

**ADAPTING CUTTING AND TISSUE CULTURE TECHNIQUES TO
CLONAL PROPAGATION OF THE SHEA TREE (*VITELLARIA
PARADOXA*) IN UGANDA**

BY

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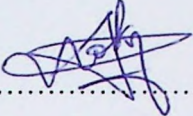
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DECLARATION

I, Okao Moses hereby declare that this dissertation is as a result of my own effort and has not been previously accepted or being concurrently submitted in candidature for any degree in any institution of higher learning.

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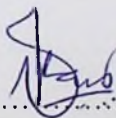
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DEDICATION

This MSc. dissertation is dedicated to my lovely wife Ms. Stella Maris, my daughter Samantha and my parents Mr. and Mrs Jimmy Okao.

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LIST OF ACRONYMS

ARF	Adventitious Root Formation
BA	6-benylaminopurine
BAP	6-benylaminopurine
2,4-D	2,4-dichlorophenoxyacetic acid
GA₃	Gibberellic acid
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
LRF	Lateral Root Formation
MS	Murashige and Skoog medium
NAA	Naphthalene acetic acid
PPM	Parts per million
NgeZARDI	Ngetta Zonal Agricultural Research and Development Institute
MUARIK	Makerere University Agricultural Research Institute, Kabanyolo
USAID	United States Agency for International Development
USITC	Unites States International Trade Commission

ABSTRACT

A study to adapt tissue culture and stem cutting techniques to vegetative propagation of *Vitellaria paradoxa* (Shea tree) was conducted between March and July, 2014. The specific objectives of the study were to: determine appropriate culture media composition for *in vitro* regeneration of *V. paradoxa*, evaluate the influence of cutting type on the rooting success of *V. paradoxa* stem cuttings, and assess the effects of mode of application of rooting hormones (IBA) on adventitious root formation of *V. paradoxa* stem cuttings. To achieve these objectives, four media compositions were evaluated for *in vitro* regeneration of *V. paradoxa* shoot tips; four modes of auxin (IBA) application at three different levels of concentration were evaluated with respect to adventitious root formation of Shea cuttings, and a third experiment was set to determine the effect(s) of cutting types on rooting success of *V. paradoxa* stem cuttings. A total of 2,556 Shea stem cuttings and 360 explants were evaluated in the study. Data were then entered in Ms Excel and analyzed using R-system software. Data on tissue culture regeneration were collected on survival rate, callus development, root and shoot formation. For the stem cutting experiments, data were collected on: number of rooted cuttings, quantity and length of developed roots including evidence of auxiliary bud break. Frequencies were derived to determine the extent, to which explants survived, rooted, callused or remained dormant. One way ANOVA was conducted to investigate the quality of rooting success between the four modes of auxin application. Where treatments were found to be significantly different, the Tukey's Honestly Significant Difference (HSD) was used to make pair wise comparisons. The linear relationship between rooting success and bud break was investigated using correlation and regression analysis. The highest level of callus formation was observed in media containing MS+BAP ($27.3 \pm 1.00\%$) and lowest ($16.7 \pm 0.58\%$) in the one composed of MS+BAP+GA₃. Root formation ($11.1 \pm 0.58\%$) was observed only for the treatment (MS+BAP + NAA). Basal cuttings dipped for 24hr at 80ppm showed the highest rooting percentage ($69.0 \pm 3.61\%$) compared to the other application methods. The basal cuttings also recorded comparatively better rooting percentage (38.1 ± 4.00 to $69.0 \pm 3.61\%$) than the tip cuttings (35.7 ± 3.61 to $57.1 \pm 6.66\%$). Results also revealed that bud break was generally lower in basal cuttings and that bud break and root number were positively correlated ($r=0.577$). It was therefore recommended that further experimentation needs to be conducted on tissue culture using more media compositions and explants so as to enhance survival rate and generate *V. paradoxa* plantlets. Also, a thorough study should be carried out on the potential for foliar application of IBA, such that if adapted well, it can be incorporated for mass production of *V. paradoxa* subsp. *nilotica*. For now the 24hr long soak method at 80ppm is recommended for large scale propagation of *V. paradoxa* using stem cuttings.

CHAPTER ONE

INTRODUCTION

1.1 Background

Vitellaria paradoxa (Shea tree) is a wild fruit-bearing tree species that plays a significant socio-economic role in Sub-Saharan Africa (Sanou *et al.*, 2004). It has the potential to improve nutrition, boost food supply in the "annual hungry season" (Masters *et al.*, 2010), in addition to fostering rural development and supporting sustainable land use (National Research Council, 2006).

Shea butter, which is the major product of the Shea tree is the fat extracted from the nut of this tree (Malachi, 2014). It serves as a source of edible oil for many households in the Shea belt (Njoku *et al.*, 2000). Shea butter is renowned for its anti-microbial properties which makes it a useful ingredient for cosmetic and pharmaceutical formulations (Akihisa *et al.*, 2010). In addition, it is traditionally used by local healers to treat rheumatism, inflammation of the nostrils, nasal congestion, leprosy, cough, and minor bone dislocation (Olaniyan and Oje, 2007). It is also used as a substitute for cocoa butter in chocolate industries and as raw material for the production margarine, soap, detergent and candle (Russo and Etherington, 2001).

Shea butter is more superior to other vegetable oils mainly because of the exceptionally high amount of unsaponifiables. A typical vegetable oil contains less than 1% of unsaponifiables, mainly phytosterols and tocopherols, whereas Shea butter can contain as much as 15% of unsaponifiable matter comprising triterpene esters as well as highly unsaturated isoprenoidal hydrocarbons (Eka, 1997). The unsaponifiable fraction is composed of bioactive substances that are responsible for Shea butter's medicinal properties (Esuoso *et al.*, 2000). On the other hand, the main Shea butter fatty acids, oleic and stearic, are sufficiently stable against oxidation. Thus, if carefully purified and correctly handled, Shea butter and its extracts show superior oxidative stability and do not require additional antioxidants (Andersson and Alander, 2015).

The Shea tree grows naturally in the wild of the dry savanna belt of West Africa, from Senegal in the West to Sudan in the East and onto the foot hills of the Ethiopian mountains. The West African trees are classified as the subspecies "*paradoxa*" and the East African one as "*nilotica*" (Samuel, 2005). It is considered a sacred tree by many communities and ethnic groups and plays important roles in religious and cultural ceremonies where is also believed to have some spiritual protective powers (Malachi, 2014).

In Uganda, *V. paradoxa* grows mainly in the West Nile, Acholi, Lango, Teso and Karamoja sub-regions where it is known locally by various names such as: Kumere, Komoro, Kamiro (Lugbara-West Nile); Awa (Madi-West Nile), Yaa (Acholi), Yao (Lango) and Ekungur (Ateso). It is mostly found in the areas with an altitude of 600-1100 m above sea level and mean annual rainfall of 600-1400 mm. Most of the areas where *Vitellaria* are found are subject to mean annual temperature of 25-29°C. The tree grows slowly taking almost 15 to 30 years to mature (Hall *et al.*, 1996). Although *V. paradoxa* came to world attention at the end of eighteenth century (Hall *et al.*, 1996), the existing *V. paradoxa* population remains essentially unmanaged and grows wild.

According to USAID (2010), statistics on production of Shea are conflicting and fragmented. This is because Shea is not publicly traded and there is not yet a well organized industry association that gathers industry data on crop production volumes, annual harvest volumes, prices, numbers of producers and their networks, volumes of raw materials exported and consumed domestically, and volumes of refined and unrefined Shea butter produced.

However, USITC (2008) previously estimated that 600,000 metric tons of Shea nuts are produced by the Sub-Saharan African countries in the Shea belt. Mali, Ghana and Nigeria are the largest producers at 150,000, 130,000 and 100,000 metric tons respectively per year; with Uganda producing only 6,000 tons. Even then, Uganda has the potential of producing between 70,000 and 300,000 metric tons per year (Bup *et al.*, 2014).

The Shea tree has many uses to man and its demand is ever increasing inversely with the productivity and increasing interest in regenerating the Shea tree has warranted research into various propagation techniques such as grafting, cutting and layering. These techniques, however, are not devoid of limitations. Although grafting has proven to be successful in reducing the juvenile phase significantly (Sanou *et al.*, 2004), the long period required to produce root stalks is a disincentive.

Even if cloning of *V. paradoxa* by means of stem cuttings has over the last decade produced substantial research progress especially as far as adventitious root formation is concerned (Yeboah *et al.*, 2009; Yeboah *et al.*, 2010; Yeboah *et al.*, 2011), there have been some difficulties in the production process, such as poor rooting which consequently affects their deployment in the

planting programme. Poor rooting of certain clones by using conventional methods has also been attributed to the maturation degree of the plant material (Sulichantini *et al.*, 2014).

Following the above, this study was designed to develop a protocol for regenerating the Shea tree through tissue culture and use of coppice cuttings derived from mature fruit bearing Shea trees. Such protocols when adapted would provide early maturing and good quality planting stock to replace the harvested and older Shea trees. Selection of superior phenotypes in a vegetative propagation technique such as this one has been a very important part of a tree improvement programme where superior genotypes can be efficiently and economically multiplied (Rosvall and Mullin, 2013). The multiplication of superior genotypes for increased fruit yield of the Shea tree would contribute to improved food security, better nutrition and valuable socio-economic benefits to farmers.

1.2 Statement of the Problem

Most of the *V. paradoxa* trees are of old age with few young ones (Okullo, 2004). Its natural regeneration is being seriously affected by the nature of the desired product collected by the users and rodents that eat the fruits before or during germination (Boffa, 1999). The natural regeneration has also been affected by *V. paradoxa* seeds losing viability within a very short time after fruit fall, estimated by Ladipo and Kio (1987), to be within three to six weeks. In addition, the successful seedling takes on average 10 to 25 years to start fruiting. This has not only reduced the Shea tree regeneration in the wild but also made most farmers to have little interest to cultivate the tree regardless of the high demand for its products.

Even if *V. paradoxa* is readily propagated sexually by use of seed (Holland, 1922), obtaining the seed is a problem since the fruits are now nearly all collected for butter production. Despite that, the Shea tree fruiting capacity also decreases with age (Hall *et al.*, 1996). This decrease in fruit production is in contrast with the increasing demand for these tree products both locally and globally. Such conditions have necessitated adapting cutting and tissue culture techniques as alternative to seed propagation. The generation of such new knowledge would lead to putting in place the most appropriate pathways for propagation of the Shea tree through cuttings and tissue culture techniques.

1.3 Objectives of the study

1.3.1 Overall Objective

The overall objective of the study was to contribute towards improved clonal propagation and regeneration of *V. paradoxa* subsp. *nilotica* in Uganda.

1.3.2 Specific Objectives

The specific objectives of this study were to:

- Determine appropriate culture media composition for *in vitro* regeneration of *V. paradoxa*.
- Evaluate the influence of cutting type on the rooting success of *V. paradoxa* stem cuttings.
- Assess the effects of mode of application of rooting hormones (IBA) on adventitious root formation of *V. paradoxa* stem cuttings.

1.3.3 Hypotheses

The study tested the following hypotheses:

- There is no difference in rooting performance of two cutting types.

$$H_0: \mu_{BC} = \mu_{TC} \text{ (BC = basal cuttings and TC = tip cuttings)}$$

$$H_1: \mu_{BC} \neq \mu_{TC} \text{ (where } \mu \text{ denotes treatment means)}$$

- There is no difference in rooting success of the different methods of IBA application

$$H_0: \mu_B = \mu_F = \mu_{QD} = \mu_{DA} \text{ (B=24hr basal soak, F=Foliar spray, QD=Quick dip and DA=Delayed IBA application)}$$

$$H_1: \mu_B \neq \mu_F \neq \mu_{QD} \neq \mu_{DA} \text{ (where } \mu \text{ denotes treatment means)}$$

1.4 Justification of the Study

Techniques for improving fruit tree productivity such as vegetative propagation are becoming increasingly important in forestry and agroforestry for multiplication of limited seed material and for multiplication of uniform planting stock. This method has been employed successfully in West Africa and some developed countries to propagate various tree species including the Shea tree (Leakey *et al.*, 1990). In Uganda, the method of vegetative propagation has been restricted mostly to domestication of citrus and mangoes. Intrinsically, a method of vegetative propagation for indigenous fruit trees such as the Shea tree among others has been lacking.

Moreover, with the decline of useful populations of economic tree species in Africa, it is important to establish seed stands and clonal banks of such species for the creation of

improved varieties. Whereas Shea tree clonal trials have been established in some countries such as Mali where propagation experiments on Shea tree have been conducted in West Africa to variable success, Uganda's Shea tree germplasm has not received as much scientific focus aimed at developing vegetative propagation techniques. This implies that much more work in this direction is still essential across the Shea tree belt including Uganda where *V. paradoxa* ssp. *nilotica* is the main sub species.

Subsequently, the development of regeneration techniques through cutting propagation and tissue culture has been postulated to have a strong impact on the current population reduction of the species and as a way of ensuring adequate availability of germplasm in the Shea parklands of Uganda. It was also expected that this study would not only lead to rapid multiplication but also shortening the long maturity period of the Shea tree. Successful adaptation of regeneration techniques could thus potentially facilitate the establishment of a clone collection thereby making way for breeding and domestication of the species for increased fruit yield at farm level in the Shea parklands of Uganda. The improved fruit yield could also contribute to increased food security, improved nutrition and income generation among the rural communities.

