In Vitro Propagation of Rubber Tree (*Hevea Brasiliensis)* Using Shoot-Tip and Nodal Cutting Explants

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ABSTRACT

Hevea brasiliensis which belongs to the Euphorbiaceae family is the primary source of natural rubber. Propagation of rubber tree by grafting on to unselected seedlings sustains intraclonal heterogeneity for vigour and productivity which could be improved via in vitro techniques. Micropropagation from rubber nodal and shoot tip explants is possible. In vitro technique is needful to mass propagate disease-free and genetically similar rubber plantlets. In vitro results in increased growth and vigour of rubber tree, However, in vitro techniques of rubber tree have not been given much critical research attention in Ghana. Propagation of H. brasiliensis by in vitro techniques was used to study alternative procedures for mass production of rubber planting materials. Murashige and Skoog (MS) basal medium amended with 30.0g/L sucrose, 100.0mg/L myo-inositol, 2.0g/L activated charcoal, 1.0mg/L silver nitrate AgNO3, 2.0mg/L GA3 and control, 2.5, 5.0, 7.5 or 10.0mg/L kinetin was used to culture both H. brasiliensis shoot-tip and nodal explants. The MS medium with 5.0mg/L kinetin significantly (P<0.05) enhanced higher shoot development (84.00%), number of shoots (3.60) and leaves (23.40) of the shoot-tip explants compared to other treatments. In nodal explants, the control medium developed higher shoots (94.00%), the height of shoot (4.80cm), number of leaves (19.20), number of shoots (6.00) and number of roots (7.00) than those with kinetin treatments. Conversely, 7.5mg/L kinetin of the nodal culture also performed significantly after the controls. Successful in vitro regeneration of plantlets was achieved using Hevea brasiliensis shoot-tip and nodal cutting explants cultured on an MS medium supplemented with kinetin.

Key words: Hevea brasiliensis, In Vitro Propagation, Shoot-Tip and Nodal Culture, MS Medium, Kinetin.

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1. INTRODUCTION

Hevea brasiliensis is a tree belonging to the family *Euphorbiaceae* and the most economically important member of the genus *Hevea* because its latex is the primary source of natural rubber. Conventionally, the rubber tree is propagated by grafting buds from selected clones on seedlings from seed orchards (1). Microcutting is employed to overcome the long years used to produce grafted plants in nurseries before planting. Also, it eliminates the incompatibility between rootstocks and scions. Grafting is a partial vegetative propagation method, and the major shortcoming of this method is the intra-clonal variation that is partially due to unpreventable rootstock/scion interaction (2). Furthermore, uniform growth and yield of rubber trees are not realized even under best managerial practices when the grafting method is used (3). This deficit in yield may be due to many reasons but much of it could be due to the heterogeneous rootstocks (4).

In vitro culture research has led to three types of micropropagation techniques and genetic modification in rubber. These are microcutting, short-term somatic embryogenesis and long-term maintained somatic embryogenesis (5). In *Hevea, in vitro* via shoot-tip cultures, nodal cultures, somatic embryogenesis and genetic modification have been successful (6). Microcutting in *Hevea brasiliensis* involves *in vitro* culture of apex and axillary buds for propagation (7). Microcutting is used to produce true-to-type clones of planting materials for the rubber industry. This involves culturing axillary buds or cotyledonary nodes to induce plantlets (8-9).

Thus, micropropagation of elite clones with their own root system could reduce intra-clonal variation due to stock-scion interaction (6). Micropropagation is indispensable for the commercial production of rubber clones (10). Shoot-tip (2-3cm long) culture was attempted for the first time using aseptically grown rubber seedlings as explants but these shoots did not root in liquid MS media (11). Thereafter, shoots from 1-3 year old rubber seedlings used as explants developed roots (12). Since then there has been a progressive level in the micropropagation of clonal *Hevea* using axillary shoot proliferation (13). *In vitro* propagation of *H. brasiliensis* through organogenesis normally involves four phases; initiation of cultures, shoot multiplication, rooting of shoots and acclimatization (14,7).

Upon all the benefits that biotechnology brings into rubber production: *in vitro* techniques have not been given critical research considerations in Ghana. Consequently, rubber-producing companies in Ghana usually import budded stump of rubber clones to establish clonal farms already characterized with scion-stock interactions. Therefore, there is limited availability of rubber planting materials of high genetic quality, thereby making the rubber companies the only producers of rubber clonal stumps in the country as well as slowing rubber tree production. Establishment of rubber plantation by individual farmers is, therefore, very expensive. The actual cost of plantation establishment and maintenance differs tremendously from one country to another ranging between \$511 and \$1,016/ha in Vietnam to \$2,180/ha in Ghana with the total cost of planting materials i.e. clonal stumps alone being \$414/ha-600unit (15). There is also an inadequate exploitation of knowledge in biotechnology of *H. brasiliensis*, which tends to affect the propagation processes of the tree plant in Ghana. *Hevea* is still propagated by grafting and budding, although the stocks produced from seeds maintain intra-clonal heterogeneity and smaller production than the mother tree.

The main objective of this study is to propagate *Hevea brasiliensis in vitro* using shoot-tip and nodal culturing explants. The effect of concentration of kinetin on shoot development from these two explants was investigated.

2. MATERIALS AND METHODS

2.1. Collection of *Hevea brasiliensis* seeds
Mature seeds of *Hevea brasiliensis* obtained from rubber outgrowers of GREL in the Western Region of Ghana were sown in nursery bags filled with sandy loam soil and were watered daily at BNARI for the harvesting of explants for this study.

2.2. Induction of shoots from *Hevea brasiliensis* shoot-tip explants
Young seedlings obtained from germinated seeds were used as explants. Shoot-tips of seedlings were harvested as explants using a scalpel blade and then put into a clean tight-lid horny jar. The explants were put under running tap water for 10 minutes and then washed with liquid detergent "sunlight" also for 20 minutes. Thereafter, they were sterilized by immersion in 0.1% mercuric chloride ($HgCl_2$) for 5 minutes followed by rinsing with three changes of sterile distilled water and cultured on a full strength Murashige and Skoog (16) basal medium amended with 30.0g/L sucrose, 100.0mg/L myo-inositol, 2.0g/L activated charcoal, 1.0mg/L silver nitrate($AgNO_3$), 2.0mg/L GA_3 and control, 2.5, 5.0, 7.5 or 10.0mg/L kinetin. The pH of the medium was adjusted to 5.8±1 using 1.0M NaOH and 1.0M HCL before the addition of 3.0g/L phytagel and then autoclaved at 121°C for 15minutes at 15psi. The cultured explants were incubated in the growth room at a temperature of 25±2°C under a 16-hour photoperiod with light supplied by fluorescent lamps/tubes at an intensity of 3000 lux.

A completely randomized design was used with five (5) explants per treatment and was replicated five (5) times. Shoot-tips were considered to have grown when their terminal buds become visible with expanded leaflets. The number of leaves, shoots, roots, height of developing shoots and number of developed explants (plantlets) produced per explant were counted 120 days after culture.

