Micropropagation of *allanblackia stuhlmannii* 'clusiaceae', an economically important wild tree species

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university

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DEDICATION

I dedicate this work to my late father whose passion for education taught me patience.

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LIST OF ABBREVIATIONS

ANR	Amani Nature Reserve
B_5	Gamborg medium
BAP	6-Benzylaminopurine
2,4-D	Dichlorophenoxyacetic acid
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
ICRAF	International Centre for Research in Agroforestry
IUCN	International Union for Conservation of Nature
JKUAT	Jomo Kenyatta University of Agriculture and Technology
KEFRI	Kenya Forestry Research Institute
KEPHIS	Kenya Plant Health Inspectorate Service
KIN	Kinetin
K.U	Kenyatta University
MS	Murashige and Skoog
SNV	Stichting Nederlandse Vrijwilligers
NAA	α-Naphthaleneacetic acid
PGRs	Plant Growth Regulators
SE	Somatic embryogenesis
SSE	Secondary somatic embryogenesis
TDZ	Thidiazuron
WPM	Woody Plant Medium (Lloyd and McCown, 1980)

ABSTRACT

Allanblackia stuhlmannii is an endangered forest tree valued for its edible nut oil which has high potential for commercialization. This tree grows naturally in the Eastern Arc Mountains of Tanzania. Regeneration of *A. stuhlmannii via* seed is slow and low. Rooting of cuttings is poor, while survival rate of grafted materials is dismal. The limited regenerative potential of *A. stuhlmannii* hinders sustainable nut harvesting from the wild to meet market demand. A private-public partnership known as '*Novella Africa*' is engaged in the domestication of members of *Allanblackia spp*. for commercial oil production. To achieve mass production, the amenability of *A. stuhlmannii* to micropropagation technique was examined in this study. A series of sterilization and micropropagtion experiments were conducted on plant material collected from Amani Nature Reserve in Tanzania. Sodium hypochlorite, formaldehyde and Redomil® were the reagents used in the sterilization protocol. Explants were best surface sterilized after subjection to 2% Redomil® solution and exposure to 8% sodium hypochlorite solution for 10 minutes. Eight basal media were tested for their suitability in micropropagation of *A. stuhlmannii*. McCown's WPM which had 88.89% explants survival rate was selected for micropropagation of *A. stuhlmannii.* Microshoots were induced from shoot tips and internodal explants of *A. stuhlmannii* cultured on WPM fortified with different treatments of PGRs, ($P<0.05$). All responding explants produced a single microshoot. Treatments $1.2mgl^{-1}$BAP and $1.2mgl^{-1}$KIN had explants with the highest mean shoot length, ($P<0.05$). Prolonged culture or subculture on the same medium

did not promote further shoot production. Callus was induced from leaf discs cultured on McCown's basal medium supplemented with Gamborg's vitamins, 3% (w/v) sucrose, $1mgl^{-1}$ KIN combined with $1.25mgl^{-1}$ 2,4-D, however no somatic embryos emerged from the callus. Success in shoot proliferation and callus induction forms a basis for further research geared to regenerating *A. stuhlmannii* clonal plantlets through micropropagtion.

CHAPTER ONE

INTRODUCTION

1.1 Background

Forests are indispensable source of food, fuel, fodder, fibres, timber, preventive and curative medicine for mankind (Lovett, 1983). Most of the forest trees susceptible to over-exploitation are those that are; habitat specific, slow growing, and destructively harvested for their seeds, barks and roots (Lovett, 1983). To prevent the loss of biodiversity, especially that of over-exploited forest tree species through over-harvesting, both *in situ* and *ex situ* management programme is necessary for conservation and improvement (Leakey and Simons, 1998; Stucki, 2008). Seed planting is still the preferred mode for propagation of most agronomic crops and forest trees. Occurrence of genetic self-incompatibility and unsuitability of uniformly homogeneous seeds (characterized by loss of hybrid vigour) in some economically important trees calls for an alternative method of propagation such as vegetative propagation (Bwojwan and Razdan, 1992).

In Tanzania, *Allanblackia stuhlmannii* locally known as 'Musambu' is a forest tree with high market potential. This species grows naturally in East and West Usambara forests, Nguru forest and Uluguru Forest Mountains (Meshack, 2004). During the First World War, German soldiers in Tanzania used fat extracted from *A. stuhlmannii* nuts as an alternative edible fat to butter (Bamps *et al*., 1978; Monela *et al*., 2001; Osemeobo, 2005; Saka, 1995). For some years now, the communities

living around the Eastern Arc Mountains, particularly farmers around Usambara forest, use the oil extracted from *A. stuhlmannii* seeds for food and soap production (Meshack, 2004; Woodcock, 1995).

Regeneration of *A. stuhlmannii* via seed is slow and low. Germination typically takes 1-7 months to start and a minimum of 18 months to complete after sowing (Mwaura and Munjuga, 2011). Rooting of cuttings is poor while survival rate of grafted materials is dismal. In reference to its limited regenerative potential and dispersal powers, sustainable harvesting of *A. stuhlmannii* nuts from natural habitat to meet market demand is not feasible.

This particular tree species is now listed as 'Vulnerable' on the IUCN Red List (IUCN, 2007) because of its restricted distribution and over-exploitation. As a result, a private-public partnership known as '*Novella Africa*' is engaged in the domestication of members of *Allanblackia* genus as a new crop in a number of African countries (Attipoe *et al.*, 2006; Ramni *et al.*, 2010).

1.2 Problem statement

Allanblackia stuhlmannii is a valuable forest tree that has attracted a lot of attention in the recent past (Munjuga *et al.*, 2008; Ramni *et al.*, 2010). Regeneration of *A. stuhlmannii* via seed is slow and low. Germination typically takes 1-7 months to start and a minimum of 18 months to complete after sowing (Mwaura *et al.*, 2011). Rooting of cuttings is poor while survival rate of grafted materials is dismal. Under natural conditions, the tree begins to bloom at the age of 12 years with the fruits taking over a year to mature (Mwaura *et al.*, 2011). Rodents and monkeys eat the fruits, and hence the only mode of seed dispersal (Meshack, 2004). The limited regenerative potential and dispersal powers of *A. stuhlmannii* raises concern regarding sustainable harvesting of its seeds from natural forests (Ramni *et al.*, 2010).

Successful long-term business in *A. stuhlmannii* by-products will depend on capability to bring this species into domestication (Atangana *et al.*, 2008). For domestication to be achieved, *A. stuhlmannii* plantations have to be established alongside development of better management techniques. Novel propagation methods are therefore needed for this newly identified forest tree species. The development of a mass propagation protocol kit (micropropagation protocol) for *A. stuhlmannii* is yet to be developed.

1.3 Justification

Allanblackia stuhlmannii seed produce edible oil which can be used in margarine production with less chemical processing and refraction than palm oil (Atangana *et al.,* 2006). Already, the oil from *A. stuhlmannii* has received the approval of the European Union (EU) Novel Food Regulations that certify safe usage as a foodstuff (Hermann, 2009), clearing an important bottleneck to high future demand in EU markets (Rammni *et al.,* 2010). This tree is traditionally valued as a medicinal plant by communities living within the habitat. As a result, the wild population of this tree species is being over-exploited. Scant knowledge about the biology of this genus is available and documented. The limited regenerative potential and dispersal powers of *A. stuhlmannii* undermine sustainable harvesting and commercial utilization of *A. stuhlmannii* seeds from natural forests.

Micropropagation by axillary shoot proliferation has been reported for many hardwood species such as *Quercus rubra* (Vietez *et al.,* 2009) and *Citrus limon* (Perez-Tornero *et al.,* 2010). These trees are difficult to propagate vegetatively due to ontogenetic maturation and also seed storage is difficult hence *in vitro* propagation represents a good alternative for propagation of these tree species. Therefore, there is need for mass production of *A. stuhlmannii* planting materials using micropropagation technique in order to introduce this tree species into gardens, commercial nurseries and farms.

1.4 General Objective

To develop a micropropagation protocol that can enhance mass production of *Allanblackia stuhlmannii* for domestication programme.

1.4.1 Specific Objectives

1. To develop sterilization protocol for explants of *A. stuhlmannii* for tissue culture
2. To induce direct or via callus, organogenesis in *A. stuhlmannii* explants
3. To induce somatic embryogenesis in *A. stuhlmannii*.

1.5 Hypothesis

Allanblackia stuhlmannii is not amenable to micropropagation technique (direct *in vitro* shoot multiplication and rooting and somatic embryogenesis).

CHAPTER TWO

LITERATURE REVIEW

2.1 Taxonomy of *Allanblackia stuhlmannii*

Allanblackia stuhlmannii locally known as 'Musambu' is a forest tree classified under the following categories; **Kingdom: Plantae, Subkingdom:** *Viridaeplantae*, **Division:** *Tracheophyta*, **SubDivision:** *Euphyllophytina*, **Class**: Magnoliopsida, **Subclass:** *Dilleniidae*, **Superorder:** *Theanae*, **Order:** *Hypericales*, **Family**: Clusiaceae, **Genus**: *Allanblackia*, **Species**: ***stuhlmannii*** Engl,

Other members of Allanblackia genus include; *A. floribunda* · *A. gabonensis* · *A. kimbiliensis* · *A. kisonghi* · *A. klaineana* · *A. klainei* · *A. klainii* · *A. marienii* · *A. monticola* · *A. parviflora* · *A. sacleuxii* · *A. staneriana* · and *A. ulugurensis* (Schulman *et al.*, 1998)

Other Vernacular names of *Allanblackia stuhlmannii* include: msambu-mbwiti (Kizigua) and Mkani (Kiluguru).