As efforts towards vegetative propagation of Shea tree via stem cuttings have usually centered on the basal quick dip method (Yeboah *et al.*, 2009; Yeboah *et al.*, 2010; Yeboah *et al.*, 2011, Akakpo *et al.*, 2014), it means that merits of other auxin application methods had not yet been explored. Besides the standard practice of the basal quick dip method, there is vast evidence in literature that uncommon methods of auxin application could potentially increase plant sensitivity to exogenous auxins thereby enhancing adventitious root formation (Blythe *et al.*, 2007). Since most research had only reported rooting response and neglected influence of high auxin concentration on the subsequent bud break and establishment of rooted cuttings, a study like this one that aimed at closing these knowledge gaps by evaluating the response of *V. paradoxa* stem cuttings with other modes of auxin application is very much needed.

CHAPTER TWO

LITERATURE REVIEW

2.1 Overview of Vegetative Propagation Techniques

The concept of vegetative propagation is derived from the fact that the exact copies of the genomes of mother plants are made and continue with new individuals. This is because plants have meristematic cells that can differentiate to various organs of a new plant; an ability that makes a piece of plant (shoot, leaf, or root) to grow into a new plant (Hannah and Beniast, 2002). This ability is also termed totipotency (Ishiuchi and Torres-Padilla, 2013).

According to Hannah and Beniast (2002), vegetative propagation allows the domestication and rapid improvement of species by capturing their important traits that would have otherwise disappeared or become diluted through sexual propagation. Traits such as straightness of the bole for timber species or fruit quality can thus be maintained and exploited through vegetative propagation. Vegetative propagation methods have also been useful in the development of high value species for lucrative export markets, in which cultivars of mangoes, oranges, macadamia and apples have been developed. Moreover, the concept of vegetative propagation has also been used in the development of uniform timber trees in plantations and the development of pest resistant plant stocks by cloning tropical timber species such as *Milicia excelsa* and the African mahogany (Ofori *et al.*, 1996).

Even if long life cycle of some plants has been an obstacle for their genetic improvement, asexual reproduction techniques have allowed the shortening of breeding cycles (Trevor, *et al.*, 2002). Cutting techniques, for instance, have been widely employed to generate quality planting materials with many forest tree species such as sweet gum (*Liquidambar styraciflua* L.) and Eucalyptus species (Wendling *et al.*, 2010).

2.2 Appropriate Culture Media Composition for *In Vitro* Regeneration

2.2.1 Categories of Micro-propagation

Depending on the plant, micro-propagation involves one of the three possible strategies namely: regeneration from callus, somatic embryogenesis (embryo formation from vegetative cells), or shoot multiplication (Jain and Minocha, 2000). Regeneration from callus was first demonstrated by Miller and Skoog (1953) who showed that stem segments taken from tobacco plants could proliferate an unorganized mass of tissue, known as callus, when placed on an appropriate nutrient

medium containing cytokinin and auxin. These calluses when divided into smaller pieces and placed on fresh media would continue to grow. According to Surendran *et al.*, (2000), the type of growth depends on the kinds and quantities of phytohormones added to the medium, especially the relative levels of cytokinin and auxin. While, high cytokinin-to-auxin concentrations do result in shoot formation from callus and low ratios result in root formation, intermediate ratios would even result in continued callus proliferation. Even though callus can be produced from practically any plant, the ability of these calluses to form shoots and roots in response to phytohormone treatments varies from species to species (Einset, 1986).

By contrast, somatic embryogenesis as reported by Sutton and Polonenko (1999) has already been utilized for species in over 25 different families. Like regeneration from callus, it involves an initial stage of callus formation, in this case using a medium containing auxin as the only phytohormone. The callus is then re-cultured on a medium lacking phytohormone or on medium with cytokinin. Often, several successive passages are required before true embryos are formed (Jain and Mocha, 2000). The technique, therefore, depends (in large part) on the proficiency of the tissue culturist.

A report by Trigiano *et al.*, (1992) also indicates that the third technique (shoot multiplication) can almost be considered as the "standard methodology" as far as woody plant micro-propagation is concerned. This method starts with a growing shoot tip and uses media with high cytokinin concentrations to promote growth and to overcome apical dominance. The result of this treatment is the production of a branched shoot system, which is subdivided. Consequently, individual shoots are then used for further shoot multiplication, or are rooted (Hussain *et al.*, 2012).

2.2.2 Stages of Micro-propagation

According to Surendran (2000), plant micro-propagation involves three basic stages designated as Stage I, II and III. During Stage I, establishment of asepsis is done where an explant is cleaned, disinfected, and placed on a tissue-culture medium. The objective of this stage is to obtain a living and growing plant tissue free from microbial contamination. However, attaining aseptic cultural conditions is usually the most difficult thing to achieve in micro-propagation (Corredoira *et al.*, 2002). Stage II, also known as the stage of propagule multiplication is the period during which rapid increase in shoots or other structures that can ultimately give rise to plants takes place (Jimenez, 2005). Often, during shoot multiplication, explants respond almost immediately to the

high cytokinin concentration of the medium by proliferating new shoots. In these cases, it is possible to produce one million shoots in a year, starting from a single growing tip (de Assis, 2001).

Stage III involves all the manipulations required for establishment of tissue-culture derived plants in soil (Surendran 2000). If, for instance, shoot multiplication is used for Stage II, then Stage III would involve a rooting treatment that produces plantlets. This is then followed by a gradual process of acclimation (hardening) of these plantlets by lowering humidity and increasing light intensity of the greenhouse or outdoor environment. Hence, depending on the tenderness of the plantlets obtained from tissue culture, hardening may last two to eight weeks (Hussain *et al.*, 2012).

2.2.3 Media Composition

According to Einset (1986), a large number of nutrients are needed by tissue culture, at least in comparison to the requirements of whole plants. In addition to the expected inorganic (mineral) nutrients, media for tissue culture needs to contain sugar (e.g., sucrose, or cane sugar), at least two vitamins, and one or more phytohormones. Although whole plants can generate all of these additional nutrients internally, their production must be restricted to specific tissues; implying that localized vitamin and phytohormone synthesis is an important mechanism coordinating growth and function within plants (Ördög, 2011).

Usually, inorganic nutrients are added to media as a standard mixture of salts. The so called Murashige and Skoog salts or MS salts (Murashige and Skoog, 1962), for example, contain about 15 different salts, carefully formulated into a mixture that furnishes all of the inorganic requirements of tissue cultures, such as nitrogen, phosphorus, potassium, and sulfur. Even though the MS salts mixture was originally devised for tobacco tissue cultures, experience has shown that it is adequate for most other plants (Einset, 1986). In addition, sucrose and vitamins (thiamine, inositol, pyridoxine, and nicotinic acid) can be added separately or, alternatively, in pre-formulated mixes. While most commercial nurseries usually prefer to add every component, including each of the MS salts, separately (de Assis, 2001), the scale at which one is working determines the most economical method of operation.

According to Hussain *et al.*, (2012), the key component of tissue culture medium is the phytohormone; and specifically, the cytokinin when micro-propagation involves shoot multiplication. Even if over 200 different cytokinins are available and all seem to have similar

effects on plants, it is usually necessary to test only a few compounds to find an effective cytokinin. This is because almost all plants, for example, respond well to a medium containing the basal components plus 1 milligram per liter (mg/l) to 5 mg/l of the cytokinin N6-benzyladenine, abbreviated as BAP, BA, or, preferably, bzl⁶Ade (Jimenez, 2005).

Likewise, N6-isopentenyladenine (2iP or i⁶Ade), kinetin, and thidiazuron are usually also effective as cytokinins, though bzl⁶Ade is generally the best choice. An exception to this phenomenon involves ericaceous species such as rhododendrons, azaleas, and kiwi fruits which respond poorly to bzl⁶Ade but exhibit extensive shoot proliferation with i⁶Ade (Prinsen *et al.*, 1997).

In general, once all nutrients have been incorporated into the medium, the mixture is first supplemented with 1 percent agar and heated to dissolve the agar, and then the medium is dispensed into the culturing container. The agar provides an inert, jelly-like support that prevents the plant tissues from sinking to the bottom of the culture vessel and suffocating from lack of oxygen (Williams and Taji, 1991).

2.3 Adventitious Root Formation in Stem Cuttings

Esau (1977) distinguished adventitious root formation (ARF) from lateral root formation (LRF), as the development of roots on excised aerial plant parts (shoots or stems), or from an unusual point of origin on the plant (old roots that have undergone secondary growth). Hence, LRF can be defined as the development of roots in a typical succession from the primary tap root of a tree and its laterals still in the early stage of growth. According to Pop *et al.*, (2011), the fundamental mechanism(s) that trigger or regulate the initiation and development of adventitious roots on stem cuttings from woody species is a complex physiological, genetic and environmental process that is still largely unknown.

2.3.1 Types of Root Formation in Stem Cuttings

Two major types of root formation (preformed and wound induced) are recognized in stem cuttings. According to Hartmann *et al.*, (2002), preformed or latent root initials are present during stem development and lie dormant until stem cuttings are made and placed in the proper environmental conditions favorable for further development and emergence as adventitious roots. Examples of plants with preformed roots include willow (*Salix*) and poplar (*Populus* whose root initials often are established by the end of the season in the current year's wood and will develop from cuttings made the following season. As has also been reported by Pijut *et al.*, (2011), species with preformed root initials generally root easily from stem cuttings.

Hartmann *et al.* (2002) also recognized wound-induced roots as the major type of ARF in stem cuttings; whereby once the stem (or shoot) is removed from the plant (wounding), a series of wound responses occur and *de novo* adventitious root regeneration proceeds. Note that, at such wounded sites, sealing off of the wound (protection from desiccation and pathogens) occurs by the production of suberized protective cells. This is followed by cell division that allows a layer of parenchyma cells (callus) to form at the wound site. The use of auxin during adventitious rooting also enhances the formation of callus in addition to inducing the formation of roots (de Klerk *et al.*, 1999). Thereafter, cells in the vicinity of the vascular cambium and phloem (near the source of hormones and carbohydrates) begin to divide and initiate adventitious roots.

2.3.2 Herbaceous Models of Root Formation

After germination, flowering plants develop through additional distinct stages or phases, including juvenile, mature (or adult), and reproductive phases (Willmann and Poethig, 2005). The shift from juvenile to mature phases is economically significant to plant propagators because, in many species, it is difficult or impossible to induce adventitious roots from mature tissues. Hence, phase change from mature to reproductive stage is economically important in both herbaceous annuals and woody perennials, although with some variations (Tan and Swain, 2006). While herbaceous plants typically make a once in a lifetime decision to flower, perennials usually have a pattern of seasonal vegetative and floral development that repeats over many years (Hsu *et al.*, 2006). According to Melzer *et al.*, (2008), the ability of meristems to switch between vegetative and floral phase may be regulated by a small number of genes that also affect growth habit and life span.

Consequently, the physiology and molecular genetics of ARF can be reduced to the genetics (of the parent plant material), and physiology of auxin (regulation) and of maturation; the former subject being better researched and understood than the latter. In addition, the maturation-related loss of adventitious rooting competence in hardwood tree species is a major limiting factor to the clonal propagation of these woody species (Pijut *et al.*, 2011).

2.4 Controllable Factors that Affect Rooting of Cuttings

2.4.1 Type of Stem Cutting and Season of Collection

Stem cuttings are classified (based on physiological age of the wood from which they are taken) into: hardwood, semi-hardwood and soft wood cuttings (Evans, 1999). According to Mishra (2005), hardwood cuttings are taken from dormant mature stems of more than one year old. The semi-hard wood cuttings are usually prepared from partially mature wood of the current season's

growth while soft wood cuttings are prepared from soft, succulent new growth of woody plants. In essence, time of the year in which cuttings are taken can have dramatic influences on rooting success and could provide the key to timing of successful rooting during the year. For instance, *Chionanthus refuses*, a notoriously hard to root plant, showed high rooting percentages when cuttings were taken in the middle of its growing season (Agbo and Obi, 2007).

Apart from the easily rooted cuttings having high correlation with carbohydrate level in the stems, in some woody plants, marked differences in the chemical composition of different parts of the shoot are known to exist from base to tip (Hartmann *et al.*, 2007). This implies that variations in root and shoot production on cuttings taken from different portions of the shoot are often observed; with the highest shooting in many cases found in cuttings taken from the basal portions of the shoot. Accordingly, the influence of carbohydrate and some root promoting substances from buds and leaves have made the basal portion of shoots the most appropriate for cuttings (Luna, 2009).

2.4.2 Phase Change from Juvenile to Adolescent and from Adolescent to Mature

The maturation-related loss of adventitious rooting competence in hardwood trees is a major limiting factor in clonal forestry, since many desirable traits or characteristics (e.g., wood quality or seed production) are not expressed until the tree matures (Pijut *et al.*, 2011). According to Hartmann *et al.*, (2002), in the already difficult-to-root species, ARF from cuttings declines with the chronological age of the tree. This means that cuttings from seedling (juvenile) trees can root more readily than cuttings from mature (reproductive) trees.

Pop *et al.*, (2011) also reported that phase change can result from the expression of certain genes at specific times in the life cycle of a plant, or in response to environmental or internal signals. The length of the juvenile period in hardwood trees can range from 5 to 40 years, therefore making ARF in cuttings from selected mature trees extremely difficult and timely. On the other hand, a juvenile to mature gradient ("cone of juvenility") exists in trees where; the physiologically mature section of the tree is at the shoot tips, while the most juvenile section is at the root-shoot junction. This phenomenon also makes ARF from cuttings variable (Hartmann *et al.*, 2002). Hence, the area near the base of the tree is considered the oldest chronologically, but youngest in terms of ontogenetic age (Hartman *et al.*, 1997). This implies that, stems and branches, therefore, are oldest in maturity (capable of reproduction), but youngest in chronological age; meaning that rooting potential decreases with increasing distance from the ground.

