PROPAGATION AND MOLECULAR CHARACTERISATION OF RUBBER TREE (*Hevea brasiliensis* Muell. Arg.) IN GHANA

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By

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DECLARATION

This thesis is the result of research work undertaken by Anthony Antwi-Wiredu in the Department of Nuclear Agriculture and Radiation Processing of the School of Nuclear and Allied Sciences, University of Ghana, under the supervision of Prof. George Y. P. Klu and Dr. Samuel Amiteye.

Signed

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Dr. Samuel Amiteye (Supervisor)
DEDICATION

This work is dedicated to my God; The Father Almighty, Jesus Christ and The Holy Spirit and to my incredible adoptive and godfather, Elder Charles Kofi Hayford.
ACKNOWLEDGEMENT

My profound gratitude goes to my Only True and Holy God of Trinity for gracing me with divine knowledge, wisdom and power to soar another ladder in my educational hierarchy.

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<tr>
<td>AFLP</td>
<td>Amplified Fragment Length Polymorphism</td>
</tr>
<tr>
<td>AP-PCR</td>
<td>Arbitrary Primed Polymerase Chain Reaction</td>
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<tr>
<td>BAP</td>
<td>6-benzylaminopurine</td>
</tr>
<tr>
<td>BNARI</td>
<td>Biotechnology and Nuclear Agricultural Research Institute</td>
</tr>
<tr>
<td>CAPS</td>
<td>Cleaved Amplified Polymorphic Sequences</td>
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<tr>
<td>cDNA</td>
<td>Complementary Deoxynucleotide Acid</td>
</tr>
<tr>
<td>CISP</td>
<td>Conserved Intron Scanning Primer</td>
</tr>
<tr>
<td>CTAB</td>
<td>Cetyltrimethyl Ammonium Bromide</td>
</tr>
<tr>
<td>2, 4-D</td>
<td>2, 4-Dichlorophenoxyacetic Acid</td>
</tr>
<tr>
<td>DAF</td>
<td>DNA amplification fingerprinting</td>
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<tr>
<td>DAP</td>
<td>Days after Planting/Propagation</td>
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<tr>
<td>dATP</td>
<td>Deoxyadenosine Triphosphate</td>
</tr>
<tr>
<td>dCTP</td>
<td>Deoxycytidine Triphosphate</td>
</tr>
<tr>
<td>dGTP</td>
<td>Deoxyguanosine Triphosphate</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>dNTPs</td>
<td>Deoxynucleotide Triphosphates</td>
</tr>
<tr>
<td>dTTP</td>
<td>Deoxythymidine Triphosphate</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
</tr>
<tr>
<td>FAO</td>
<td>Food and Agriculture Organisation</td>
</tr>
<tr>
<td>GA$_3$</td>
<td>Gibberellic Acid</td>
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<tr>
<td>GAEC</td>
<td>Ghana Atomic Energy Commission</td>
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<tr>
<td>IAA</td>
<td>Indole-3-Acetic Acid</td>
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<tr>
<td>IAEA</td>
<td>International Atomic Energy Agency</td>
</tr>
<tr>
<td>IBA</td>
<td>Indolebutyric Acid</td>
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<tr>
<td>Kin</td>
<td>6-furfurylaminopurine</td>
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<td>MAS</td>
<td>Marker Assisted Selection</td>
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<td>NAA</td>
<td>α-Naphthalene Acetic Acid</td>
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<td>Polyacrylamide Gel Electrophoresis</td>
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<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<td>PSG</td>
<td>Plantation Socfinaf Ghana Limited</td>
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<td>QTL</td>
<td>Quantitative Trait Loci</td>
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<td>RAPDs</td>
<td>Randomly Amplified Polymorphic DNA</td>
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<td>RFLP</td>
<td>Restriction Fragment Length Polymorphism</td>
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<td>SALB</td>
<td>South American Leaf Blight</td>
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<td>SCARs</td>
<td>Sequence Characterized Regions</td>
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<td>SNPs</td>
<td>Single Nucleotide Polymorphisms</td>
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<td>SRAPs</td>
<td>Sequence Related Amplified Polymorphisms</td>
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<td>SSR</td>
<td>Simple Sequence Repeats</td>
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<td>STS</td>
<td>Sequence Tagged Sites</td>
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<td>TRAP</td>
<td>Targeted Region Amplification Polymorphism</td>
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ABSTRACT

The study was aimed at the propagation and molecular characterisation of some introduced clones of *Hevea brasiliensis* in Ghana. Propagation of *H. brasiliensis* by stem cuttings and *in vitro* techniques was used to study alternative procedures for mass production of rubber planting materials. Brown and green stem cuttings of *Clone I* and *Clone II* were soaked for 6 hours in 0.0-22.5g/L NAA followed by propagation in a nursery bag filled with nutrient-rich soil. Only the brown stem cuttings of *H. brasiliensis* survived. The % survival, length of shoots, number of roots as well as length of roots of *Clone II* was significantly (P<0.05) higher than *Clone I*. Stem cuttings treated with 15.0g/L NAA significantly (P<0.05) developed higher shoots (83.33%), number of roots (6.167), length of shoots (15.38cm) and length of roots (6.00cm) than the remaining treatments. There was significant (P<0.05) effects of NAA and *Clone II* in sprouting and rooting growth of the brown stem cuttings. The addition of 5.0mg/L kinetin in the MS culture medium 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 cultured on a medium without kinetin developed higher shoots (94.00%), 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. However, 7.5mg/L kinetin of the nodal culture also performed significantly after the controls. A dendrogram derived from the UPGMA distinguished the rubber clones from four areas of Ghana into two clusters. The five SSR markers showed high degree of relatedness among the rubber clones which suggested high genetic similarity (IRCA317-5, IRCA41-2, IRCA331-6, IRCA230-4, IRCA109-3, B8-23) and some degree of diversity/variation (K2-18,
IRCA840-7, PB217-8, PB217-10, IRCA317-16) among the clones. Thus, clones of interest could be selected for future breeding and propagation programmes. Successful in vivo and in vitro propagation as well as molecular characterisation (genetic similarity and diversity) of rubber tree (Hevea brasiliensis) in Ghana were achieved.
CHAPTER ONE

1.0. INTRODUCTION

Rubber tree, *Hevea brasiliensis* (Wild) Muell.-Arg., is a member of the family *Euphorbiaceae* and a major economic tree crop because of its timber and latex. The tree, also called Para rubber, produces latex, which contains cis-1, 4-polyisoprene, an industrial raw material of high commercial importance producing about 99% of the world’s natural rubber (Vinod, 2002). There are eleven species of the genus, *Hevea* among which *H. brasiliensis* is the only species commercially exploited for natural rubber (Pires et al., 2002).

The genus, *Hevea* originated from the Amazon River basin in South America including Brazil, Venezuela, Ecuador, Surinam, Guyana, Colombia, Peru and Bolivia (Vinod, 2002). The main rubber production zone worldwide is, however, concentrated between 15°N and S. for South East Asia and the South Pacific. There are prominent plantations also in Central and West Africa (Congo, Cameroon, Cote d’Ivoire, Ghana, Nigeria, and Liberia) while rubber production in tropical America is concentrated over a small area between 10°N and S. (Verheye, 2010).

The importance of rubber tree to producing countries can be categorized into three broad areas namely, natural rubber (latex), rubber wood (timber) and agroforestry establishment. Natural rubber which coagulated latex- is used in the manufacture of some indispensable industrial products such as tyres, balls, containers, shoes and bands (Edna,
2000). In developing countries, the rubber tree has become an important developmental fulcrum as well as a good venture in combating soil erosion and deforestation. In consequence, the production of rubber trees reduces the pressure of human on natural forests (Diaby et al., 2011).

Currently, two main propagation methods are employed in ensuring the production of *Hevea* just as any other crop. These are:

- conventional propagation via cuttings, budding/grafting, seedlings from seeds;
- tissue culture techniques such as shoot-tip, axillary bud, embryo culture and somatic embryogenesis.

Stem cutting is an asexual means of propagation which preserves the genetic traits and transfer of superior and genetically similar traits of parent plants to progenies. Cuttings are also used to propagate plants that do not produce viable seeds, seeds which have germination difficulty and high recalcitrant nature. Vegetative propagation via cuttings in tree species which have a longer flowering and fruiting time can help to bypass the juvenile stage of plant growth. The use of rubber cuttings as planting material is a feasible option (Nayanakantha and Seneviratne, 2007), worthy of investigation.

It has been reported that due to the increase in demand for rubber products, there is the need for the *in vitro* technique to be used in the mass propagation of rubber since it produces disease-free plantlets and can be carried to different places in large quantities easily (Ighere et al., 2011). *In vitro* propagation of tree species does not only lead to rapid
multiplication of unique genotypes but also provides genetically uniform plantlets. The guarantee for the exchange of tissue cultured germplasm between countries is fully ensured with less stringent phyto-sanitary measures when an in vitro technique is used. In *Hevea*, increased growth and vigour have already been reported for plants regenerated through tissue culture (Carron *et al*., 2000).

Just like other perennial crops, conventional breeding of *Hevea* has been slow and time consuming, the major hindrances being its very narrow genetic base, long gestation time, heterozygous nature, non-synchronous flowering, low fruit set, lack of fully reliable early selection parameters and inadequate land availability for field experiments (Venkatachalam *et al*., 2007). Marker-assisted selection (MAS) is used to ease and quicken the selection process of plant traits and to determine the genetic profile of a clone or variety (ISAAA, 2014). Molecular markers help in providing better understanding of genetics of *Hevea brasiliensis* and also play prominent roles in the clonal identification of *Hevea* (Sirisom and Te-chato, 2014). Additionally, advance genetic techniques such as RFLP, AFLP, RAPDs and SSRs have been used to quantify the genetic variability/diversity in the *Hevea* species (Vinod, 2002).

Microsatellite markers have become the marker system of choice owing to merits as low quantities of template DNA required (10-100ng), high genomic abundance, random distribution throughout the genome and high levels of polymorphism. It allows for band profiles to be interpreted in terms of loci, alleles and co-dominance inheritance. It also allows for alleles to be determined with an accuracy of 1bp, allows accurate comparison
across different gels as well as high reproducibility. Different SSRs could be multiplexed in PCR or on gel, allow for a wide range of applications and are amenable to automation and multi-allelic and hyper variability (Singh et al., 2014). In Ghana, genetic studies using molecular markers to diagnose, quantify and characterize genetic variations/diversities in rubber clones have received meagre attention, thereby hindering rubber tree propagation and breeding.

The ability to successfully regenerate plantlets from Hevea either through tissue culture or in vivo locally would go a long way in rubber improvement and not only of rubber trees but also stimulate interest in attempts at in vitro or in vivo propagation of some tropical woody species in the country. All the aforementioned constraints encountered by the rubber breeding industry in the country would be counteracted if at the end of the experiments, efficient in vitro and in vivo protocols are established and genetic diversity (distances) among the various rubber clones are determined using molecular markers.

Upon all the benefits that biotechnology brings into rubber production: in vitro techniques, stem cutting propagation and molecular systems 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 (Delarue, 2009).

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 the country. *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. This 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 (Seneviratne, 1996).

Furthermore, uniform growth and yield of rubber trees are not realized even under best managerial practices when the grafting method is used (Combe, 1975). This shortfall in yield may be due to many reasons, among which soil heterogeneity may be one, but much of it could be due to the heterogeneous rootstocks (Senanayake and Wijewantha, 1968). It has also been deduced that some trees observed by selection, produced yield of 15-20kg of dry rubber per tree yearly while the grafted clones produce only an average of about 4-6kg/tree/year. The difference represents a potential yield increase by more than 200%, which thus demands the assistance of a better vegetative propagation method (Seveviratne, 1996). Thus, micropropagation of elite clones with their own root system
could reduce intra-clonal variation due to stock-scion interaction (Nayanakantha and Seneviratne, 2007).

There would be quite significant contribution of knowledge on methods of propagation and diversity studies on the rubber tree. Huge sums of foreign exchange needed to import rubber planting materials into the country can be avoided. Apart from these benefits, the creation of employment and the good use of indigenous biotechnological knowledge in the genetic improvement and propagation of the rubber tree would be realized in the country. There would be the production of vigorous and high yielding clones to increase productivity at an affordable price to local farmers.

Consequently, it has become necessary to use tissue culture and stem cutting techniques to produce rubber planting materials to supply these companies with clones to either grow directly or graft unto locally generated rootstocks and to genetically analyse the diversity/variation among some introduced rubber clones in Ghana. The outcome of this work will go a long way to resolve the problem associated with the shortage of rubber planting materials usually in the off-season and lead to the large-scale production of rubber propagules affordable to rubber outgrowers. Thus, this study is aimed at propagation and genetic diversity studies of some introduced clones of Para rubber (Hevea brasiliensis) in Ghana. Specific objectives are to;

- vegetatively propagate Hevea brasiliensis using stem cuttings
- micropropagate Hevea brasiliensis using shoot-tip and nodal culture
- determine the genetic diversity/similarity among some introduced Hevea clones in Ghana using SSR marker systems.
CHAPTER TWO

2.0. LITERATURE REVIEW

2.1. Origin and Distribution of *Hevea brasiliensis*

Genus of *Hevea* species occurs in the wild in an area which covers the whole of the Amazon basin and extends southwards into the foothills of the Matto Grosso region of Brazil and northwards into the upper part of the Orinoco basin, the lower slopes of the Guiana Highlands and parts of the lowlands of the Guianas (Webster and Paardekooper, 1989). Rubber tree is a native plant to the Amazon region (7°N and 15°S) covering areas from Brazil, Bolivia, Peru, Colombia, Ecuador, Venezuela, French Guiana, Surinam and Guyana (Silvio and Julio, 2007; Vinod, 2002).

![Distribution of rubber tree (H. brasiliensis) in the world where this species has been planted (adapted from Orwa et al., 2009).](image)

**NATIVE RANGE:** Bolivia, Brazil, Colombia, Peru and Venezuela; **EXOTIC RANGE:** Brunei, Cambodia, China, Ethiopia, India, Indonesia, Laos, Liberia, Malaysia, Myanmar, Philippines, Singapore, Sri Lanka, Thailand, Uganda, Vietnam, Guatemala, Cameroon, Ivory Coast, Ghana, Guinea, Liberia, Nigeria, Congo, Bangladesh, Papua New Guinea, and Mexico.
2.1.2. Hevea History and Distribution

*Hevea* was introduced to Africa early in the twentieth century in Uganda and Nigeria in 1903, Congo in 1904 and Liberia in 1924 by the Firestone Tyre and Rubber Company. In Africa rubber trees are grown mainly in the forest regions of Congo, Cameroon, Cote d’Ivoire, Ghana, Nigeria, Mali, Central African Republic and Liberia while rubber production in tropical America is concentrated over a small area between 10°N and S (Verheye, 2010; FAO, 1977). Natural rubber is produced in south-east Asia (92%), in Africa (6%), and in Latin America (2%). Mainly rubber producing countries are Thailand, Indonesia, Vietnam, India and Malaysia, China and also Côte d’Ivoire, Liberia, Sri-Lanka, Brazil, Philippines, Cameroon, Nigeria, Cambodia, Guatemala, Myanmar, Ghana, D.R. of Congo, Gabon and Papua New Guinea (Venkatachalam *et al.*, 2013). Thailand (34%) produces the highest quantity of natural rubber in the world, followed by Indonesia (25%) with three African countries; Liberia (1%), Cameroun (1%) and Nigeria (0%) producing the least amount of rubber (Owusu, 2014).