2.3. Induction of shoots from *Hevea brasiliensis* nodal explants
Young shoots with axillary buds of the rubber seedlings were harvested as explants using a scalpel blade and then put into a clean tight-lib horny jar. Nodal cuttings were prepared from the seedlings of two nodes. The nodal explants were sterilized and cultured as previously described (**Section 2.2**). Cultures were kept under growth room conditions (**Section 2.2**).

Nodal cuttings were considered sprouted when the buds ruptured with at least one leaf. The number of cuttings that developed plantlets was counted. The number of leaves, shoots, roots and height of developing shoots produced per explant were counted 120 days after culture.

2.4. Data analysis
The data collected were analysed using the Statgraphics® Centurion XVI. Fisher's least significant difference (LSD) was used for the separation of means where appropriate at 5%.

3. RESULTS

3.1. Effect of concentrations of Kinetin on shoot formation from shoot-tips of *Hevea brasiliensis*
In vitro shoot-tip explants of *Hevea brasiliensis* seedlings cultured on MS medium supplemented with different concentrations (0.0-10.0mg/L) of kinetin developed into plantlets 120 days after culture irrespective of the concentrations of kinetin present in the medium. However, there were differences in the growth of the shoots depending on the concentration of the kinetin. Morphologically, the sub-adjacent leaves of the shoot tips expanded indicating growth followed by the elongation of the internodes. Irrespective of the concentration of the kinetin in the culture medium, all the shoot-tip explants showed signs of growth two to three weeks after culture. Due to contaminations from the phenolic compounds and latex of the explants as well as the laboratory environment, most of the shoot-tip explants failed to grow and develop further (Fig. 1).

Figure 1. Contaminated MS medium of developing shoot-tip explant of *H. brasiliensis*
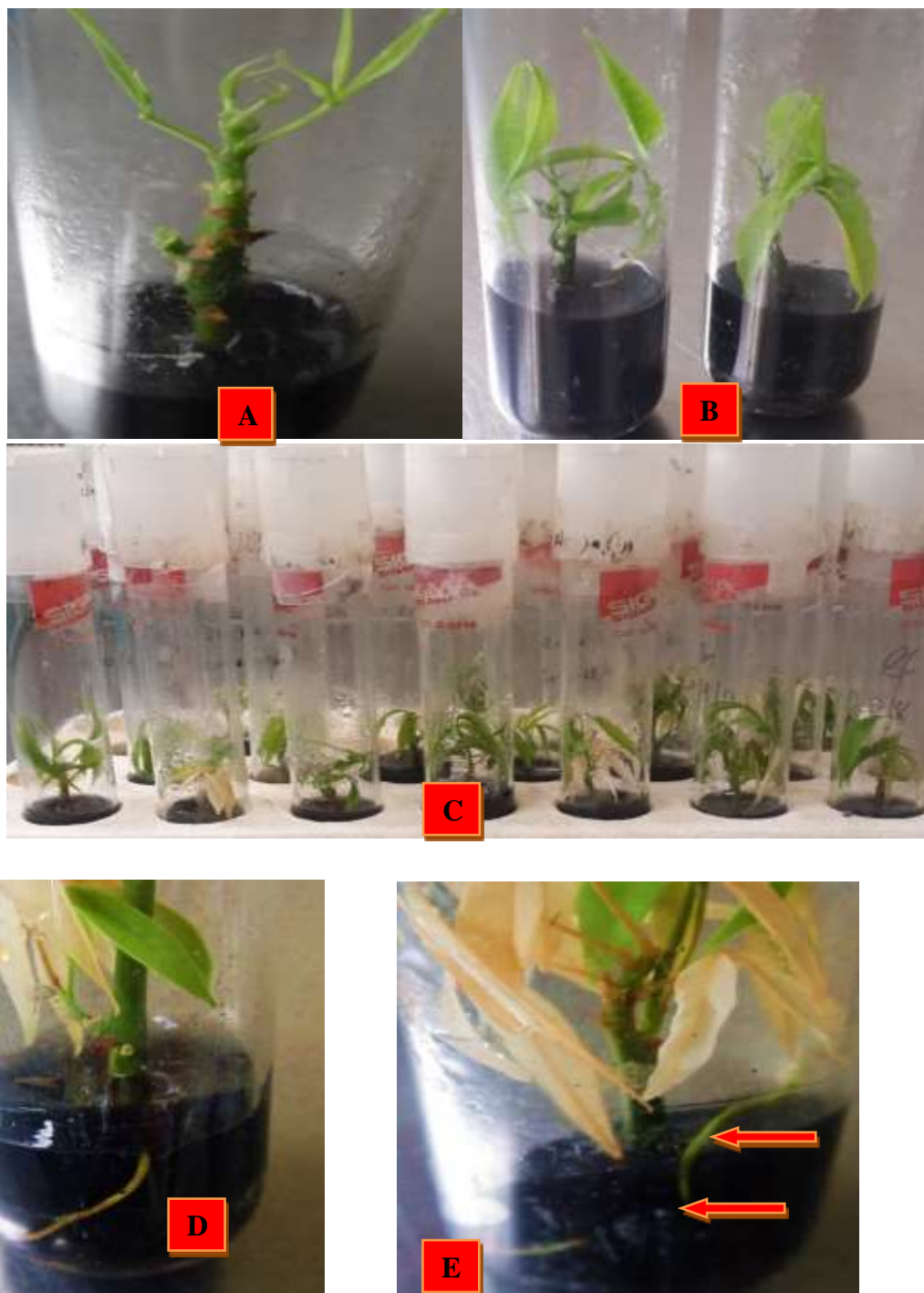
Figure 2 (A-E): Shoot-tip Explants of *H. brasiliensis* developing into plantlets on MS medium amended with concentration of kinetin