2.4.3 Horticultural Practices for Enhancing Re-sprouting and Rooting

Several horticultural or forestry-related practices and techniques can be utilized to help promote physiological juvenility (improve adventitious root formation from cuttings) in difficult to-root tree species (Pijut *et al.*, 2011). One such practice is pruning or hedging. This is the process of cutting back the original stock plant from which cutting material is to be taken, in order to produce new physiologically invigorated (juvenile) shoots or stump sprouts that root more readily (Howard 1994). A case in point is the work of Pijut and Moore (2002) in which it was necessary to prune back a 5- to 6-year-old *Juglans cinerea*, in order to encourage sprouting of softwood stems in which 77-88% rooting success was realized.

Another practice of major importance is the manipulation of light. This is because light or the exclusion of light can be a major factor that influences the physiological and anatomical status of the stock plant (photosynthesis, levels of endogenous hormones, carbohydrate levels, cell formation and development (root primordia, lignification, etc.), and subsequent adventitious root formation of cuttings collected from these plants (Blythe *et al.*, 2007). According to Hartmann *et al.*, (2002), reducing light levels (irradiance), modifying light quality (red and far-red), and the use of supplemental lighting to extend the photoperiod (fluorescent, metal halide, or high-pressure sodium) on stock plants prior to cutting collection are several ways to increase the rooting potential of cuttings.

In addition, etiolation (forcing new shoot growth under conditions of heavy shade), banding (excluding light from that portion of the stem that is to become the cutting base), and shading (reducing light intensity) have been shown to improve the rooting potential of cuttings taken from treated stock plants (Howard, 1994). For instance, in a study conducted on 3 to 5 year old oak seedlings of *Quercus bicolor*, *Quercus macrocarpa*, and *Quercus palustris* cut back to a 2 – 4cm stem stump and subjected to; ~98% light exclusion, an IBA treatment (basal paint) and modified stool-bed technique, produced etiolated shoots. As a result, etiolation used in conjunction with this modified stool-bed technique improved adventitious root formation of all three oak species from 29%, 52%, and 25% to 64%, 83%, and 100%, respectively (Hawver and Bassuk, 2000).

2.4.4 Rooting Medium

According to Khayyat *et al* (2007), besides the type of rooting media in use which has a direct influence on rooting success of stem cuttings, choice of medium also depends on availability, size and type of container, method of watering and cost among others. Even then, the medium should

be free of infectious pathogens, weeds, pests, nematodes in addition to having good water holding capacity and good drainage (Kakoei and Salehi, 2013).

Generally, the commonly utilized rooting media are: peat moss, perlite, vermiculite, pine bark and sand (Kakoei and Salehi, 2013). Peat, as reported by Raviv *et al.*, (2002), is formed as a result of the partial decomposition of plants (such as *Sphagnum* or *Carex* species) typical of poorly drained areas (peat bogs), with low nutrients and pH, under low temperatures and anaerobic conditions. Hence, peat is particularly suitable as a rooting medium due its physical properties (slow degradation rate, low bulk density, high porosity and high water holding capacity) and its chemical characteristic of having a relatively high Cation Exchange Capacity – CEC (Bohlin and Holmberg, 2004). In addition to the above, peat can also be a conducive substrate for numerous soil-borne diseases and its sterilization does not solve the problem as it leaves a biological vacuum that can be easily filled by pathogenic fungi.

In particular, perlite and vermiculite are inert potting media that can be used to improve moisture retention and aeration. Vermiculite is an aluminum-iron-magnesium silicate that can soak up 3 to 4 times its volume in water; while at the same time attracting plant nutrients such as potassium, magnesium, calcium and phosphorus (Handreck, 2005). Because it is an amorphous volcanic rock that is rich in silicon and due to its porosity; perlite usually allows excess water to drain more readily than vermiculite thereby improving soil aeration (Schundler Company, 2003). Despite having good attributes, the cost and availability of perlite and vermiculite limit their usage as rooting media (Landis and Morgan, 2009).

According to Dickens *et al.*, (2009), the use of either pine bark or sand has been suggested in instances, where the cost of peat, perlite or vermiculite is prohibitive. While pine bark is a growing medium capable of suppressing root-rot fungi; implying a reduced need for fungicides (Castillo 2004), it also has low reserve water and drains very slowly. Indeed, characteristics such as; low cost, relatively high bulk density as well as ability to improve drainage and aeration make sand a favorable growing medium.

Typically, different rooting media are used singly or in mixed ratios depending on their properties. This is so because a medium with small pore spaces becomes highly saturated with water which blocks the pathways by which gases could exchange with the atmosphere (Brady and Weil, 1999). Thus, to improve the aeration of such a medium, Carter and Slee (1991) recommended the inclusion of coarse mineral components such as perlite or coarse sand.

2.4.5 Propagation Chamber Parameters

Greenhouse parameters such as humidity, temperature, mineral nutrition and pH can be important factors in the success or failure of adventitious root formation (ARF) in cuttings (Pijut *et al.*, 2011). According to Mateja *et al.*, (2007), maintaining an adequate level of humidity can minimize physiological stress of leafy cuttings of some woody species. In particular, green house air temperatures ranging from 21°C to 27°C and rooting media temperatures ranging from 18°C to 25°C have been successfully employed for rooting cuttings of most tree species (Hartmann *et al.*, 2002).

Although a pH range of 5.5 to 7.5 has been reported to be optimum for root growth, specific plants, such as *Azalea* or *Pinus* seedlings, actually require low soil pH. Such plants are often iron inefficient, meaning that they require low soil pH to aid in the uptake of iron from the soil (Goatley and Hensler, 2011).

2.5 The Use of Auxin as a Plant Growth Regulator

2.5.1 The Invention of Auxins

The discovery of auxins as plant growth regulating chemicals and early research into their physiological effects and practical applications form a fascinating story of horticultural research (Tincker, 1936). From these studies, the finding that auxins could stimulate adventitious rooting in cuttings was a major breakthrough for commercial plant propagation (Arteca, 1996.). In the 1930s. Thimann and Koepfli (1935) reported the synthetic preparation of indole-3-acetic acid (IAA), a naturally occurring auxin that had been found to have root-forming properties, and demonstrated its practical use in stimulating root formation on cuttings. In the same year, the synthetic auxins indole-3-butyric acid (IBA) and 1-naphthaleneacetic acid (NAA) were shown to be more effective than IAA for rooting of stem cuttings (Zimmerman and Wilcoxon, 1935). Indole-3-butyric acid was later found to occur naturally in plants as a conversion product of IAA, at lower concentrations than IAA (Ludwig-Müller and Epstein, 1994; Ludwig-Müller *et al.*, 1993).

At present, IBA and NAA are the most widely used auxins for promoting root formation on stem cuttings. Apart from promoting root formation, auxin treatments are commonly used in commercial plant propagation to increase overall rooting percentages, hasten root initiation, increase the number and quality of roots, and encourage uniformity of rooting (Hartmann *et al.*, 2002; Macdonald, 2006). Chadwick and Kiplinger (1938) demonstrated that, depending upon species, auxin treatment could enhance rooting percentage and quality of the root system.

Even with or without IBA and NAA application, plants normally propagated by softwood cuttings could still be rooted in less time than non-treated cuttings implying that auxin treatment may not similarly benefit difficult to propagate species; thereby providing an opportunity for further research (Blythe *et al.*, 2007).

2.5.2 Physiological Response of Cuttings to Applied Auxin

a) **Timing and concentration of auxin:** Optimal rooting response by cuttings typically occurs when auxin is supplied soon after cutting preparation. In contrast to the standard practice of applying auxin to cuttings soon after their preparation, Luckman and Menary (2002) established that root initiation can also occur if auxin is applied several weeks after placement of cuttings in a growing medium. This observation was made when IBA was applied 4-5 weeks after difficult-to-root cuttings of shining gum (*Eucalyptus nitens*) were inserted into the rooting substrate. In the same experiment, auxin treatment immediately after preparation of the cuttings had no significant effect in comparison with non-treated cuttings.

According to Grossmann (2000), high concentrations of applied auxin are often damaging and can produce a variety of growth abnormalities within 24 hrs after treatment. These defects include: leaf epinasty (downward curvature), stem curvature, intensified green leaf pigmentation, and growth inhibition and reductions in stomatal aperture. These changes are followed by accelerated foliar senescence, chloroplast damage, destruction of membranes and vascular system integrity, necrosis, and plant death.

b) **Absorption of auxin:** Effectiveness of exogenous applications of auxin in promoting rooting on stem cuttings is dependent on adequate absorption by plant tissue. Absorption of auxin solutions at the base of stem cuttings can be influenced by auxin concentration and treatment duration, with increasing concentration and duration providing greater uptake (Howard, 1985). According to Gouws *et al.*, (1990), the amount of time between cutting collection/preparation and treatment can have an influence on absorption, with greater water loss from the cutting base prior to treatment increasing the suction that develops at the base of the cutting. Furthermore, depth of treatment can also affect absorption and subsequent rooting response, with increased depth providing additional solution that can run down the epidermis and be absorbed through the cut base (Blythe *et al.*, 2007).

On the other hand, absorption of auxin solutions by plant foliage can be strongly affected by the physical structure of the foliage itself. As has also been reported by Xu *et al.*, (2011), waxy leaf

surfaces are more difficult to wet, causing applications of auxin at a given concentration to be less effective than on plants with leaves that wet easily.

c) Translocation of auxin: Effectiveness of exogenous applications of auxin in promoting rooting on stem cuttings depends mainly on adequate translocation from the site of application to the site of adventitious root initiation. Accordingly, auxin translocation can occur in three primary manners: basipetally by polar transport; passively through the phloem; and acropetally in the xylem with exogenous application. This shows that auxin is unique amongst plant hormones in exhibiting a polarity (unidirectional nature) in its movement within plants (Bennett *et al.*, 1998). As reported by Taiz and Zeiger (1998), the polar transport of auxin occurs primarily in a basipetal direction, with the shoot apices serving as the primary sources of endogenous auxin for the entire plant.

Subsequently, polar transport can contribute to the creation of an auxin gradient from the shoot to the root, with the auxin gradient affecting various physiological processes. Among the naturally occurring auxins, only IAA and IBA are translocated via polar transport (Srivastava, 2002). While biologically active synthetic auxins (such as 1-NAA) also show polar transport, biologically inactive analogs (such as 2-NAA and 2, 6-dichlorophenoxyacetic acid) do not exhibit polar transport (Blythe *et al.*, 2007).

Recently, however, it has been demonstrated that most of the auxin synthesized in mature leaves appears to be transported to the rest of the plant in a non-polar direction through the phloem along with carbohydrates and other components of the phloem. This means that phloem transport of auxin is primarily passive and occurs at greater velocities than with polar transport in other plant tissues (Taiz and Zeiger, 2010).

2.6 Common Methods of Auxin Application

According to Blythe (2007), three methods of applying auxin to stem cuttings have been most common in commercial horticulture over the past 70 years. These are: the basal quick-dip (concentrated-solution dip or quick-dip) method; the powder (talc or dust) application method; and the dilute soak (dilute-solution soaking) method. The basal quick-dip and powder application methods tend to be most common, with the quick-dip generally considered to be the superior method of the two (Dirr and Heuser, 1987).

In the basal quick-dip method, the bottom 0.5 to 2cm of the stem cutting is dipped into a concentrated solution (500 to 10,000 ppm) of synthetic auxin(s) for 1 to 5 sec prior to inserting the cutting into the rooting substrate (Macdonald, 2006). Thus, advantages of quick-dips include: speed and simplicity of application; uniformity of application (with individual or bundled cuttings); greater economy (particularly if a large quantity of cuttings is to be treated); ability to prepare a variety of final concentrations; and general uniformity of results (Hartman and Kester, 2011). On the other hand, disadvantages of quick-dips include: preparation techniques that requires experience and equipment; and auxin concentration which can change during extended use due to evaporation of alcohol or dilution with water – from wet cuttings or other sources (Blazich, 1988).

As reported by Blythe, (2007), the powder application method usually involves dipping the basal portion (0.5 to 2 cm) of a stem cutting (often pre-moistened to enhance adhesion) into a blend of auxin and talcum powder, followed by a light tap to remove excess powder prior to inserting the cutting into the rooting substrate. The powder application method has many advantages ranging from ease of application; no preparation required with commercial formulations; easy storage; and visible evidence of application for workers. The disadvantages of powder application include: less uniformity of application; loss of powder when inserting the cutting into the rooting substrate; limited selection of concentrations; and higher cost compared to a quick-dip when a large quantity of cuttings must be treated (Hartmann *et al.*, 2002).

Dirr (1998) described the dilute soak method as one that involves placing the bottom 1 to 3 cm of a stem cutting into a dilute solution (20 to 250 ppm) of auxin for an extended period (2 to 48 hr) in a warm (20°C) location with indirect illumination. An advantage of a dilute soak method is that it allows greater uptake of auxin by the cuttings, which may enhance rooting of some difficult-to-root species. A dilute soak method tends to be unpopular due to several disadvantages such as the process being cumbersome and requiring more time; additional space and equipment required for treating cuttings; additional planning required to ensure removal of cuttings from the solution; and results can be variable because the environmental conditions can affect the amount of solution absorbed (Macdonald, 2006).

2.7 Uncommon Methods of Auxin Application

Some uncommon methods of auxin application include: injection; acid/base pre-treatment; foliar sprays; and substrate application methods. According to Leakey (1992), auxins can also be

administered by injecting solutions of IBA or NAA into the xylem at either 10 cm from the plant base or 10 cm below the uppermost lateral shoot at 1 to 8 weeks before the cuttings are severed from the plants. Lee *et al.*, (1977) also found that rooting of hardwood cuttings can be promoted if they are dipped in either 2N sulphuric acid for 20 sec or in sodium hydroxide for 10 min prior to auxin application and placement into a rooting substrate. For instance, when stem cuttings of *Chrysanthemum*, *Begonia*, *Dieffenbachia* and *Hibiscus* were treated with foliar sprays containing aqueous solutions of IBA at 50 to 250 ppm, high quality symmetrical root systems were produced (Kroin, 1992).