As of 2013 West Africa was accounting for only 4% of the world’s natural rubber production. Out of that, Côte d’Ivoire (56%) produced the largest amount of natural rubber followed by Liberia (15%), Cameroun (11%) and Nigeria (10%) whilst the remaining 8% comprising rubber producing countries with low natural rubber production including Ghana (SIPH, 2013).
2.1.3. Production and Development of Rubber industry in Ghana

The rubber sector grows but only marginally in Ghana. For instance, rubber production increased by 28.1% between 2000 and 2006 (MoFA, 2010). Rubber production area has been expanding steadily since the 1980s. Production of rubber has increased from 9,300 metric tons in 2000 to 19,134 metric tons in 2009 recording an increase of 74% over the period. The Ghana rubber master plan estimates production of natural rubber to reach 52,000 metric tons by 2020 and finally to 70,000 metric tons by 2030 (MoFA, 2012).

Ecological areas favourable in Ghana for rubber production include forest zones of Western, Central, Eastern and Ashanti regions characterized by minimum rainfall of 1,200 millimetres per annum, evenly distributed on lower slopes, uplands and flatlands. Besides cocoa, oil palm and coconut, rubber production now stands out as one of the most profitable farming activities, even though it takes a long time, about six years to mature. Areas with coconut trees affected by the Lethal Yellowing Disease (LYD) are being used for the development of rubber plantations in the Western Region. Africa produces less than six percent of the world’s natural rubber. Ghana produces about 15,000 tonnes of natural rubber per annum and could reach 50,000 tonnes by the year 2020 (Marfo, 2009).

Chronology of rubber tree introduction and production in Ghana, Ghana Rubber Estate Limited, GREL (2013):
*Hevea* was introduced into Ghana at Aburi botanic garden, near Accra in 1898. Way back in the 1930’s, the United African Company (UAC) carried out tests on a small scale in the Western Region. In 1957 the East Asiatic Company, a Danish company, proceeded with rubber plantation at Dixcove covering a land of 923 hectares (ha) which was then nationalized in 1960 and became a state farm in 1962. The Ghana Government produced 3,500 hectares (20ha on the average per unit) cooperative rubber plantations in 1960. Thereafter in 1968 the Government of Ghana signed a joint venture with Firestone to build a tyre plant at Bonsaso and thus developed the Ghana Rubber Estate Limited (GREL) plantations.

Firestone in 1981 left out of the joint venture with Ghana Government, therefore GREL became a state farm. The first rehabilitation phase of GREL (3,000 ha and infrastructure) was performed by SODECI between 1988 and 1990. In 1991, another rehabilitation phase (6,175 ha old plantations rehabilitated; 2,700 ha extension and construction of a new processing plant at Agona) was carried out again by SODECI. GREL took back the purchase of rubber from the cooperatives in 1992. The launch of the first programme of outgrower project by GREL was done in 1995.

### 2.2. Taxonomy of *Hevea brasiliensis*

Natural rubber is synthesized in over 2500 plant species confined to 300 genera of seven families namely, *Euphorbiaceae, Apocynaceae, Asclepiadaceae, Asteraceae, Moraceae, Papaveraceae* and *Sapotaceae* (Venkatachalam *et al.*, 2013). *Hevea brasiliensis* is a
deciduous perennial dicotyledonous tree species which is highly cross pollinated and exhibits monoecy (Vinod, 2002). *Hevea brasiliensis* belongs to the family *Euphorbiaceae*. Other members of the family are *Croton tiglium*, *Aleurites moluccana*, *Aleurites fordii*, *Sapium sebiferum*, *Sapium biloculare*, *Sebastiana pavoniana*, *Euphorbia pulcherrima*, *Hippomane mancinella*. Others include; *Hura crepitans*, *Cnidoscolus angustidens*, *Euphorbia antisyphilitica* and *Manihot glaziovii* (Ceara rubber tree i.e. lesser known new world source of rubber latex); *Manihot esculenta* (Cassava) and *Ricinus communis* (Castor Bean) (Armstrong, 2008).

The genus *Hevea* encompasses species of which all are natives of the Amazon region and are strongly outcrossing and monoecious (Schultes, 1990). The species of the genus *Hevea* are *H. benthamiana*, *H. camporum*, *H. camargoana*, *H. guianensis*, *H. microphylla*, *H. nitida*, *H. pauciflora*, *H. rigidifolia*, *H. spruceana* and *H. brasiliensis*. Genetically, *Hevea brasiliensis* has a chromosome number of 2n = 36. All of these species possess latex in their plant parts but *H. brasiliensis* is the only species commercially exploited for natural rubber-sap-like extract (Feng et al., 2012).

### 2.2.1. Botany and Development of *H. brasiliensis* Plant

*Hevea* species are intercrossable and hence a wide variability of the interspecific offspring exists in the primary centre of diversity. Morphologically, *H. brasiliensis* have trifoliate leaves with long petioles bearing nectaries which grow into pinnately veined leaflets, which are folded back on emergence but subsequently, assume various positions from reclinate through horizontal to nearly erect (Webster and Paardekooper, 1989).
Separate male and female flowers are borne in the same inflorescences with the females at the ends of the main branches of the panicles. Flowers of both sexes have a 5-lobed calyx borne on a basal disc of 5 free or united glands, but no petals. Male flowers have 5-10 stamens with their filaments united into a column and their anthers sessile. The ovary is 3-celled with one ovule per cell. The fruit is a trilocular capsule, usually containing 3 seeds which in all species except *H. spruceana* and *H. microphylla*, dehisces explosively to scatter the seeds (Webster and Paardekooper, 1989).

Flowers are monoecious and are found in raceme inflorescences (Silvio and Julio, 2007). The female flowers are bold and situated on the tip of the inflorescences whilst the male flowers are in numerous numbers beneath them. Moreover, the rubber tree starts to flower 3-4 years after planting with an economic life span of 25-30 years (Vinod, 2002).

The mature fruit is a dehiscent capsule usually containing three oil seeds. *H. brasiliensis* seeds possess a brilliant spotted coat showing a prominent caruncle and globate in shape (Silvio and Julio, 2007). Seeds are oval in shape, 1-2cm long and weigh between 3 and 6g and are viable for a short time. Viable seeds germinate within 3-25 days in a hypogeal form (Verheye, 2010). The primary tap-root is 5.0-10.0 m long and lateral roots usually reach 6.0 to 9.0 m in lateral growth (Silvio and Julio, 2007). The tap-root can penetrate 4 to 5m deep in the soil (Verheye, 2010).

Rubber tree has a conical or cylindrical stem/trunk in shape with alternate and trifoliate leaves (Silvio and Julio, 2007). Young rubber leaves are dark red in colour whilst older
leaves are green on top and greyish-green underneath with a diameter of a fully grown leaf ranging between 15 and 20cm (Verheye, 2010).

### 2.3. Plant Propagation

Propagation of plants is the art and science of the multiplication of plants by both sexual and asexual means. Sexual propagation is the production of new plants by seed. Seeds are used in large-scale agriculture and forestry operations for growing tree seedlings for reforestation projects. Sexual propagation is the cheapest and easiest method of propagation known. It requires the fewest skills with no specialized equipment or facilities; and also can be stored for several years without loss of viability. Additionally, seeds are cheaply and easily shipped or transported and if properly cleaned and stored, seeds are less likely to carry diseases. In spite of these advantages, seedlings produced are liable to be genetically different from the parent plants and may not have the same desirable characteristics due to outcrossing. Also, some plants do not produce viable seeds and may also have long propagation cycle or take a long time to grow into mature plants (Enslin, 2006).

Contrarily, asexual or vegetative propagation is the production of plants using the vegetative parts of a plant. The vegetative parts may be stems, leaves, roots, bulbs, corms, tubers, tuberous roots, rhizomes, and undifferentiated tissue often used in micropropagation. Propagation by cuttings, layering and grafting are all forms of asexual propagation. When compared to sexual methods, asexual methods have certain advantages namely; plants are genetically identical to the parents (clones). Also
vegetative propagation preserves the genetic characteristics of a particular plant and allows for propagation of plants that do not produce seeds at all or do not produce viable seeds, or are difficult to grow from seeds. Matured plants are more quickly produced from vegetative propagation for many plants. Asexual production bypass the juvenile stage of plant growth when the plants will not flower and bear fruits for a long time and disease tolerance is obtained by using tolerant rootstocks during grafting and budding.

Some disadvantages of vegetative propagation include: being generally more expensive; requiring greater skill, and/or special equipment or facilities; increased likelihood of spreading or perpetuating certain diseases and clones becoming weakened and losing vigour after years of propagation, although this is by no means a general rule (Enslin, 2006). Macropropagation of tree crops most commonly makes use of both sexual (seed) and vegetative propagation. This is because vegetative reproduction allows the propagator to make an exact copy of the parent plant, while propagating with seed combines the properties of two parent plants and results in a completely new individual plant with completely new characteristics.

2.3.1. Propagation of H. brasiliensis Tree

Propagation of rubber can be achieved either by conventional methods involving seedlings from nurseries, cuttings and budding/grafting or in vitro procedures. Each mode of propagation has its limitations.
2.3.2. Propagation of *H. brasiliensis* by Seeds

Rubber plantations were originally established with unselected seedlings which led to considerable heterogeneity (Nayanakantha and Seneviratne, 2007). During the early years, rubber propagation was made through seeds only. Vegetative propagation by budding became very common after 1917. Presently, seeds are utilized mainly for the production of rootstocks (Cardinal *et al.*, 2007). Nursery seedlings from ordinary/illegitimate seeds of rubber tree (Edna, 2000) can be used as planting stocks for rubber plantation establishment, although it is genetically improper since they pose problems like late maturity, lack of true to type and non-uniformity.

Rootstocks influence the performance of scion after bud grafting (Noordin *et al.*, 2012). A good rooting system of rubber seedlings influences the efficiency of water and nutrient uptake from the soil (Bastiah *et al.*, 1996; Soong, 1976). *Hevea* seeds have short viability, declining drastically when exposed to sunlight and high temperatures (Noordin *et al.*, 2012).

2.3.3. Propagation by *H. brasiliensis* Cuttings

Vegetative propagation provides the best opportunity to multiply valuable trees for cultivation. The rooting of stem cuttings on a large scale could be expected to provide a simple means of propagating clones on their own roots or on genetically uniform clonal rootstocks. Rooted cutting of rubber is the only mode of vegetable propagation available for establishment of rubber plantations (Seveviratne, 1996). Rooting of cuttings may occur 2-3 months under a permanent mist spray with root-inducing compounds
(Mialoundama et al., 2002). The developed roots are either fibrous or adventitious (Seveviratne, 1996). *Hevea* is a typically cross-pollinated plant and hence there is a high degree of variability in the species and even among individuals of the same mother tree. It is for this reason that rubber is propagated vegetatively (Satchuthananthavale, 1973).

Cuttings were different to root and thus only juvenile shoots were used. Thus, in the late 1870s, cuttings from the basal juvenile part of young seedlings were rooted only at Kew Botanical Gardens and Singapore (Webster, 1989). However, in the mid-1950s mist propagation was successfully used to root leafy matured cuttings (Levandowski, 1959). By modifying Levandowski’s techniques, Tinley and Garner (1960) improved rooting of cuttings of most clones. However, rate of rooting varied considerably depending on the clone; 60-80 percent of most clones were successfully rooted.

### 2.3.4. Propagation of *H. brasiliensis* by Budding and Grafting

Grafting and budding techniques are currently used to produce almost all the planting materials of rubber for large scale plantation development. The technique is associated with chimera or intra-clonal differences of the scion-rootstocks plantlets for transplantation. Since traditional propagation of *Hevea* from ordinary/illegitimate seeds is not a recommended approach, budded rubber stump is normally achieved by grafting/budding a scion of a desired rubber clone to rootstocks obtained from seedlings of unselected seeds (Edna, 2000).
Even though, bud grafting of *Hevea* leads to intraclonal variation and production of few planting materials, it is still employed for plantation development (Nayanakantha and Seneviratne, 2007). It has been reported that much of the lower yield in rubber is as a result of rootstock variability (Senanayake and Wijewantha, 1968). However, rubber is presently propagated through bud-grafting in the nursery to develop high-yielding clones. The bud-grafted planting materials are anticipated to ensure a high level of homogeneity and reduced intra-clonal variation in yield. However, most breeding efforts are centered on the bud-wood, aerial part of tree while leaving the rootstock traits which affect growth and yield (Hayashi, 2009). The development of grafting techniques led to efficient plant selection procedures for more productive clones used in seedling formation compared to previous direct sowing techniques (Silvio and Julio, 2007).

### 2.3.5. Plant Tissue Culture

Plant tissue culture encompasses a variety of *in vitro* manipulations of plant cells, tissues and organs that direct the de-differentiation of the parental cells into meristematic or embryogenic cells, which then divide and differentiate into whole plants. The morphogenic routes through which cells regenerate into plants are organogenesis and embryogenesis, but both can occur via callus stage. In practice, all micropropagation protocols are established empirically by determining the medium components and environmental conditions for each stage of morphogenesis (Watt, 2012). The most important organogenesis used in plant propagation is shoot-tip, nodal or root cultures. Embryo cultures involve the excisions where zygotic embryos are also used for propagation. Additionally, meristem cultures comprising very small apical dome or
meristematic dome excised from shoot tip are also used for shoot development. Somatic embryogenesis in plant propagation includes callus, protoplast, anther and pollen cultures (George, 2008).

Micropropagation is the technique use to produce clones of plant species on a large scale. The technique involves the following stages (IAEA, 2004):

- Stage 0 - selection of explants.
- Stage 1 - initiation of explants - surface sterilization, establishment of mother explants.
- Stage 2 - proliferation stage.
- Stage 3 - shooting and rooting of the explants.
- Stage 4 - acclimatization.

This is the culture of shoot tips and its sub-adjacent leaves on nutrient medium to regenerate a new plant. The growing points of shoot initials cultured can continue uninterrupted and organised growth which can be rooted. Larger stem apices or lateral buds (between 5 and 20 mm) explants are used for culture. Even though large explants have advantages under \textit{in vitro} conditions, they are more difficult to decontaminate from micro-organisms. Additionally, they are associated with virus transmission from donor plant to the regenerated explant. Shoot-tip explants have shoot meristem which undergoes rapid multiplication followed by differentiation to produce shoots. Shoot regeneration can serve as explants for further multiplication through serial subculture. Plantlets regenerated can be rooted on a medium supplemented with naphthalene acetic acid or indole butyric acid and thereafter acclimatized (George and Debergh, 2008).
Nodal culture involves the culture of excised lateral buds/nodes (either single or multiple nodes) on MS medium supplemented with growth regulators. Nodal culture is used for propagating species that have long internodes e.g. potato. Shoots produced from nodal cuttings are more genetically stable. Though the rate of multiplication is generally low, there is less likelihood of associated callus development. Thus, it reduces the risk of induced genetic irregularity. Thus, nodal culture is a micropropagation method least likely to induce somaclonal variation (George and Debergh, 2008).