2.2 Floral and fruit phenology of *Allanblackia stuhlmannii*

Knowledge about the various components of plant reproduction, such as flowering and fruiting, is essential in order to understand a plant's breeding characteristics (Mathayo *et al.*, 2009; Rodgers *et al.*, 2004). Such basic information is, however, scant for many African trees that are of high economic potential (Rodger *et al.*, 2004). In sub-Saharan Africa, a number of native forest tree species

of economic importance such as *A. stuhlmannii* are dioecious and this poses peculiar challenges for management, which requires investigation of reproductive strategies and regeneration potential (Mathayo *et al.*, 2009). Dioecious trees are more common in the tropics than in temperate regions (Bawa and Opler, 1975) and require pollination between individual trees of opposite sexes through wind or animal dispersal (Bawa and Opler, 1975; Osunkoya, 1999; Renner and Feil, 1993).

The leaves of *A. stuhlmannii* male tree are thinner compared to the female one. Branches of the female tree incline down while those of the male tree are slightly up. The male tree has buttresses and the trunk is wrinkled and grooved whereas the female tree has no buttresses and the trunk is smooth and not grooved. However this is only conspicuous when the trees are mature enough (Mbuya *et al.*, 1994; Meshack, 2004; Ndemu, 2002). The bark of the *A. stuhlmannii* tree is dark grey or black, sometimes smooth or with rough square-like scales. The slash is red with white stripes, fibrous and exuding clear exudates latex, which later turns yellowish (Gynn and Ritzl, 2000).

Under natural conditions, the trees begin to bloom at the age of 12 years (Mwaura and Munjuga, 2011). Flowering occurs during the short rainy season in November-February. Pollination is effected by short-tongued insects, birds and bats (Mwaura and Munjuga, 2011). Generally, physical resemblance of female flowers to male flowers exists, the latter of which offer multiple floral cues for attracting pollinators hence exhibit pollination-by-deceit strategy (Agren *et al.*, 1986). The fruits take over a year to mature and the fruiting period is pronounced from January

to March preceded by a smaller peak in October (Mbuya *et al*., 1994). One can tell by looking at the gum or fluids on the fruit, the ripe fruit has a crack and the colour of a ripe fruit is gray, brown. Rodents and monkeys eat the fruits and hence the significant mode of seed dispersal (Meshack, 2004).

2.3 Ecology and Distribution of *Allanblackia stuhlmannii*

A. stuhlmannii is a medium to tall sized (12-36m) evergreen mountain rain forest tree (Meshack, 2004). Its branches are drooping and often conspicuously whorled. This tree species grows naturally in East and West Usambara forests, Nguru forest and Uluguru forest Mountains (El Tahir and Mlowe, 2002; Meshack, 2004) characterized by highly weathered and leached acidic clay soils derived from granite, gneiss or siliceous rock (Glynn and Ritzl, 2000). These forests lie within an altitude range of 500-1600m above sea level and receive an annual rainfall of between 1100-2400mm (Van Rompaey, 2005). The mean annual temperature in the Eastern Usambara Mountains is 18˚C, maximum temperature range from 25˚C to 35˚C while minima are occasionally as low as 3˚C (El Tahir and Mlowe, 2002; Meshack, 2004).

2.4 Economic importance of *Allanblackia stuhlmannii*

Communities living around the Eastern Arc Mountains, particularly farmers around Usambara forest, use the oil extracted from *A. stuhlmannii* seeds for food and soap production (Meshack, 2004; Harkonen and Vainio-Mattila, 1998). They also

use dry leaves of *A. stuhlmannii* as medicinal tea against chest pain and smear heated seed oil on aching joints, rashes and wounds (Meshack, 2004). Phytochemical analysis of *A. stuhlmannnii* extracts showed that *Guttiferone F*, a prenylated benzophenone, a compound related to a group of compounds that have anti-HIV property, was present (Fuller *et al.*, 2003).

This tree species also has a great commercial potential for margarine production from its edible seed oil whose extraction requires less chemical processing and refraction than palm oil (Atangana *et al.*, 2006; Leakey and Simons, 1998). Oil from *Allanblackia* seeds consists mostly of oleic and stearic acid, 39-45% and 52-58% respectively, (Hilditch, 1958). Oleic and stearic acids are reported to lower plasma cholesterol levels thus reducing the risks of heart attack (Bonanome and Grundy, 1988). Already, the oil from *A. stuhlmannii* has received the approval of the European Union Novel Food Regulations that certify safe usage as a foodstuff, clearing an important bottleneck to high future demand in EU market (Hermann, 2009).

2.5 Propagation of *Allanblackia stuhlmannii*

A. stuhlmannii can be propagated by seed, even though the seeds are recalcitrant (Elinge and Ndayishiminye, 2003). Regeneration of *A. stuhlmannii via* seed is slow and low. Normally, well-matured fruits are kept for about 2 weeks to allow the pulp to become soft and to make extraction of the seed easy. These fruits may be kept for up to 3 months if covered with banana leaves. Clean seeds are then

placed in a nursery where they take about 3 months to start germinating. However germination may take more than 7 months to start and another 18 months to complete (Muriuki, 2006; Mwaura and Munjuga, 2011).

Due to the fact that propagation by seed is difficult and also male and female trees are very difficult to distinguish until they flower, methods of vegetative propagation are being developed (Atangana *et al.*, 2006). Vegetative propagation is possible by cuttings, marcotting and grafting. Cuttings are placed a few cm deep in soil at a 45° angle in polythene tubes with at least one node above the growth medium (Muriuki, 2006). Cuttings strike root in 8–12 weeks, after which sprouted and rooted cuttings are transferred to polybags. Methods of layering and budding are being developed (Mbuya *et al.,* 1994; Munjuga *et al.,* 2008). Domestication and establishment techniques for this genus on trials utilize the variability within species by selecting trees with desirable traits and propagating them sexually or asexually (Atangana *et al.,* 2006; Kameswara 2004).

2.6 Conservation of *Allanblackia stuhlmannii*

Propagation of *A. stuhlmannii* is actually a priority for conservation given its poor regeneration in the wild coupled with prolonged seeds germination period (Mathayo, 2009; Meshack, 2004; Newmark, 2002). Presently, female trees are often being retained when clearing land for agricultural use. It is estimated that one male tree for every ten female trees is needed to ensure adequate pollination (Atangana *et al.*, 2006; Mbuya *et al.,* 1994).

This particular tree species is now listed as 'Vulnerable' on the IUCN Red List (IUCN, 2007) because of its restricted distribution and over-exploitation. As a result, a private-public partnership known as '*Novella Africa*' is engaged in the domestication of *A. stuhlmannii* (Attipoe *et al.,* 2006 and Ramni *et al.*, 2010). Through this partnership, forest reserves in the Usambara Mountains have placed sizeable stock of *A. stuhlmannii* trees under protection (Atangana *et al.*, 2006; Hamilton and Bensted-smith, 1989). Massive input has also been channeled to seedlings production in tree nurseries in the same reserves. A complete seed marketing chain is also being developed.

2.7 The *Novella* Project and the context of the guidelines

To exploit these opportunities, the World Conservation Union (IUCN), Netherlands Development Organisation (SNV), the World Agroforestry Centre and Unilever *plc* have formed a partnership called '*Novella Africa'* to produce nuts of *Allanblackia* species' on a commercial scale (Amanor *et al.,* 2003).

Novella Africa, as a pilot project, focuses on the sustainable use of *Allanblackia* seeds. Unilever's objective is to secure a sustainable supply of *Allanblackia* seeds and also achieve the coperate goal of promoting a socially acceptable and environmentally sound market-based financing mechanism in order to safeguard biodiversity and livelihoods in the tropical forest belt in Africa (Attipoe *et al.,* 2006). *Allanblackia* has the potential to provide a novel source of household

income for rural populations while contributing to forest landscape restoration (Attipoe *et al.*, 2006).

The main objectives of the *Novella* project guidelines have been categorized as; Production of high quality products while maintaining the viability, diversity and yield of existing source trees; Enable local communities to protect and improve their well-being and environment; Minimize adverse effects on biodiversity, making a positive contribution where possible (Amanor *et al.*, 2003; Egyir, 2007).

The *Allanblackia* population is likely to be a vulnerable element of the local biodiversity to the extent that the quality of the harvested product may decline when mismanaged and also there may be erosion of the *Allanblackia* population diversity that does not have any impact on the seed production or quality thus attracting mitigation strategies (Amanor *et al.*, 2003; Nyame, 2008). Harvesting from the wild is particularly desirable if it can limit degradation of reserved ecosystems, whilst local communities can gain benefit and therefore value its survival (Cordeiro and Ndangalasi, 2007). *A. stuhlmannii* harvesting should therefore be built on existing local cultural practices that encourage sustainable management. Local initiatives to manage natural resources strengthen local stewardship of resources and encourage sustainable development (Pye-Smith, 2009).