According to Blythe *et al.*, (2003), although auxin-containing substrates are commonly utilized in micro-propagation (tissue culture), such a technique also has potential for propagation by stem cuttings. To demonstrate this potential, single node cuttings of *Hedera helix* and *Rosa chinensis* were inserted into plugs containing a stabilized organic rooting substrate. Results showed that cuttings inserted in rooting media treated with a low concentration of auxin (below 100 ppm) responded similarly well to cuttings that received a conventional basal quick-dip (Blythe *et al.*, 2003).

Although various odd methods of auxin application have been reported (since 1930s) to increase rooting success in a few difficult-to-root species, some of these methods still have limited application in commercial horticulture. This is because some of them are less effective or efficient than the more common techniques; having served a useful purpose in early research studies (Blythe *et al.*, 2007). Even then, such methods still remain of historical interest and a source of inspiration for further research.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Location

Tissue culture experiments (experiment 1) were conducted at Makerere University Agricultural Research Institute (MUARIK) situated in Kabanyolo (Wakiso district). Kabanyolo lies at latitude 0.4500° and longitude 32.6167° . The study on stem cuttings (experiments 2 and 3) was conducted at Ngetta Zonal Agricultural Research and Development Institute (NgeZARDI) located in Lira district, Northern Uganda ($2^{\circ}17'N$, $32^{\circ}55'E$). The district is 1,100m above sea level. Ngetta (Fig. 1) is located in the mid-northern agro-ecological zone which has an average rainfall of 1,197mm and temperatures ranging from 15 to $32.5^{\circ}C$ (UBOS, 2009). All experiments were executed between March and July, 2014.

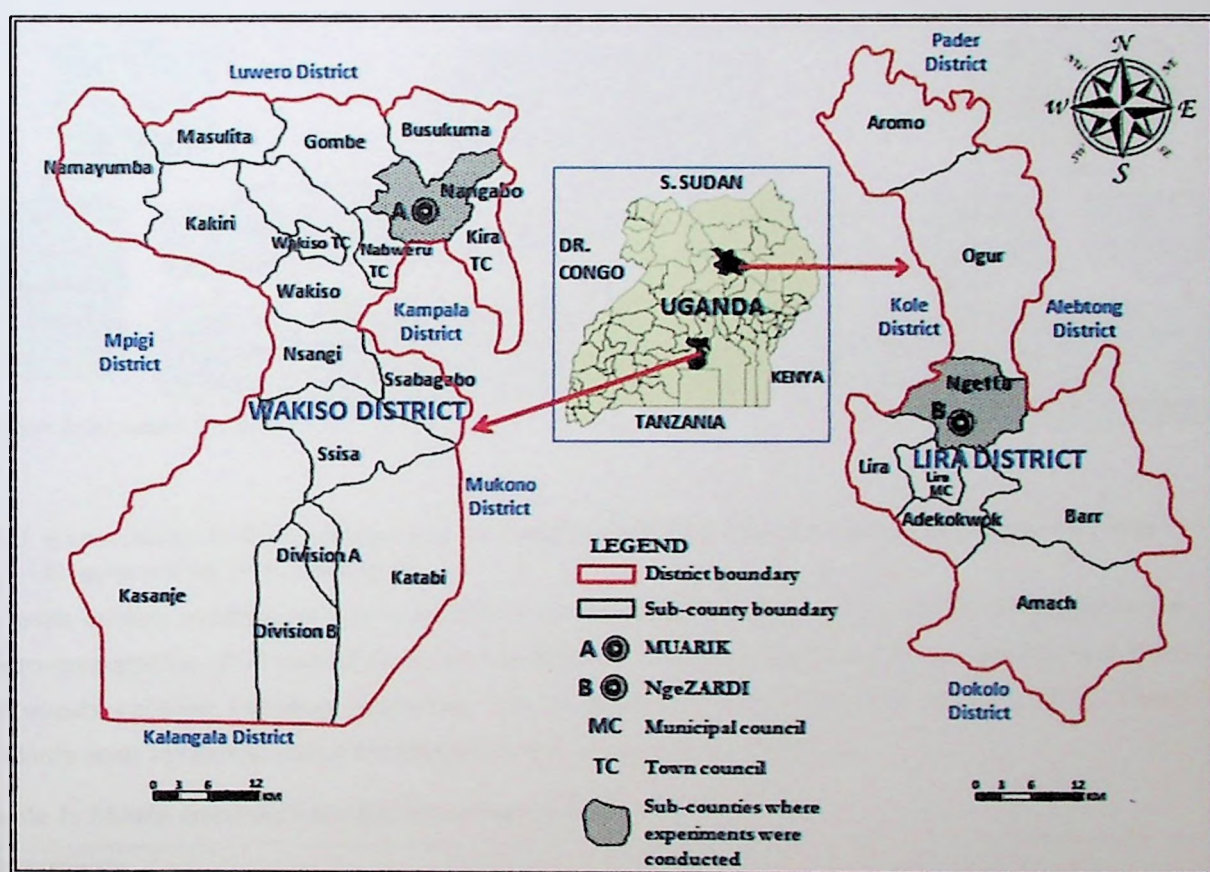


Figure 1: Map of Uganda with locations of research institutes where experiments were conducted in Wakiso and Lira districts.

3.2 Methods

3. 2.1 Research Design

In order to address the set objectives, three different experiments were designed. In the first experiment, determination of appropriate media compositions for *in vitro* regeneration of *V. paradoxa* shoot tips was carried out. For the second experiment, four modes of auxin (IBA) application at three different levels of concentration were evaluated with respect to adventitious root formation of Shea cuttings (Agbo & Obi, 2007). In the third experiment, the effectiveness of cutting type on rooting success of *V. paradoxa* stem cuttings was determined where two cutting types (basal and tip) were subjected to four different kinds of IBA treatment; 24 hr soak, foliar spray, basal quick dip and delayed IBA application (Fig. 2).

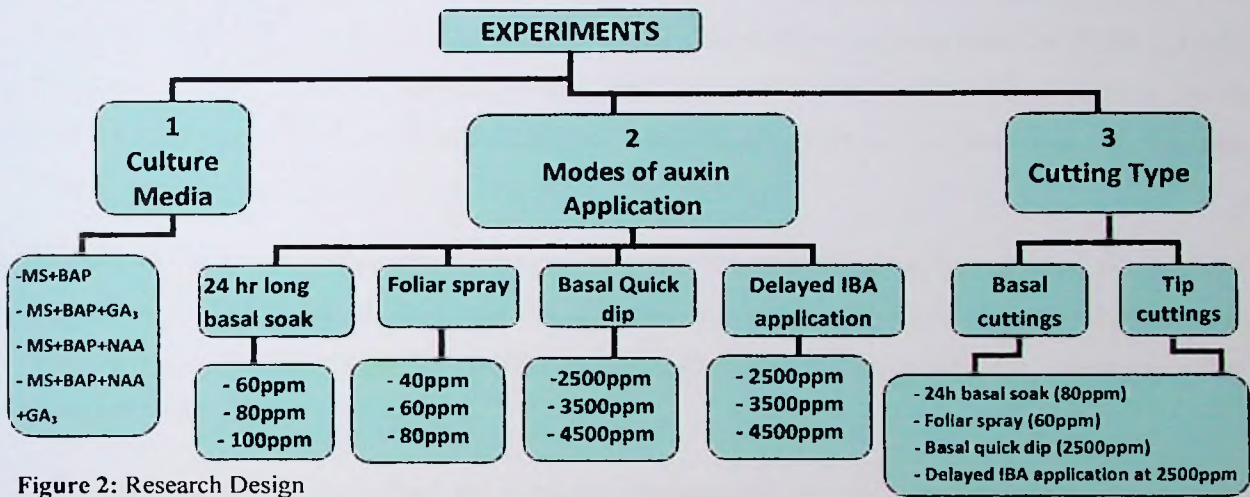


Figure 2: Research Design

3.2.2 Experiment 1: Determining the Appropriate Culture Media Composition for *In Vitro* Regeneration of *V. paradoxa*

A tissue culture experiment was established to determine the appropriate media composition for micro-propagation of *V. paradoxa* using shoot tips as explants. Stem explants were excised from vigorously growing coppices according to a procedure described by Park *et al.*, (1998). These explants were evaluated under 4 different media compositions (Table 1).

Table 1: Media compositions for experimentation

Treatments	
i) MS+1mg/l BAP,	iii) MS+0.5mg/ l BAP +0.5mg /INAA
ii) MS+ 0.5mg/l BAP + 0.5mg/l GA ₃	iv) MS+0.5mg /l BAP + 0.5M/l NAA + 0.5M/l GA ₃

Where; MS = Murashige and Skoog medium, BAP = 6-benylaminopurine, NAA = Naphthalene acetic acid

In this experiment, shoot tip explants were subjected to primary and secondary surface sterilization (Cassells and Boyle, 2005). During primary surface sterilization, explants were washed with liquid detergent (Jireh) and wiped with a piece of cotton wool for 3-4 minutes then rinsed with running tap water for 20 minutes. This was followed by placing them upright in a solution containing a fungicide at 2gm/l in sterile distilled water overnight. After that, the explants were placed in distilled water containing ampicillin at a concentration of 3 capsules per liter as an antibacterial for 2 hours (Jena and Samal, 2011).

In carrying out secondary surface sterilization, the explants were immersed in 70%v/v ethanol for 1 min, rinsed with sterile distilled water three times and placed in fungicide (mancozeb) at 3gm/l of distilled water for 30 minutes and also rinsed three times (Krishnapuram and Pandey, 2010). The shoot tips were then immersed in distilled water containing ampicillin (3 capsules per liter) for 25 minutes, rinsed three times with distilled water followed by soaking them in distilled water containing 15% commercial sodium chlorite (jik). Thereafter, the shoots were soaked in sterile distilled water containing tween-20 at a concentration of 20 drops per liter and subsequently rinsing was done three times (Smith, 2013).

The pH of the tissue culture medium was also adjusted to 5.8 before the addition of agar and autoclaved at 121°C for 20 min. For the different treatments, 20 ml of culture medium were dispensed into 25 x150 mm culture bottles, closed with aluminum foil and fastened using parafilm (Girijashankar, 2011).

After attaining aseptic conditions, the explants were reduced to 1cm in length under a laminar air flow hood where the basal (3mm) portion was inserted into an agar-based culture medium. A Completely Randomized Design (CRD) was used and the experiment was set in such a way that each culture bottle contained 2 explants and every treatment consisted of 15 culture bottles replicated 3 times. The first transfer into fresh media was carried out after 3 weeks and subsequent transfers into respective fresh media were done at 2 week intervals (Hussain *et al.*, 2012).

3.2.3 Experimental Setup and Environmental Conditions for Experiments 2 and 3

a) Preparation of Stem Cuttings and IBA

Coppice cuttings of *V. paradoxa* measuring 25cm in length were obtained from 3 months old coppices of mature *V. paradoxa* trees; wrapped in damp manila paper (Fig. 3a), placed firmly in buckets and transported to the research station. Coppice cuttings have been chosen because coppicing helps to promote physiological juvenility in difficult-to-root tree species by producing

shoots that root more readily (Howard, 1994). The cuttings were then reduced to 12cm and subjected to various IBA treatments prior to placing them firmly in a polythene propagator (Fig. 3b) containing rooting substrate. The various concentrations were prepared by dissolving IBA in 1N NaOH.



Figure 3: Illustrations: a) Packaging of cuttings in the field; b) Arrangement of the cuttings inside the propagators.

b) Rooting Media

The rooting substrate comprised of a sterile vermiculite + perlite mixture (1:2 v/v). Vermiculite was used because of its ability to hold water whereas perlite was included to improve aeration of the rooting substrate. The addition of perlite increases the amount of air and water retained by substrate mixture which in turn improves the growing conditions of the cuttings (Miller and Donahue, 1990). Since vermiculite also possesses cation exchange properties, it can hold and enhance availability of ammonium, potassium, calcium and magnesium to the growing plants. Thus, the incorporation of vermiculite promoted faster root growth and gave quick anchorage to young plant (Hartmann *et al.*, 2007).

C) Environmental Conditions

The stem cuttings were housed in wooden propagation boxes lined with black polythene. These propagators were set up beneath a black shade net that provides 75% light intensity. Because

humidity was a crucial factor during experimentation, watering was done only when humidity levels fell below 70%. This is because at high levels of humidity, cuttings appeared to deteriorate at the basal end (Fig. 4), hence affecting the survival rate of the cuttings. To this end, dehumidification was achieved by opening the propagators for about 5 – 10 minutes to obtain optimum humidity levels of 70-80%.



Figure 4: Detrimental effect of very high relative humidity

3.2.4 Experiment 2: Examining the Effects of Various Modes of Auxin Application on Adventitious Root Formation of Shea Tree Coppice Cuttings

Shoot tip cuttings measuring 12cm in length were used in a 4x3 factorial experiment (4 modes of IBA application x 3 concentration levels) with 42 cuttings per treatment (Table 2). A Completely Randomized Design (CRD) was used with three replications (Kirk, 2013).

Table 2: Treatments for the four modes of auxin application

Treatments in IBA		Concentrations of IBA (ppm)		
i)	24hr extended basal soak	60	80	100
ii)	Bi-weekly foliar spray with IBA	40	60	80
iii)	Basal quick dip	2500	3500	4500
iv)	Delayed IBA treatment	2500	3500	4500

Where IBA = Indole-3-butyric acid

The extended basal soak treatment involved bundling 21 stem cuttings together using rubber bands (Fig. 5) and placing the basal portion (2cm) of the cuttings into dilute solutions of IBA for 24hr in a room with indirect lighting prior to insertion into the rooting substrate.