2.3.6. Plant Growth Regulators

Plant growth regulators are substances produce in small quantities by plants then translocated to the other plant parts for use which thus have the capacity to stimulate and/or inhibit physiological activities. Five major plant growth regulators are auxins, cytokinins, gibberellins, ethylene and abscisic acids. The two mainstream growth regulators in plant tissue culture are auxins and cytokinins which activate different metabolic pathways in plant physiology (Abiri et al., 2016). Auxins (Naphthalene Acetic Acid; Indolebutyric Acid; 2, 4-Dichlorophenoxyacetic Acid; Indoleacetic Acid; Tryptophan) are responsible for the enlargement in the plasticity of plant cell walls, elongation of cells and stems, promotion of vascular cambium and tissue activity and formation of roots. On the other hand, cytokinins (kinetin, 6-benzylamino purine and zeatin) result in the promotion of cell division, formation of shoots, morphogenesis and development of lateral buds (Yokoya et al., 2014).
Growth regulators directly influence the growth, development and in vitro propagation time of explants and also have pleiotropic effects in gene activation shifts which may affect essential cellular metabolism. Thus, normal cell functioning requires optimal concentrations of plant growth regulators (Khan et al., 2015). Synthetic auxins and cytokinins are the two main plant growth regulators which result in cell division and differentiation (Féher et al., 2003). The growth of girth and bark thickness of rubber tree can be accelerated by the use of exogenous plant growth regulators so as to shorten the productive lifespan. Plant growth regulators are very important in the enhancement of growth and development of Hevea brasiliensis (Koryati et al., 2015).

Successful in vitro regeneration depends on rooting percentage and survival of the plantlets. The auxins improve rooting efficiency and rhizogenesis of plantlets (de Oliveira et al., 2015). Sirisom and Te-chato (2014) performed a nodal culture of rubber trees on multiple shoot induction medium of MS supplemented with 5.0mg/L cytokinin gave 100% shoot formation with mean number of 3±0.63 shoots per explants. Shoot and root were obtained from the embryo culture on MS medium supplemented with BAP and NAA by (Ighere et al., 2011). In vitro shoot formation from cultured shoot tips of rubber seedlings was achieved on MS medium with 5.0mg/L cytokinins (Sirisom and Te-chato, 2012)

2.3.7. Tissue Culture for H. brasiliensis Propagation

Several in vitro techniques have been applied in producing more planting materials of H. brasiliensis for plantation establishment. Tissue culture regenerated Hevea plants have
shown increased growth and vigour (Carron et al., 1995). In 1953, Bouychou of the Institut Francais Caoutchouc was the first to apply *in vitro* technique to *Hevea* species. He used calli to study the laticiferous system in *H. brasiliensis*. It was then, taken up by Chua (1966) of the Rubber Research Institute of Malaysia and later by Wilson and Street (1975).

There has not been any report of mass propagation of clonal rubber tree yet whether through somatic embryogenesis or microcutting. However, recent reports indicate progress in the development of *Hevea brasiliensis* through tissue culture (Nayanakantha and Seneviratne, 2007). Since rubber seeds are very recalcitrant and hence, lose their viability during storage, tissue culture can be used to micropropagate and even store them *in vitro*. Micropropagation of *Hevea* using different explants mostly derived from seedlings has been reported (Thulaseedharan et al., 2000). Paranjothy and Gandhimathi, (1976) reported that shoots derived from shoot-tip culture of rubber rooted in liquid MS medium, failed to grow on solid medium.

Only limited reports are available on successful micropropagation using clonal material of *Hevea*. Explants derived from elite clones of mature *Hevea* trees are highly recalcitrant (Nayanakantha and Seneviratne, 2007). The problem lies in the failure to produce an adequate root system with taproot quality necessary for tree stability (Carron and Enjalric, 1982).
2.4. Biotic and Abiotic Stresses Affecting \textit{H. brasiliensis} Tree

Rubber production and development is greatly influenced by biotic stresses such as diseases as well as abiotic stresses, notably environmental factors that affect any crop. Environmental stress parameters such as temperature, wind, humidity, water availability and light have a great influence on the growth and development of rubber trees (Hayashi, 2009). Temperature and relative humidity, according to Silvio and Julio (2007) are the most important climatic conditions affecting plant developmental stages, latex production and seed yield. They proposed that temperatures ranging between 27°C and 30°C are the most favourable for rubber tree growth. For root proliferation, a well-drained (1.2m deep), sandy-loam and well-aerated permeable subsoil is suitable for rubber tree production (Edna, 2000). Moreover, Edna (2000) reported that a well distributed annual rainfall of at least 1700mm, soil rich in nitrogen and a temperature between 24°C and 35°C or more are ideal for the proper growth and development of the rubber tree.

Major adverse abiotic factors affecting rubber production include temperature stress, moisture stress and mechanical stress such as wind (Vinod, 2002). Humid tropical climate with sunlight and rainfall throughout the year affects the viability of rubber seeds (Noordin \textit{et al.}, 2012). Diseases are the major biotic factors that adversely influence \textit{Hevea} cultivation and productivity. Major diseases include South American Leaf Blight-\textit{Microcyclus ulei}, Abnormal Leaf Fall-\textit{Phytophthora spp.}, Powdery Mildew-\textit{Oidium heveae}, Corynespora Leaf Fall-\textit{Corynespora cassiicola} and \textit{Gloeosporium} Leaf Spot-\textit{Colletotrichum gloeosporioides} (Vinod, 2002).
The low production percentile of the rubber tree is as a result of the occurrence of an endemic disease called South American leaf blight (SALB) caused by an ascomycete fungus- *Microcyclus ulei* (Le Guen *et al*., 2002). SALB which shows a permanent major threat to rubber production in Asia and Africa prevents South America from developing its rubber industry (Davis, 1997). Some elite *Hevea* clones are vulnerable to undesirable traits such as the physiological disorder called Tapping Panel Dryness (TPD), drought, leaf fall diseases caused by *Corynospora* and *Phytophthora* (Nayanakantha and Seneviratne, 2007). Chen *et al.* (2003) estimated that 20-40% of annual rubber production is affected by TPD which cause partial or sheer stoppage of latex biosynthesis in high latex yielding rubber trees (Venkatachalam *et al*., 2007). Latex productivity, which is the major breeding objective of rubber, is affected by the growth of mature and immature tree trunk, wind damage, diseases, TPD and wintering (Hayashi, 2009).

2.5. Uses and Socio-Economic Importance of *H. brasiliensis* Tree

The latex was named ‘rubber’ after it was discovered by Joseph Priestly in 1770 that it could rub out pencil marks (Noordin *et al*., 2012). Natural rubber is deemed a suitable raw material for the manufacture of heavy-duty automobile tyres due to its higher strength, low heat build-up, better resistance to wear and flex cracking (Nayanakantha and Seneviratne, 2007). Even though, synthetic rubber from petroleum is often blended with natural rubber to manufacture certain products, some for instance airplane tyres solely require natural rubber (Venkatachalam *et al*., 2007).
The rubber tree produces good quality natural rubber and is preferred to synthetic rubber due to its unique mechanical features such as resistance to tear, elasticity, resilience and good heat transfer potentials. Natural rubber and synthetic rubber content, in a relatively small-size truck tyre, are 27% and 14% respectively, while those in a passenger tyre are 14% and 27% respectively (de Fray et al., 2010; Venkatachalam et al., 2007; Clement-Demange et al., 2007).

Over 40,000 products such as vehicle and plane tyres, gaskets, belts, waterproof clothing, gloves, hats, shoes, bottles, gloves, syringes, tapes, oxygen tents, hearing aids, swimmers’ goggles, caps, flippers, balls, seal jars, toys, paints, sponge, foam, furniture, cushions, pillows, mattresses, erasers, glues and adhesives are made from natural rubber (Duan, 2011; Thomas and Sydenham, 2005; Mooibroek and Cornish, 2000). Tyre represents over 50% of natural rubber consumption (Hayashi, 2009).

Rubber tree is a white gold tree species due to its high-priced latex product (Corpuz, 2013). Rubber plantations provide gainful self-employment and sustainable livelihoods and serves as a foreign exchange earner besides ecological benefits including carbon sequestration (Omo-Ikerodah et al., 2009). The rubber tree is a prolific producer of honey which is found on the extra floral nectarines located on the *Hevea* leaves (Vinod, 2002). Beyond the 30 year tapping period, latex production economically declines, thus the tree becomes a good source of timber (Omo-Ikerodah et al., 2009; FAO, 2001; Killmann and Hong, 2000).
Rubberwood are used as a cheap source of wood fuel in some rubber producing either for industrial brick burning, tobacco curing or for fuelling locomotive engines. In Southeast Asia, rubberwood is one of the most successful export timbers due to applied research on durability especially with wood seasoning and preservation (FAO, 2001). Some products made from rubberwood are furniture and its parts, parquet, panelling, wood-based panels (particleboard, cement and gypsum-bonded panels, medium-density fibrewood), kitchen and novelty items, sawn timber for general utility and fuel (Salleh, 1984).

In some countries like Malaysia and Thailand, and also India, Vietnam, Indonesia and Cambodia, rubberwood has proven to be a profitable industry (Venkatachalam et al., 2013). There are some advantages of rubberwood over conventional timbers from the natural forest because is a plantation by-product and is also available at a relatively low cost (FAO, 2001). The demand for and popularity of rubber timber and its products in traditional timber-importing countries such as Japan and USA stimulated the growth of the downstream processing industry in Malaysia (Hong, 1995).

### 2.6. Molecular Characterisation

Plant germplasm characterisation verifies the expression of highly heritable traits from agronomical or morphological characters to seed proteins or molecular makers. Therefore, plant germplasm characterisation may be phenotypical, molecular, protein polymorphism based on DNA (nucleotide sequence or genotypes), seed storage protein, allozymes and isozymes. Genetic biodiversity can be determined when species are characterised to distinguish numerous varieties/clones to select desirable traits of interest. Desirable genetic variations are observed for efficient utilization through characterisation.
Molecular characterization uses genetic markers for molecular analysis and genotyping useful for studying genetic variability in living things.

According to Stansfield (1986), the term marker is used for “locus marker”. Each gene has a unique place on the chromosome called ‘locus’. Genes can be modified in several mutually-exclusive forms called alleles (allelic forms) as a result of either spontaneous or artificial mutations. All allelic forms of a gene occur at the same locus on homologous chromosomes. Genotypic homozygosity (at this locus) is achieved when allelic forms of one locus are identical whereas genotypic heterozygosity is when different allelic forms are constituted. For instance, in triploid organisms, genotype is formed by three allelic forms of the homologous chromosomes. Moreover, genetic molecular markers are all loci markers related to DNA.

Molecular markers are employed in crop improvement programmes, ecological, physiological, genetic studies of plants as well as to improve the efficiency and precision of conventional plant breeding through marker-assisted selection. They are vital tools for crop improvement through marker assisted selection (MAS) and reliably estimating the production of desirable traits. Molecular markers may also improve efficient screening for many (polygenic) traits through their linkage with alleles with small (quantitative traits) and with large (qualitative traits) effects. They are vital in facilitating the initial understanding of biology and architecture of quantitative traits at the DNA level, as well as in studying plant genetics and diversity analysis. Molecular markers also provide
critical tools in aiding the comprehension of phylogenetic relationships among closely related, as well distantly related species/genera of plants. Other uses include the construction of molecular maps, QTL analysis, gene tagging, map-based cloning and germplasm characterisation. Therefore, molecular markers solely might be helpful in the process of assembling the desirable traits to develop an ideal variety (Singh et al., 2014).

2.6.1. Considerations for the Use of DNA Markers in Marker-Assisted Selection

Desirable genetic markers should meet the following considerations: high level of genetic polymorphism, co-dominance to distinguish heterozygote from homozygote, clear distinct allelic feature to easily identify different alleles, even distribution on the entire genome, neutral selection without pleiotropic effect, easy detection for the automation of processes and high duplicability to accumulate and share data between laboratories (Xu, 2010).

Considerations for the use of DNA markers in marker-assisted selection (MAS) are categorized into six (Mackill and Ni, 2000):

1. **Reliability**: Markers should be tightly linked to target loci. The use of flanking markers or intragenic markers will greatly increase the reliability of the markers to predict phenotype.

2. **DNA Quantity and Quality**: Some marker techniques require large amounts and high quality DNA, which may sometimes be difficult to obtain in practice, adding to the cost of the procedures.
3. **Technical Procedure:** The level of simplicity and the time required for the technique are critical considerations.

4. **High-throughput:** Simple and quick methods are highly desirable.

5. **Level of Polymorphism:** Ideally, the marker should be highly polymorphic in breeding material (i.e. it should discriminate between different genotypes), especially in core breeding material.

6. **Cost:** The marker assay must be cost-effective in order for MAS to be feasible.

Low *et al.* (1996) reported different marker index value i.e. number of polymorphic products per sample for some marker techniques: RFLPs (0.10), RAPD (0.23), SSRs (0.60) and AFLPs (6.08). Molecular markers can be classified into different groups based on: mode of transmission (biparental nuclear inheritance, maternal nuclear inheritance, maternal organelle inheritance, or paternal organelle inheritance); mode of gene action (dominant or co-dominant markers) and method of analysis (hybridization-based or PCR-based markers) (Singh *et al.*, 2014).

There are various types of molecular-marker techniques with each having substantial advantages and disadvantages. These techniques are restriction fragment length polymorphisms (RFLPs), amplified fragment length polymorphisms (AFLPs) and random amplified polymorphic DNAs (RAPDs). Others are microsatellites or simple sequences repeats (SSRs), inter simple sequence repeats (ISSRs), sequence characterized regions (SCARs) and cleaved amplified polymorphic sequences (CAPS). The rest are, single nucleotide polymorphisms (SNPs), targeted Region amplification polymorphism
(TRAP), single strand conformation polymorphism (SSCP), sequence related amplified polymorphisms (SRAPs) and conserved intron scanning primer (CISP).

2.6.2. Simple Sequence Repeats (SSRs) Marker

SSR markers are based on tandem repeats of short (2-6bp) DNA sequences (Litt and Luty, 1989) that are widely dispersed in the eukaryotic genomes. Their high degree of allelic variation is as a result of differences in the number of repeat units caused by slippage of DNA polymerase during replication (Moxon and Willis, 1999). They are ultimate markers for assessing genetic diversity (Brown et al., 2007), high density genetic map construction (Xue et al., 2008), population and conservation genetic studies (Powell et al., 1995). They are also appropriate for assessing, clonal identification (Dayanandan et al., 1998), controlled crosses certification, species and hybrid identification and paternity determination (Van de Ven and McNicol, 1996), marker assisted selection (Wiesing et al., 1998), genotyping (Parida et al., 2009) and providing insight into fine-scale ecological questions due to their hypervariability and efficiency in detecting polymorphisms.

SSR markers are of two classes based on their origin. These are genomic developed from enriched DNA libraries and genic or expressed sequence tags (EST)-SSRs, derived from EST sequences originating from the expressed region of the genome (Chagne et al., 2004). (EST)-SSRs are relatively less expensive to develop, represent transcribed genes which often have assigned putative function, and are more transferable across taxonomic boundaries than traditional genomic SSRs (Arnold et al., 2002). These advantages out-
balance putative disadvantages of EST-SSR like lower levels of polymorphism (Silfverberg-Dilworth et al., 2006). Polymerase chain reaction (Mullis and Faloona, 1987; Mullis et al., 1986) is used to amplify microsatellite SSR markers. Therefore, rapid data generation can be obtained from a relatively small amount of plant tissues. SSR markers are also expensive to generate and labour intensive to certain species. Consequently, many researchers have endeavoured to use SSR primers developed from one species for studies on related species and genera.