This project aims at ensuring that; exploitation of *Allanblackia* will contribute to the maintenance of the network of protected areas and enactment of protective legislation; Coordinate sensitization programmes on the critical biodiversity conservation issues in such areas; Utilization of *Allanblackia* should not

erode the local *Allanblackia* gene pool to a position where genes are likely to be lost or the population is reduced to an unsustainable structure and promote natural and artificial *Allanblackia* regeneration (Amanor *et al.*, 2003; Attipoe *et al.*, 2006).

2.8 Micropropagation

Micropropagation is used mainly in reference to the mass production *in vitro* of clonal plants for commercial purposes, thus excluding the limited-scale regeneration of plants by embryo rescue or from ovules, microspores and transgenic tissues (Bornman, 1993). It encompasses three types of vegetative propagation. One of them is axillary shoot production from tissues containing pre-existing meristems comprising *de novo* meristem formation from callus tissue or directly from organized tissues such as epidermal or sub epidermal cells. The second one is adventitious shoot production following induction of adventitious meristems whereby axillary buds and meristems give rise to shoots that are excised and used to produce additional shoots (Pankaj and Toshiyuki, 2001). The third one is somatic embryogenesis in which structures are formed containing a shoot and root connected by a close vascular system, directly analogous to zygotic embryos in regards to histology, physiology, and biochemistry (Bornman, 1993; Lin *et al.*, 1991).

The process of micropropagation is usually divided into several stages (Stage 0 to IV) i.e. sterilization, initiation cultures, subculture of explants for shoot proliferation and root induction, and finally hardening (George *et al.*, 1984; McCown and Sellmer, 1987). Recognition of the contamination problems often

associated with inoculation of primary explants prompted Debergh and Maene (1981) to include a Stage 0. This stage describes specific cultural practices which maintain the hygiene of stock plants and decrease the contamination frequency during initial establishment of primary explants. Quality of the explants and subsequent responsiveness *in vitro* are significantly influenced by the phytosanitary and physiological conditions of the donor plant (Debergh and Maene, 1981). Induction of cellular differentiation *in vitro* however depends on totipotency, culture medium formulation, and incubation conditions (Gasper and Coumnas, 1987).

Micropropagation of most plants using explants from mature plants is not frequently accomplished mainly due to the high level of contamination (Drew, 1988), reduced or absence of morphogenetic ability (Bonga, 1982) and poor rooting of the regenerated shoots (George, 1993). Prior to culture establishment, careful attention is therefore given to the selection and maintenance of the plants used as the source of explants. Stock plants are maintained in clean, controlled conditions that allow active growth but reduce the probability of disease infection (Drew, 1988). Initiation and aseptic establishment of pathogen-free responsive terminal or lateral shoot meristems explants is very important at Stage I (Pankaj and Toshiyuki 2001).

The cultured cells and tissue can take several pathways (McCown *et al.*, 1987; Salvi *et al.*, 2001). The pathways that lead to the production of true-to-type plants in large numbers are the preferred options for commercial plant multiplication (George and Sherington, 1984). This system has been adopted by most commercial

and research laboratories as it simplifies production scheduling, accounting, and cost analysis (Pankaj and Toshiyuki, 2001).

Numerous examples of successful application have been reported for; meristem culture; organogenic micropropagation from undifferentiated tissues; cells or protoplasts; zygotic embryo culture; somatic embryogenesis; and gametic embryogenesis (Thorpe *et al.*, 1990). Meristem cultures have been employed to eliminate plant virus in some asexually propagated species and commercially (both meristem and bud cultures) for multiplication of some high value genotypes (Bhajwan and Razdann, 1992). Zygotic embryo culture has been successfully employed to circumvent post-fertilization and cross-incompatibility during interspecific transfer of genes among related plant species for many years (Pankaj and Toshiyuki, 2001).

Recently embryo rescue techniques have also proven useful in recovery of haploid plants that emerge from chromosome elimination in zygotic embryos following interspecific crosses (Bajaj, 1983). Regeneration of plants from callus, individual cells and protoplasts has involved organogenic or embryogenic differentiation (Gamborg *et al.*, 1974; Scowcraft *et al.*, 1987). These techniques have been useful in providing both spontaneous (Scowcraft *et al.*, 1987) and mutagen induced genetic variation (somaclonal/gametoclonal/protocol variance). Embryogenesis has played a significant role in recovery of haploid plants from culture anthers or microspores (Scowcraft *et al.*, 1987). Thus, considerable progress in micropropagation technologies is evident and their applications in crop plants are

diverse and significant (Goyal *et al.*, 1985; Pankaj and Toshiyuki, 2001; Zimmerman and Jones, 1991).

2.9.1 Plant Growth Regulators used in Plant micropropagation

The use of plant growth regulators in plant micropropagation is of fundamental importance (Bennet *et al.*, 1994; Kumar *et al.*, 1997). Knowledge of the specific sites of hormone biosynthesis in intact plants provides insight into the relationship between explant size and dependence on exogenous growth regulators in the medium (Einset and Skoog, 1973; Zaerr and Mapes, 1982). Endogenous cytokinins and auxins are synthesized primarily in root tips and leaf primordia respectively (Zaerr and Mapes 1982). Consequently, smaller explants, especially cultured apical meristem domes exhibit greater dependence on medium supplemented with exogenous cytokinin and auxin for maximum shoot survival and development (Murashige, 1977). Larger shoot tip explants usually do not require the addition of auxin in stage I medium for establishment. For many years, particularly herbaceous and woody perennials, consistency in growth rate and shoot multiplication is achieved only after multiple subculture on stage I medium (Murashige, 1977).

Physiological stabilization may require from 3 to 24 months and 4 to 6 subcultures depending on plant species (Murashige, 1977). Failure to allow culture stabilization, before transfer to stage II medium containing higher cytokinin levels, may result in diminished shoot multiplication rates or production of undesirable

basal callus and adventitious shoots (Murashige, 1977). In some species, the time required for stabilization can be reduced by initial culture in liquid medium (McCown and Sellmer, 1987).

Multiple shoot formation in tissue culture can be achieved in media containing average levels of cytokinins as demonstrated in *M. alternifolia* by List *et al.,* (1996). Low cytokinins levels result in production of relatively fewer shoots whereas stem segments fail to form multiple shoots in cytokinin-free media (List *et al.,* 1996). Superiority of 6-Benzylaminopurine over Kinetin in micropropagation has been shown in *Maytenus emarginata,* (Rathore *et al*., 1992) and *Tecomella undulate,* (Rathore *et al*., 1991). *Morus alba*, a Japanese woody species multiplied readily on Woody plant medium supplemented with BAP (Karkonen *et al.,* 1999). The effectiveness of cytokinin in promoting axillary bud development *in vitro* in forest trees is well documented by Joshi (1991).

2.9.2 Regeneration from meristem

This is rather a direct pattern involving the excision of a shoot tip or meristem explants from either terminal or lateral bud of the desired parent plant followed by surface sterilization and subsequent culture of explants on a defined medium (Nehra and Kartha, 1994). As explants in culture develop into new shoots, they adopt a different inherent programming that determines the subsequent pattern of growth (George and Sherrington, 1984).

Plant growth regulators added into the best nutrient medium can also induce this programming (Von Arnold and Hakman, 1988). The entire method of micropropagation using shoot tips is based on cytokinin-induced outgrowth of preformed axillary bud until the desired number of shoots is formed (Nehra and Kartha, 1994). Once a cluster of shoots is formed, it's dissected into single shoots and each transferred to a fresh medium to continue the cycle of multiplication (Nehra and Kartha, 1994). The rate of multiplication varies greatly from species to the other, but it is often possible to obtain several millions of plants in one year starting from a single isolated shoot tips (Williams and Dodds, 1983). Cytokinins applied to whole plant rarely produces anything more than small and transient effects on the growth of axillary shoots but its inclusion into nutrient media generally has marked effect of promoting the outgrowth of axillary buds on the cultured shoot tips (Yeoman,1986).

The application of shoot proliferation technique for clonal multiplication was first realized by Morel in 1960 during his research on the propagation of orchid cymbidium. His technique has since then been modified and is currently used for commercial production of orchids. The progress in micropropagation technique using multiple shoot formation is largely attributed to Murashige (1974). Today, tissue culture technology is in its widest application with over 300 commercial operations worldwide producing ornamentals (Pankaj and Toshiyuki, 2001).

2.9.3 Regeneration from callus tissues and somatic embryos

Regeneration of plants from callus is an indirect pattern involving initiation and induction of callus formation on an explant, a non-meristematic part of a plant (Merkle *et al.,* 1998). In nature, callus formation is often a plant's reaction to a wounded plant tissue (Braun, 1954) or by insect feeding on the wounded plant tissue (Pellet *et al.,* 1960). A callus consists of an amorphous mass of loosely arranged, thin walled parenchyma cells arising from the proliferation of cells of the cultured explants (Yeoman and Macleod, 1977). Callus formation may be induced in many different plant parts even those that do not form callus in response to injury (Merkle *et al.,* 1998).

Establishment of callus from explants can be divide into three developmental steps namely; induction, cell division and differentiation (Gomez and Segura, 1994; Yeoman and Macleod, 1977). In the induction phase, metabolic activities are stimulated prior to mitotic activity. A phase of active cell division follows as the explant cells revert back to meristematic state. Finally, cellular differentiation phase appears and certain metabolic pathways leading to the formation of secondary products are expressed (Yeoman and Macleod, 1977).