Figure 5: Bundling the cuttings prior to dipping in IBA

For the basal quick dips, the basal 1 – 2.5cm portions of the cuttings were dipped for ten (10) seconds in the respective IBA concentrations before insertion into the rooting substrate. The different auxin concentrations and application techniques evaluated were based on the work of Blythe *et al.*, (2007) who explored several methods of auxin application and their effect on adventitious root formation.

Treatments did not include a control (with a no IBA treatment) mainly because previous studies conducted on Shea nut have shown poor rooting (1%) without the use of rooting hormones (Opoku-Ameyaw *et al.*, 2000; Yidana, 2004). The different concentrations were prepared by dissolving IBA in 1N NaOH.

3.2.5 Experiment 3: Effect of Cutting Type on Rooting and Bud break of Shea nut Stem Cuttings

Another set of experiment was set to assess the effect of stem cutting type (basal/tip cuttings) on the rooting ability of Shea nut stem cuttings. In this experiment, 12cm long *V. paradoxa* stem cuttings were categorized into; shoot tip cuttings and basal cuttings.

The respective cuttings were subjected to various IBA treatments (Table 3) prior to placing them firmly in a polythene propagator containing rooting substrate.

Table 3: Treatments for the two cutting types

Treatments with IBA			
i)	24h extended basal soak at 80ppm	iii)	Basal quick dip (2500ppm IBA)
ii)	Bi-weekly foliar spray with IBA at 60ppm	iv)	Delayed IBA application (with a quick dip of 2500ppm IBA) after 1 month.

Where IBA = Indole-3-butyric acid

The treatments comprised of 42 cuttings each replicated three times and set in a Randomized Completely Block design (Kirk, 2013). According to Andreini *et al* (2009) a bud break is the opening or sprouting of dormant buds. Hence, percentage bud break of cuttings was recorded at 15 day intervals and lateral bud ≥ 0.5 cm in length was counted as broken (Sun and Bassuk, 1991).

3.2.6 Data Collection

Data on tissue culture regeneration was collected on survival rate, callus development, root and shoot formation (Ling *et al.*, 2013). For experiments 2 and 3, cuttings were evaluated for rooting 120 days upon establishment and data were collected on; number of rooted cuttings, quantity and length of developed roots as described by Akakpo *et al* (2014) and occurrence of auxiliary bud break as described by Quan and Bassuk (1991) at 15 day intervals up to 120 days. All data were entered and stored into Ms Excel (2007) computer package. All cuttings with roots ≥ 1.0 cm were considered as rooted (Stenvall, 2006). Stem cuttings that remained green but unresponsive at the end of the experimental period were regarded as dormant whereas dead cuttings appeared dry and dark brown in color.

3.2.7 Data Analysis

On the whole, data were analyzed using R-system software, version 3.2.0 (Venables *et al.*, 2009). Due to high contamination and low survival rates in the tissue culture experiment, variances between the treatments were not analyzed. Instead, descriptive statistics such as proportions of dormant, callusing, rooting and surviving explants were determined (Lovett and Haq, 2013). In order to investigate the quality of rooting success (root number and root length) between four modes of auxin application on Shea stem cuttings, analysis of variance (ANOVA) was conducted (Kirk, 2013).

ANOVA was also used to examine rooting success between tip and basal cutting types. Where treatments were found to be significantly different, the Tukey's Honestly Significant Difference (HSD) was used to make pair wise comparisons (Zar, 1999). Since HSD test is able to compute

differences between means of unequal sample size, it was preferred over Fisher's LSD test (Abdi and Williams, 2010) which requires equal sample sizes. The linear relationship between rooting success and bud break was also investigated using correlation and regression analysis (Alexopoulos, 2010).

CHAPTER FOUR

RESULTS

4.1 Appropriate Culture Media Composition for *In Vitro* Regeneration of *V. paradoxa*

Survival rate of 6.7-12.2% was rather low in all the treatments (Table 4). The highest level of callus formation was observed in media containing MS+BAP (27.3±1.00%) and lowest (16.7±0.58%) in the one composed of MS+BAP+GA₃ (Table 4). The medium containing MS+BAP+GA₃ gave the highest number and percentage of dormant explants (83.33±1.15%). This was followed by culture media containing MS salts supplemented with BAP + NAA +GA₃, BAP and BAP + NAA respectively in descending order (Table 4).

Table 4: Effect of media composition on callus induction and shoot regeneration of *V. paradoxa* explants

Treatments containing MS	Dormant explants ^b (%)	Explants having callus ^b (%)	Survival ^a (%)	Rooted explants ^b (%)
BAP	72.73±2.08	27.3±1.00	12.2±1.53	-
BAP + GA ₃	83.33±1.15	16.7±0.58	6.7±2.65	-
BAP + NAA	66.67±1.53	22.2±0.58	10.0±2.00	11.1±0.58
BAP + NAA +GA ₃	75.00±1.73	25.0±1.15	8.9±2.08	-

Values represent mean±standard deviation (SD) of three replicates per treatment. ^asymbolizes percentage of original number of explants after 49 days in culture. ^brepresents percentage of all surviving explants

After two subsequent transfers, shoot formation (Fig 6b and 6c) was observed in all treatments except the one containing only BAP where several tiny whitish buds were observed. Regarding shoot formation, it is only the treatments containing GA₃ where more than one shoot was observed (Fig. 6b). In addition, these shoots appeared bigger than those of the other treatments.

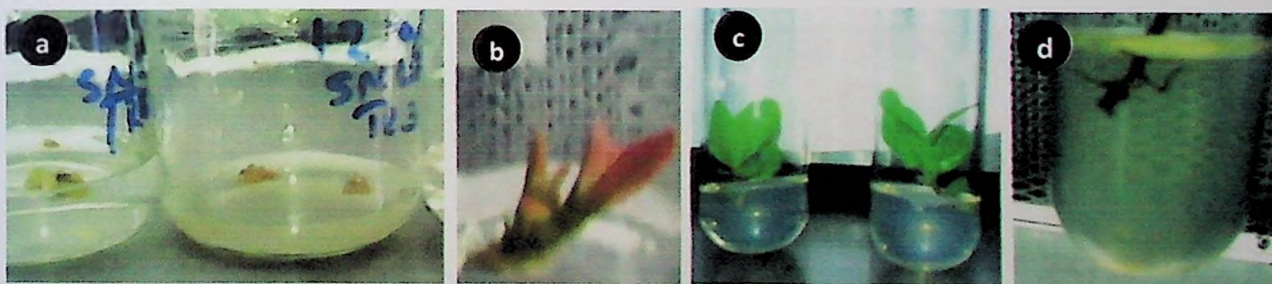


Figure 6: Responses of Shea tree explants to four media compositions (a) Callusing (b) and (c) Shoot proliferation (d) Root formation

On the other hand, root formation (11.1±0.58%) was observed only for the treatment (MS+BAP + NAA). In this treatment, tiny healthy roots were observed (Table 4, Fig. 6d)

4.2 Evaluating Effects of Cutting Type on Rooting and Bud break in Shea nut Stem Cuttings

Basal cuttings dipped for 24hr at 80ppm showed the highest rooting percentage ($69.0 \pm 3.61\%$) compared to other application methods. The basal cuttings also recorded comparatively better rooting percentage (38.1 ± 4.00 to $69.0 \pm 3.61\%$) than the tip cuttings (35.7 ± 3.61 to $57.1 \pm 6.66\%$) with the various treatments (Table 5). Mean root length for basal cuttings was higher than that of apical cuttings except for the delayed IBA treatment method and foliar spray application (Table 5).

Table 5: Effect of stem type on rooting and bud break of *V. paradoxa* stem cuttings

Treatments		% cuttings after 120 days			ARL	MRC	% bud break
		Rooted	Dormant	Dead			
Extended soak	80ppm (BC)	69.0 ± 3.61	9.5 ± 2.52	21.4 ± 4.04	14.3 ± 2.22	3.8 ± 1.40	46.2 ± 6.24
	80ppm (TC)	61.9 ± 6.24	7.1 ± 0.58	31.0 ± 3.61	13.5 ± 1.73	3.3 ± 1.16	57.6 ± 3.61
Foliar spray	60ppm (BC)	38.1 ± 4.00	21.4 ± 2.52	40.5 ± 5.57	4.5 ± 0.68	3.4 ± 1.50	3.2 ± 0.00
	60ppm (TC)	35.7 ± 3.61	31.0 ± 4.51	33.3 ± 4.93	5.0 ± 0.74	4.3 ± 0.75	8.4 ± 2.65
Quick dip	3500ppm (BC)	54.8 ± 8.62	16.7 ± 2.00	28.6 ± 3.21	8.4 ± 1.96	5.5 ± 2.39	55.2 ± 6.00
	3500ppm (TC)	47.6 ± 3.06	31.0 ± 3.06	21.4 ± 3.51	7.8 ± 3.39	5.9 ± 2.37	62.4 ± 5.57
Delayed IBA application	3500ppm (BC)	61.9 ± 6.43	28.6 ± 3.46	9.5 ± 2.65	10.3 ± 3.47	7.3 ± 2.46	67.1 ± 4.51
	3500ppm (TC)	57.1 ± 6.66	31.0 ± 4.00	11.9 ± 1.73	12.1 ± 2.90	6.5 ± 2.89	75.7 ± 6.08

All data represents means $\pm$ SD (standard deviation) of 3 replications per treatment. ARL: Average root length per cutting; MRC: Mean root count per cutting; BC: Basal cuttings; TC: Tip cuttings.

Cuttings subjected to delayed IBA application recorded the greatest degree of bud break after 120 days for tip and basal cuttings which stood at 75.7 ± 1.53 and $67.1 \pm 2.65\%$ respectively. On the other hand, foliar sprayed cuttings had the lowest level of bud break throughout the experimentation period. After 120 days, 16.7 ± 1.73 and $4.8 \pm 1.15\%$ bud break (Fig. 7) was observed for basal and tip cuttings respectively (Table 5).

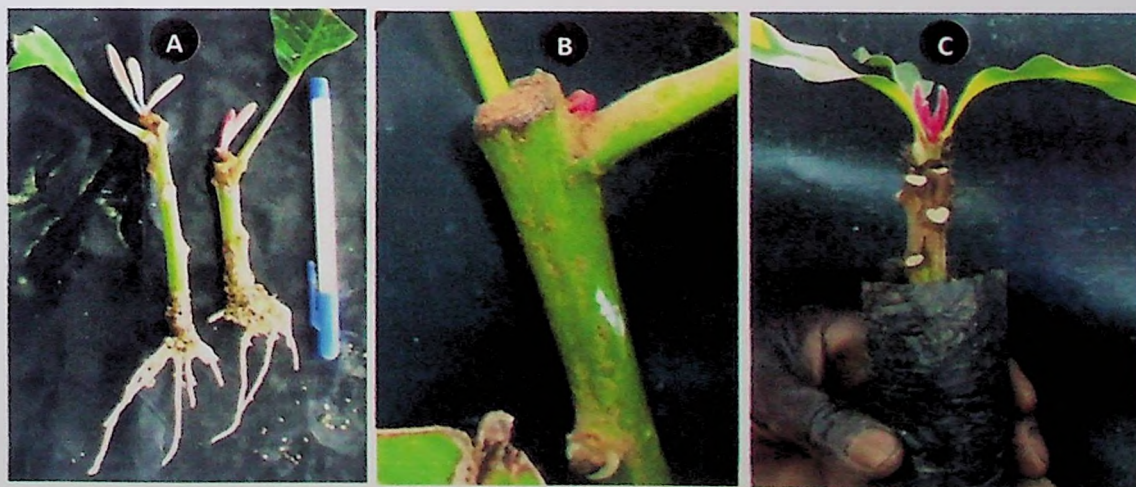


Figure 7: Rooting (A) and budbreak in basal (B) and tip cuttings (C) of *V. paradoxa*

Significant difference ($p < 0.05$) was observed between the two stem cutting types (BC and TC) with respect to MRC (Table 6).

Table 6: Analysis of variance between mean root numbers of basal and tip cuttings using four different modes of IBA application

Source	Df	Sum Sq	Mean Sq	F value	P value
Auxin mode of application	3	335.7	111.90	25.308	2.21×10^{-13}
Cutting type	1	2.1	2.10	0.476	0.491
Auxin_Mode: Cutting type	3	12.8	4.25	0.962	0.413
Residuals	155	685.3	4.42		

Generally, basal cuttings had better rooting percentage compared to tip cuttings with the four modes of auxin application evaluated (Fig. 8). On the other hand, tip cuttings recorded a higher percentage of dormant stem cuttings than basal cuttings (Fig. 8b, 8c and 8d) except with the extended soak method (Fig. 8a). Tip cuttings also recorded higher proportions of dead cuttings than basal cuttings (Fig. 8a, 8b and 8d) except for those subjected to basal quick dip method (Fig. 8c).