A-Shoot-tip explant of *H. brasiliensis* showing shoot elongation

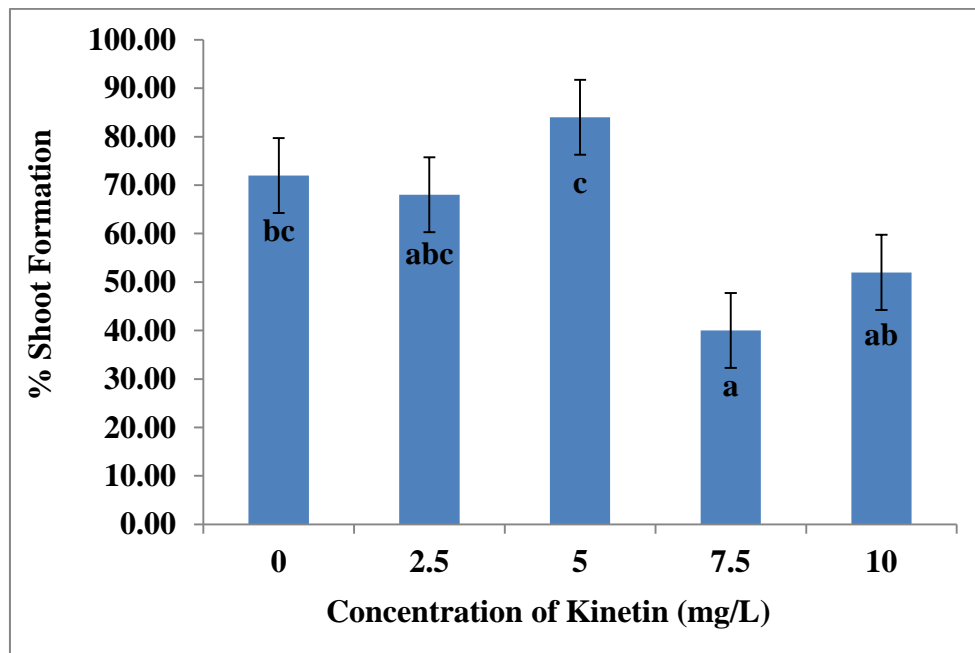**B-**Shoot-tip explants of *H. brasiliensis* showing leaf formation

C-Shoot-tip explants of *H. brasiliensis* on MS medium supplemented with 5.0mg/L

D&E- Rooted shoot-tip plantlets of *H. brasiliensis* on the control MS medium

All the four different kinetin concentrations had the potential of ensuring growth of the shoot-tip explants. Apart from the effects of contamination on the shoot formation of the shoot-tip explants, higher concentrations of kinetin (7.5mg/L and 10.0mg/L) also tended to have the least sprouting percentages. Not only did the higher kinetin concentrations have a reduced sprouting percentage, but they also showed less effect in the number of leaves, number of plantlets with roots and number of shoots.

The number of shoot-tips that developed into shoots/plantlets ranged from 40.0-84.0%. The number of explants that developed plantlets marginally increased from 70.0% in the controls to 84.0% on a medium supplemented with 5.0mg/L kinetin (Fig. 3). After the optimal concentration, shoot development decreased gradually to 40.0% on a medium supplemented with 7.5mg/L kinetin suggesting that higher concentrations of kinetin were detrimental to shoot development. The medium supplemented with

5.0mg/L (Fig. 2C) of kinetin produced the highest shoots (84.0%) percentage followed by the control and 2.5mg/L of kinetin concentrations (Fig. 3). Statistical analysis showed significant difference in the response of the shoot-tip to the growth regulator. Explants cultured on 0.0 to 5.0mg/L kinetin significantly ($P<0.05$) developed into plantlets than those cultured on 7.5mg/L kinetin to 10.0mg/L kinetin.

Figure 3. Percentage sprouting of *Hevea brasiliensis* shoot-tip explants cultured on MS basal medium supplemented with 0.0-10.0 mg/L Kinetin. Means with the same letter are not significantly different ($P\geq0.05$). The bars indicate standard errors of the means.

The mean height of shoots (Fig. 2A) produced per shoot-tip was also greatly influenced by the concentrations of cytokinin added to the MS medium. However, the effect of the cytokinin did not follow any particular trend (Fig. 4). The height of shoots development on a medium with 2.5, 5.0 and 10.0mg/L kinetin was significantly higher than the controls (1.98cm) and those cultured on 7.5mg/L kinetin (1.08cm) (Fig. 4). The lower length of shoots developed by shoot-tip explants cultured on 7.5mg/L kinetin cannot be explained.
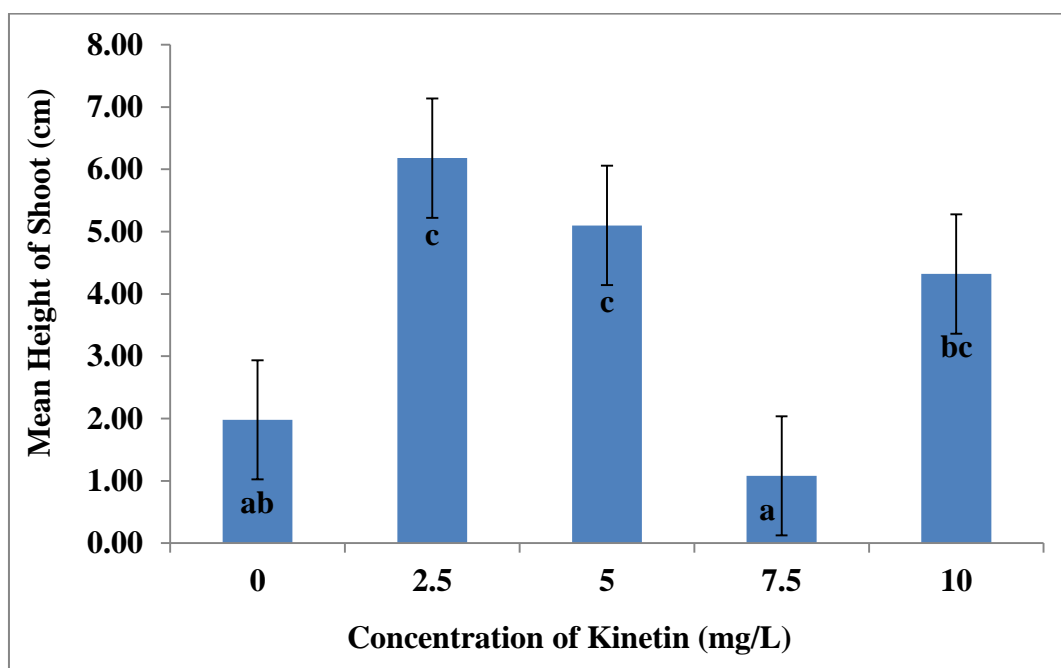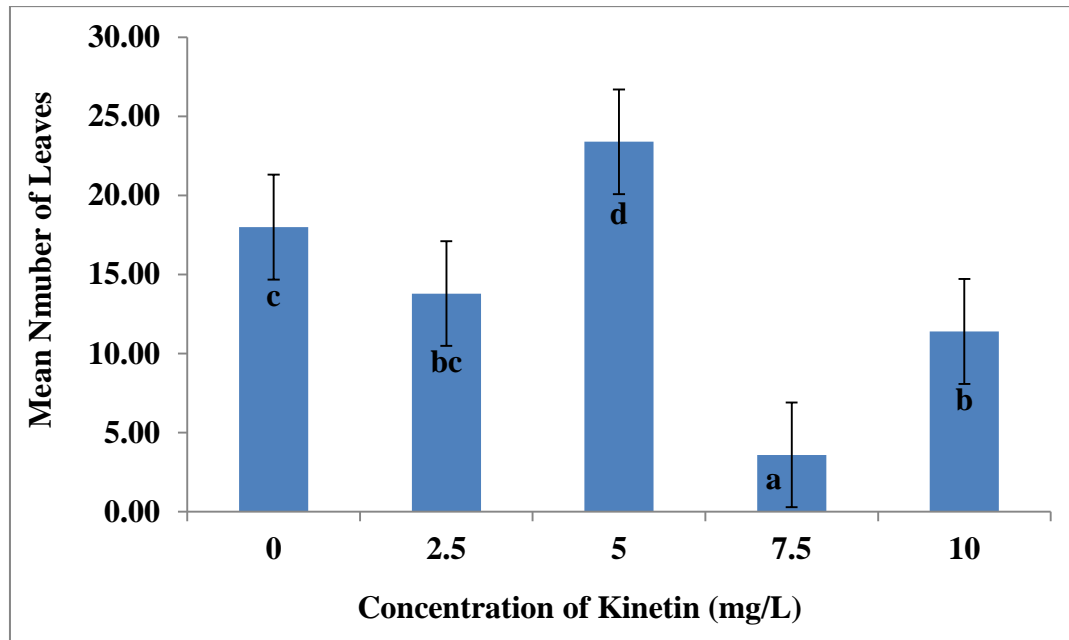