The growth characteristics of callus involves a complex interaction of the plant tissues used to initiate callus, medium composition and the environmental conditions during the incubation period (Atchison *et al.,* 1997). It is important to recognize that the culture conditions act in a selective manner, favouring certain cells which are best suited to that particular culture environment (Murashige, 1974). Calli

of different species and even of the same species differ markedly in texture and coloration. For instance, callus initiated in light can be pale yellow or pigmented while callus initiated in the dark is white to pale yellow (Murashige, 1974). The texture can also vary from compact to friable and from smooth to very nodular.

The hormonal requirements for the initiation of the callus depend on the origin of the explants tissue (Kevers *et al.*, 2002; Yemets *et al.*, 2003). Most excised tissues require the addition of one or more growth regulators to initiate callus formation (Atchison *et al.*, 1997). Generally, levels of PGRs particularly auxin higher than those necessary for stimulating the direct formation of adventitious shoots give rise to proliferation of callus from the explant material (Bennet *et al.*, 1994). Adventitious shoots or embryos may be formed from callus if the concentrations of growth regulators especially auxins is lowered (Becwar *et al.*, 1988). There is no clear line of difference between the formation of adventitious shoots via intermediate callus or via embryogenesis, both processes may occur simultaneously on the same explant or different explants from the same organ (Yeoman, 1986).

Growth of callus on the same media for a period of time results in the depletion of the essential nutrients and a gradual desiccation of the gelling agar (Dyson and Hall, 1972). Metabolites are also released from the callus and may accumulate to toxic levels in the medium and end up killing them (Dyson and Hall, 1972). Therefore, successive sub culturing is usually performed after every four to six weeks with cultures maintained in agar at the appropriate environmental

conditions (Yeoman and Mcleod, 1977). With continued sub culture of callus, certain changes often occur. One such change is the loss of a phytohormone requirement for growth. Some tissues are auxin-independent while others are cytokinin-independent or both (Dyson and Hall, 1972; Einset and Skoog, 1973). Although callus can be maintained in a proliferative state indefinately, reorganization can be experimentally induced (Nair and Gupta, 2006). These include tracheary element formation or primordia and embryo formation, which ultimately lead to plantlet regeneration *in vitro* (Einset and Skoog, 1973). To produce organs or embryos *de novo,* manipulation of the cultures is required.

Somatic embryogenesis (SE) is usually favoured over other methods of plant regeneration *in vitro* as it allows propagation to be scaled up using bioreactors and cryopreservation of somatic embryos and/ or whole embryogenic cultures, which in turn makes it possible to establish gene banks. Besides its practical value, SE is useful in basic research on totipotency and on the fundamental processes underlying plant morphogenesis. In the genus Trifolium, comprising about 250 species (Gillet 1985), numerous protocols for plant regeneration via SE have been developed. Somatic embryos have been induced from different explants of almost all economically important clovers, including T. repens (Maheswaran and Williams 1984; Parrot 1991), T. pratense (Phillips and Collins 1980; Maheswaran and Williams 1986), T. fragiferum (Rybczyn´ski 1997), T. incarnatum (Pederson 1986), T. vesiculosum (Pederson 1986), T. ambiguum (Pederson 1986), T. rubens (Parrot and Collins 1983), T. medium (Choo 1988), T. respupinatum and T. subterraneum

(Maheswaran and Williams 1986). Webb et al. (1987) were the first to report the production of embryo-like structures in callus cultures of T. nigrescens. Later, Konieczny (1995) presented a method for whole plant regeneration via SE from cotyledon- and hypocotyl-derived callus of young T. nigrescens seedlings.

In somatic embryogenesis, auxin and nitrogen content play crucial roles (Kohlenbach, 1978). Studies have shown that the process of somatic embryogenesis normally takes place in two stages. First, induction of cells with embryogenic competence (proembryos or proembryogenic tissue) in the presence of high concentrations of auxins is initiated. Secondly, development of embryogenic masses into embryos in the absence of or presence of low auxin concentration preceeds (Nadar *et al.*, 1978; Sung *et al.*, 1979).

In a survey of media used to bring about somatic embryogenesis, Evans *et al.*, (1981) found that Murashige and Skoog medium or a modification was used in 70% of the successful cases and that 2,4-D in a concentration of 0.5-27.6µM was used in 57% of the primary cultures (induction of embryogenetic tissue). NAA was used in about 25% of the cases and other auxins much less frequently. Regarding the role of nitrogen, Helperin (1966) was the first to recognize the importance of reduced nitrogen in the form of NH_4^+ for somatic embryogenesis. Wetherell and Dougall (1976) showed that as little as 0.1mM ammonium chloride added to nitrate significantly enhanced embryogenesis in carrot. In organogenesis *via* callus, localized active cell division (presumed to begin in a single cell in the callus mass) leading to the organization of a meristemoid is usually observed (Torrey, 1966).

These meristem-like cells, under light microscope appear non-vacuolated (Murashige, 1974 and Torrey, 1966).

2.10 *In vitro* propagation of woody plant species

Tissue culture has been used for cloning superior genotypes and breeding programmes of woody perennials (Karnoski, 1981). Micropropagation of tree species offers a rapid means of producing clonal planting stocks, wood biomass production of elite and rare genotypes thereby boosting production gains (Bonga, 1987 and Watt *et al.*, 1995). Micropropagation is a useful tool for selection and development of new cultivars (Iriondo *et al.*, 1995) and also conservation of germplasm (Scott *et al.*, 1995). Micropropagation is also a potentially important tool for genetic manipulation (Gill and Gosal, 1999; Timmis *et al.*, 1987).

Micropropagation technique has found application in the propagation of some tree species that are difficult to establish conventionally (Evers *et al.*, 1988; Warrang *et al.*, 1990). They include trees that do not produce seeds or have recalcitrant seeds (low viability) or are difficult to propagate through cuttings or are difficult to root (Karkonen *et al.*, 1999). This technique can also be used to propagate species whose demand for seedlings surpasses the supply (Odee, 1989).

Many tree species are propagated from tissues collected from juvenile explants whereas some can be multiplied from explants obtained from mature tree (Bonga, 1982). Micropropagation of a wide range of species has been successfully achieved (Bonga and Von Aderkas, 1992). Lakshmi and Bhopava (1993) described plant regeneration from shoot-derived callus of *Dalbergia latifolia*. Other similar

successful research work include; *Fragrala fragans,* a slow growing species, indigenous to South East Asia, whose wood provides valuable timber of the heavy hard wood (Lec and Rao 1986). Muriithi *et al.* in 1982 reported on *Ficus* plantlets regeneration.

In vitro propagation through somatic embryogenesis is also important tool for improvement of forest trees and has been reported in a number of crops and woody species. Chema (1989) achieved successful somatic embryogenesis and plant regeneration from cell suspensions derived from a 40 year old *Populus ciliana*, (Himalayans poplar). Regeneration of *Melia azedarach,* has been achieved in which the requirement of TDZ was similar to that found in numerous crop and woody plant species (Huetteman and Preece, 1993). In some cases, TDZ was used for induction of somatic embryogenesis from cultures of immature zygotic embryos (Goffreda *et al.,* 1995; Norgaard and Krogstrup, 1991), cotyledons of mature seeds (Neumann *et al.,* 1993), leaf discs (Harst, 1995; Kahia *et al.,* 1999), floral explants (Li *et al.*, 1998) inflorescences and seed cultures (Merkle *et al.,* 1998). In *Azadirachta indica*, TDZ was very effective in inducing somatic embryogenesis from mature seeds across a wide range of concentrations between 0 and 50 µM (Murthy and Saxena, 1998). Kahia *et al.,* (1999) reported somatic embryogenesis of *Coffea Arabica*, from leaf discs using TDZ. Ipekei and Gozukirmizi (2004) reported on indirect somatic embryogenesis and plant regeneration from leaf and internode explants of *Paulownia elongate*, however its mode of action is still poorly understood (Hutchinson *et al.*, 1997 and Murthy *et al.,* 1998).

2.10.1 Necrosis and phenols in micropropagation of woody plants

Shoot-tip necrosis (STN) is a phenomenon whereby *in vitro* apical shoots initially becomes brown and later dies (McCown and Sellmer, 1987). Due to the symptoms of STN, i.e. browning of buds and youngest leaves, one would automatically assume the cause to be nutrient deficiency. The symptoms of nutrient deficiency of less mobile minerals such as Calcium and Boron (Raven, 1977) often appear first in the meristematic regions and young leaves while symptoms of excessive presence of these minerals are observed in the older leaves (Barghchi and Alderson, 1996). However, STN *in vitro* is a function of complex set of factors. Piagnani *et al.*, (1996) suggested that the involvement of these numerous factors in STN could contribute to the many discrepancies seen in *in vitro* cultures affecting a wide range of plants.

The absence of roots which are one of the main sites of endogenous cytokinin biosynthesis leads to a decrease in endogenous cytokinins which in turn causes the cessation of cell division and necrosis in the apical meristem (Kataeva *et al.*, 1991). This is followed by the reduction of auxin and abscisic acid (ABA) synthesis, which normally takes place in the apical meristem (Kataeva *et al.*, 1991). Dipping the shoot tips in BAP solution prior to transferring to a rooting medium helped overcome STN in cultures of apricot cultivars (Perez-Tornero and Burgos, 2000). BAP was also successfully used to control STN in chestnut cultures (Piagnani *et al.*, 1996).