Some demerits associated with SSR makers are: high development cost in case primers are not yet available species specific primers and misclassification of heterozygotes as homozygotes when null-alleles occurs due to mutation in primer annealing sites. Other demerits are stutter bands on gels which may complicate accurate scoring of polymorphism; and largely unknown underlying mutation model (infinite allele’s model or stepwise mutation model). Another disadvantage is the presence of homoplasy which develops due to different forward and backward mutations which may underestimate genetic divergence (Singh et al., 2014).

2.6.3. Simple Sequence Repeats (Microsatellites) in Hevea

Microsatellites in the Hevea genome through the database search of some Hevea gene sequence was detected for the first time by Low et al. (1996). DNA fingerprints in H. brasiliensis using heterologous minisatellite probes from humans were reported by Besse et al. (1993a). Atan et al. (1996) reported on the construction of a microsatellite-enriched library from H. brasiliensis. Moreover, Lespinasse et al. (2000) reported on a saturated
genetic linkage map of rubber tree (*Hevea spp*) based on RFLP, AFLP, microsatellite and isozyme markers. Genetic diversity analysis of wild germplasm and cultivated clones of *H. brasiliensis* by using microsatellite markers was reported by Lekawipat *et al.* (2003). Simple sequence repeats was identified in rubber (*H. brasiliensis*) by Bindu Roy *et al.* (2004).

Development, characterisation and cross-species/genera transferability of EST-SSR markers for rubber tree was reported by Feng *et al.* (2009). Also, analysis of genetic diversity and SSR allelic variation in rubber tree was reported by Feng *et al.* (2012). Genetic divergence of rubber tree estimated by multivariate techniques and microsatellite markers was reported by Gouvea *et al.* (2010). Saha *et al.* (2005) reported on microsatellite variability and its use in the characterisation of cultivated clones of *H. brasiliensis*. Somaclonal variations of *in vitro*-plants derived from nodal culture of rubber tree by SSR markers was assessed by Sirisom and Te-chato (2014). Nakkanong *et al.* (2008) analysed 53 early introduced clones of rubber trees using RAPDs and microsatellite primer pairs (*hmac 4, hmct 1, hmct 5* and *hmac 5*) producing a total of 44 amplified fragments with an average of 14.67 fragment per primer.

### 2.6.4. Molecular Techniques in *Hevea* Breeding

Modern molecular techniques such as random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and restriction fragment length polymorphism (RFLP) are also used to analyse the genetic variability, sequence genome and develop cDNA libraries in the *Hevea* germplasm (Vinod, 2002). Molecular markers
have served as a useful aid in comprehending the genetics of *Hevea brasiliensis* with an important role in assisting *Hevea* clonal identification and origin. Marker-assisted selection (MAS) provides large quantum benefits in the genetic improvement of crops (Venkatachalam *et al.*, 2007).

Efficiency and precision of traditional plant breeding can be improved with marker-assisted selection (MAS) using DNA markers (Collard and Mackill, 2008). Neutral DNA markers have been used in *Hevea* in different applications (clonal conformity, parentage identification, diversity analysis) (Duan, 2011). At first, isozymes were utilized for clonal identification (Chevallier, 1988). Other tools developed and used in the detection of molecular markers in *Hevea brasiliensis* include RFLPs (Besse *et al.*, 1994; 1993b), RAPDs and DAFs (Venkatachalam *et al.*, 2002; Verghese *et al.*, 1997), AFLPs (Lespinasse *et al.*, 2000).
REFERENCE


CHAPTER THREE

3.0. Ex Vitro Propagation of *Hevea brasiliensis* using Stem Cuttings

3.1. Introduction

Rubber seeds are not only very recalcitrant but also of low quality, low vigour potency and low germination frequency. Low quality seed and poor germination rates affect the availability of planting stocks (Palanisamy and Subramanian, 2000). The time taken to raise seedlings of *H. brasiliensis* in nursery before transplantation can be shortened by stem cut nursery techniques. In addition, cost and effort of raising planting materials can be minimised by putting tree parts into mass propagation during the seed-off year or even during peak seasons of rubber trees by utilizing the stems being cut after budding successes (Corpuz, 2013). Clonal plantations of genetically improved *Eucalyptus* raised through cuttings enhanced the productivity 4 to 7 fold compared to plantations of unimproved seed sources (Lal *et al*., 1993; Zobel and Ikemori, 1983).

*Hevea brasiliensis* can be propagated by various methods including *in vitro* techniques, seedlings from nursery, budding and grafting all of which have their own merits and demerits in production of the plant species. Vegetative propagation facilitates rapid and large scale production of planting materials not only in rubber but also other tree species and can play a key role in tree improvement programmes for multiplication of superior clones or tested plus trees (Palanisamy and Subramanian, 2000).

Intra-clonal heterogeneity emanates from different genetic variations imposed by both the rootstocks and scion-stock of the budded/grafted plantlets. *Hevea brasiliensis* is currently
propagated by grafting and budding techniques. Budding and grafting of different plants or clones cause intra scion-rootstock variability. Thus, for genetic stability the budding technique should be avoided. In view of this, stem cuttings and micropropagation are recommended for production of planting materials. A stem cutting inherits all the traits of the donor plant and it is a good substitute for budding and grafting. A stem cut inherits all the traits of the tree source (prototype) (Corpuz, 2013).

Propagation by rooted cuttings has been tried as an alternative to overcome the stock/scion effect. Satchuthananthavale, (1973) has reported that cuttings grow better than budding. Furthermore, stem cuttings require limited space. The technique is also rapid, inexpensive and does not require special techniques used in grafting or budding. There is no problem of compatibility with rootstocks or of poor graft unions.

The most common treatment to enhance rooting in stem cuttings is the use of growth regulators (Hartmann et al., 1997). These substances enhance cell differentiation, starch hydrolysis, sugar and nutrient mobilization to the basal end of the cuttings resulting in root initiation (Das et al., 1997). There is rapid root formation and development and higher percentage of roots on cuttings treated with auxins (Leakey, 2004). Of all the growth regulators (auxins, cytokinins, gibberellins, abscisic acids and ethylene), auxins have the greatest effect on root formation in cuttings. Auxins are not only involved in rooting but also enhance stem growth, lateral bud inhibition, abscission of leaves and fruits and activation of cambial cells. Besides, the naturally occurring indole-3-acetic acids (IAA), there are more effective synthetic auxins such as indolebutyric acid-IBA,
naphthaleneacetic acid-NAA (Hartmann and Kester, 1975) which is used in rooting of rubber stem cuttings.

Since juvenile rubber shoots are very sensitive to desiccation and high temperature, they require immediate use of their cuttings for propagation vegetatively. Rubber is a seasonal tree species which does not produce fruits regularly, thus production of planting materials is seriously hindered.

This study is aimed at using rubber stem cuttings influenced by clonal types, stem types and growth regulators as an alternative to budding and grafting technique for planting material development. Specific objectives are to:

- determine the survival rate of lignified (brown) and non-lignified (green) stem cuttings of rubber as affected by clones and NAA levels;
- determine the sprouting (shoot development) and rooting growth of H. brasiliensis stem cuttings as influenced by the clones and the rooting compound;
- determine the survival rate, length of sprouts and length of roots among H. brasiliensis stem cuttings as influenced by the clones and the NAA at varying concentrations.

3.2. Materials and Methods

3.2.1. Collection of Hevea brasiliensis Stem Cuttings

Rubber cuttings of two Hevea clones (Clone I and Clone II) were used in this study. They were collected from rubber outgrowers in the Western Region of Ghana.
3.2.2. Propagation of *Hevea brasiliensis* by Stem Cuttings

One hundred and twenty (120) lignified (brown) and non-lignified (green) stem cuttings of rubber of each of the two *Hevea* clones (*Clone I* and *Clone II*) were prepared from harvested stems. Each cutting was trimmed to a uniform length of 40cm with each cutting consisting of 4 or 5 nodes.

The basal portions of the cuttings were then immersed in 7.5, 15.0 or 22.5g/L NAA for 6 hours and thereafter planted in nursery bags filled with sandy-loam soil mixed with manure in a ratio of 3:1. The experiment was arranged in a 2x4x5 factorial experiment with three (3) replications. The number of stem cuttings of each clone is determined as (2 clones x 4 treatments x 3 replications x 5 cuttings per treatment =120).

Stem cuttings were considered potentially sprouted when their buds ruptured or became visible. The number of cuttings that sprouted was counted at 20-day intervals after planting for four times (20, 40, 60 and 80 days). The number of cuttings that developed shoots (survival), number of roots and number of shoots were counted. The length of shoots as well as the length of roots measured using a metre rule 80 days after planting.

3.2.3. Data analysis

Data was subjected to analysis of variance using the Statgraphics® Centurion XVI. Fisher's least significant difference (LSD) procedure was used for the separation of means where appropriate at 5%.
3.3. Results

3.3.1. Effects of Clonal Types and NAA Concentrations on Shoot Development

None of the green stem cuttings survived irrespective of the clonal types and the concentrations of NAA suggesting the stem cuttings have an influence on sprouting or shoot development. Contrarily, *Hevea brasiliensis* brown stem cuttings (Fig. 3.0) survived irrespective of the clonal types and NAA levels. A higher significant difference was observed in the survival rate (cuttings that did not die), sprouting and rooting between brown and green stem cuttings of *Hevea brasiliensis* clones.

Total number of brown stem cuttings planted=120. Number of brown stem sprouted=87.

This implies that percentage of brown stem cuttings sprouted=$\frac{87}{120} \times 100\%$.

Therefore, percentage of *H. brasiliensis* brown stem cuttings sprouted is **72.5%**.

*Figure. 3.0: H. brasiliensis* brown stem cuttings used for propagation
Figure 3.1 (A-E): Morphological characteristics, growth and developmental stages of stem cuttings of two *H. brasiliensis* clones treated with NAA.

A- Stem cutting of *H. brasiliensis* showing shoots 20 days after planting  
B- Stem cutting of *H. brasiliensis* showing shoots 40 days after planting  
C- Rubber stems cuttings showing well-developed shoots 60 days after planting  
D- Rubber stem cuttings showing well-developed shoots 80 days after planting  
E- Uprooted rubber stem cuttings with root and shoot systems
3.3.2. Effects of Types of Clone on Sprouting Growth, Survival Rate, Length of Sprouts, Number of Roots and Length of Roots of *H. brasiliensis* Stem Cuttings

Table 3.1 depicts effect of types of clone on shoot development, survival rate, length of sprouts, length of roots and number of roots of *H. brasiliensis* stem cuttings 80 days after planting (DAP). The clonal types in 20 (Fig. 3.1A) and 60 (Fig. 3.1C) days after planting had no significant effects on the sprouting growth of the *Hevea brasiliensis* stem cuttings between the clones (Table 3.1). Although, the clonal types on the 40th (Fig. 3.1B) and 80th (Fig. 3.1D) days after planting had no significant effect on the sprouting growth of the stem cuttings, *Clone II* showed higher performance than *Clone I* (Table 3.1).

The clonal types, however, showed a significant difference (P<0.05) in the survivability of the brown stem cuttings (Table 3.1). *Clone II* showed a higher mean number of survived stem cuttings representing more than half of the number of brown stem cuttings that survived (Table 3.1). The survivability of cuttings is very important in the establishment of planting materials since the growth and rooting depend on the ability of the cuttings to survive.

After 80 (Fig. 3.1D) days of propagation, brown stem cuttings of *Clone II* of *Hevea brasiliensis* had a higher survival percentage of 98.333% as compared to 55.000% of *Clone I*. Thus, there was a significant difference (P<0.05) between *Clone I* and *Clone II* (Table 3.1).
Table 3.1. Effect of Types of Clone on the Sprouting Growth/Shoot Development, Survival Rate, Length of Sprouts, Number of Roots and Length of Roots of Brown *H. brasiliensis* Stem Cuttings 80 Days after Planting (DAP)

<table>
<thead>
<tr>
<th>TYPE OF CLONES</th>
<th>NUMBER OF CUTTINGS SPROUTED DAYS AFTER PLANTING</th>
<th>PERCENT SURVIVAL (%)</th>
<th>SHOOT LENGTH/CM</th>
<th>ROOT NUMBER</th>
<th>ROOT LENGTH/CM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20-DAY  40-DAY  60-DAY  80-DAY</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CLONE I</strong></td>
<td>0.500±0.905ᵃ  0.500±0.905ᵃ  0.667±0.778ᵃ  1.333±0.985ᵃ</td>
<td>55.000±33.166ᵃ</td>
<td>6.167±3.228ᵃ</td>
<td>2.917±0.996ᵃ</td>
<td>4.000±1.414ᵃ</td>
</tr>
<tr>
<td><strong>CLONE II</strong></td>
<td>0.500±0.522ᵃ  0.500±0.522ᵃ  1.250±1.055ᵃ  2.000±0.853ᵃ</td>
<td>98.333±5.774ᵇ</td>
<td>11.950±7.133ᵇ</td>
<td>5.000±2.663ᵇ</td>
<td>5.667±2.146ᵇ</td>
</tr>
</tbody>
</table>

Means in the same column with the same letter superscript are not significantly different (P≥0.05)
Regardless of the concentration of NAA, almost all the survived brown stem cuttings showed significant (P<0.05) length of shoots/sprouts between the two clones of *Hevea brasiliensis*. With the length of shoots measured after 80 (Fig. 3.1D) days of propagation, *Clone II* of the brown stem cuttings performed significantly higher in mean length of 11.950cm than that of *Clone I* in mean length of 6.167cm (Table 3.1). Therefore, this signifies that *Clone II* brown stem cuttings grow and develop faster in shoot length up to 80 days of planting (Table 3.1).

Clonal types had a significant difference (P<0.05) in both the number of roots and the length of roots of the brown stem cuttings. The number of roots developed varied significantly as influenced by the clones of the brown stem cuttings. The *Clone II* brown stem cuttings statistically were higher in the mean number of roots (5.000) compared with (2.917) in *Clone I* brown stem cuttings (Table 3.1).

In the case of the length of roots, *Clone II* of the brown stem cuttings once again was statistically longer in mean length of 5.667cm compared to the 4.000cm of the *Clone I* brown stem cuttings (Table 3.1).
3.3.3. Effects of NAA Concentrations on Sprouting Growth, Survival Rate, Length of Shoots, Length of Roots and Number of Root of *H. brasiliensis* Stem Cuttings

Table 3.2 shows the effects of concentration of NAA on shoot development, survival rate, length of sprouts, length of roots and number of roots of *H. brasiliensis* stem cuttings 80 days after planting (DAP). The concentration of NAA did not have significant effects on the sprouting of the brown stem cuttings 20th, 40th, 60th and 80th days after planting (Table 3.2). Similarly, no significant difference (P≥0.05) was observed in all the days after planting among the NAA treatments in the sprouting growth of the brown stem cuttings of *H. brasiliensis* (Table 3.2).