Figure 4. Mean height shoot of *Hevea brasiliensis* shoot-tip explants cultured on MS basal medium supplemented with 0.0-10.0 mg/L Kinetin. Means with the same letter are not significantly different ($P\geq0.05$). The bars indicate standard errors of the means.

The number of leaves (Fig. 2B) formed varied considerably depending on the concentrations of kinetin in the culture medium. However, the highest mean number of leaves of the explants was achieved on a medium amended with 5.0mg/L kinetin and this was significant than the controls and the other remaining treatments (Fig. 5).

The mean number of leaves (Fig. 2B) formed on the medium with 5.0mg/L kinetin is statistically (P<0.05) higher than the other treatments. However, insignificant difference (P≥0.05) existed between the medium supplemented with 2.5mg/L kinetin and the controls. Neither was the medium with 2.5mg/L kinetin significantly different from 10.0mg/L kinetin of the number of leaves formed by the shoot-tip explants (Fig. 5). Also, shoot-tips cultured on 7.5 or 10.0mg/L kinetin had significantly lower leaf development (less than 12 leaves) than the rest of the treatments. The highest mean number of leaves (23.4) formed by the shoot-tip explants was achieved on the medium supplemented with 5.0mg/L of kinetin (Fig. 5).

Figure 5. Mean number of leaves of developed by shoot-tip explants cultured on MS basal medium supplemented with 0.0-10.0 mg/L Kinetin. Means with the same letter are not significantly different (P≥0.05). The bars indicate standard errors of the means.

The presence of kinetin in the MS medium resulted in multiple shoots development from shoot-tip explants. The mean number of shoots produced ranged from 1.6-3.6 shoots per shoot-tip explants (Table 1). Although the medium with 5.0mg/L of kinetin produced the highest mean number of shoots (3.6), it showed no significant difference (P≥0.05) from the controls and the medium with 2.5mg/L kinetin. Explants cultured on the medium supplemented with 7.5mg/L of kinetin produced the lowest mean number of shoots (1.2), but it was not significantly different (P≥0.05) from the controls and the medium with 10.0mg/L kinetin (Table 1).

The shoot produced also developed roots even without the influence of exogenous auxins in the MS medium (Fig. 2D&E).The number of cultured shoots with roots was significantly higher in the controls (8.0) than the kinetin treated explants. Among the explants treated with kinetin, the concentrations of the cytokinins had significant effect on shoot development (Table 1). The highest number of roots was obtained from shoots growing on the medium amended with 7.5mg/L kinetin while the lowest number (1.0) of roots was observed on a medium with 5.0mg/L and 10.0mg/L kinetin. All shoots developing on MS medium amended with 2.5mg/L kinetin did not develop roots (Table 1).

Table 1. Mean Number of Shoots and Plantlets with Roots of *Hevea brasiliensis* Shoot-Tip Explants Cultured on MS Basal Medium Supplemented with 0.0-10.0 Mg/L Kinetin.

Concentration of Kinetin (mg/L)	Mean Number of Shoots	Mean Number of Plantlets with Roots
0.0	2.600±1.140[bc]	**8.000±5.701[b]**
2.5	2.800±1.483[bc]	0.000±0.000[a]
5.0	**3.600±1.140[c]**	1.000±2.236[a]
7.5	1.200±0.447[a]	3.000±2.739[a]
10.0	1.600±0.548[ab]	1.000±2.236[a]

Means in the same column with the same letter superscript are not significantly different (P≥0.05)

3.2. Effect of Kinetin on shoot formation from nodal cuttings

Nodal cutting explants cultured on MS medium supplemented with kinetin developed shoots irrespective of the concentration of the kinetin (Fig. 6A). After two to three weeks of culture, there was shoot emergence from the buds with some nodal explants of *H. brasiliensis* consequently developing roots. Differences in growth and development of shoots in the number of developed shoots, length of shoots, number of leaves and roots were observed among the nodal explants of *H. brasiliensis* depending on the concentration of the kinetin added to the MS medium.

Just like the shoot-tip explants of the *H. brasiliensis*, the nodal explants culture on MS medium supplemented with four different concentrations of kinetin (2.5, 5.0, 7.5 and 10.0mg/L) also showed development of plantlets 120 days after culture no matter the concentrations of kinetin added to the MS medium. Elongation of internodes preceded the breaking of the axillary buds of the nodal explants of *H. brasiliensis.* After stem elongation of the nodal explants of *H. brasiliensis*, the newly developing shoots started forming leaves.
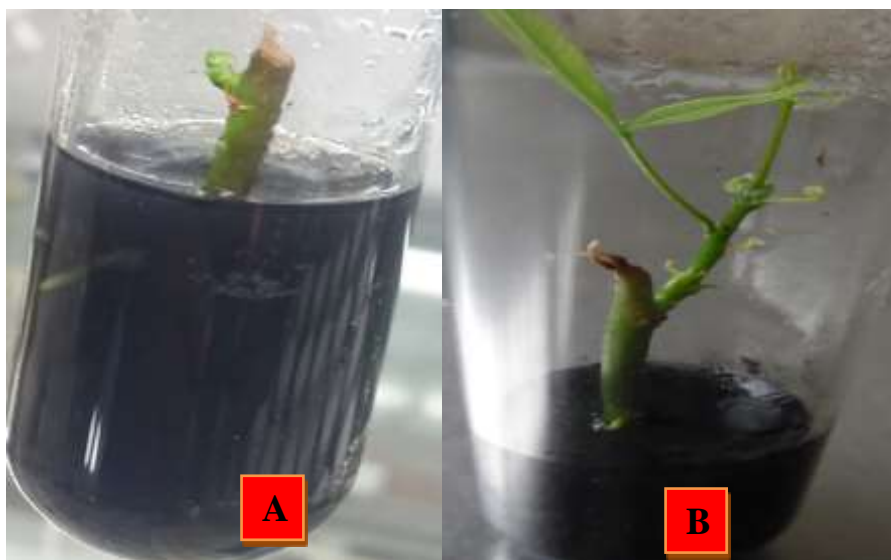
Figure 6(A-D): Nodal explants of *H. brasiliensis* cultured on MS medium amended with concentrations of kinetin showing developing shoots:

A-twenty (20) days after culture

B-forty (40) days after culture

C-elongation of stem and formation of multiple shoots

D-formation of cluster of roots

All the nodal explants sprouted after four (4) weeks of culture on MS medium supplemented with kinetin (Fig. 6B). The number of shoots (92.0%) developed was significantly higher on the control medium than on medium with 2.5mg/L (60.0%) and 10.0mg/L (56.0%) kinetin (Fig. 7). The concentrations of the kinetin in the culture medium had influence on the percentage shoot development, number of leaves, shoots as well as roots and height of shoots.
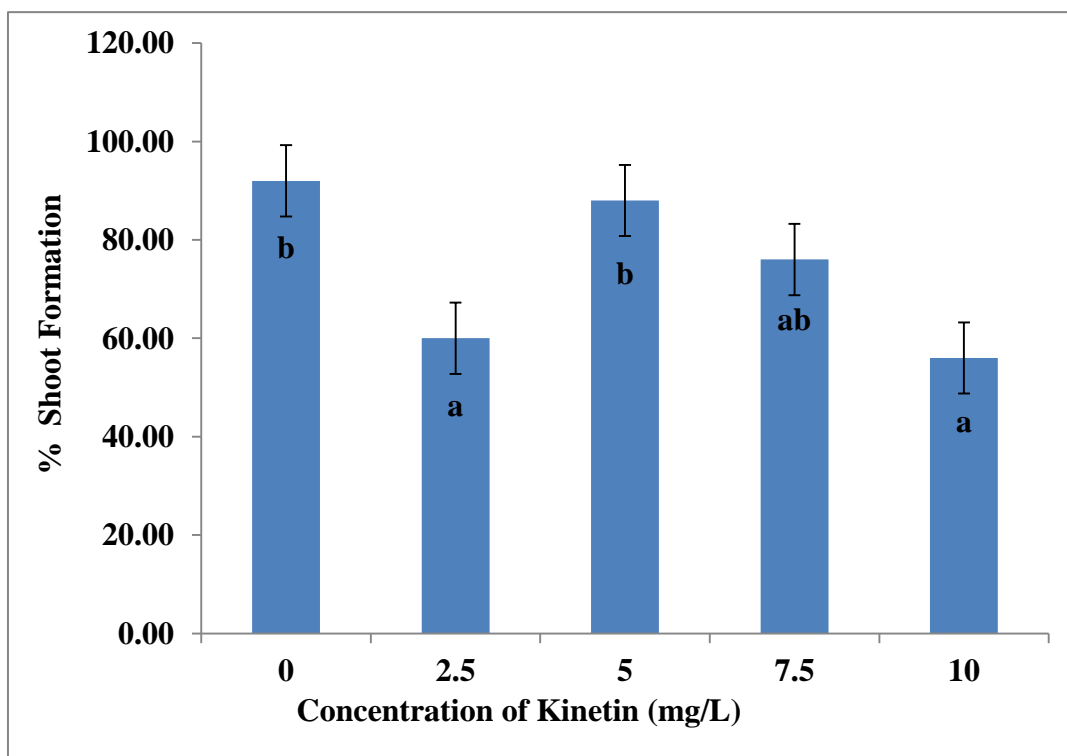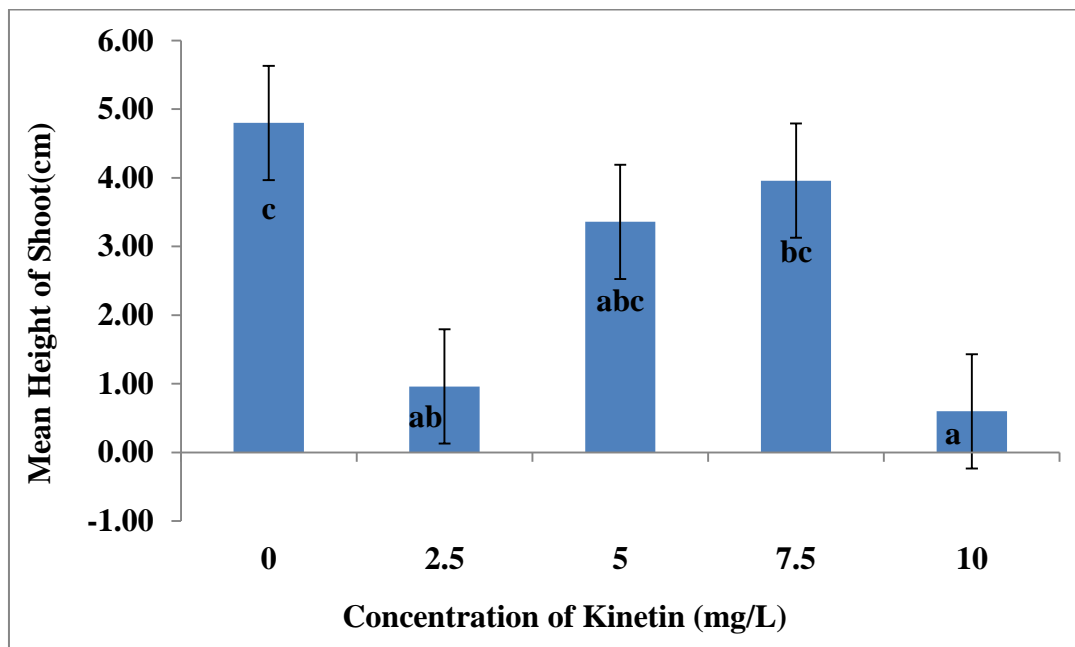

Figure 7. Effect of concentration kinetin on shoot development from nodal cutting explants. Means with the same letter are not significantly different ($P \geq 0.05$). The bars indicate standard errors of the means.

Similarly, the concentration of the kinetin in the culture medium significantly affected the growth of the developing shoot height (Fig. 6C). Again, nodal explants cultured on a medium without growth regulator developed shoots which grew taller (4.80cm)

than the kinetin treated explants (Fig. 8). Among the treated explants, the height of shoots increased from (0.96cm) on 2.5mg/L as the concentration of the kinetin increased to 3.36cm on a medium with 7.5mg/L, after which the height declined to 0.6cm on a medium with 10.0mg/L kinetin (Fig. 8).

Figure 8. Mean height of developed shoots of nodal explants cultured on MS basal medium supplemented with 0.0-10.0 mg/L Kinetin. Means with the same letter are not significantly different (P≥0.05). The bars indicate standard errors of the means.