Tissue culture of woody plant species is often faced with problems of phenolic exudations (Preece and Compton, 1991), lack of shoot elongation, poor rooting ability hyperhydricity (vitrification) of the explants and high contamination rate especially when field materials are used (Drew, 1988). During the *in vitro* culture process, undesirable or inhibitory compounds such as excess phenolic metabolites (Carlberg *et al.*, 1983; Mensuali-Sodi *et al.*, 1993), ethylene (Mensuali-Sodi *et al.,* 1993) and 5-hydroxy methyl, an inhibitory by-product of autoclaving sucrose can be produced during medium preparation. These compounds hinder successful *in vitro* induction of cellular differentiation. As a result, there is need for preliminary experiments for selecting or modifying the known basal media that will be suitable for micropropagation of the plant species (Lakshmi and Raghawa, 1993).

A wide range of techniques has been employed to obtain healthy cultures. In auxillary bud culture, selection of explants from coppiced shoots or basal lateral branches at specific times of the year and changing them several times to a fresh medium reduce phenol exudation (Dan and Mitra, 1990). Addition of antioxidants such as cystein-Hcl, polyvinyl pyrrolidone (PVP), ascorbic acid, citric acid and activated charcoal to the medium is recommended to rescue explants from toxic effects of the oxidized phenols (Gupta *et al.*, 1981; Sondahl and Sharp, 1977).

2.10.2 *In vitro* rooting of woody plant species'

Many authors have demonstrated that, rooting process comprises of a series of independent physiological phases, which are associated with changes in

peroxidase activity and in endogenous auxin concentration (Naija *et.al.*, 2009). A number of plants root spontaneously in culture system, however some monocots, herbaceous species and shoots of most tree species multiplied *in vitro* lack a rooting system (Moncousin, 1991). Greater variation occurs between species with regard to the ease with which shoots can be rooted. Thus systematic trials are often needed to determine the optimal conditions for rooting. In some species, rooting occurs on the shoots after transfer to a media with or without plant growth regulators while others require an additional elongation step prior to transfer to the rooting medium (Moncousin, 1991). In some species it is possible to remove the shoots from *in vitro* conditions and root them directly *ex vitro* via conventional methods (Moncousin, 1991).

It has been demonstrated that, all cytokinins inhibit rooting and BAP used in shoot multiplication does so strongly even after transferring the shoots to a cytokinin free medium (Moncousin, 1991). Rooting in many herbaceous and woody species has been shown to improve by lowering the concentrations of macro salts to half or less and sucrose from 2 or 3 % to 0.5 or 1% (Joshi, 1991). In *Boswellia serrata* for instance, quarter strength MS media containing 1% sucrose helped in callus-free rooting (Purohit *et al.*, 1995). The concentration of the rooting hormones required is often critical to provide sufficient stimuli to initiate roots while preventing the excessive callus formation (Heloir *et al.*, 1996; Joshi, 1991). Most species require IBA and NAA to stimulate root formation. IAA is rapidly broken-down in culture and usually induces thick roots.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Collection and management of propagation material

Plant materials used in this study were collected in October 2009 at Amani Nature Reserve (ANR) in the southern part of East Usambara Mountains in Tanzania. The soil in this region is well developed laterized red earth with a shallow organic top soil (Tahir, 2002). Annual rainfall at ANR is 1918 mm and 2262 mm at Kwamkoro and is generally sustained throughout the year by constant flow of moist currents from the nearby Indean Ocean (Hamilton and Bensten-Smith, 1989).

A phytosanitary certificate was obtained from KEPHIS before travelling to Tanzania where plant materials were collected. The plant materials collected consisted of mature seeds derived from ripe fruits, seedlings and cuttings from coppices. They were thoroughly washed under running tap water at site and then stored in cool boxes before transportation to KEFRI (Muguga). On arrival, the seedlings were transplanted in potting bags containing well mixed loam soil and kept in a glasshouse at KEFRI. The seeds were germinated on growth trays half of them containing sawdust mixed with sand (1:1) and the other half containing sawdust mixed with decomposing manure and kept in same glasshouse. The cuttings were dipped vertically in sawdust mixed with sand (1:1) in a non-mist propagator at KEFRI. They were all watered (to soil saturation level) twice a week using a

spraying can. Seeds that germinated were transplanted into potting bags as described above. Well adapted seedlings were used as stock plants in subsequent experiments.

The following plate 1 to plate 6 are photographs of *A. stuhlmannii* fruit, tree trunk, seeds and seedlings collected at Amani Nature Reserve in Tanzania

Plate 1 Collecting of *A. stuhlmannii* fruit at ANR

Plate 2 A mature *A.stuhlmannii* in ANR (27th,10, 2009)

Plate 3 *A. stuhlmannii* tree nursery in ANR 29th ,10,2009

Plate 4 Trans section of *A. stuhlmannii* fruit

Plate 5 *A. stuhlmannii* seedlings collected from ANR

Plate 6 Potted *A. stuhlmannii* seedlings at KEFRI

Plate 1: Harvesting of *A. stuhlmannii* fruit at ANR in Tanzania. These fruits were later used to establish *A. stuhlmannii* plant stock at KEFRI.

Plate 2: A giant buttressed mature *A. stuhlmannii* tree (approximately 40 years) growing at ANR at Tanzania.

Plate 3: *A. stuhlmannii* seedlings in a Tree Nursery at ANR. This Nursery is used to conserve *A. stuhlmannii* seedlings only.

Plate 4: The arrangement of seeds in *A. stuhlmannii* fruit.

Plate 5: *A. stuhlmannii* seedlings used to establish plant stock at KEFRI nursery.

Plate 6: Three months old potted *A. stuhlmannii* seedlings at KEFRI Nursery. These seedlings were used as stock plants in this study.

3.2 Establishment of sterilization protocol

Young emerging leaves shoot apices and slender branches (section 3.1) were harvested from stock plants and used as explants. The explants were put in a glass jar containing 500ml of water into which 3 drops of Tween® 20 and 5 drops of Dettol soap detergent had been added. The jar was swirled gently for 15 minutes before washing the explants with running tap water for 10 minutes. All the glassware and metallic equipment used were sterilized by autoclaving at 121˚C at 1.06 kg/cm² pressure for 15 minutes before use. Under a clean Lamina flow hood, the explants were separately subjected to two sterilants (sodium hypochlorite and formaldehyde) at varying concentration and exposure times using the following experimental designs;

In Experiment 1: Two sterilants (sodium hypochlorite and formaldehyde) at four concentration levels (0, 10, 15 and 20) % each subjected to two exposure times (10 minutes and 20 minutes) was set.

In Experiment 2: Explants were exposed to 2% Redomil® solution prior to immersion in sodium hypochlorite at three concentration levels (6, 8 and 10) % with each level being subjected to three exposure times (6 minutes, 8 minutes and 10 minutes) was set. All the explants were rinsed three times using sterile distilled water and put on sterile blotting paper.

The shoots were trimmed to a length of 1cm and leaves cut into squares of 1cm dimension before culturing in MS medium. Explants exhibiting symptoms of

fungal and bacterial contamination (morphological descriptors) were scored as 1 while those with no infection were scored as 0.

3.3 Effect of different basal media on explants survival

Eight nutrient media namely; Murashige and Skoog medium, Gamborg (B_5) medium, Lloyd and McCown's Woody Plant medium, White's medium, Preece Hybrid medium, Driver and Kiniyuki medium, Anderson's medium, and Quorin and Lepoivre medium were tested for their suitability in micropropagation of *A. stuhlmannii*. **Appendix 1** show composition of these media and the amount of individual element. All the media were supplemented with $30g1^{-1}$sucrose and $0.8gl^{-1}$ *in vitro* agar. The pH value of each media was adjusted to 5.75 using 0.1M Hcl or 0.1M NaOH and dispensed in 150mm (height) by 25mm (diameter) culture tubes (10ml of medium per tube). The media was then sterilized in an autoclave at 121˚C at 1.06 kg/cm² pressure for 15 minutes before use. Cultures were maintained in a growth chamber at 25±1˚C and a 16hour photoperiod by cool white fluorescent tubes (36 lmol s^{-1} m^{-2}). Because *Allanblackia stuhlmannii* is an episodic woody plant, the calcium ion content of the selected medium is adjusted slightly (increased) while that of Boron is slightly reduced to achieved the most appropriate required ion concentration (Bairu *et al.,* 2009).

3.4 Effect of PGRs and their concentrations on shoot proliferation

Modified Lloyd and McCown's Woody plant medium (the new quantities were; 76mgl^{-1} $CaCl_2$ and 6.0mgl^{-1} H_3BO_3) was used to test the effect of different PGRs at varying concentrations on shoot proliferation. In the single PGRs applications, 1.2mgl^{-1}, 2.4mgl^{-1} and 3.6mgl^{-1} separately for both BAP and KIN and 1.2mgl^{-1} and 2.4 mgl^{-1} for TDZ and a control totaling to nine treatments were set.