Though, 15.0g/L NAA was higher (1.000) in sprouting of the brown stem cuttings in both the 20th (Fig. 3.1A) and 40th (Fig. 3.1B) days after planting, statistically, it showed no significant difference (P≥0.05) with the other NAA concentrations. At 60 (Fig. 3.1C) days, both 7.5g/L and 22.5g/L NAA had similar number of cuttings sprouted (1.333) which were not significantly different (P≥0.05) from the remaining treatments (Table 3.2).

Although at 80 days, no significant difference existed among the treatments, the trend increased with the control treatments and the 7.5g/L NAA showing a higher and equal number of sprouted cuttings of 1.833. At 80 days after planting, almost all the living cuttings sprouted (Fig. 3.1D) and at this propagation time, a large number of stem cuttings sprouted (Table 3.2).
Table 3.2. Effect of NAA Levels Applied on the Sprouting Growth /Shoot development, Survival Rate, Length of Sprouts, Number of Roots and Length of Roots of *H. brasiliensis* Stem Cuttings 80 Days after Planting (DAP)

<table>
<thead>
<tr>
<th>CONC. OF NAA (g/L)</th>
<th>NUMBER OF CUTTINGS SPROUTED DAYS AFTER PLANTING</th>
<th>PERCENT SURVIVAL (%)</th>
<th>SHOOT LENGTH/CM</th>
<th>ROOT NUMBER</th>
<th>ROOT LENGTH/CM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20-DAY 40-DAY 60-DAY 80-DAY</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.333±0.516ᵃ 0.333±0.516ᵃ 0.500±0.548ᵃ 1.833±1.472ᵃ</td>
<td>63.333±42.739ᵃ</td>
<td>3.067±2.013ᵃ</td>
<td>1.833±0.753ᵃ</td>
<td>2.167±0.408ᵃ</td>
</tr>
<tr>
<td>7.5</td>
<td>0.333±0.516ᵃ 0.333±0.516ᵃ 1.333±0.516ᵃ 1.833±0.753ᵃ</td>
<td>76.667±32.042ᵃ</td>
<td>6.833±2.406ᵇ</td>
<td>3.833±1.169ᵇ</td>
<td>5.833±1.169ᵇ</td>
</tr>
<tr>
<td>15.0</td>
<td>1.000±1.095ᵃ 1.000±1.095ᵃ 0.667±0.516ᵃ 1.333±0.516ᵃ</td>
<td>83.333±32.042ᵃ</td>
<td>15.383±6.656ᶜ</td>
<td>6.167±2.787ᶜ</td>
<td>6.000±1.673ᵇ</td>
</tr>
<tr>
<td>22.5</td>
<td>0.333±0.516ᵃ 0.333±0.516ᵃ 1.333±1.633ᵃ 1.667±1.033ᵃ</td>
<td>83.333±23.381ᵃ</td>
<td>10.950±4.418ᵇᶜ</td>
<td>4.000±1.414ᵇ</td>
<td>5.333±1.366ᵇ</td>
</tr>
</tbody>
</table>

Means in the same column with the same letter superscript are not significantly different (P≥0.05)
The level of NAA concentrations showed no significant difference \( (P \geq 0.05) \) in the survivability (cuttings that did not die) of the brown stem cuttings. The controls are significantly lower in survival (63.333\%) compared with 76.667\%, 83.333\% and 83.333\% of 7.5g/L, 15.0g/L and 22.5g/L NAA respectively (Table 3.2).

The concentration of NAA had effect on length of shoots/sprouts and this effect was highly significant \( (P < 0.05) \) (Table 3.2) suggesting NAA had a significant influence on shoot development. The length of shoots ranged from 15.383cm to 3.067cm with 15.0g/L NAA having the highest significant mean length of shoots (Table 3.2).

Similarly, the concentration of NAA had effect on the root growth of brown stem cuttings and the effect was highly significant \( (P < 0.05) \) (Table 3.2). The effect of NAA on the length of roots varied significantly \( (P < 0.05) \) between the controls and the NAA treatments (Table 3.2). The 15.0g/L NAA significantly had a higher mean number of roots (6.167) and root length (6.000cm) compared to the control treatments with number of roots (1.833) and mean length of roots (2.167cm) (Table 3.2).
3.3.4. Effects of Clonal Types and NAA Levels on Sprouting Growth, Survival Rate, Length of Sprouts, Number of Root and Length of Roots of *H. brasiliensis* Stem Cuttings

Table 3.3 depicts the effects of type of clones and level of NAA applied on shoot development, survival rate, length of sprouts, length of roots and number of root of *H. brasiliensis* brown stem cuttings 80 days after planting (DAP). The interaction between *Clone I* and NAA levels statistically showed no significance (*P*>0.05) in the sprouting of the brown stem cuttings in the number of days after planting (Table 3.3). Also, brown stem cuttings showed no significant difference (*P*>0.05) in the number of cuttings sprouted in all the days after planting in the interaction between *Clone II* and NAA levels (Table 3.3). On the 20th (Fig. 3.1A) and 40th (Fig. 3.1B) day after planting, though no significant difference existed between 15g/L NAA and the other treatments, it significantly (*P*<0.05) showed high number of sprouted stem cuttings (1.333).

On the 60th (Fig. 3.1C) and 80th (Fig. 3.1D) days after planting, 22.5g/L NAA and the control treatments significantly had a high number of sprouted stem cuttings of 2.000 and 2.667 respectively (Table 3.3). It was observed that a few number of cuttings sprouted in the first three data collection days. In both clones, a large number of stem cuttings sprouted on the 80th day after planting (Table 3.3).
<table>
<thead>
<tr>
<th>TYPE OF CLONES</th>
<th>CONC. OF NAA (g/L)</th>
<th>20-DAY</th>
<th>40-DAY</th>
<th>60-DAY</th>
<th>80-DAY</th>
<th>PERCENT SURVIVAL (%)</th>
<th>SHOOT LENGTH/CM</th>
<th>ROOT NUMBER</th>
<th>ROOT LENGTH/CM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CLONE I</strong></td>
<td>0.0</td>
<td>0.333±0.577&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.333±0.577&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.333±0.577&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1.000±1.732&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>33.33±41.633&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.633±2.715</td>
<td>1.667±0.577&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.000±0.000&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>0.000±0.000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.000±0.000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.333±0.577&lt;sup&gt;abcd&lt;/sup&gt;</td>
<td>1.667±0.577&lt;sup&gt;bcd&lt;/sup&gt;</td>
<td>53.33±30.551&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>5.333±2.454</td>
<td>3.333±0.577&lt;sup&gt;babc&lt;/sup&gt;</td>
<td>5.000±1.000&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>15.0</td>
<td>1.333±1.528&lt;sup&gt;abcd&lt;/sup&gt;</td>
<td>1.333±1.528&lt;sup&gt;abcd&lt;/sup&gt;</td>
<td>0.333±0.577&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1.333±0.577&lt;sup&gt;abcd&lt;/sup&gt;</td>
<td>66.66±41.633&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>9.567±1.701</td>
<td>3.667±0.577&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.667±0.577&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>22.5</td>
<td>0.333±0.577&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.333±0.577&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.667±1.155&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>1.333±1.155&lt;sup&gt;abcd&lt;/sup&gt;</td>
<td>66.66±23.094&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.133±1.589</td>
<td>3.000±1.000&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.333±1.155&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>CLONE II</strong></td>
<td>0.0</td>
<td>0.333±0.577&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.333±0.577&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.667±0.577&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>2.667±0.577&lt;sup&gt;d&lt;/sup&gt;</td>
<td>93.33±11.547&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>3.500±1.479</td>
<td>2.000±1.000&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.333±0.577&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>0.667±0.577&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>0.667±0.577&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>1.333±0.577&lt;sup&gt;abcd&lt;/sup&gt;</td>
<td>2.000±1.000&lt;sup&gt;d&lt;/sup&gt;</td>
<td>100.00±0.000&lt;sup&gt;c&lt;/sup&gt;</td>
<td>8.333±1.305</td>
<td>4.333±1.528&lt;sup&gt;d&lt;/sup&gt;</td>
<td>6.667±0.577&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>15.0</td>
<td>0.667±0.577&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>0.667±0.577&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>1.000±0.000&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>1.333±0.577&lt;sup&gt;abcd&lt;/sup&gt;</td>
<td>100.00±0.000&lt;sup&gt;c&lt;/sup&gt;</td>
<td>21.200±2.524</td>
<td>8.667±0.577&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.333±1.155&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>22.5</td>
<td>0.333±0.577&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.333±0.577&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>2.000±1.000&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.000±1.000&lt;sup&gt;d&lt;/sup&gt;</td>
<td>100.00±0.000&lt;sup&gt;c&lt;/sup&gt;</td>
<td>14.767±1.607</td>
<td>5.000±1.000&lt;sup&gt;d&lt;/sup&gt;</td>
<td>6.333±0.577&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Means in the same column with the same letter superscript are not significantly different (P≥0.05)
Statistical analysis showed significant interactions between clones and NAA on the survivability of the brown stem cuttings. Although, no significant difference ($P \geq 0.05$) existed among the NAA concentrations within *Clone I*, 15.0g/L and 22.5g/L NAA showed an equal and higher survival rate (66.667%) of the brown stem cuttings as compared with the control treatments producing the least rate of 33.333% (Table 3.3).

Also, no significant difference ($P \geq 0.05$) was shown in *Clone II* among NAA concentrations. However, 7.5, 15.0 and 22.5g/L NAA showed equal and higher brown stem cutting survivability (100.000%) with the control treatments having lower survival rate of 93.333% (Table 3.3).

The interaction between clones and NAA on the shoot length of the *H. brasiliensis* brown stem cuttings was statistically different ($P < 0.05$) (Table 3.3). In both clones, there was a significant difference ($P < 0.05$) in the concentration of NAA on the length of shoots of *H. brasiliensis* brown stem cuttings with 15.0g/L NAA showing the highest shoot length (9.567cm and 21.200cm) (Table 3.3). This was followed by 22.5g/L NAA of shoot length whilst the control treatments showing the least shoot length of 2.633cm (Table 3.3).

For the number of roots (Fig. 3.1E) produced, significant difference ($P < 0.05$) existed in the interaction between the two clones and NAA treated brown stem cuttings with 15.0g/L NAA providing high number of roots (3.66 7 and 8.667). The control treatments had lower number of roots (Table 3.3). The interaction between the two clones and NAA concentrations showed significance ($P < 0.05$) in the root length (Fig. 3.1E) with both
7.5g/L and 15.0g/L NAA producing high mean length of roots of 5.000cm and 7.333cm.

The lowest length of shoots was provided by the control treatments (Table 3.3).
3.4. Discussion

Vegetative propagation provides the best opportunity to ensure the multiplication of valuable trees for production (Mialoundauma et al., 2002). A particular importance is the use of stem cuttings which impact significantly on the production of this important economic tree species. The use of stem cutting results in the production of true to type planting materials (clones) with genetic constitution similar to that of the mother plant and thus reliably clone plants with desirable traits (Enslin, 2006). Additionally, the technique leads to early maturity of new plants and the production of large scale planting materials within a short time for field establishment.

None of the green stem cuttings survived even under the same propagation conditions of the brown stem cuttings. Most of the brown stem cuttings of *Hevea* sprouted regardless of the concentration of NAA and the type of clones. For the surviving brown stem cuttings, there was significant difference (P<0.05) between the two clones in the number of roots, length of roots, length of shoots, survival rate and shoot growth. With the large number of both brown and green stem cuttings propagated more than half of them did not survive. Also, of the two clones studied, *Clone II* had a higher survival rate, number of shoots and roots as well as length of shoots and roots than *Clone I*. This could be attributed to both biotic and abiotic stresses especially an extremely high temperature and a very low relative humidity during the early stages of planting as well as fungal attacks on the cuttings. The reason for the successful sprouting and growth of brown stem cuttings could be due to the hydrolysis and availability of carbohydrates stored within the stem tissues of hardwood cuttings (Leakey, 2004). Cuttings from green stems because
they are slightly lignified and succulent are extremely susceptible to attack by soil-borne pathogens (Nestel, 1976). Fungal attack could be a serious threat to the failure and dying of the rubber stem cuttings. Rubber production, as is in the case of other crops is affected by various plant physiological conditions and pathogenic diseases such as South American Leaf Blight (SALB) and *Microcyclus ulei* (Le Guen *et al.*, 2003).

The concentrations of NAA had significant influence on length of shoots and roots and the number of roots, except the survival rate and shooting growth of the brown cuttings. The treatment of stem cuttings chemically by the application of auxins such as NAA or other growth regulators and fungicides strongly influence the ability of stem cuttings to develop roots. Root initiation and elongation are influenced by genetic, physiological and environmental factors (Leakey, 1985). It is established that stem cuttings with a single node are better propagules or planting materials for large and successful survival plum. A large sour plum cutting which response to hormone application in small effects could be as a result of high endogenous auxins within the cutting tissues which could have negative interaction with the applied NAA growth regulator (Owuor *et al.*, 2009).

Variations in soil moisture caused by ions such as Sodium, Calcium and Magnesium, aeration; pH and temperature could affect physical properties, absorption rates, and breakdown of soil as well as plant injury or death (Hartmann and Kester, 1975). Alpha naphthalene acetic acid (ANAA) has been found to be reliable in rooting cuttings (Corpuz, 2013) and there are compounds within stem cuttings such as phenolic that interact with auxins to promote rooting and increase root length (Hartmann *et al.*, 1997).
Root development differs between tree species and among plants within clones. Thus, variation in rooting may be attributed to lack of endogenous auxins, phenolic or other rooting co-factors in the cutting or lack of enzymes or their activators for synthesis of auxin-phenol complexes (Leakey, 1985).

Also, although some showed signs of growth in the early stage, they could not live on till the end of the planting duration which could be attributed to lack of root to translocate nutrients and moisture through the stem tissues. Some cuttings sprouted vigorously for a long time but later wilted or died due to lack of root formation and rotting of the basal part of the cuttings. Uncontrolled heat and light which cause an increase in temperature may cause plants to suffer from heat injury. Also, insufficient supply of water and drought are detrimental to all plants. Over-supply of water suffocates the plant roots and can cause diseases such as root rot, damping off and collar rot (Enslin, 2006). Auxins, NAA when applied at high rates to Oxalis plants as a foliar spray was revealed to be phytotoxic (Holt and Chism, 1988). The capacity of the stem cuttings to form roots can be determined by the percentage of cutting rooted, the number of roots per rooted cutting and the speed with which roots emerge and grow (Leakey, 1985).