The growth of the shoots led to a corresponding increase in the development of leaves. However, the number of leaves depended on the concentration of the kinetin in the culture medium. There was profuse leaf formation from most of the developing shoots of nodal explants of *H. brasiliensis* on the MS medium. The control treatment produced the highest mean number of leaves followed by 7.5mg/L kinetin (Fig. 9) while the other levels of kinetin concentration seemed to produce a few number of leaves. At the hormone-free treatment, the mean number of leaves (19.2) of the nodal explants was significantly higher than those of the remaining treatments (Fig. 9).
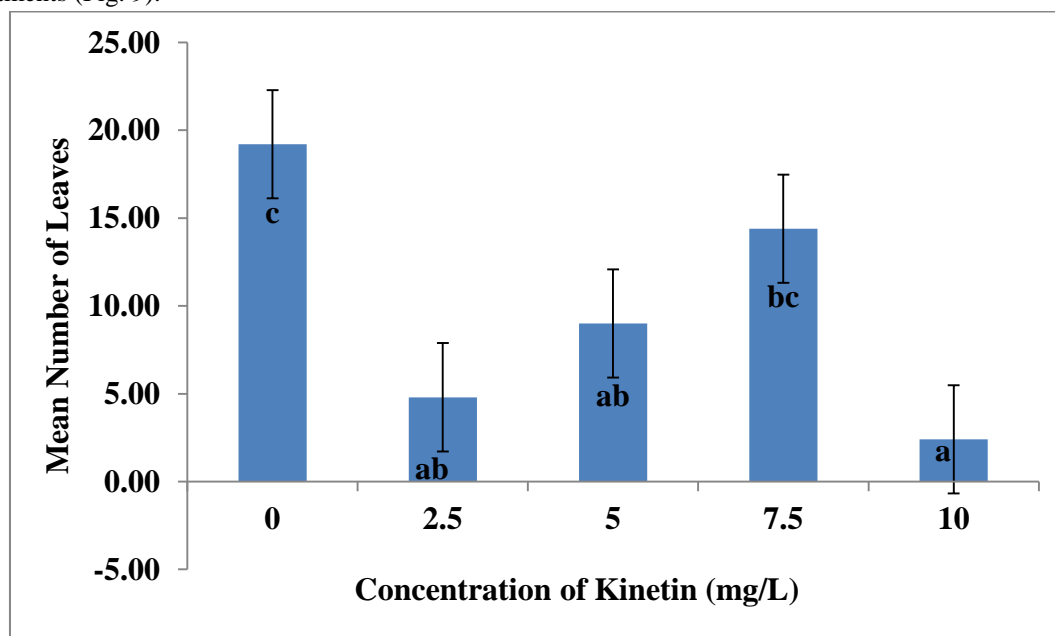
Figure 9. Mean number of leaves of developing shoots of nodal explants cultured on MS basal medium supplemented with 0.0-10.0 mg/L Kinetin. Means with the same letter are not significantly different (P≥0.05). The bars indicate standard errors of the means.

The treatments produced more than one shoot (Fig. 6C). The highest number (6.0) of shoots was produced by explants cultured on MS medium without growth regulator (Table 2). The number of shoots developed varied considerably among the treatments. The number of shoots produced on medium supplemented with 10.0mg/L kinetin was as low as ten times those produced on the hormone-free medium (Table 2). A significant difference (P<0.05) existed between the medium with 5.0mg/L, 7.5mg/L kinetin and 10.0mg/L in the mean number of shoots. It was seen that 5.0mg/L and 7.5mg/L kinetin had equal mean numbers of shoots, 3.0 whilst the other concentrations (2.5 and 10.0mg/L) had a decreased mean number of shoots (Table 2).

The shoots produced also developed roots. However, among the five different culture media studied, two of them (2.5mg/L and 10.0mg/L) did not enhance root formation while the remaining media enhanced multiple root development (Fig. 6D). The highest mean number (7.0) of plantlets with roots was achieved on the control treatments (Table 2). Shoots growing on a medium supplemented with 5.0mg/L and 7.5mg/L kinetin produced equal number (3.0) of roots (Table 2). Thus, there was a statistical difference (P<0.05) in the mean number of nodal plantlets with roots among all the treatments of the MS media (Table 2).

Table 2. Mean Number of Shoots and Plantlets with Roots of *Hevea brasiliensis* Nodal Explants Cultured on MS Basal Medium Supplemented with 0.0-10.0 Mg/L Kinetin.

Concentration of Kinetin (mg/L)	Mean Number of Shoots	Mean Number of Plantlets with Roots
0.0	**6.000±2.121[c]**	**7.000±2.739[b]**
2.5	1.800±1.643[ab]	0.000±0.000[a]
5.0	3.000±2.121[b]	3.000±2.739[a]
7.5	3.000±0.000[b]	3.000±4.472[a]
10.0	0.600±1.342[a]	0.000±0.000[a]

Means in the same column with the same letter superscript are not significantly different (P≥0.05)

4. DISCUSSION

Successful micropropagation of *Hevea brasiliensis* locally will tremendously lead to the propagation of this important economic plant species. Using this techniques, large numbers of planting materials will be produced for nurseries for the establishment of rubber plantations. The effect of concentration of kinetin on shoot development from shoot-tip and nodal explants was investigated. In this study, the presence of kinetin in the culture media led to successful *in vitro* propagation of *H. brasiliensis* from shoot-tip and nodal explants. The plantlets developed had multiple shoots with roots independent of the explant. However, the concentration of kinetin significantly affected plant development from both shoot-tip and nodal explants.

4.1. Effect of concentrations of Kinetin on shoot formation, leaf development and shoot elongation from shoot-tips and nodes of *Hevea brasiliensis*

In vitro propagation of plant species is influenced by genotype, age and source of donor tissues and exogenous application of growth regulators (17). In plant tissue culture, the activity of cytokinins oxidase is enhanced by exogenous application of cytokinins suggesting that treatment of explants with synthetic cytokinins could decrease the concentration of natural endogenous compounds (18). In this study, only low concentration of kinetin enhanced shoot development from both shoot tip and nodal culturing explants. In a similar study 0.5-5.0mg/L kinetin induced shoot proliferation. Also, there was shoot and root induction of sugar beet on MS medium with 0.5mg/L kinetin without auxin (19). There had been a significant increase in the average shoot length and the number of shoot per sugarcane explants with low concentrations of Kinetin (below 0.5mg/L) (20). Successful *in vitro* shoot regeneration of *Hevea* was achieved on MS medium amended with only cytokinins growth regulator. Also, there was 100% shoot regeneration from nodal explants of *H. brasiliensis* cultured on MS medium with only cytokinins (21).

The presence of kinetin in the regeneration medium influenced the development of the plantlets. Kinetin had a significant influence (P<0.05) in the response of shoot-tip explants to culture conditions and subsequent development of both shoots and leaves. This shows that cytokinins are responsible for the enhancement of cell division, organogenesis and adventitious shoot development in plant species (22-23). The low response of explants to shoot regeneration may be due to the effect of phenolic compounds from the explants. *In vitro* proliferation is negatively affected by secondary metabolites such as phenol which are secreted and oxidized in the culture medium (24).