In the combination sets, three different concentrations of BAP (1.2mgl^{-1}, 2.4mgl^{-1} and 3.6mgl^{-1}) and two concentrations of NAA (0.2mgl^{-1} and 0.4mgl^{-1}) were combined in six different treatments. In another combination, three different concentrations of KIN (1.2mgl^{-1}, 2.4mgl^{-1} and 3.6mgl^{-1}) and two concentrations of NAA (0.2mgl^{-1} and 0.4mgl^{-1}) were combined in 6 different treatments. In 3rd combination, two concentrations of TDZ (1.2mgl^{-1} and 2.4mgl^{-1}) and two concentrations of NAA (0.2mgl^{-1} and 0.4mgl^{-1}) were combined in four different treatments. The media were supplemented with 30mgl^{-1} sucrose and 8mgl^{-1} agar. Medium sterilization and culture conditions were as described in section 3.4 of methodology. The length of induced microshoots was measured at an interval of four weeks.

3.5 Effect of PGRs and their concentrations on callus induction

Modified McCown's WPM basal salts (76 mgl^{-1} $CaCl_2$, 6.0 mgl^{-1} H_3BO_3) and Gamborg's vitamins (McCown *et al.,* 1987 and Gamborg *et al.,* 1974) were used to test the effect of different PGRs at varying concentrations on induction of callus from leaf discs. Three different concentrations of KIN (0.5mgl^{-1}, 1mgl^{-1} and 2mgl^{-1})

and four concentrations of 2,4-D ($1mgl^{-1}$ $1.25mgl^{-1}$, $1.5mgl^{-1}$ and $2mgl^{-1}$) were combined in 5 different treatments including a control (media without PGRs). All the media were supplemented with $30mgl^{-1}$ sucrose and $8mgl^{-1}$ agar. Medium sterilization and culture conditions were as described in section 3.4. The proportion (%) of callus induction around the leaf discs was evaluated at an interval of 4 weeks.

3.6 Induction of somatic embryogenesis

To induce somatic embryogenesis, explants were sourced from cotyledon, hypocotyl and primary root. The explants were excised into sections of approximately 8 mm and cultured on MS medium containing 3.0% sucrose supplemented with 1.0 mg l^{-1} Dichlorophenoxyacetic acid (2,4-D) and others in 0.5 mg l^{-1} α-Naphthaleneacetic Acid (NAA) and 0.5 mg l^{-1} Kinetin (KIN). Five explants were cultured in each flask with 10 replications. The cultures were maintained in the dark (Dan and Mitra, 1990; Harst, 1995; Sairam *et al.*, 2001)

3.7 Effect of PGRs and their concentrations on rooting of shoots

Different PGRs were selected and used to design treatments for root initiation media.

In experiment 1: Half strength MS and WPM media in which half of each media contained $30mgl^{-1}$ and the other half $1mgl^{-1}$ sucrose and $8mgl^{-1}$ agar. All the media were fortified with IBA, IAA and NAA at various concentrations (0.0, 0.05, 0.1 and 2.5) mgl^{-1}.

In experiment 2: A two-step procedure (Gasper and Coumans, 1987) was used. Stable shoot were initially cultured in half-strength MS medium containing $30mgl^{-1}$sucrose and $8mgl^{-1}$ agar and separately supplemented with IBA, IAA and

NAA at 0.0, 0.03, 0.05 and 0.1mgl^{-1} concentration levels. The shootlets were then transferred into the same medium but without PGRs after three weeks and regularly.

3.8 Data collection and Analysis

Data was collected in *MS* Excel spreadsheets and analyzed using Statistical Analysis System (SAS) and Genstat 12th Edition, statistical softwares. The number of explants infected by either fungi or bacteria or both and also the number of explants that died due to bleaching when cultured on basal nutrient of Murashige and Skoog medium were recorded after one week for two consecutive weeks. Mean number of explants that were infected by bacteria and fungi as well as the number of explants that died due to bleaching was determined. The best sterilant and the suitable concentration and the preferred time of exposure were deduced from the analyzed means, hence adopted as the sterilization protocol. In medium selection experiment, the number of explants that survived on each of the eight media was recorded. The medium with the highest explant survival rate was also determined. The shoot lengths of all explants used in the shoot induction and proliferation experiments were recorded at an interval of four weeks for a period of twelve weeks using binoculars microscope. ANOVA tests showing the effects of variations and interactions of the various plant growth regulators used and duration (weeks) on induction of microshoots, roots and callus were compared at $P<0.05$ (Duncan's multiple test). At twelfth week, three out of the nine explants per treatment were subjected to rooting experiments.

CHAPTER FOUR

4.0 RESULTS

4.1. Sterilization protocol for *A. stuhlmannii* explants used in tissue culture

Results on the effectiveness of *A. stuhlmannii* explants sterilization by sodium hypochlorite and formaldehyde show that increase in sterilant concentration and exposure time, resulted to high explants mortality rate and decrease in bacterial contamination rate, $P<0.05$. Fungal contamination level on the other hand remained inconsistent and unpredictable (**Table 1**).

For instance, when the concentration of sodium hypochlorite was increased from 10% to 20% at a constant exposure time of 10 minutes, fungal contamination level remained constant i.e. 16.7% and bacterial contamination rate reduced from 33.3% to 16.7% while mortality of explants increased from 33.3% to 100% (**Table 1**). When the exposure time for concentration 10% was increased from 10 minutes to 20 minutes, fungal contamination level increased from 16.7% to 33.3% and bacterial contamination rate remained constant i.e. 33.3% while explant mortality rate increased from 33.3% to 83.30% (**Table 1**). When the exposure time for concentration 20% was increased from 10 minutes to 20 minutes, contamination levels for both fungi and bacteria reduced from 16.7% to 0.00%, however no explant survived this concentration level and 100% explant mortality was experienced (**Table 1**).

In the case of Formaldehyde, when concentration was increased from 10% to 20% at a constant exposure time of 10 minutes, both fungal and bacterial contamination levels reduced from 67.0% to 33.0% while death of explants increased from 67.0% to 100% (**Table 1**). When the exposure time for concentration 10% was increased from 10 minutes to 20 minutes, both fungal and bacterial contamination levels reduced from 67.0% to 33.0% while explant mortality rate remained constant at 67.0% (**Table 1**). When the exposure time for 20% concentration level was increased from 10 minutes to 20 minutes, both fungal and bacterial contamination levels reduced from 33.0% to 0.00% however no explant survived this concentration level (**Table 1**).

Table 1 Fungal and bacterial contamination levels and explants mortality when exposed to different concentrations of Sodium hypochlorite and Formaldehyde at varying exposure times *(n=132)*

Sterilant	Concentration ml(v/v)%	Exposure time (minutes)	Contamination (%)		Explant Mortality(%)
			Fungi	Bacteria	
Sodium	0	10	100.00	96.00	0.00
Hypochlorite	0	20	100.00	92.10	0.00
	10	10	16.70	33.30	33.30
	10	20	33.30	33.30	83.30
	15	10	33.30	16.70	100.00
	15	20	0.00	0.00	100.00
	20	10	16.70	16.70	100.00
	20	20	0.00	0.00	100.00
Formaldehyde	10	10	67.00	67.00	67.00
	10	20	33.00	33.00	67.00
	15	10	33.00	33.00	33.00
	15	20	0.00	33.00	100.00
	20	10	33.00	33.00	100.00
	20	20	0.00	0.00	100.00
$L.S.D_{0.05}$			57.71	57.71	36.50

Sodium hypochlorite was more effective in the eradication of fungal and bacterial contamination (77.2% and 73.4% effectiveness respectively) from explants compared to Formaldehyde (66.7% and 64.4% effectiveness respectively) and hence the preferred sterilant for further work (**Table 2**).

Table 2 Effectiveness of the two sterilants in sterilization (%), *n=180*

	% effectiveness in eradication of		
Variate	Fungi	Bacteria	Explant mortality (%)
Formalin	66.70	64.4	24.4
Sodium hypochlorite	77.20	73.3	24.4
$L.S.D_{0.05}$	1.883	1.952	1.997

In the final sterilization experiment (**Table 3**), when sodium hypochlorite concentration level was increased from 6% to 10% at a constant exposure time of six minutes, there was no change in fungal contamination level (66.7%) while bacterial contamination level reduced from 66.7% to 33.3%. Explant mortality on the other hand increased from 0.00% to 33.3% (**Table 3**). When concentration level was increased from 8% to 10% at a constant exposure time of eight minutes, fungal contamination rate reduced from 66.7% to 33.3% while bacterial contamination level reduced from 33.3% to 0.00% (**Table 3**). However explant mortality rate increased from 0.00% to 66.7% (**Table 3**). When the exposure time for concentration 8% was increased from 8 minutes to 10 minutes, fungal and bacterial contamination levels reduced from 66.7% and 33.3% to 33.3% and 0.00% respectively (**Table 3**). Mortality of explants on the other hand remained constant at 0.00% (**Table 3**). To

arrest fungal contamination at this level, explants were subjected to 2% Redomil® solution (Perez *et al.,* 2009) prior exposure to sodium hypochlorite. It was noted that, *A. stuhlmannii* explants were best surface sterilized when exposed to 2% Redomil® solution for 15 minutes followed by 10 minute exposure to 8% sodium hypochlorite (**Table 3**) and finally rinsed three times with sterile distilled water.