Comparatively, Clone II significantly developed more shoots and roots than Clone I. In addition, the length of roots in Clone II was significantly longer than Clone I. The difference in performance between the two clones could be due to the drastic environmental changes which had adverse effect on Clone I than Clone II.
3.5. Conclusion

The sprouting and rooting growth success of the *Hevea brasiliensis* clones, particularly *Clone II*, out-performed and survived significantly better and higher than *Clone I* of the brown stem cuttings. Also, there was higher significant (P<0.05) survival rate of the *Hevea brasiliensis* brown stem cuttings than the green stem cuttings of which none sprouted. In the case of the levels of NAA influence on the sprouting and rooting growth on the *Hevea brasiliensis* brown stem cuttings, 15g/L of NAA out-performed better and higher than all the other NAA treatments and the control treatments. The interaction effects between *Clone II* and 15.0g/L NAA levels statistically (P<0.05) performed higher and better than the other interactions. Therefore, an alternative method to ensure rapid and mass propagation of *Hevea brasiliensis* planting materials for plantation is established. In using stem cuttings for the propagation of *Hevea brasiliensis* trees, these findings can be considered to shape the propagation procedures.
REFERENCE:


CHAPTER FOUR

4.0. *In vitro* Propagation of *Hevea brasiliensis* using Shoot-Tip and Nodal Cutting Explants

4.1. Introduction

*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 (Montoro *et al.*, 2010). In *Hevea*, *in vitro* via shoot-tip cultures, nodal cultures, somatic embryogenesis and genetic modification have been successful (Nayanakantha and Seneviratne, 2007).

Microcutting in *Hevea brasiliensis* involves *in vitro* culture of apex and axillary buds for propagation (Duan, 2011). 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 (Venkatachalam *et al.*, 2007; Thulaseedharan *et al.*, 2000). One powerful tissue culture technique for mass propagation of *Hevea* is somatic embryogenesis. The utilization of somatic embryogenesis has created avenues for molecular work through genetic engineering in *Hevea* (Zhao *et al.*, 2015). *Hevea* somatic embryogenesis was first developed in China and Malaysia using anther wall (Venkatachalam *et al.* 2007)

Traditionally, the rubber tree is propagated by grafting buds from selected clones on seedlings from seed orchards (Hua *et al.*, 2009). 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. Thus, micropropagation is a promising technique for the commercial production of selected clones (Mendanha et al., 1998). 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 (Paranjothy and Gandhimathi, 1976). Thereafter, shoots from 1-3 year old rubber seedlings used as explants developed roots (Enjalric and Carron, 1982). Since then there has been a progressive level in the micropropagation of clonal Hevea using axillary shoot proliferation (Seneviratne, 1991).

*In vitro* propagation of *H. brasiliensis* through organogenesis normally involves four phases; initiation of cultures, shoot multiplication, rooting of shoots and acclimatization (Trigiano et al., 1992; Duan, 2011). Mendanla et al. 1998 successfully produced shoots with roots using rubber axillary bud culture on MS medium supplemented with 1.0 mg/L kinetin, 1.0 mg/L 2, 4-D and 20 g/L sucrose. Successful *in vitro* shoot regeneration of *Hevea* was achieved on MS medium amended with only cytokinins growth regulator. For instance, Sirisom and Te-chato (2012) obtained multiple shoot from rubber shoot-tips cultured on MS medium with 5.0mg/L BA. Also, there was 100% shoot regeneration from nodal explants of *H. brasiliensis* cultured on MS medium with only cytokinins (Sirisom and Te-chato, 2014).

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.
4.2. Materials and Method

4.2.1. 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.

4.2.3. 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 (HgCl₂) for 5 minutes followed by rinsing with three changes of sterile distilled water and cultured on a full strength Murashige and Skoog (1962) 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. 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.

4.2.4. 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 4.2.3). Cultures were kept under growth room conditions (Section 4.2.3).

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.

4.2.5. 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%.
4.3. Results

4.3.1. 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. 4.0).

Figure 4.0. Contaminated MS medium of developing shoot-tip explant of *H. brasiliensis*
Figure 4.1 (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
Rooted shoot-tip plantlets of _H. brasiliensis_ on the control MS medium

**Figure 4.2.** 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 ≥ 0.05). The bars indicate standard errors of the means.

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 explant 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. 4.2). 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. 4.1C) of kinetin produced the highest shoots (84.0%) percentage followed by the control and 2.5mg/L of kinetin concentrations (Fig. 4.2). 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 (see appendix).

![Figure 4.3](https://example.com/figure4_3.png)

**Figure 4.3.** 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≥0.05). The bars indicate standard errors of the means.

The mean height of shoots (Fig. 4.1A) 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. 4.3). 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.4.3). The lower length of shoots developed by shoot-tip explants cultured on 7.5mg/L kinetin cannot be explained.

The number of leaves (Fig. 4.1B) 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. 4.4).

The mean number of leaves (Fig. 4.1B) 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. 4.4). 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. 4.4).

Table 4.0. Mean number of shoots and explants with roots of developed by shoot-tip explants cultured on MS basal medium supplemented with Kinetin.

<table>
<thead>
<tr>
<th>Concentration of Kinetin (mg/L)</th>
<th>Mean Number of Shoots</th>
<th>Mean Number of Explant with Roots</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>2.600±1.140&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>8.000±5.701&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>2.5</td>
<td>2.800±1.483&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.000±0.000&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>5.0</td>
<td>3.600±1.140&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.000±2.236&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>7.5</td>
<td>1.200±0.447&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.000±2.739&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>10.0</td>
<td>1.600±0.548&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1.000±2.236&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

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

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 4.0). 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 4.0).

The shoot produced also developed roots even without the influence of exogenous auxins in the MS medium (Fig. 4.1 D&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 4.0). 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 4.0).
4.3.2. 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. 4.5A). 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 4.5 (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. 4.5B). 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. 4.6). 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. 4.7. 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.

Similarly, the concentration of the kinetin in the culture medium significantly affected the growth of the developing shoot height (Fig. 4.5C). Again, nodal explants cultured on a medium without growth regulator developed shoots which grew taller (4.80cm) than the kinetin treated explants (Fig. 4.7). 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. 4.7).
Figure 4.8. 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 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. 4.8) 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. 4.8).
Table 4.1. Mean number of shoots and explants with roots of *Hevea brasiliensis* nodal explants cultured on MS basal medium supplemented with 0.0-10.0 mg/L Kinetin.

<table>
<thead>
<tr>
<th>Concentration of Kinetin (mg/L)</th>
<th>Mean number of shoots</th>
<th>Mean number of explant with roots</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>6.000±2.121&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.000±2.739&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>2.5</td>
<td>1.800±1.643&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.000±0.000&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>5.0</td>
<td>3.000±2.121&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.000±2.739&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>7.5</td>
<td>3.000±0.000&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.000±4.472&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>10.0</td>
<td>0.600±1.342&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.000±0.000&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

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

The treatments produced more than one shoot (Fig. 4.5C). The highest number (6.0) of shoots was produced by explants cultured on MS medium without growth regulator (Table 4.1). 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 4.1). 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 4.1).
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. 4.5D). The highest mean number (7.0) of explants with roots was achieved on the control treatments (Table 4.1). Shoots growing on a medium supplemented with 5.0mg/L and 7.5mg/L kinetin produced equal number (3.0) of roots (Table 4.1). Thus, there was a statistical difference (P<0.05) in the mean number of nodal explants with roots among all the treatments of the MS media (Table 4.1).
4.4. 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.

*In vitro* propagation of plant species is influenced by genotype, age and source of donor tissues and exogenous application of growth regulators (George, 1993). In plant tissue culture, the activity of cytokinin oxidase is enhanced by exogenous application of cytokinin suggesting that treatment of explants with synthetic cytokinins could decrease the concentration of natural endogenous compounds (Motyka and Kaminek, 1990). 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 (Konwar and Coutts, 1990).
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 and adventitious shoot development in plant species (Schmulling, 2002). 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 (Ozyigit, 2008).

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. 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 necrosis, browning and the subsequent death of explants (Abdelwahd *et al.*, 2008; Arnaldos *et al.*, 2001). 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 (Hard *et al.*, 2015). 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.
The limited success achieved in regeneration from explants could be due to the addition of activated charcoal in the culture medium. Activated charcoal serves as an absorbent of many inhibitors, especially oxidized polyphenols which are exuded into the culture medium by woody tissues (Monnier, 1990).

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. Harb *et al.*, 2015 achieved the highest increase in shoot length of jackfruit on medium with 3.0mg/L GA₃.

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 (Kalidass and Mohan, 2009) 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). 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. Sirisom and Te-chato (2014) added 1.0mg/L AgNO₃ to MS medium to achieve excess of 5.0 shoots per explants in *Hevea brasiliensis* cultures. 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 (Zhang *et al.*, 2001), achiote (Parimalam *et al.*, 2010) and turnip (Cogbill *et al.*, 2010). Silver nitrate in plant
tissue culture inhibits ethylene synthesis (Zhang et al., 2001) which often builds up in culture vessels thereby inhibiting shoot development.

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 (Murashige, 1974). The number of explants with roots reduced as the concentrations of kinetin increased in the MS culture medium. Concentrations of cytokinin between 0.5 and 10mg/L generally inhibit or delay root formation (Ben-Jaacov et al., 1991) and also prevent the promotive effects of auxins on root initiation and subsequent root growth (Humphries, 1960).

The ability of the explants to root under the influence of cytokinin 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.
4.5. 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.
REFERENCE:


CHAPTER FIVE

5.0. Microsatellite Variation in *Hevea brasiliensis* Clones Cultivated in Ghana

5.1. Introduction

The fundamental components in plant breeding are the germplasm diversity and genetic relatedness among the best breeding materials (Mukhtar *et al.*, 2002) which are obtainable from pedigree analysis, morphological traits or by means of molecular markers (Mohammadi and Prasanna, 2003). Since morphological characterisation based on vegetative, reproductive and yield attributes, has the limitation of differentiating among some accessions that are very similar; molecular characterisation can resolve this varietal variation easily.

The development of molecular markers is as a result of the lack of useful morphological traits of the rubber tree for clonal distinction (Besse *et al.*, 1993a). Inheritance and mutations are the two determinants of genetic uniqueness and all genetic diversities among species are found in the primary sequence of their DNA genomes. Thus, an efficient procedure is necessary to identify an individual sequence for genomes under comparison (Krawczak and Schmidtke, 1994). Genetic markers are useful in labeling and amplifying DNA and highlighting its variations among individual organisms. When DNA is extracted, the variation in the plant samples can be identified using polymerase chain reaction (PCR), hybridization or capillary electrophoresis (CE) to identify unique molecule sizes, charges and chemical compositions as well as to tag and track genetic diversity in DNA samples (Xu, 2010).
According to Karp et al. (1997), molecular markers provide genetic information on genetic distances to identify particular divergent sub-populations harbouring valuable genetic traits, identification of duplicate accessions, monitor changes in genetic structure; and characterisation with respect to genetic diversity within collections. Markers may extend and complement characterisation based on morphological or biochemical descriptions with more detailed passport data and accuracy than classical phenotypic data (Hodgkin et al., 1995). Molecular markers may, therefore, be used in four types of measurements needed for effectively resolving the numerous operational, logistical and biological questions (Kresovich et al. 1992).

Investigations into the genetic diversity of the rubber tree using of molecular markers have been reported extensively (Oktavia et al., 2011; Gouvéa et al., 2010; Lam et al., 2009; Saha et al., 2005; Lekawipat et al., 2003; Feng et al., 2012). In recent past, genetic markers have served as powerful aids in comprehending the genetics of Hevea brasiliensis as well as assisted in Hevea clonal origin and identification.

Among all the markers, microsatellite markers are deemed the most suitable for genetic studies because they combine co-dominance and high polymorphism with abundance, locus specificity and uniform dispersion in plant genomes (Kalia et al., 2011). A combination of four microsatellite markers was used to discriminate uniquely 27 Hevea clones and also to generate some clone-specific allelic profiles (Saha et al., 2005). The rubber breeding objective is to provide superior and mature budded clones for latex production, rubberwood production, quality rubber products and environmental
protection through carbon sequestration (Venkatachalam et al., 2013). *Hevea* breeding is time-consuming and expensive. Thus, molecular markers such as microsatellites can be used to reduce the time required for breeding the tree crop (Mantello et al., 2012).

Since the introduction of rubber in Ghana in 1898, there has not been any critical research into genetic variation analysis and relatedness among various clones grown in the country. It is therefore necessary to use SSR markers to study the genetic diversity and similarity of the various local *Hevea* clones being grown by outgrowers in the country. The study will be useful in parent-tree selection for *Hevea* breeding and propagation programme, germplasm evaluation and genetic characterisation of traits of agronomic interest. The objective of this study is to use microsatellite markers to identify and assess genetic variability and also evaluate the genetic relationships among some introduced clones of *Hevea brasiliensis* cultivated in Ghana.

### 5.2. Materials and Methods

Twenty-three (23) *H. brasiliensis* clones, collected from Plantations Socfinaf Ghana Ltd (PSG), Takoradi (GT1, PB 217, RRIM 703, RRIC 100, IRCA 230, IRCA 41, IRCA 317, IRCA 331); Ghana Rubber Estate Ltd (GREL), Western Region (GT1, IRCA 41, IRCA 109, IRCA 230, IRCA 317, IRCA 331, IRCA 840, PB 217); Forest and Horticultural Crops Research Centre (FOHCREC), Kade, University of Ghana (K1, K2, K3); Plant Genetic Resource Research Institute (PGRRI), Bunso (B1, B5, B7, B8) were used for this study. Fresh leaves were collected from each clone and stored at -20°C after thoroughly washing them under sterile distilled water.
5.2.1. DNA Extraction

Total genomic DNA was extracted from fresh rubber leaves following the Cetyltrimethyl Ammonium Bromide (CTAB) protocol of Doyle and Doyle (1990) with some modifications. Hundred (100) mg of the leaves were ground to fine powder in approximately 500 μl of CTAB buffer and transferred to a microcentrifuge tube for incubation at 60°C in a water bath for 45 minutes. After incubation, the extracted mixtures were centrifuged at 10,000 rpm for 15 minutes and 700μl supernatant transferred to a new clean microcentrifuge tube. Five hundred (500) μl of chloroform-isoamylalcohol (24:1) was added to each of the tubes and the solution mixed by inversion. After mixing, the tube was centrifuged at 12,000 rpm for 10 minutes and 700μl upper aqueous phase (contains the DNA) transferred to a clean microcentrifuge tube. The solution was added to 500μl isopropanol and the tube inverted slowly for several times to precipitate the DNA overnight. The sample was spun at 12000rpm for 10minutes and the solution poured out from the tube leaving the pellet. The DNA pellet was washed with 70% ethanol two times and dried at room temperature.

5.2.2. Quality Assurance Test

The quality of DNA was confirmed by agarose gel electrophoresis (0.8% agarose) with ethidium bromide in TAE buffer (40 mM Tris-acetate pH 8.0, 1 mM EDTA) and exposed to UV light and photographed. Samples were loaded into agarose gel with 0.25% bromophenol blue, 0.25% Xylene cyanol FF and 30% glycerol in water as loading buffer. Thereafter, 20μl of each DNA was diluted with sterilize distilled water to 100μl for the running of the PCR.
5.2.3. PCR Amplification

PCR amplification of DNA was performed following a protocol described by Saha et al. (2005). The amplification reaction was carried out in 25μl final volume containing 20 ng of genomic DNA (1μl), 25 mM MgCl$_2$ (50μl), 10x Taq buffer (50μl), 0.2 μM (25μl) each of the forward and reverse primers, 200 μM dNTPs (5μl) and 0.7 units of Taq polymerase (5μl). Three out of the four most applied SSR marker pairs in _H. brasiliensis_ genetic variability analysis identified from the genomic library of _H. brasiliensis_ (Saha et al. 2005; Roy et al., 2004) together with two other primer pairs reported by Pootakham et al. (2012) were used for the study. These five (5) different SSR primers are hmct1, hmac4, hmct5, gSSR212 and gSSR194.