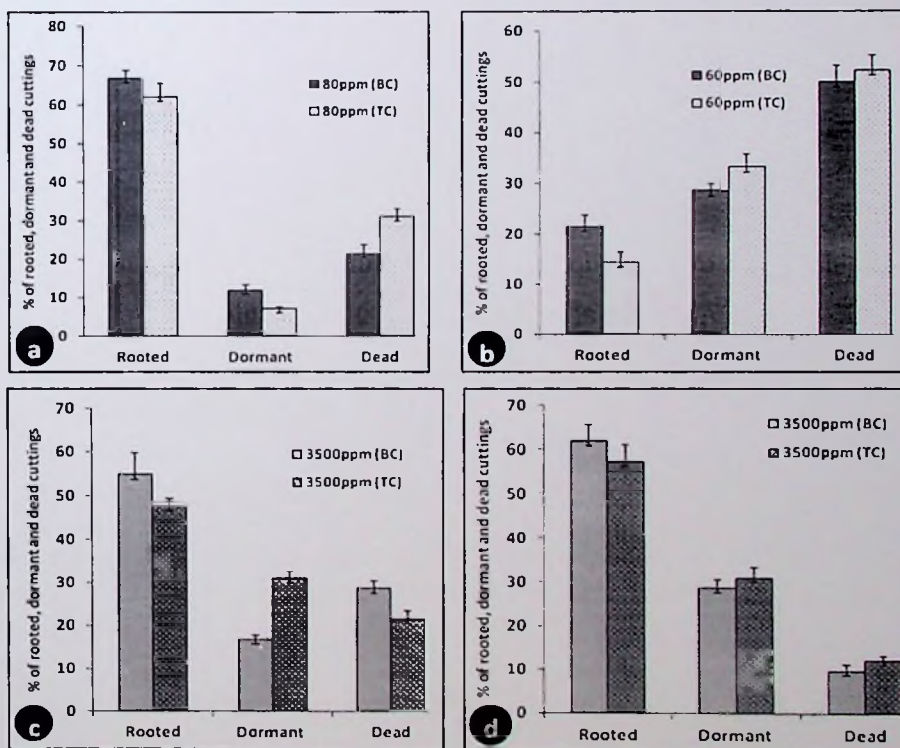


Figure 8: Effect of cutting type on rooting success of Shea stem cuttings: (A) Extended soak (B) Foliar spray (C) Quick dip method (D) Delayed IBA application. Values are means $\pm$ SE (standard error) of 3 replicates.

On the whole, percentage bud break was lower for basal cuttings compared to tip cuttings. At respective 15 day observations, but break was higher for tip cuttings implying that bud break was delayed more in basal cuttings (Fig. 9).

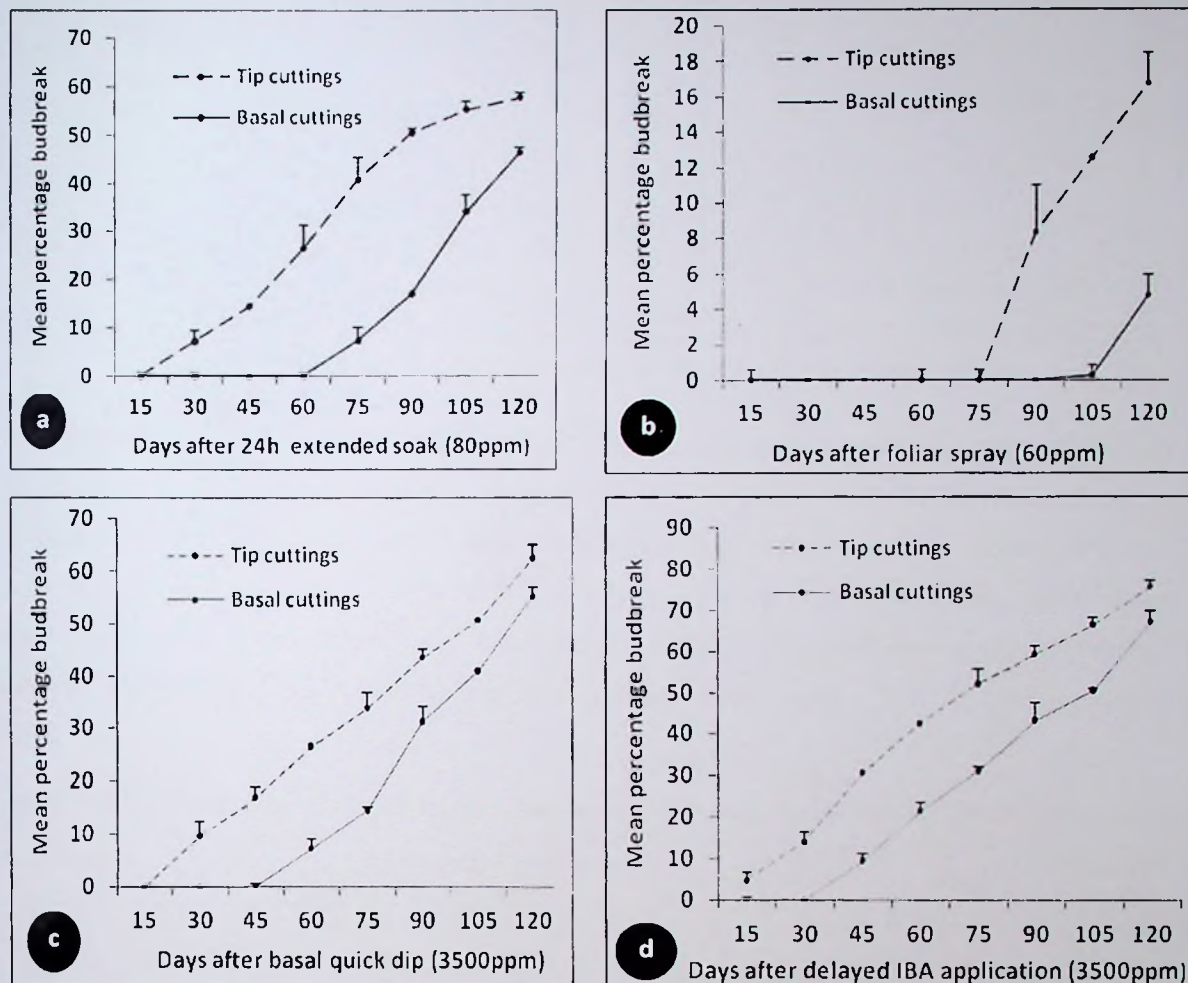


Figure 9: Effect of mode of IBA application on subsequent bud break at 15-day intervals. Each point is the average value $\pm$ SE of 3 replications per treatment.

The greatest delay in bud break was recorded for cuttings subjected to foliar sprays at 60ppm where bud break was observed after 75 days and 105 days for tip and basal cuttings respectively (Fig. 9b). Stem cuttings that were subjected to delayed IBA treatment at 3500ppm experienced the earliest bud break at 15 and 30 days for tip and basal cuttings respectively (Fig. 9d).

A correlation analysis showed that root number and bud break were positively correlated ($r=0.577$). A positive correlation ($r=0.819$) was also observed between root length and bud break (Figure 10).

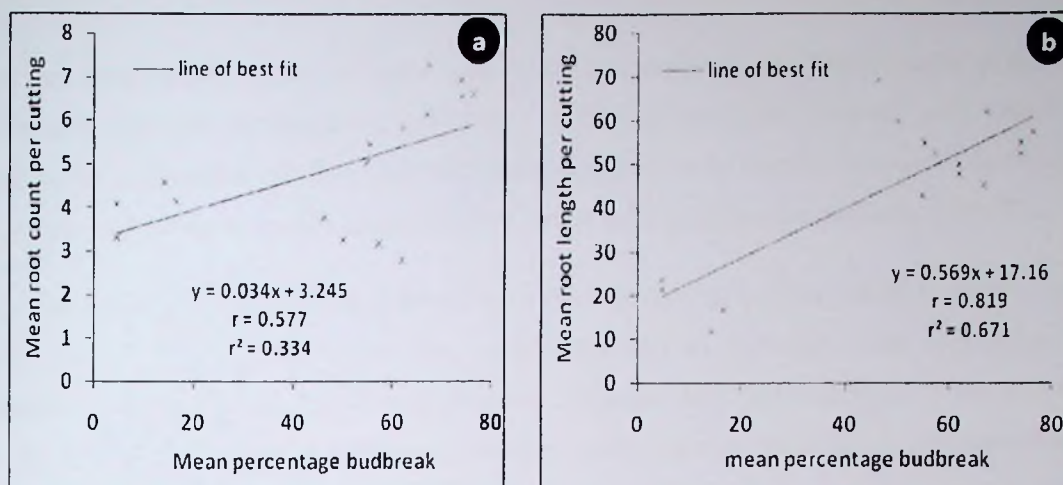


Figure 10: Scatter plots showing correlation between rooting success and budbreak A): Root number against mean percentage budbreak ($r^2=0.334$); B): root length against percentage budbreak ($r^2=0.671$).

4.3 Effect of IBA Modes of Application on Rooting Success in *V. paradoxa* Stem Cuttings

Of all the treatments, stem cuttings subjected to 24h extended soak at 100ppm rooted best ($59.5 \pm 8.33\%$). This was followed by delayed IBA application ($52.4 \pm 4.36 - 54.8 \pm 6.08\%$), basal quick dip ($42.9 \pm 7.23 - 47.6 \pm 4.73\%$) and foliar sprays ($11.9 \pm 3.06 - 23.8 \pm 4.16\%$) in descending order. At the end of the experiment, foliar sprayed cuttings exhibited the largest proportion of dormant ($26.2 \pm 3.00 - 35.7 \pm 4.04\%$) and dead cuttings ($42.9 \pm 8.96 - 57.1 \pm 11.79\%$) altogether (Table 7).

Table 7: Effect of auxin mode of application on rooting and bud break of Shea stem cuttings

Treatments		% cuttings after 120 days			ARL	MRC	% bud break
		Rooted	Dormant	Dead			
Extended soak (24h)	60ppm	50.0 ± 5.29	28.6 ± 5.00	21.4 ± 4.93	11.4 ± 2.42	2.8 ± 0.68	61.2 ± 4.58
	80ppm	52.4 ± 7.94	23.8 ± 5.29	23.8 ± 6.51	13.9 ± 1.16	3.2 ± 0.73	57.6 ± 7.55
	100ppm	59.5 ± 8.33	21.4 ± 4.00	19.1 ± 4.04	15.5 ± 1.24	3.3 ± 0.68	51.0 ± 5.57
Foliar spray	40ppm	11.9 ± 3.06	35.7 ± 4.04	52.4 ± 6.56	4.7 ± 0.87	4.5 ± 0.55	13.1 ± 3.61
	60ppm	16.7 ± 4.36	26.2 ± 3.00	57.1 ± 11.79	5.4 ± 0.85	4.1 ± 1.35	8.4 ± 0.00
	80ppm	23.8 ± 4.16	33.3 ± 4.58	42.9 ± 8.96	5.1 ± 1.00	4.1 ± 1.29	5.3 ± 0.58
Quick dip	2500ppm	45.2 ± 4.58	26.2 ± 4.36	28.6 ± 5.03	10.6 ± 2.67	6.2 ± 1.61	66.7 ± 5.57
	3500ppm	47.6 ± 4.73	21.4 ± 2.65	31.0 ± 4.36	8.2 ± 2.65	5.9 ± 2.18	62.4 ± 4.58
	4500ppm	42.9 ± 7.23	16.7 ± 5.00	40.5 ± 6.66	6.9 ± 2.36	5.1 ± 1.86	56.2 ± 3.61
Delayed IBA application	2500ppm	52.4 ± 4.36	35.7 ± 7.55	11.9 ± 2.65	11.6 ± 3.15	7.0 ± 2.01	74.1 ± 4.36
	3500ppm	57.1 ± 7.55	23.8 ± 6.56	19.0 ± 2.08	12.3 ± 2.79	6.6 ± 2.37	75.7 ± 2.65
	4500ppm	54.8 ± 6.08	21.4 ± 5.29	23.8 ± 5.86	12.7 ± 2.98	6.6 ± 2.19	73.8 ± 5.03

All data represents means $\pm$ SD of 3 replications per treatment. ARL: Average root length per cutting; MRC: Mean root count per cutting.

Cuttings that received the extended basal soak treatment produced the fewest roots ($2.8 \pm 0.68 - 3.3 \pm 0.68$), and also had the longest ($11.4 \pm 2.42 - 15.5 \pm 1.24\text{cm}$) roots. Overall, root length was observed to be a function of IBA concentration; whereby root length increased significantly ($P \leq 0.05$) with an increment in IBA concentration except for the quick dip method (Table 7).

Cuttings that exhibited the highest bud break were those subjected to delayed IBA treatment after 1 month. On the whole, bud break (Fig. 11b) appeared to decrease with increasing IBA concentration (Table 7). Delaying IBA application enhanced rooting percentage of the quick dip method by 7.1, 9.5 and 11.9% at 2500ppm, 3500ppm and 4500ppm respectively. It also enhanced mean root length and roots per cutting (Table 7).



Figure 11: Rooting response and budbreak. (a) Root proliferation in stem cuttings subjected to a quick dip in IBA at 2500ppm (b) Buddbreak of Shea stem cuttings prior to root formation (c) Root proliferation in stem cuttings treated with a 24hr basal soak of IBA at 80ppm.

Cuttings subjected delayed IBA application had the lowest number of dead stem cuttings (11.9 - 23.8%), followed by the extended soak method (19.1 - 23.8%), the basal quick dip (Fig. 11a) and foliar spraying in descending order (Fig. 12).

With the exception of foliar sprayed stem cuttings, percentage of dormant cuttings appeared to decrease with increase in IBA concentration (Fig. 12a, 12c and 12d).