Table 3 Fungal and Bacterial contamination levels and mortality of explants exposed to concentrations at different exposure durations of Sodium hypochlorite (%), *n=54*.

Concentration (ml)v/v)%	Time (minutes)	Contamination (%)		Explant mortality(%)
		Fungi	Bacteria	
6	6	66.70	66.70	0.00
6	8	100.00	66.70	0.00
6	10	100.00	66.70	0.00
8	6	66.70	33.30	0.00
8	8	66.70	33.30	0.00
8	10	33.30	0.00	0.00
10	6	66.70	33.30	33.30
10	8	33.30	0.00	66.70
10	10	0.00	0.00	100.00
$L.S.D_{0.05}$		73.82	87.34	46.69

4.2 Media Selection

When explant survival rate (%) of the selected media (without PGRs) were compared using $LSD_{0.05}$ test, significant differences were observed (**Table 4**). Media with low explant survival rates comprised of Anderson's medium (22.22%), White's medium (27.78%) and Murashige and Skoog medium, 33.33%, **Table 4**. Nutrient media with average explant survival rates consisted of Preece Hybrid medium

(55.56%), Quorin and Lepoivre medium (61.11%), Gamborg medium (61.11%) and Driver and Kiniyuki medium, 66.67, **Table 4**. McCown's (WPM) medium (88.89%) had the highest explants survival rate and thus selected for subsequent experiments (**Table 4**). The modification introduced in WPM significantly reduced shoot-tip necrosis and phenol exudation by *in vitro* explants.

Table 4 Explants survival rate on various nutrient media

Type of media	No. of cultured explants	No. of live explants	Explant survival (%)	Grade
Anderson's medium	18	4	22.22	Low
White's Medium	18	5	27.78	Low
Murashige and Skoog	18	6	33.33	Low
Preece Hybrid medium	18	10	55.56	Average
Quoirin & Lepoivre	18	11	61.11	Average
Gamborg	18	11	61.11	Average
Driver and kiniyuki	18	12	66.67	Average
McCown's(WPM)	18	16	88.89	High
$L.S.D_{0.05}$			11.27	

4.3 Direct shoot induction/Proliferation

When the response of PGRs treatments (mean shoot length of *in vitro* nodal explants) were compared using Duncan's multiple range test, significant differences between and within treatments at three time intervals were observed (**Table 5**);

At week 4: Explants cultured in treatments; $2mgl^{-1}$2,4-D + $6mgl^{-1}$BAP; $2.4mgl^{-1}$ BAP + $0.2mgl^{-1}$NAA; $2.4mgl^{-1}$ KIN + $0.2mgl^{-1}$NAA; $2.4mgl^{-1}$ TDZ + $0.4mgl^{-1}$NAA (combined PGRs application) and $3.6mgl^{-1}$KIN (single PGR application) exhibited the highest mean shoot length (1.30cm, 1.30cm, 1.30cm, 1.28cm and 1.28cm respectively). Explants cultured in treatment $1.2mgl^{-1}$KIN

(single PGR application) and 3.6mgl^{-1}KIN + 0.4mgl^{-1}NAA (combined PGRs application) exhibited the lowest mean shoot length (1.20cm and 1.13cm respectively). The control treatment had the least mean shoot length of1.04cm (**Table 5**).

At week 8: Explants cultured in treatments; 1.2mgl^{-1}BAP and 1.2mgl^{-1}KIN (single PGR application) and 1.2mgl^{-1}TDZ + 0.4mgl^{-1}NAA (combined PGRs application) exhibited the highest mean shoot length (1.40cm, 1.39cm and 1.42cm respectively). The mean shoot length of explants cultured in treatment 1.2mgl^{-1} TDZ (single PGR application) exhibited the lowest mean shoot length (1.24cm). Explants cultured in 1.2mgl^{-1}TDZ + 0.2mgl^{-1}NAA (combined PGRs application) and the control exhibited the least mean shoot length (1.11cm and 1.10cm respectively) as recorded in **Table 5**.

At week 12: Explants cultured in treatments 1.2mgl^{-1}BAP and 1.2mgl^{-1}KIN (single PGR application) and 1mgl^{-1}2,4-D+6mgl^{-1}BAP (combined PGRs application) exhibited the highest mean shoot length (1.43cm, 1.44cm and 1.37cm respectively). Explants cultured in 1.2mgl^{-1}TDZ had low mean shoot length (1.2cm) followed by control (1.15cm). Explants cultured in treatment 2.4mgl^{-1}TDZ + 0.4mgl^{-1}NAA (combined PGRs application) had the least mean shoot length, 1.14cm, **Table 5**.

Table 5 Mean shoot length of microshoots induced from nodal explants cultured on WPM supplemented with various PGRs treatments, *n=279*

	Shoot length (cm)		
Treatment (mg/l)	**Week4**	**Week8**	**Week12**
Ctrl	1.04±0.010g	1.10±0.006g	1.15±0.030jk
2,2,4-D+6BAP	1.30±0.026a	1.36±0.028±abc	1.35±0.041abcdefg
2.4BAP+0.2NAA	1.30±0.026a	1.36±0.034±abc	1.34±0.032abcdefg
2.4KIN+0.2NAA	1.30±0.021a	1.36±0.030abc	1.35±0.030abcdef
2.4TDZ+0.4NAA	1.28±0.024a	1.36±0.032abc	1.14±0.023k
3.6KIN	1.28±0.027a	1.36±0.013abc	1.38±0.036abc
1.2TDZ+0.2NAA	1.27±0.027ab	1.11±0.006g	1.27±0.018defghi
3.6BAP	1.26±0.026abc	1.36±0.031abc	1.38±0.035abc
1,2,4-D+6BAP	1.25±0.021abcd	1.34±0.026abcde	1.37±0.025abcd
1.2TDZ	1.21±0.022bcde	1.24±0.017def	1.20±0.022ijk
2.4BAP	1.21±0.026bcde	1.37±0.058ab	1.36±0.052abcde
2.4KIN	1.20±0.024bcdef	1.35±0.058abcd	1.38±0.055abc
2.4TDZ	1.20±0.028bcdef	1.17±0.015fg	1.35±0.030abcdef
3.6KIN+0.2NAA	1.20±0.021bcdef	1.24±0.025cdef	1.28±0.016cdefghi
1.2BAP	1.20±0.021bcdef	1.40±0.070a	1.43±0.077a
1.2KIN	1.20±0.021cdef	1.39±0.071a	1.44±0.066a
1,2,4-D+2BAP	1.19±0.020def	1.25±0.021cdef	1.33±0.025bcdefg
1,2,4-D+4BAP	1.19±0.024def	1.26±0.030bcdef	1.30±0.027cdefghi
3.6BAP+0.2NAA	1.19±0.018def	1.24±0.025cdef	1.25±0.027fghij
2.4KIN+0.4NAA	1.19±0.014def	1.238±0.017def	1.23±0.019hijk
2.4BAP+0.4NAA	1.19±0.014def	1.24±0.017def	1.23±0.016hijk
1.2KIN+0.2NAA	1.16±0.015ef	1.23±0.016ef	1.28±0.009cdefghi
2,2,4-D+2BAP	1.16±0.014ef	1.21±0.014fg	1.24±0.010ghij
1.2TDZ+0.4NAA	1.16±0.012ef	1.42±0.067a	1.26±0.018efghi
1.2BAP+0.2NAA	1.15±0.015ef	1.22±0.013fg	1.25±0.013fghi
3.6BAP+0.4NAA	1.15±0.013ef	1.21±0.013fg	1.21±0.015ijk
2,2,4-D+4BAP	1.15±0.016ef	1.22±0.016fg	1.25±0.020fghij
1.2KIN+0.4NAA	1.15±0.017ef	1.21±0.023fg	1.22±0.018ijk
2.4TDZ+0.2NAA	1.15±0.015ef	1.36±0.055abc	1.21±0.013ijk
1.2BAP+0.4NAA	1.14±0.015ef	1.21±0.023fg	1.21±0.019ijk
3.6KIN+0.4NAA	1.13±0.021f	1.21±0.014fg	1.21±0.014ijk
L.S.D$_{0.05}$	0.060	0.099	0.091

Mean values (±SE) within a column followed by the same letter are not significantly different by Duncan's multiple range test (*P≥0.05*)

When the response of PGRs treatments (mean shoot length of *in vitro* shoot tip explants) were compared using Duncan's multiple range test, significant differences between and within treatments at three time intervals were observed (**Table 6**);

At week 4: Explants cultured in treatments; 2.4mgl^{-1}BAP + 0.2mgl^{-1}NAA; 2.4mgl^{-1}KIN + 0.2mgl^{-1}NAA; 2mgl^{-1}2,4-D + 6mgl^{-1}BAP and 2.4mgl^{-1}TDZ + 0.4mgl^{-1}NAA (combined PGRs application) exhibited the highest mean shoot length (1.31cm). Single PGR applications with the highest mean shoot length were 3.6mgl^{-1}BAP and 3.6mgl^{-1}KIN (1.28cm). Explants cultured in treatment 3.6mgl^{-1}KIN + 0.2mgl^{-1}NAA and 3.6mgl^{-1}BAP + 0.2mgl^{-1}NAA exhibited the lowest mean shoot length (1.09cm) while the control treatment had the least mean shoot length, 1.02cm, **Table 6**.