The temperature profile involved an initial denaturation step of 5 minutes at 95°C followed by a touch-down PCR programme. Temperature profiles of touch-down PCR for seven cycles were as follows: 94°C for 30 sec, annealing at (63°C for 1 minute and 53°C for 30sec each depends on primer pairs), and decreased 1°C for seven cycles, and finally 72°C for 1 minute. It was further followed by a normal cycling of 94°C for 30 sec, 56°C for 1 minute, 72°C for 1 minute for 23 cycles and a final extension at 72°C for 10 minutes. The touch-down protocol is used to eliminate stuttering and artifact bands. PCR amplicons were ran on 3% agarose gel matrix stained with ethidium bromide to a final concentration of 0.5μg/ml. Eight (8μl) PCR products were loaded into the wells and ran at 100V for 1hour and were visualized using a UV transilluminator.
5.3. Results

All the five different microsatellites produced bands for the evaluation of genetic diversity/similarity among the 23 rubber clones from four (4) different geographical areas of Ghana. The primers provided monomorphic patterns and the number of bands for each primer varied from 11 to 16 with an average of 13.75 fragments per primer. The amplified products ranged from 100 to 650 base pairs in size which was in consonant with the findings of Sirisom and Te-chato (2014).

![Figure 5.1](image1.png)

**Figure. 5.1.** The banding pattern of primer hmct1 on rubber clones numbered 1-23. Forward primer: aaccagaaggtgtcatg, Reverse primer: ggaatcccatgacaatccac.

![Figure 5.2](image2.png)

**Figure. 5.2.** The banding pattern of primer hmac4 on rubber clones numbered 1-23. Forward primer: gtttctcgcgacactcag, Reverse primer: atccacaaataaggcatga.

**GREL** (GT1-1, IRCA 41-2, IRCA 109-3, IRCA 230-4, IRCA 317-5, IRCA 331-6, IRCA 840-7, PB 217-8); **PSG** (GT1-9, PB 217-10, RRIM 703-11, RRIC 100-12, IRCA 230-13, IRCA 41-14, IRCA 317-15, IRCA 331-16); **Kade** (K1-17, K2-18, K3-19) and **Bunso** (B1-20, B5-21, B7-22, B8-23) and M-1kb ladder.
5.3.1 Genetic Diversity/Similarity and Cluster Analysis

Figure 5.3 shows a dendrogram generated using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) from NYSYSpc version 2.02i. The bands generated from SSR (Section 5.3) were used to plot the dendrogram/cluster to study the genetic similarity and diversity or variation among the clones collected. A dendrogram generated from the UPGMA cluster analysis distinguished 23 clones of *H. brasiliensis* from four (4) different geographical areas of Ghana into two clusters (A and B) at genetic distance of 36%. Cluster A was further divided into two sub-clusters (AI and AII) at genetic distance of 53.6%. Sub-cluster AI consisted of *H. brasiliensis* clones K2-18 (Kade) and AII of clones GT1-9 (PSG), IRCA317-5 and IRCA41-2 (GREL) (Fig.5.3).

Contrarily, cluster B was further divided into three sub-clusters at 44.8% genetic distance. With this, the first sub-cluster B (III-IV) was made up of RRIC100-12 and RRIM703-11 (PSG). The second sub-cluster B (V-VII) comprised K1-17, K3-19 (Kade), B1-20, B5-21, B7-22, B8-23 (Bunso), IRCA317-15, IRCA230-12, IRCA41-14 (PSG), IRCA840-7 and IRCA331-6 (GREL). The final sub-cluster B (VIII-XI) consisted of such rubber clones as PB217-8, IRCA230-4, IRCA109-3, GT1-1 (GREL), PB217-10 and IRCA317-16 (PSG) (Fig.5.3).

At genetic distance of 100%; IRCA317-5, IRCA41-2, IRCA331-6, IRCA230-4, IRCA109-3 (GREL) and B8-23 (Bunso) were genetically similar. Moreover, rubber clones consisting of K1-17, K3-19 (Kade), IRCA317-15, IRCA230-12 (PSG), B1-20, B5-21 and B7-22 (Bunso) were genetically similar at genetic distance of 70.4%. Also, at
genetic distance of 80.0% rubber clones GT1-9, RRIC100-12, RRIM703-11, IRCA41-14 (PSG) and GT1-1 (GREL) were similar genetically (Fig.5.3).

In contrast, *H. brasiliensis* clones including K2-18 (Kade), IRCA840-7, PB217-8 (GREL), PB217-10 and IRCA317-16 (PSG) at different genetic distances of 53.6%, 73.6%, 64%, 65.6% and 66.8% respectively were genetically dissimilar (Fig.5.3).

The farthest genetic distance was shown between *Hevea brasiliensis* clones K2-18 (Kade) and GT1-1 (GREL) (Fig. 5.3).
Figure 5.3. A dendrogram showing genetic diversity/similarity revealed by 5 simple sequence repeat primers among 23 H. brasiliensis clones cultivated in Ghana.
5.4. Discussion

There was genetic diversity and similarity present in *H. brasiliensis* clones from the four different geographical areas of rubber production in Ghana as been shown by the dendrogram (Fig. 5.3). The dendrogram generated a linkage among the *H. brasiliensis* clones at genetic distance of 36% where two clusters were formed.

It was observed that some rubber clones were genetically similar irrespective of their geographical location of growth which could be as a result of their development from budding of clones with scions of similar genetic constitutions. These possible similarities were revealed in rubber clones IRCA317-5, 1RCA41-2, IRCA331-6, IRCA230-4, IRCA109-3 (GREL) and B8-23 (Bunso) which were linked at 100% genetic similarity level. This supports a controversial results reported by Cesar *et al.* (2006) and Lekawipat *et al.* (2003) that some relationship existed between geographical areas and sample collection. Introduction of planting materials into Ghana from other rubber-producing countries could have facilitated the distribution of genes in various clones irrespective of the country where they were initially bred.

At a genetic distance of 70.4%, *H. brasiliensis* clones K1-17, K3-19 from Kade; B1-20, B5-21, B7-22 from Bunso and IRCA317-15, IRCA230-12 from PSG showed genetic similarity. Furthermore, at 78.4% level of similarity, *H. brasiliensis* clones; GT1-9, RRIC100-12, RRIM703-11, IRCA41-14 from the same growing area, PSG and GT1-1 from GREL showed to be genetically similar. High genetic similarity among clones could be as a result of the same parent plants selected for breeding programme over the years. The high genetic similarity suggests a narrow genetic diversity among the clones and this may be explained by clonal propagation employed
in establishing plantations (Nakkanong et al., 2008). Genetic similarity could occur because generally vegetative propagation is known to maintain traits of parental lines (Varghese et al., 1997). Genetic similarity assessment might be useful in selecting the parents for a rubber breeding and propagation programme aimed at obtaining heterosis effects and high productivity. Microsatellite primers provide a large quantity of polymorphic information, for this reason they are ideal for differentiating genotypes that are genetically very similar (Nakkanong et al., 2008). This was very much shown in this current study.

Contrarily, *H. brasiliensis* clones K2-18, IRCA 840-7, PB217-8, PB217-10 and IRCA317-16 from different geographical zones did not show any genetic similarity with other clones indicating genetic variability among the genotypes. Genetic diversity observed could be attributed to cross-pollination between the genotypes since rubber tree is outcrossing. Genetic differences emanate from gene flow among populations through seed and pollen dispersal. The genetic variability may have been influenced by gene mutation, although it has low incidence rate in *H. brasiliensis* species (Nakkanong et al., 2008). Varghese et al. (1997) reported that it could happen because the rubber tree is a cross pollinated plant and very heterozygous. Also, segregation causes proportion of hybrid alleles from parents to vary.

However, genetic variability/diversity assessment within the germplasm is of interest to practical applications in breeding where it can rapidly identify the breeding materials. Genetic difference is indispensable for genetic improvement and elite gene exploitation where high yield related genes have been found (Pethin et al., 2015).
5.5. Conclusion

Microsatellite primers amplified their corresponding DNA sequence in the genomic DNA. The band size ranged from 100 to 650 base pairs. UPGMA dendrogram based on the amplification band clustered the clones into two main groups based on genetic make-up independent of the geographical origin. There was high genetic similarity among the *H. brasiliensis* clones than genetic diversity regardless of their geographical origin of collection. Therefore for the purposes of propagation and breeding programme of *H. brasiliensis*, some local clones could be selected based on their genetic differences and relatedness.
REFERENCE:


CHAPTER SIX

6.0. General Conclusions and Recommendations

6.1. Conclusions

*Hevea brasiliensis* was introduced into Ghana in 1898 and the country has since benefitted economically from the exploitation of its latex. This study was aimed at propagating rubber tree using both *in vivo* and *in vitro* propagation techniques as well as the use of SSR primers to genetically analyse some clones.

- Sprouting on shoot development from cuttings used for propagation *in vivo* was influenced by the lignification of cuttings. Lignified brown cuttings easily developed shoots in the presence of NAA while all non-lignified green cuttings did not develop any shoot.
- *Clone II* of *H. brasiliensis* brown stem cuttings treated with concentrations of NAA significantly developed more shoots than *Clone I*. Also, 15.0g/L NAA was optimal for shoot and root development of *H. brasiliensis* stem cuttings.
- The effects of 15.0g/L NAA and *Clone II* showed significant performance in the sprouting and rooting growth of *H. brasiliensis* brown stem cuttings.
- Murashige and Skoog basal medium supplemented with 5.0mg/L kinetin was the optimum concentration of kinetin for shoot regeneration because 84.0% of the shoot tip explants of *H. brasiliensis* developed plantlets.
- Murashige and Skoog basal medium without kinetin was optimum for shoot and root formation because 92.0% of the nodal explants of *H. brasiliensis* regenerated. It was followed by 7.5mg/L kinetin in performance.
- High genetic similarity was observed in *H. brasiliensis* clones; IRCA317-5, 1RCA41-2, IRCA331-6, IRCA230-4, IRCA109-3 and B8-23.
• Genetic diversity was showed in *H. brasiliensis* clones; K2-18, IRCA840-7, PB217-8, PB217-10 and IRCA317-16. The widest genetic diversity among the *H. brasiliensis* clones was between K2-18 and GT1-1

• SSR primers identified all the rubber clones as separate entries to determine both genetic diversity and similarity

6.2. Recommendations

Based on the outcomes of this study, these recommendations could be considered in future research works:

• Root formation could be induced on both shoot-tip and nodal explants of *Hevea brasiliensis* on a root induction medium. Other cytokinin growth regulators such as 6-benzylaminopurine (BAP) and auxin-cytokinin combinations such as BAP and NAA could be considered on both shoot-tip and nodal explants of *Hevea brasiliensis* for the assessment of shoot and root induction.

• Future research could consider other types of growth media such as Linsmaier and Skoog (LS), Gamborg (B5), Nitsch and Nitsch (NN) and White’s medium as well as other explants of *Hevea brasiliensis* such as the whole seed, seed embryo, seed integument, meristem, root, anther, pollen and others.

• Other methods of propagation, for instance marcottage that can lead into effective production of roots on stem cuttings before transferring them into nurseries are recommended.

• The type of growth medium (soil) and its various components as well as the season and time of the year for the collection and propagation of *Hevea brasiliensis* stem cuttings must be taken into consideration since they influence
survivability and growth. Treatment of the stem cuttings with fungicides to promote survival rate and root growth should be considered.

- DNA extraction protocol of *H. brasiliensis* should be modified, especially by adding polivinylpolipyrolidon (PVPP) to obtain high quality and pure DNA for amplification. Also, PCR amplicons could be ran using polyacrylamide gel (PAGE) to find out if quality bands would be obtainable.

- Other types of primers such as RAPDs and SNPs and a combination of different markers could be taken into consideration to genetically analyse *H. brasiliensis* clones in Ghana.
APPENDICES

7.0. Appendices

Appendix 7.1 Microsatellite patterns of rubber clones

Microsatellite patterns of rubber clones (lane1-23) consist of **GREL** (GT1-1, IRCA 41-2, IRCA 109-3, IRCA 230-4, IRCA 317-5, IRCA 331-6, IRCA 840-7, PB 217-8); **PSG** (GT1-9, PB 217-10, RRIM 703-11, RRIC 100-12, IRCA 230-13, IRCA 41-14, IRCA 317-15, IRCA 331-16); **Kade** (K1-17, K2-18, K3-19) and **Bunso** (B1-20, B5-21, B7-22, B8-23) amplified by primer hmct5 (A) and gSSR212 (B). Lane M = 1kb ladder

Appendix 7.2. ANOVA for factorial analysis of the effect of concentration of NAA for the survivability rate of rubber brown stem cuts 80 days after planting

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F-Ratio</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between groups</td>
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<td>533.333</td>
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<td>0.6985</td>
</tr>
<tr>
<td>Within groups</td>
<td>22133.3</td>
<td>20</td>
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<tr>
<td>Total (Corr.)</td>
<td>23733.3</td>
<td>23</td>
<td></td>
<td></td>
<td></td>
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Appendix 7.3. ANOVA for factorial analysis of the effect of concentration of NAA for the shoot length of rubber brown stem cuttings 80 days after planting

<table>
<thead>
<tr>
<th>Source</th>
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<td>Within groups</td>
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<td>Total (Corr.)</td>
<td>874.958</td>
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### Appendix 7.4. ANOVA for factorial analysis of the effect of concentration of NAA for the root length of rubber brown stem cuttings 80 days after planting

<table>
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<th>F-Ratio</th>
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</tr>
<tr>
<td>Within groups</td>
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<td>20</td>
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<td>Total (Corr.)</td>
<td>89.3333</td>
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</table>

### Appendix 7.5. ANOVA for factorial analysis of the effect of concentration of NAA for the number of roots of rubber brown stem cuttings 80 days after planting

<table>
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<tr>
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<td>Total (Corr.)</td>
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### Appendix 7.6. ANOVA for factorial analysis of the effect of concentration of NAA on sprouting time of rubber brown stem cuttings 20 days after planting

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### Appendix 7.7. ANOVA for factorial analysis of the effect of concentration of NAA on sprouting time of rubber brown stem cuttings 40 days after planting

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<th>MS</th>
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<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between groups</td>
<td>2.0</td>
<td>3</td>
<td>0.66667</td>
<td>1.33</td>
<td>0.2916</td>
</tr>
<tr>
<td>Within groups</td>
<td>10.0</td>
<td>20</td>
<td>0.5</td>
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<td></td>
</tr>
<tr>
<td>Total (Corr.)</td>
<td>12.0</td>
<td>23</td>
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<td></td>
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</tr>
</tbody>
</table>

### Appendix 7.8. ANOVA for factorial analysis of the effect of concentration of NAA on sprouting time of rubber brown stem cuttings 60 days after planting

<table>
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<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between groups</td>
<td>3.4583</td>
<td>3</td>
<td>1.15278</td>
<td>1.32</td>
<td>0.2965</td>
</tr>
<tr>
<td>Within groups</td>
<td>17.5</td>
<td>20</td>
<td>0.875</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total (Corr.)</td>
<td>20.9583</td>
<td>23</td>
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</tbody>
</table>

