nano silver as an antioxidant and polyphenol adsorbent in overcoming browning of field-derived *H. brasiliensis* explants.

In this study, MB medium was comparatively found to be more suitable than WPM and MS media on *in vitro* shoot induction of *H. brasiliensis*. MB medium had earlier been specifically suggested for *in vitro* culture of *Hevea* by Enjalric and Carron [31]. There are numerous reports on the better performance of woody plant species grown on low salt media including WPM, however it was not ideally the case with *H. brasiliensis*. This study showed that the mean percentage of shoot formation from cultures grown on MB medium supplemented with 3%, 6% and 9% sucrose was better than that obtained on WPM with 6% sucrose. WPM contains much less macro elements in comparison with MB medium. Earlier, Seneviratne et al. [32] showed better growth and survival of *H. brasiliensis* axillary bud on WPM supplemented with 10% sucrose in comparison with MS medium [33-35].

## Conclusion

This research study indicates that the successful establishment of *in vitro* culture of *H. brasiliensis* requires the understanding of the species leaf developmental stages, the interaction of the explant source with the environment and the effective application of the non-toxic silver nano particles in reducing microbial contamination and browning of *Hevea* explants. An efficient initial culture establishment developed in this study thus opens the way for future application of the microcutting technique for propagation as well as genetic improvement of *H. brasiliensis*.

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