Though auxins were not added to the culture medium, kinetin concentrations of 5.0mg/L significantly showed shoot elongation of 3.6cm, indicating acceleration of growth of the *H. brasiliensis* shoot-tip explants. This could be attributed to the addition of gibberellic acids to the MS medium which was meant to break dormancy. In jackfruit the highest increase in shoot length was achieved on medium with 3.0mg/L GA_3 (25).

4.2. The effect of contaminations on shoot regeneration of *H. brasiliensis* explants

The failure of some explants of *H. brasiliensis* to respond to culture could be due to contaminations from the phenolic compounds and latex of the explants as well as the laboratory environment irrespective of concentration of Kinetin. In this study*,* the rate of exudation of phenolic compounds was very high because some of the cultures turned brown and became contaminated despite the addition of activated charcoal into the culture medium. Phenolic compounds exuded during the growth of plant development cause shoot formation inhibition, necrosis, browning and the subsequent death of explants (26-28).

Additionally, the explants contain large quantities of latex in lactiferous tissues which are exuded from cut surfaces of explants which resulted in contamination of shoots. The phenolic substances on oxidation are converted into quinines, which cause tissue blackening and inhibit new *in vitro* morphogenetic responses in plants (25). The explant health and sterilize conditions are critical *in vitro* requisites for eliminating all contaminants to obtain high-frequency shoot regeneration (29). The contamination of the medium few weeks after culture with whitish exudates observed on the cut ends of some explants could be due to the presence of latex. There would have been over 90 percent of shoot regeneration of both shoot-tip and nodal explants of *H. brasiliensis*, if it had not been the high contamination effects. The limited success achieved in regeneration from explants could be due to the addition of activated charcoal in the culture medium. Activated charcoal tends to eliminate browning and thus enhances shoot regeneration and also serves as an absorbent of many inhibitors, especially oxidized polyphenols which are exuded into the culture medium by woody tissues (26, 30).

4.3. Mean number of shoots and plantlets with roots of *Hevea brasiliensis* shoot-tip and nodal explants

An interesting observation was the development of multiple shoots from the explants. The development of multiple shoots has the potential to speed up regeneration of *H. brasiliensis* for plantation establishment. Phytotoxic effects of cytokinins on shoot regeneration and on growth of some plants (31) could have had negative effects on shoot height, number of leaves and even number of shoots at higher kinetin concentrations (7.5mg/L and 10.0mg/L). For instance, increase in kinetin concentration (above 1.5mg/L) significantly reduced shoot regeneration, number of shoot per explants and average shoot length of sugarcane shoot-tip cultures (20). The addition of silver nitrate in the culture medium could be an influencing factor for the multiple developments of shoots per shoot-tip and nodal explants. Addition of 1.0mg/L $AgNO_3$ to MS medium produced excess of 5.0 shoots per explants in *Hevea brasiliensis* cultures (21). Silver nitrate has been proven effective in improving plantlet regeneration, not only in *H. brasiliensis* but also in a number of crop species such as cassava, achiote and turnip (32-34). Silver nitrate in plant tissue culture inhibits ethylene synthesis (32) which often builds up in culture vessels thereby inhibiting shoot development. For instance, multiple shoot from rubber shoot-tips cultured was obtained on MS medium with 5.0mg/L BA (35).

Shoot regeneration was associated with root formation in both nodal and shoot-tip explants. However, root formation was significantly higher in the controls of both explants than kinetin amended medium. The high root formation on media free from growth regulators could be due to the presence of endogenous auxins found in the young shoots. Also, shoot-tips are known to contain high concentrations of endogenous auxin which may even inhibit virus multiplication (36). The number of plantlets with roots reduced as the concentrations of kinetin increased in the MS culture medium. Concentration of cytokinins between 0.5 and 10mg/L generally inhibit or delay root formation (37) and also prevent the promotive effects of auxins on root initiation and subsequent root growth (38).

The ability of the explants to root under the influence of cytokinins leading to successful regeneration of plantlets *in vitro* can be used to solve the problem of intra clonal heterogeneity associated with grafting thereby raising large number of plantlets for plantation development of this economically important tree crop.

5. CONCLUSION

Successful *in vitro* regeneration of plantlets was achieved using shoot-tip and nodal cutting explants cultured on an MS medium supplemented with kinetin. Lower concentrations of kinetin (0.5 to 5.0mg/L) enhanced shoot development from both shoot-tip and nodal cutting explants while as higher concentrations were phytotoxic to explant tissues. Regenerated shoots developed adventitious roots especially on a medium without kinetin amendment whilst the growth regulator significantly decreased root

development. Successful *in vitro* plantlets regeneration using shoot-tip and nodal explants will enhance nursery establishment of this economically important *H. brasiliensis* tree for plantation and reforestation programmes.

ACKNOWLEDGMENTS

REFERENCE

[1]. Hua, Y., Huang, T., and Huang, H. Micropropagation of self-rooting juvenile clones by secondary somatic embryogenesis in *Hevea brasiliensis*. *Plant Breeding,* 2009, vol. 129, pp.202-207.

[2]. Seneviratne, P. tissue culture for rubber. *Bulletin of the Rubber Research Institute of Sri Lanka,* 1996, vol. 34, pp.26-31.

[3]. Combe, J. Demonstration of intraclonal variability in young graft trees. *Revue Generale des Caouth Plast.,* 1975, vol. 52, pp.91-94.

[4]. Senanayake, Y., and Wijewantha, T. Synthesis of *Hevea* Cultivars:A New Approach. *Journal of Rubber research Institute of Ceylon,* 1968, vol. 44, pp.16-24.

[5]. Montoro, P., Carron, M.-P., Lardet, L., Clément-Demange, A., and Leclercq, J. Biotechnologies in rubber tree (*Hevea brasiliensis*). *AsPac Journal Molecular Biology and Biotechnology*, 2010, vol. 18 (1), pp.81-83.

[6]. Nayanakantha, N., and Seneviratne, P. Review tissue culture of rubber: past, present and future prospects. *Ceylon. Journal Science (Biological Science)*, 2007, vol. 36(2), pp.116-125.

[7]. Duan, C. Etude de l'interaction entre l'éthylène et le jasmonate, hormones impliquées dans la production de caoutchouc naturel chez *Hevea brasiliensis*. *These de Doctorat. Biologie Integrative des Plantes*, 2011.

[8]. Venkatachalam, P., Jayasree, K., Sushmakumari, S., Jayasree, R., Rekha, K., Sobha, S., Priya, P., Kala, R. G. and Thulaseedharan, A. Current perspectives on application of biotechnology to assist the genetic improvement of rubber tree (*Hevea brasiliensis* Muell. Arg.). *An Overview. Functional Plant Science and Biotechnology*, 2007, vol. 1(1), 1-17.

[9]. Thulaseedharan, A., Kumari Jayasree, P., and Venkatachalam, P. Biotechnological approaches for crop improvement in rubber. In: Nayanakantha, N. M. C. and Seneviratne, P. (2007). Tissue culture of rubber: past, present and future prospects, 2000, pp.116-125.