### Appendix 7.9. ANOVA for factorial analysis of the effect of concentration of NAA on sprouting time of rubber brown stem cuttings 80 days after planting

<table>
<thead>
<tr>
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<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between groups</td>
<td>1.0</td>
<td>3</td>
<td>0.333333</td>
<td>0.33</td>
<td>0.8052</td>
</tr>
<tr>
<td>Within groups</td>
<td>20.3333</td>
<td>20</td>
<td>1.01667</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total (Corr.)</td>
<td>21.3333</td>
<td>23</td>
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</tr>
</tbody>
</table>

### Appendix 7.10. ANOVA for factorial analysis of the effect of type of clones for the survivability rate of rubber brown stem cuts 80 days after planting

<table>
<thead>
<tr>
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<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between groups</td>
<td>11266.7</td>
<td>1</td>
<td>11266.7</td>
<td>19.88</td>
<td>0.0002</td>
</tr>
<tr>
<td>Within groups</td>
<td>12466.7</td>
<td>22</td>
<td>566.667</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total (Corr.)</td>
<td>23733.3</td>
<td>23</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Appendix 7.11. ANOVA for factorial analysis of the effect of type of clones for the sprout length of rubber brown stem cuts 80 days after planting

<table>
<thead>
<tr>
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<th>MS</th>
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<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between groups</td>
<td>200.682</td>
<td>1</td>
<td>200.682</td>
<td>6.55</td>
<td>0.0179</td>
</tr>
<tr>
<td>Within groups</td>
<td>674.277</td>
<td>22</td>
<td>30.6489</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total (Corr.)</td>
<td>874.958</td>
<td>23</td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

### Appendix 7.12. ANOVA for factorial analysis of the effect of type of clones for the root length of rubber brown stem cuts 80 days after planting

<table>
<thead>
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<th>MS</th>
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<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between groups</td>
<td>16.6667</td>
<td>1</td>
<td>16.6667</td>
<td>5.05</td>
<td>0.0351</td>
</tr>
<tr>
<td>Within groups</td>
<td>72.6667</td>
<td>22</td>
<td>3.30303</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total (Corr.)</td>
<td>89.3333</td>
<td>23</td>
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<td></td>
</tr>
</tbody>
</table>

### Appendix 7.13. ANOVA for factorial analysis of the effect of type of clones for the number of roots of rubber brown stem cuts 80 days after planting

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
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<th>MS</th>
<th>F-Ratio</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between groups</td>
<td>26.0417</td>
<td>1</td>
<td>26.0417</td>
<td>6.44</td>
<td>0.0187</td>
</tr>
<tr>
<td>Within groups</td>
<td>88.9167</td>
<td>22</td>
<td>4.04167</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total (Corr.)</td>
<td>114.958</td>
<td>23</td>
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<td></td>
<td></td>
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</tbody>
</table>

### Appendix 7.14. ANOVA for factorial analysis of the effect of type of clones on sprouting time of rubber brown stem cuttings 20 days after planting

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F-Ratio</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between groups</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0.00</td>
<td>1.0000</td>
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<tr>
<td>Within groups</td>
<td>12.0</td>
<td>22</td>
<td>0.5455</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total (Corr.)</td>
<td>12.0</td>
<td>23</td>
<td></td>
<td></td>
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</tbody>
</table>

### Appendix 7.15. ANOVA for factorial analysis of the effect of type of clones on sprouting time of rubber brown stem cuttings 40 days after planting

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
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<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between groups</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0.00</td>
<td>1.0000</td>
</tr>
<tr>
<td>Within groups</td>
<td>12.0</td>
<td>22</td>
<td>0.5455</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total (Corr.)</td>
<td>12.0</td>
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</table>

### Appendix 7.16. ANOVA for factorial analysis of the effect of type of clones on sprouting time of rubber brown stem cuttings 60 days after planting

<table>
<thead>
<tr>
<th>Source</th>
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<th>MS</th>
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<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between groups</td>
<td>2.04167</td>
<td>1</td>
<td>2.04167</td>
<td>2.37</td>
<td>0.1376</td>
</tr>
<tr>
<td>Within groups</td>
<td>18.9167</td>
<td>22</td>
<td>0.85984</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total (Corr.)</td>
<td>20.9583</td>
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</table>

### Appendix 7.17. ANOVA for factorial analysis of the effect of type of clones on sprouting time of rubber brown stem cuttings 80 days after planting

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
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<th>P-Value</th>
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</thead>
<tbody>
<tr>
<td>Between groups</td>
<td>2.66667</td>
<td>1</td>
<td>2.66667</td>
<td>3.14</td>
<td>0.0901</td>
</tr>
<tr>
<td>Within groups</td>
<td>18.6667</td>
<td>22</td>
<td>0.84845</td>
<td></td>
<td></td>
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<tr>
<td>Total (Corr.)</td>
<td>21.3333</td>
<td>23</td>
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<td></td>
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</table>
**Appendix 7.18.** ANOVA for factorial analysis of the effect of type of clones and concentration of NAA for the survivability rate of rubber brown stem cuts 80 days after planting

<table>
<thead>
<tr>
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<th>MS</th>
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<th>P-Value</th>
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<tbody>
<tr>
<td>Between groups</td>
<td>13600.0</td>
<td>7</td>
<td>1942.86</td>
<td>3.07</td>
<td>0.0300</td>
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<tr>
<td>Within groups</td>
<td>10133.3</td>
<td>16</td>
<td>633.333</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total (Corr.)</td>
<td>23733.3</td>
<td>23</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

**Appendix 7.19.** ANOVA for factorial analysis of the effect of type of clones and concentration of NAA for the sprout length of rubber brown stem cuts 80 days after planting

<table>
<thead>
<tr>
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<th>SS</th>
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<th>P-Value</th>
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</thead>
<tbody>
<tr>
<td>Between groups</td>
<td>811.638</td>
<td>7</td>
<td>115.948</td>
<td>29.30</td>
<td>0.0000</td>
</tr>
<tr>
<td>Within groups</td>
<td>63.32</td>
<td>16</td>
<td>3.9575</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total (Corr.)</td>
<td>874.958</td>
<td>23</td>
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<td></td>
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</tbody>
</table>

**Appendix 7.20.** ANOVA for factorial analysis of the effect of type of clones and concentration of NAA for the root length of rubber brown stem cuts 80 days after planting

<table>
<thead>
<tr>
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<th>SS</th>
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<th>P-Value</th>
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<tr>
<td>Between groups</td>
<td>79.3333</td>
<td>7</td>
<td>11.3333</td>
<td>18.13</td>
<td>0.0000</td>
</tr>
<tr>
<td>Within groups</td>
<td>10.0</td>
<td>16</td>
<td>0.625</td>
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<td></td>
</tr>
<tr>
<td>Total (Corr.)</td>
<td>89.3333</td>
<td>23</td>
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</table>

**Appendix 7.21.** ANOVA for factorial analysis of the effect of type of clones and concentration of NAA for the number of roots of rubber brown stem cuts 80 days after planting

<table>
<thead>
<tr>
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<th>SS</th>
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<th>P-Value</th>
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<tr>
<td>Between groups</td>
<td>101.625</td>
<td>7</td>
<td>14.5179</td>
<td>17.42</td>
<td>0.0000</td>
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<tr>
<td>Within groups</td>
<td>13.3333</td>
<td>16</td>
<td>0.83333</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total (Corr.)</td>
<td>114.958</td>
<td>23</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Appendix 7.22.** ANOVA for factorial analysis of the effect of type of clones and concentration of NAA on sprouting time of rubber brown stem cuttings 20 days after planting

<table>
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<th>MS</th>
<th>F-Ratio</th>
<th>P-Value</th>
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</thead>
<tbody>
<tr>
<td>Between groups</td>
<td>3.33333</td>
<td>7</td>
<td>0.47619</td>
<td>0.88</td>
<td>0.5438</td>
</tr>
<tr>
<td>Within groups</td>
<td>8.66667</td>
<td>16</td>
<td>0.541667</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total (Corr.)</td>
<td>12.0</td>
<td>23</td>
<td></td>
<td></td>
<td></td>
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**Appendix 7.23.** ANOVA for factorial analysis of the effect of type of clones and concentration of NAA on sprouting time of rubber brown stem cuttings 40 days after planting

<table>
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<th>F-Ratio</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between groups</td>
<td>3.33333</td>
<td>7</td>
<td>0.47619</td>
<td>0.88</td>
<td>0.5438</td>
</tr>
<tr>
<td>Within groups</td>
<td>8.66667</td>
<td>16</td>
<td>0.541667</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total (Corr.)</td>
<td>12.0</td>
<td>23</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
Appendix 7.24. ANOVA for factorial analysis of the effect of type of clones and concentration of NAA on sprouting time of rubber brown stem cuttings 60 days after planting

<table>
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<th>MS</th>
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<th>P-Value</th>
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<tbody>
<tr>
<td>Between groups</td>
<td>6.95833</td>
<td>7</td>
<td>0.994048</td>
<td>1.14</td>
<td>0.3895</td>
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<tr>
<td>Within groups</td>
<td>14.0</td>
<td>16</td>
<td>0.875</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total (Corr.)</td>
<td>20.9583</td>
<td>23</td>
<td></td>
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</table>

Appendix 7.25. ANOVA for factorial analysis of the effect of type of clones and concentration of NAA on sprouting time of rubber brown stem cuttings 80 days after planting

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
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<th>MS</th>
<th>F-Ratio</th>
<th>P-Value</th>
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</thead>
<tbody>
<tr>
<td>Between groups</td>
<td>6.0</td>
<td>7</td>
<td>0.857143</td>
<td>0.89</td>
<td>0.5336</td>
</tr>
<tr>
<td>Within groups</td>
<td>15.3333</td>
<td>16</td>
<td>0.958333</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total (Corr.)</td>
<td>21.3333</td>
<td>23</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

Appendix 7.26. ANOVA for the effect of concentration of Kinetin in the culture medium on regeneration of shoots (percentage sprouting) of shoot tip explants of *H. brasiliensis*

<table>
<thead>
<tr>
<th>Source</th>
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<th>MS</th>
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<th>P-Value</th>
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<tbody>
<tr>
<td>Between groups</td>
<td>5984.0</td>
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<td>1496.0</td>
<td>3.07</td>
<td>0.0402</td>
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<tr>
<td>Within groups</td>
<td>9760.0</td>
<td>20</td>
<td>488.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total (Corr.)</td>
<td>15744.0</td>
<td>24</td>
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</tbody>
</table>

Appendix 7.27. ANOVA for the effect of concentration of Kinetin in the culture medium on the height of shoots of shoot tip explants of *H. brasiliensis*

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F-Ratio</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between groups</td>
<td>91.5624</td>
<td>4</td>
<td>22.8906</td>
<td>5.11</td>
<td>0.0053</td>
</tr>
<tr>
<td>Within groups</td>
<td>89.532</td>
<td>20</td>
<td>4.4766</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total (Corr.)</td>
<td>181.094</td>
<td>24</td>
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</tbody>
</table>

Appendix 7.28. ANOVA for the effect of concentration of Kinetin in the culture medium on the number of leaves of shoot tip explants of *H. brasiliensis*

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
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<th>MS</th>
<th>F-Ratio</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between groups</td>
<td>1096.56</td>
<td>4</td>
<td>274.14</td>
<td>22.07</td>
<td>0.0000</td>
</tr>
<tr>
<td>Within groups</td>
<td>248.4</td>
<td>20</td>
<td>12.42</td>
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<td></td>
</tr>
<tr>
<td>Total (Corr.)</td>
<td>1344.96</td>
<td>24</td>
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</table>

Appendix 7.29. ANOVA for the effect of concentration of Kinetin in the culture medium on the number of shoots of shoot tip explants of *H. brasiliensis*

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
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<th>MS</th>
<th>F-Ratio</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between groups</td>
<td>18.56</td>
<td>4</td>
<td>4.64</td>
<td>4.38</td>
<td>0.0105</td>
</tr>
<tr>
<td>Within groups</td>
<td>21.2</td>
<td>20</td>
<td>1.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total (Corr.)</td>
<td>39.76</td>
<td>24</td>
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</table>
**Appendix 7.30.** ANOVA for the effect of concentration of Kinetin in the culture medium on the number of shoot tip explants with roots

<table>
<thead>
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<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between groups</td>
<td>206.0</td>
<td>4</td>
<td>51.5</td>
<td>5.15</td>
<td>0.0051</td>
</tr>
<tr>
<td>Within groups</td>
<td>200.0</td>
<td>20</td>
<td>10.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total (Corr.)</td>
<td>406.0</td>
<td>24</td>
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</tbody>
</table>

**Appendix 7.31.** ANOVA for the effect of concentration of Kinetin in the culture medium on regeneration of shoots (percentage sprouting) of nodal explants of *H. brasiliensis*

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F-Ratio</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between groups</td>
<td>5216.0</td>
<td>4</td>
<td>1304.0</td>
<td>4.66</td>
<td>0.0081</td>
</tr>
<tr>
<td>Within groups</td>
<td>5600.0</td>
<td>20</td>
<td>280.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total (Corr.)</td>
<td>10816.0</td>
<td>24</td>
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</tr>
</tbody>
</table>

**Appendix 7.32.** ANOVA for the effect of concentration of Kinetin in the culture medium on the height of shoots of nodal explants of *H. brasiliensis*

<table>
<thead>
<tr>
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<th>SS</th>
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<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between groups</td>
<td>69.3216</td>
<td>4</td>
<td>17.3304</td>
<td>2.78</td>
<td>0.0551</td>
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<tr>
<td>Within groups</td>
<td>124.776</td>
<td>20</td>
<td>6.2388</td>
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<td></td>
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<tr>
<td>Total (Corr.)</td>
<td>194.098</td>
<td>24</td>
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</tbody>
</table>

**Appendix 7.33.** ANOVA for the effect of concentration of Kinetin in the culture medium on the number of leaves of nodal explants of *H. brasiliensis*

<table>
<thead>
<tr>
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<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between groups</td>
<td>948.96</td>
<td>4</td>
<td>237.24</td>
<td>4.32</td>
<td>0.0111</td>
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<tr>
<td>Within groups</td>
<td>1098.0</td>
<td>20</td>
<td>54.9</td>
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</tr>
<tr>
<td>Total (Corr.)</td>
<td>2046.96</td>
<td>24</td>
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</tbody>
</table>

**Appendix 7.34.** ANOVA for the effect of concentration of Kinetin in the culture medium on the number of shoots of nodal explants of *H. brasiliensis*

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
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<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between groups</td>
<td>80.64</td>
<td>4</td>
<td>20.16</td>
<td>7.47</td>
<td>0.0008</td>
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<tr>
<td>Within groups</td>
<td>54.0</td>
<td>20</td>
<td>2.7</td>
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<td></td>
</tr>
<tr>
<td>Total (Corr.)</td>
<td>134.64</td>
<td>24</td>
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</tbody>
</table>

**Appendix 7.35.** ANOVA for the effect of concentration of Kinetin in the culture medium on the number of nodal explants with roots

<table>
<thead>
<tr>
<th>Source</th>
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<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between groups</td>
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<td>41.5</td>
<td>5.93</td>
<td>0.0026</td>
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<tr>
<td>Within groups</td>
<td>140.0</td>
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<tr>
<td>Total (Corr.)</td>
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</tbody>
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