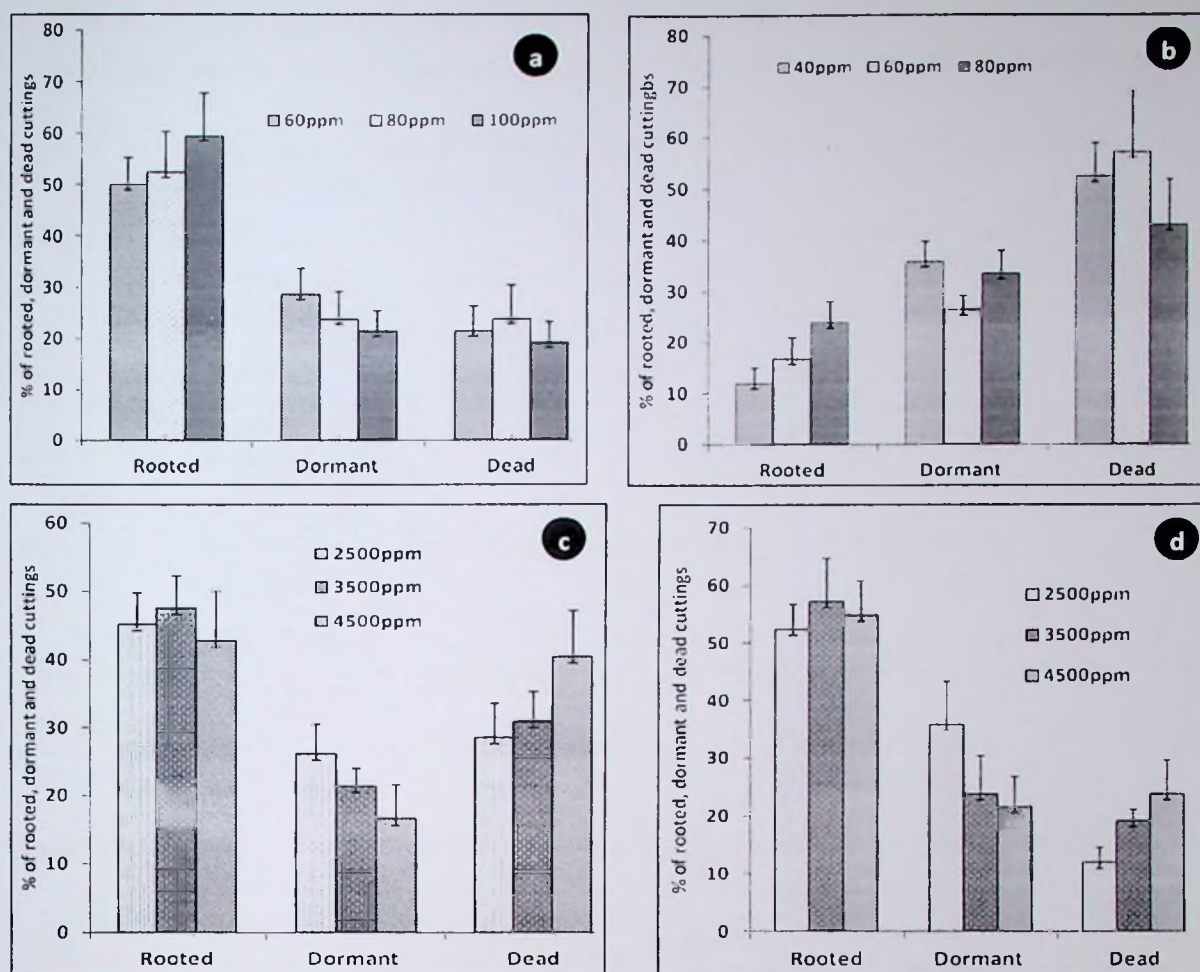


Figure 12: Effect of auxin mode of application on rooting success of shea stem cuttings: (a) Extended soak (b) Foliar spray (c) Quick dip method (d) Delayed IBA application. Each point is the average value $\pm$ SE of 3 replications per treatment.

There was a significant difference ($P \leq 0.05$) in the modes of auxin application as far as mean root count is concerned (Table 8).

Table 8: Analysis of variance between mean root numbers with four different modes of IBA application

Source	Df	Sum Sq	Mean Sq	F value	P value
Auxin mode	3	476.38	158.79	55.99	1.41×10^{-26}
Residuals	208	589.82	2.84		
Total	211	1066.20			

The differences between mean root numbers of the four modes of auxin application were significantly larger than the calculated HSD (0.93). The highest significant difference (3.61) was

recorded between the extended soak method and delayed IBA application; whereas the lowest (1.01) was between the quick dip method and delayed IBA application method (Table 9).

Table 9: Pair wise comparisons of between mean root numbers for the four modes of IBA application (Tukeys HSD test)

Treatment means	Experimental Group			
	M ₁ 3.10	M ₂ 4.23	M ₃ 5.70	M ₄ 6.71
24hr_soak (M ₁ =3.10)	0.00	1.13*	2.6 *	3.61*
Foliar spray (M ₂ =4.23)		0.00	1.47*	2.48*
Quick dip (M ₃ =5.70)			0.00	1.01*
Delayed IBA applicn (M ₄ =6.71)				0.00

*Differences larger than 0.93 are significant at $\alpha = 0.05$ level and are indicated with *.*

Mean root numbers between the four modes of auxin application were significantly different at $\alpha = 0.05$ level (Table 10 and 11).

Table 10: Analysis of variance between root lengths with four different modes of IBA application

Source	Df	Sum Sq	Mean Sq	F value	P value
Auxin mode	3	1647.09	549.03	78.86	4.17×10^{-34}
Residuals	208	1448.17	6.96		
Total	211	3095.26			

Table 11: Difference between means and significance of pair wise comparisons of root lengths with four modes of IBA application (Tukeys HSD test)

Treatment means	Experimental Group			
	M ₁ 13.7	M ₂ 5.07	M ₃ 8.57	M ₄ 12.24
24hr_soak (M ₁ =13.7)	0.00	8.63*	5.13*	1.46
Foliar spray (M ₂ =5.07)		0.00	3.50*	7.17*
Quick dip (M ₃ =8.57)			0.00*	3.67*
Delayed IBA applicn (M ₄ =12.24)				0.00

*Differences larger than 1.47 are significant at $\alpha = 0.05$ level and are indicated with *.*

The differences between mean root lengths of the four modes of auxin application were significantly larger than the calculated HSD (1.47) except the comparison between extended basal soak and delayed IBA application (1.46).

The highest significant difference was recorded between the foliar spray method and the delayed IBA application (7.17); whereas the lowest (3.50) was observed between the foliar spray and the quick dip method (Table 11).

CHAPTER FIVE

DISCUSSION

5.1 Determination of Appropriate Culture Media Composition for *In Vitro* Regeneration of *V. paradoxa*

The Shea culture survival rate of 6.7 ± 2.65 to $12.2 \pm 1.53\%$ was poor in the various media compositions investigated (Table 4). This low success rate could be attributed to the vast amount of sap that exudes from excised parts of *V. paradoxa* shoot explants. It is probable that the sap may have prevented sufficient uptake of nutrients and growth regulators into the tissues of the explants. The effect of this sap can be related with the negative influence of browning on explants' survival and response. According to Titov *et al.*, (2006), browning of surface plant tissues that emanates from the oxidation of phenolic compounds usually leads to the formation of quinones. These resulting quinones are highly reactive and toxic to plant tissue thereby forming a barrier around the tissues, preventing nutrient uptake and hindering growth (Strosse *et al.*, 2004).

In the present study, the role of the cytokinin (BAP) towards proliferation of *V. paradoxa* shoot tips was found to be essential. According to George *et al.*, (2008), when cytokinins are added to shoot culture media, apical dominance is overcome and lateral buds are released from dormancy by induction of cell division which in turn stimulates multiple shoot formation from embryos, shoot apices, or auxiliary buds. This probably explains why shoot formation in Shea culture was observed in all the media compositions investigated. Shea shoot initiation in the media containing MS+BAP was characterized by the development of tiny whitish buds. Indeed, shoot proliferation appeared to have been enhanced by the presence of other growth regulators including auxin (NAA) and (or) gibberellins (GA_3). Accordingly, fully developed shoots were observed in the Shea culture media having MS salts supplemented with BAP+ GA_3 , BAP + NAA and BAP + NAA + GA_3 .

The formation of adventitious shoots, whether directly from Shea explanted tissues or indirectly from callus seems to have been regulated by an interaction between auxins and cytokinins. It also shows that cell division has been regulated by the joint action of auxins and cytokinins, each of which could have influenced the different phases of the cell cycle. While auxins influence DNA replication, cytokinins seem to exert some control over the events leading to mitosis; implying that auxins are considered to be 'inducers' of the cell cycle while cytokinins are regarded as its 'promoters' (Pasternak *et al.*, 2000). Therefore, when cytokinins are applied in an appropriate concentration along with auxins, shoot regeneration can take place (Prinsen *et al.*, 1997).

It has also been reported by Haberer and Kieber (2002) that a high cytokinin concentration (0.5-10 mg/l) may result in proliferation of many small shoots, which typically fail to elongate. This could be the reason as to why many tiny buds were observed in the Shea culture media containing BAP only. This phenomenon can also be attributed to the absence of auxin in the growth media. While auxins can potentially inhibit cytokinin accumulation, the former can inhibit at least some of the action of the latter (Hansen *et al.*, 1985),

Even if it is well known that gibberellins are primarily used in plant tissue culture to stimulate cell division, thereby producing elongated shoots (Little and MacDonald, 2003; Hadeer *et al.*, 2014), maximum shoot proliferation has been found in Shea culture media supplemented with either 0.5 mg/l NAA, 0.5 mg/l BAP and 0.5 mg/l GA₃ or 0.5 mg/l BAP and 0.5 mg/l GA₃ in this study. A possible explanation for this is the presence of GA₃. A similar view point was also reported by Valles and Boxus (1987) where they noticed that while shoot regeneration from *Rosa hybrida* could be induced by 1-5mg/l BAP, adding 0.3-1mg/l GA₃ also can increase the number of shoots.

In addition, adding GA₃ to culture medium during the shoot elongation phase can also help to convert buds into functioning buds (Trigiano and Gray, 2005). A study carried out by Gaba (2005) on some varieties of melon and squash following induction of shoots on medium with BAP (5µM) revealed that shoot elongation was best stimulated by a medium with reduced cytokinin concentration (0.5µM) and the addition of GA₃ (1.5-3 µM). He further explained that the reduction of cytokinin concentration can permit buds to elongate when aided by the cell elongating effect of the GA₃.

Although the media containing the NAA (0.5mg/l) and BAP (0.5 mg/l) produced some roots, other media combinations did not respond in a similar way due to the presence of the auxin NAA which is capable of inducing root initiation and producing a positive rooting response on Shea stem explants (Ling *et al.*, 2013). Conversely, the presence of NAA in media containing GA₃ (0.5mg/l) and BAP (0.5 mg/l) did not produce roots probably due to the presence of GA₃ which can inhibit root formation especially when applied with auxin at the same time (George *et al.*, 2008).

Even if it is well documented that BAP is the most effective form of cytokinin (Haberer and Kieber, 2002), a considerable amount of Shea explants (66.67 ± 1.53 – $83.33 \pm 1.15\%$) were still dormant at the end of the experimentation period (Table 4), suggesting a possibility of cytokinin specificity. This means that for a particular species, different responses can be obtained if various cytokinins are applied either singly or in combination. A case in point is the work of Trigiano and

Gray (2005) on the specificity in cytokinin action when kinetin, BAP and zeatin application were not effective in the instigation of shoot cultures of *Browallia viscosa*, whereas the application of 2-ip (2-isopentenyladenine) was successful. In another experiment with *Gerbera spp.* a better shoot quality was obtained with kinetin although more rapid propagation was achieved with BAP (Trigiano and Gray, 2005). Such variations need to be tried with Shea explants too.

5.2 Evaluating Influence of Cutting Type on Rooting and Bud break in the Shea Stem Cuttings

Rooting success in the Shea tree stem cuttings varied significantly ($p < 0.05$) between the two stem cutting types. Basal cuttings recorded comparatively better rooting than tip cuttings in the extended soak and basal quick dip application methods. In addition, basal cuttings dipped for 24hr at 80ppm gave the highest rooting percentage (66.7%) compared to other application methods (Table 5; Fig. 6). The results of this present study where higher rooting percentages were obtained with cuttings taken from the basal position concurs with the work of other authors. For instance, in the work of Saifuddin *et al.*, (2013), basal cuttings of *Peltophorum pterocarpum* gave higher root biomass, root length and volume than apical cuttings. In a related study, root length and root number were significantly greater in basal cuttings than tip cuttings of *Hevea brasiliensis* (Corpuz, 2013).

This trend can be attributed to physiological differences between the two cutting types. To elucidate this point, Hartmann *et al* (2011) noted that, in many tree species, rooting ability increases from apical to basal parts of shoots: attributing this increase to the accumulation of carbohydrates at the base of the shoot. This implies that basal portions of stem cuttings have higher photosynthetic rate, stomatal conductance and transpirational rate which is largely associated with higher carbohydrate content and water absorption capacity of the new roots. Since the low photosynthetic rates and comparatively poor rooting of apical cuttings might be caused by nutritional stress (Ezekiel, 2010), higher levels of stored carbohydrates and nutrients could be the reason as to why basal cuttings in the present study gave a better rooting success than tip cuttings.

Variation in rooting percentage in this study can also be explained by the difference in chronological age between the two cutting types. According to Hartmann *et al.*, (1997), the stem area near the base of the tree is considered the oldest chronologically, but youngest in terms of ontogenetic age. The fact that stems and branches are oldest in maturity (capable of reproduction), but youngest in chronological age implies that rooting potential would decrease with increasing

distance from the ground (Pijut *et al.*, 2011). This means that the higher chronological age of the basal cuttings may have enhanced their rooting performance compared to tip cuttings in this study.

In addition, better rooting might also have been favored in basal cuttings in this study by the absence of the terminal bud. These buds are mostly located at the stem tips and have special tissue called apical meristem that consists of cells capable of dividing indefinitely (Dirk and Keil, 1996). Hence, due to apical dominance, shoot tips usually have an intense demand for sugar which limits its availability to other parts (Beveridge *et al.*, 2014). This high demand for sugar in the tip cuttings probably explains why they initiated fewer roots than basal cuttings in the current study.

As terminal buds (which are usually present in tip cuttings) may have influenced rooting success through their competition with developing roots for metabolites (Shaheed, 2002); the resulting lower rooting performance of tip cuttings in this study could have emanated from such competition. Along with having lower amounts of stored nutrients and carbohydrates, terminal cuttings may also contain flower buds that are usually unnecessary for propagation and may end up consuming valuable tissue-stored nutrients (Hartman *et al.*, 2011). That could be the reason as to why tip cuttings had a lower rooting percentage than basal cuttings in the current study.