[10]. Mendanha, A. B., Torres, R. A., and Freire, A. d. Micropropagation of rubber trees (*Hevea brasiliensis* Muell. Arg.). *Genetic and Molecular Biology*, 1998, vol. 21(3).

[11]. Paranjothy, K., and Gandimathi, H. tissue and organ culture of *Hevea. Proceedings of international Rubber Conference, Kuala Lumpur, Malaysia*, 1976, vol. 59-84.

[12]. Enjalric, F., and Carron, M. Microbouturage *in vitro* de jeunes plants d'*Hevea brasiliensis* (Kunth) Müll. Arg. *C. R. Acad. Sci. Paris.Série,* 1982, vol. 295 (3)*:* 259-264.

[13]. Seneviratne, P. Micropropagation of juvenile and mature *Hevea brasiliensis*. PhD. Thesis, University of Bath, UK. 1991, pp.48-167. In: review tissue culture of rubber: past, present and future prospects. Nayanakantha, N.M.C. and Seneviratne, P (2007).

[14]. Trigiano, R. N., Geneve, R. L., Merkle, S. A., and Preece, J. E. Tissue and cell culture of woody legumes. *Horticulture Review*, 1992, vol. 14, pp.265-332. In: Minocha, R. and Jain, S. M. Tissue culture of woody plants and its relevance to molecular biology.

[15]. Delarue, J. Developing smallholder rubber production, evaluation and capitalisation unit. *Agence Francaise de Developpement (AFD), France*, 2009.

[16]. Murashige, T., and Skoog, F. A revised medium for rapid growth and bio-assays with tobacco tissue culture. *Physiologia Plantarum*, 1962, vol. 15, pp.473-497.

[17]. George, E. F. Plant propagation by tissue culture. Part I: *The Technology Exegetics Ltd., England,* 1993, pp.23-98

[18]. Motyka, V., and Kaminek, M. Regulation of cytokinin catabolism in tobacco callus cultures, 1990, pp.492-497.

[19]. Konwar, B., and Coutts, R. Rapid regeneration of sugar beet (*Beta vulgaris* L.) plants from *in vitro* cultures. Pp.114-118 in Nijkamp et al. (eds.), 1990, pp.114-118.

[20]. Tolera, B.; Diro, M. and Belew, D. *In vitro* aseptic culture establishment of sugarcane (*Saccharum officinarum L.*) varieties using shoot tip explants. *Advance Crop Science Technology,* 2014, vol. 2, pp.128. doi:10.4172/2329-8863.1000128

[21]. Sirisom, Y., and Te-chato, S. Assessment of somaclonal variations of *in vitro*-plants derived from nodal culture of rubber trees by SSR markers. *Songklanakarian Journal of Plant Science*, 2014, vol. 1(2), pp.7-12.

[22]. George, E. F.; Machakova, I. and Zazimalova, E. Plant propagation by tissue culture. Third Edition, 2008, Pp: 175-205.

[23]. Schmulling, T. New insights into the functions of cytokinins in plant development. *Journal of Plant Growth Regulation*, 2002, pp.40-49.

[24]. Ozyigit, I. K. Phenolic changes during *in vitro* organogenesis of cotton (*Gossypium hirsutum* L.) shoot tips. *African Journal Biotechnology*, 2008, vol. 7(8), pp.1145-1150.

[25]. Hard, M. E.; Alhady, M. R. A. A.; Elsalam, N. A. A. *In vitro* rapid propagation of Jackfruit (*ARtcarpus heterophyllus Lam*). *American-Eurasian Journal Agriculture and Environmental Science*, 2015, vol. 15(2), pp.147-153.

[26]. Moradpour, M., Aziz, M. A. and Abdullah, S. N. A. Establishment of *in vitro* culture of rubber (*Hevea brasiliensis*) from field-derived explants: effective role of silver nanoparticles in reducing contamination and browning. *Journal Nanomed Nanotechnology*, 2016, vol. 7, pp.375. doi:10.4172/2157-7439.1000375

[27]. Abdelwahd, R., Najat, H., Mustapha, L., and Sripada, M. Use of an absorbent and antioxidants to reduce the effects of leached phenolics in *in vitro* plantlet regeneration of faba bean. *African journal of biotechnology*, 2008, vol. 7(8), pp.997-1002.

[28]. Arnaldos, T., Munoz, R., Ferrer, M., and Calderon, A. Changes in phenol content during strawberry (*Fragaria xananasa*, cv. Chandler) callus culture. *Physiologia Plantarum,* 2001, vol. 113, pp.315-322.

[29]. Yildiz, M. The prerequisite of the success in plant tissue culture: high frequency shoot regeneration. *INTECH, World's largest Science, Technology & Medicine Open Access book publisher*, 2012, Pp.64-90.

[30]. Monnier, M. Induction of embryogenesis in callus culture. Methods in molecular biology. *Plant cell and tissue culture,* 1990, vol. 6, pp.141-148.

[31]. Kalidass, C., and Mohan, V. *In vitro* rapid clonal propagation of *Phyllanthus urinaria* L. (*Euphorbiaceae*): A medicinal plant. *Researcher,* 2009, vol. 1(14), pp.56-61.

[32]. Zhang, P., Phansiri, S. and Puonti, K. Improvements of cassava shoot organogenesis by the use of silver nitrate *in vitro*. *Plant Cell, Tissue and Organ Culture,* 2001, vol. 67, pp.47-54.

[33]. Parimalan, R., Giridhar, P., and Ravishankar, G. Enhanced shoot organogenesis in *Bixa orellana* L. in the presence of putrescine and silver nitrate. *Plant Cell, Tissue and Organ Culture,* 2010, vol. 105, pp.285-290.

[34]. Cogbill, S., Faulcon, T., Jones, G., McDaniel, M., Harmon, G., Blackmon, R., Young, M. Adventitious shoot regeneration from cotyledonary explants of rapid-cycling fast plants of *Brassica rapa* L. *Plant Cell, Tissue and Organ Culture,* 2010, vol. 101, pp.127-133.

[35]. Sirisom, Y., and Te-chato, S. The effect of peptone and silver nitrate on *In vitro* shoot formation in *Hevea brasiliensis* Muell Arg. *Journal of Agricultural Technology,* 2012, vol. 8(4), 1509-1516.

[36]. Murashige, T. Plant propagation through tissue cultures. *Annual review of plant physiology*, 1974.

[37]. Ben-Jaacov, J., Ackerman, A., Tal, E., and Jacobs, G. Vegetative propagation of *Alberta magna* by tissue culture and grafting. *Horticulture Science*, 1991, vol. 26, pp.74.

[38]. Humphries, E. Kinetin inhibited root formation on leaf petioles of detached leaves of *Phaseolus vulgaris* (dwarf bean). *Physiology Plant,* 1960, vol. 13, pp.659-663.