Much as basal cuttings showed better rooting compared to the tip cuttings, percentage bud break was lower for basal cuttings at respective observations of 15 day intervals. This means that bud break (Figure 11) was generally delayed in the former than in the latter. This disparity could be due to the less-developed vascular system (in the basal cuttings) connecting to the buds and the existing xylem vessels (Sun *et al.*, 1991). A well developed vascular system is crucial to the establishment and survival of stem cuttings. Thus, establishment will usually fail if cuttings are unable to produce adventitious roots from preformed or induced primordia, expand their leaves from dormant buds, and connect vascular tissues that allocate carbohydrates alongside hormones between roots and shoots (Dickmann *et al.*, 2001). Even in the absence of terminal buds, the axillary buds present in basal cuttings need to form a vascular connection before they can grow. As this process is relatively slow in comparison with the system that connects with the terminal bud, basal cuttings in this current study delayed to bud break. Indeed, their vascular system needed more time to develop for axillary bud growth to proceed (Zalesny and Zalesny, 2009).

Although basal cuttings showed better mean root length with the extended soak and the quick dip method in this study, this trend was reversed where apical cuttings subjected to delayed IBA application and foliar spray gave higher mean root length than basal cuttings (Table 5; Fig. 7). A

similar trend was also observed by Bona *et al.*, (2012) when working with stem cuttings of *Lavandula dentata*. Thus, cuttings prepared from apical portions would have good rooting indices due to the presence of endogenous auxins and other cofactors which are concentrated at stem tips where meristematic cells abound (Hartmann *et al.*, 2002; Pio *et al.*, 2003). Subsequently, the effect of having high amount of endogenous auxins could have outweighed the low carbohydrate level of apical stem cuttings subjected to extended basal soak and foliar spray methods in this study.

Regardless of the cutting type, percentage bud break correlated positively with both the mean number of roots per cutting ($r=0.577$) and mean root length per cutting ($r=0.819$). As has also been reported by Pio *et al.*, (2006), there is usually a positive correlation between bud break and rooting in which, the higher the degree of bud break, the greater the number and length of roots. According to Sousa (2013), a strong correlation between bud break and rooting success is an indication that development of buds promotes adventitious root formation and growth. The buds are also thought to be sites of auxin synthesis. From an economic standpoint, the immediate release of buds is essential for shortening the production time and hence reducing ultimate costs of woody plants when propagated vegetatively (Hartmann *et al.*, 1997).

Significant differences ($p \leq 0.05$) obtained for rooting successes between basal and tip cuttings in the present study (Table 6) are an indication of variations between cutting types. Such variations have also been reported by a number of authors (Pio *et al.*, 2003; Bona *et al.*, 2012; Saifuddin *et al.*, 2013; Corpuz, 2013). Accordingly, decapitation of stem cuttings (which characterizes basal cuttings) usually leads to increased levels of endogenous cytokinins in the stem and/or xylem sap (Li *et al.*, 1995) and increased delivery of cytokinins to axillary buds (Turnbull *et al.*, 1997; Tanaka *et al.*, 2006). Eventually, the translocation of cytokinins to axillary buds ends up enhancing bud outgrowth leading to more bud breaks in such cuttings (Beveridge, 2006).

Even then, cytokinin synthesis is sometimes directly affected by auxin whereby increase in cytokinin content following removal of an endogenous supply is partially prevented by auxin application (Nordstrom *et al.*, 2004). This notion supports findings from the current investigation where delaying IBA application appeared to prevent inhibition of bud break by auxin even if bud break was higher in the tip cuttings compared to cuttings taken from the basal portion of the stem (Fig. 8; Table 5).

Much as cutting type has been shown to have a strong bearing on rooting success, the divergence in responses for adventitious rooting in the current investigation and other studies can also be

associated with other factors involved in the rooting process. Such factors include among others: the age of the tissue used, level of carbohydrate reserves, hormonal balance in the tissue, cell sensitivity, the presence of other molecules in the tissue and the nutritional and phytosanitary conditions of the mother plant (Sousa *et al.*, 2013), which should always be taken into account when conducting such experiments.

5.3 Examining the Effects of IBA Modes of Application on Rooting Success and Bud break in Shea Tree Stem Cuttings

The Shea tree stem cuttings subjected to 24h extended soak exhibited the best rooting performance in terms of rooting percentage and root length growth at the end of the experimental period (Table 7, Fig. 12). Generally, root length was observed to be a function of IBA concentration: whereby root length increased with an increment in IBA concentration (except for the quick dip method). This finding concurs with a report by Howard (1985) who asserted that effectiveness of exogenous application of auxin in promoting rooting on stem cuttings is dependent on adequate absorption by plant tissue. Accordingly, the absorption of auxin solutions at the base of stem cuttings can be influenced by auxin concentration, with increasing concentration providing greater uptake. Indeed, the overall superiority of the extended basal soak over other application methods in terms of rooting percentage and root length can be attributed to increased uptake of auxin by the cuttings which may have enhanced these rooting parameters (Blythe *et al.*, 2007).

Variations in rooting success following the long soak method have also been reported in other studies. Gehlot *et al.*, (2014) working with *Azadirachta indica*, reported increased rooting percentage with increased IBA concentration. Even then, root number and root length declined when IBA concentration was increased from 100 to 1,500ppm, an observation which differed from the findings of the current study. In another investigation, administration of auxin by a quick dip method was found to be superior to that of soaking stem cuttings of *Castuarina sumatrana* in IBA solution for extended periods (Tanaka, 1995). Thus, these variations in rooting success can be attributed not only to the environmental conditions during experimentation but also to differences in the species under study.

The basal quick dip method is considered as the most common way of applying auxin (Luckman and Menary, 2002; Blythe *et al.*, 2004). The results of the rooting percentage, mean length and mean root count that ranged between 42.9 – 47.6%, 6.9 – 10.6cm and 5.1 – 6.2 roots respectively for the quick dip method (Figure 13, Table 7) was found to be contrary to the finding by Akakpo *et*

al., (2014) whose work on rooting success of *Vitellaria paradoxa* stem cuttings indicated that the number of roots per cutting had increased with higher IBA concentration.

Although this was contrary to findings in the current investigation, rooting percentage in both experiments recorded similar results. The disparity could be a result of genetic variation between the different stem cuttings in the two studies. Cuttings used in the present investigation were of *V. paradoxa* ssp. *nilotica* whereas the previous study evaluated stem cuttings of *V. paradoxa* ssp. *paradoxa* (Allal *et al.*, 2011). Accordingly, varying responses to plant growth regulators can be due to intrinsic factors based on the genetics of plant materials used (Bennett *et al.*, 2003).

On the other hand, the lowest rooting percentage of between 35.7 and 38.1 %, exhibited by the foliar sprayed cuttings (Table 7), could be due to inadequate translocation of auxin (IBA) from the site of application (stem tips and leaves) to the site of adventitious root initiation. This is because exogenous auxins usually move acropetally in the xylem meaning that translocation in the xylem is unlikely to occur to any great extent when auxins are applied as foliar sprays unless when auxin is applied directly to the soil or to a cut plant part, providing direct access to the transpiration stream (Blythe *et al.*, 2007). Another line of argument by Blythe *et al.*, (2004) is that auxins applied as foliar sprays are usually not retained long enough for adequate absorption to take place.

Despite the above, the use of foliar sprays is becoming more relevant as far as mass production of planting materials from cuttings is concerned in order to minimize production costs (Eugene *et al.*, 2004). For example, at the Bailey Nurseries in Minnesota USA, single IBA applications of between 250 to 2000ppm gave satisfactory results with foliar sprays 24h after the cuttings had been stuck into propagation trays or beds. Much as applications made several days after sticking resulted in reduced rooting success, a number of species that have also responded well to foliar sprays include *acer*, *Berberis*, *Cornus*, *Diervilla*, *Euonymus*, *Forsythia*, *Hydrangea*, *Juniperus*, *Lonicera*, *Philadelphus*, *Rhus* and *Rosa* among others. Apart from reducing labour costs, foliar application of auxin at this commercial establishment has also minimized direct exposure to chemicals and the transfer of contaminants and pathogens in a communal solution of hormones (Drahn, 2007); implying that when adapted well, use of foliar sprays can also work for *V. paradoxa* subsp. *nilotica*.

Comparatively, it can be seen that foliar spray applications elsewhere (Hamza, 2014; Drahn, 2007), gave better rooting success than those recorded in this study with *V. paradoxa* cuttings. A possible explanation could arise from the frequency of auxin application. In the other

investigations, single foliar sprays were applied within 24h of sticking the cuttings into the rooting substrate whereas in the current study, bi-weekly foliar sprays were conducted for one month during the experimental period. Since repeated spraying was found to produce negative effects on root and shoot development of *Rosa spp.* (red cascade rose) cuttings (Blythe *et al.*, 2004b). This suggests that continued application of auxin may have had a negative influence on rooting success of *V. paradoxa* cuttings in this study.

Subsequently, rooting success with foliar sprays can also be related to the manner in which auxins are translocated. According to Davies (2004), when auxin solutions enter the plant through the stomata, they are captured and stored in air spaces beneath the guard cells and stomata. Eventually, the stored auxin is translocated through the vascular system where movement from cell to cell is from the leaf source towards the basal end of the cuttings. During this process, IBA is converted by the plant to IAA by slow bio-transformation and is then utilized by the cutting to produce roots at the basal end (Gao and Zhao, 2014).

Even when the use of delayed auxin treatment with stem cuttings has been rarely reported in most studies, in the current investigation: delayed IBA treatment delivered better rooting percentage ($52.4 \pm 4.36 - 57.1 \pm 7.55\%$) than foliar application ($11.9 \pm 3.06 - 23.8 \pm 4.16\%$) and quick dip method ($42.9 \pm 7.23 - 47.6 \pm 4.73\%$). It also gave the highest mean number of roots per cutting ($6.6 \pm 2.19 - 7.0 \pm 2.01$). A similar run of results was obtained by Luckman and Menary (2002) when working with difficult-to-root cuttings of *Eucalyptus nitens*. In that study, root initiation occurred in response to a 48-hr basal soak in a solution of IBA at 20 mg/liter (ppm), with the greatest response obtained when auxin was applied 4 or 5 weeks after the cuttings were inserted into the rooting substrates since early bud break (Figure 14) and shoot growth are vital for survival of propagated cuttings (Hartmann, *et al.*, 1990).

The decrease in bud break with increase in IBA concentration for all the treatments evaluated (Table 7) indicates that IBA strongly influences shoot development. A similar trend was observed by Quan and Bassuk (1991) in a study to examine the relationship between IBA concentration and bud break in *Rosa hybrida* cuttings whereby increase in IBA concentration (from 0 – 1200ppm) subsequently reduced bud break. In fact, IBA treatment remarkably reduced bud break of cuttings such that, a concentration of IBA ≥ 600 ppm almost completely inhibited bud break of cuttings during the first four weeks in that study. This decrease in bud break could be attributed to ethylene synthesis stimulated by IBA. This is so because at 1000ppm IBA can stimulate more ethylene production that is responsible for delaying bud break to a greater extent than the 500ppm

treatment. As control cuttings had the lowest ethylene level and highest percent bud break, it implies that subsequent increase of IBA concentration in the present study might have resulted in ethylene production that eventually suppressed bud break.

Significant differences ($P \leq 0.05$) that were obtained between rooting success of the four IBA application methods (Tables 8, 9, 10 and 11) is an indication that variations in rooting success can result from use of different concentrations, formulations or forms of plant growth regulators (Wendling *et al.*, 2000; Fogaça and Fett-Neto, 2005; Wendling and Xavier 2005; Almeida *et al.* 2007; Schwambach *et al.*, 2008). Such findings are usually associated with the role that auxins play in adventitious root formation.

In general, exogenous auxin application usually influences rooting success by auxins exerting their effects in plant tissues located near the region of contact with the plant growth regulator (Husen and Pal, 2007). These effects can also be related to the timing of IBA applications (Luckman and Menary, 2002) as well as its concentration (Wendling *et al.*, 2010).

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

Based on the results of this study, the following conclusions have been drawn:

- a) The use of culture media comprising of MS salts, 0.5mg/l BAP and 0.5mg/l NAA produced the most satisfactory result with *V. paradoxa* explants where both shoots and roots were formed.
- b) The extended basal soak method/mode of auxin application studied produced the best results as far as *V. paradoxa* stem cutting rooting percentage and root length are concerned.
- c) *V. paradoxa* stem cuttings subjected to bi-weekly foliar sprays produced the poorest rooting success.
- d) *V. paradoxa* basal cuttings generally rooted better than the tip cuttings and also showed delayed bud break in comparison with the latter.
- e) Delaying IBA application to *V. paradoxa* stem cuttings did not only improve the rooting performance of cuttings subjected to basal quick dips but also appeared to enhance bud break in such cuttings.
- f) The release of buds correlated positively with rooting percentage and root count implying that root formation of *V. paradoxa* stem cuttings is also enhanced by bud break.

6.2 Recommendations

The following recommendations have been made:

- i. Based on the findings from this study, it is clear that the medium containing MS salts supplemented with 0.5mg/l BAP and 0.5mg/l NAA is suitable for *in vitro* regeneration using shoot explants. However, to enhance the success rate for micro-propagation of the Shea tree, more trials need to be conducted with different media compositions.
- ii. Following the percentage rooting, mean root number per cutting and average root length of *V. paradoxa* stem cuttings, the 24h long soak method at 80ppm and delayed auxin application at 3500ppm are techniques that can be applied for mass propagation of *V. paradoxa* using stem cutting methods.
- iii. Given the economic significance of foliar spraying, use of single applications rather than bi-weekly sprays (which was experimented in this study) needs to be evaluated as well.

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