At week 8: Explants cultured in the following single and combined PGRs application treatments; 1.2mgl^{-1}BAP; 1.2mgl^{-1}KIN and 1.2mgl^{-1}TDZ + 0.4mgl^{-1}NAA exhibited the highest mean shoot length (1.37cm). Explants cultured in treatment 1.2mgl^{-1}TDZ (single PGR application) exhibited low mean shoot length (1.09cm). For combined PGRs application, explants cultured in 1.2mgl^{-1}TDZ + 0.2mgl^{-1}NAA exhibited the lowest and the same mean shoot length as in the control, 0.82cm, **Table 6**.

At week 12: Explants cultured in the following single and combined PGRs applications, 1.2mgl^{-1}BAP and 1.2mgl^{-1}KIN and 1mgl^{-1}2,4-D + 6mgl^{-1}BAP exhibited the highest mean shoot length (1.41cm, 1.41cm and 1.35cm respectively). For single PGR application, 2.4mgl^{-1}BAP, 2.4mgl^{-1}KIN, 3.6mgl^{-1}BAP and 3.6mgl^{-1}KIN had explants with low mean shoot length (1.37cm, 1.37cm, 1.36cm and 1.36cm respectively). Explants cultured in treatment 2.4mgl^{-1}TDZ + 0.4mgl^{-1}NAA

and the control treatment exhibited equal and the lowest mean shoot length of 0.87cm (**Table 6**).

Table 6 The mean shoot length of microshoots induced from shoot tips cultured on WPM supplemented with KIN,BAP, TDZ alone or in combination with NAA and 2,4-D, *n=279*

	Shoot length (cm)		
Treatment (mg/l)	**Week4**	**Week8**	**Week12**
Ctrl	1.02±0.003d	0.82±0.155d	0.87±0.166d
1.2BAP	1.21±0.023abc	1.37±0.068a	1.41±0.070a
2.4BAP	1.21±0.027abc	1.33±0.056abc	1.37±0.060ab
3.6BAP	1.28±0.028ab	1.34±0.031ab	1.36±0.035ab
1.2KIN	1.21±0.023abc	1.37±0.068a	1.41±0.070a
2.4KIN	1.21±0.027abc	1.33±0.056abc	1.37±0.060ab
3.6KIN	1.28±0.028ab	1.34±0.031ab	1.36±0.035ab
1.2TDZ	1.21±0.023abc	1.09±0.137c	1.19±0.021abc
2.4TDZ	1.21±0.027abc	1.15±0.015abc	1.34±0.030abc
1.2BAP+0.2NAA	1.16±0.014bc	1.20±0.014abc	1.23±0.013abc
1.2BAP+0.4NAA	1.15±0.017bc	1.19±0.021abc	1.19±0.020abc
2.4BAP+0.2NAA	1.31±0.028a	1.34±0.030ab	1.34±0.030abc
2.4BAP+0.4NAA	1.19±0.014abc	1.22±0.017abc	1.22±0.017abc
3.6BAP+0.2NAA	1.09±0.137dc	1.22±0.017bc	1.12±0.141bc
3.6BAP+0.4NAA	1.15±0.015bc	1.19±0.015abc	1.20±0.014abc
1.2KIN+0.2NAA	1.16±0.014bc	1.20±0.014abc	1.23±0.013abc
1.2KIN+0.4NAA	1.15±0.017bc	1.19±0.021abc	1.19±0.020abc
2.4KIN+0.2NAA	1.31±0.028a	1.34±0.030ab	1.34±0.030abc
2.4KIN+0.4NAA	1.19±0.014abc	1.22±0.017abc	1.22±0.017abc
3.6KIN+0.2NAA	1.09±0.137dc	1.11±0.140bc	1.12±0.141bc
3.6KIN+0.4NAA	1.15±0.015bc	1.19±0.015abc	1.20±0.014abc
1,2,4-D+2BAP	1.21±0.023abc	1.24±0.024abc	1.28±0.023abc
1,2,4-D+4BAP	1.21±0.027abc	1.24±0.026abc	1.28±0.026abc
1,2,4-D+6BAP	1.28±0.028ab	1.32±0.027abc	1.35±0.025b
2,2,4-D+2BAP	1.16±0.014bc	1.18±0.013abc	1.21±0.014abc
2,2,4-D+4BAP	1.15±0.017bc	1.18±0.018abc	1.22±0.020abc
2,2,4-D+6BAP	1.31±0.028a	1.19±0.150abc	1.22±0.155abc
1.2TDZ+0.2NAA	1.28±0.028ab	0.82±0.155d	1.22±0.017abc
1.2TDZ+0.4NAA	1.16±0.014bc	1.37±0.068a	1.11±0.140c
2.4TDZ+0.2NAA	1.15±0.017bc	1.33±0.056abc	1.19±0.015abc
2.4TDZ+0.4NAA	1.31±0.028a	1.34±0.031ab	0.87±0.166d
$L.S.D_{0.05}$	0.117	0.201	0.211

Mean values (±SE) within a column followed by the same letter are not significantly different by Duncan's multiple range test ($P \geq 0.05$)

When the response of PGRs treatments (mean shoot length of *in vitro* explants) were compared using Duncan's multiple range test, significant differences between and within treatments after 12 weeks were recorded (**Plate 7** and **Table 7**).

Explants cultured in the following treatments 1.2mgl^{-1}KIN, 1.2mgl^{-1}BAP, 3.6mgl^{-1}KIN (single PGR application) and 2mgl^{-1}2,4-D + 6mgl^{-1}BAP (combined PGRs application) exhibited high mean shoot length (1.34cm). Explants cultured in treatments 3.6mgl^{-1}KIN + 0.4mgl^{-1}NAA, 1.2mgl^{-1}BAP + 0.4mgl^{-1}NAA and 3.6mgl^{-1}BAP + 0.4mgl^{-1}NAA (combined PGRs application) and 1.2mgl^{-1}TDZ (combined PGRs application) exhibited the low mean shoot length (1.19cm, 1.19cm, 1.19cm and 1.22cm respectively). Control treatment had explants with least mean shoot length of 1.10cm (**Table 7**).

The mean shoot length of microshoots induced from *in vitro* shoot tip explants also differed significantly between different treatments of PGRs after 12 weeks in culture. Explants cultured in the following single and combined PGRs application treatments; 1.2mgl^{-1}KIN, 1.2mgl^{-1}BAP, 3.6mgl^{-1}KIN, 3.6mgl^{-1}BAP, 2.4mgl^{-1}BAP + 0.2mgl^{-1}NAA and 2.4mgl^{-1}KIN + 0.2mgl^{-1}NAA exhibited high mean shoot length (1.33cm). Explants cultured in treatments 1.2mgl^{-1}TDZ and 3.6mgl^{-1}BAP + 0.2mgl^{-1}NAA exhibited the lowest mean shoot length with the control treatment having the least mean shoot length (1.16cm and 1.10cm respectively). Control treatment had explants with the least mean shoot length, 0.90cm, **Table 7**.

Table 7 The mean shoot length of microshoots induced from nodal and shoot tip explants cultured on WPM supplemented with KIN, BAP, TDZ alone or in combination with NAA and 2,4-D after 12 weeks, *n=558*.

	Nodal explants (length, cm)	Shoot tip explants (length, cm)
Treatment (mg/l)	**Mean**	**Mean**
Ctrl	1.096a	0.9000g
3.6KIN+0.4NAA	1.185b	1.180cdef
1.2BAP+0.4NAA	1.187b	1.172cdef
3.6BAP+0.4NAA	1.189b	1.170cdef
1.2KIN+0.4NAA	1.194bc	1.178cdef
2,2,4-D+2BAP	1.204bcd	1.183bcdef
2,2,4-D+4BAP	1.206bcd	1.184bcdef
1.2BAP+0.2NAA	1.208bcd	1.178cdef
1.2TDZ+0.2NAA	1.213bcd	1.108def
2.4BAP+0.4NAA	1.216bcde	1.207abcdef
2.4KIN+0.4NAA	1.217bcde	1.211abcdef
1.2TDZ	1.217bcde	1.163def
1.2KIN+0.2NAA	1.223bcde	1.195abcdef
3.6BAP+0.2NAA	1.227bcde	1.096f
2.4TDZ+0.2NAA	1.236bcde	1.225abcdef
2.4TDZ	1.24bcde	1.236abcde
3.6KIN+0.2NAA	1.24bcde	1.103ef
1,2,4-D+4BAP	1.252cde	1.246abcd
2.4TDZ+0.4NAA	1.258def	1.173cdef
1,2,4-D+2BAP	1.259def	1.244abcd
1.2TDZ+0.4NAA	1.277efg	1.209abcdef
2.4KIN	1.311fgh	1.306abc
2.4BAP	1.316gh	1.306bc
1,2,4-D+6BAP	1.321gh	1.317ab
2.4BAP+0.2NAA	1.333gh	1.326a
3.6BAP	1.334gh	1.330a
2.4KIN+0.2NAA	1.336gh	1.331a
2,2,4-D+6BAP	1.337h	1.238abcde
3.6KIN	1.339h	1.330a
1.2BAP	1.341h	1.329a
1.2KIN	1.342h	1.329a
L.S.D$_{0.05}$	0.056	0.120

Mean values within a column followed by the same letter are not significantly different by Duncan's multiple range test (*P≥0.05*)

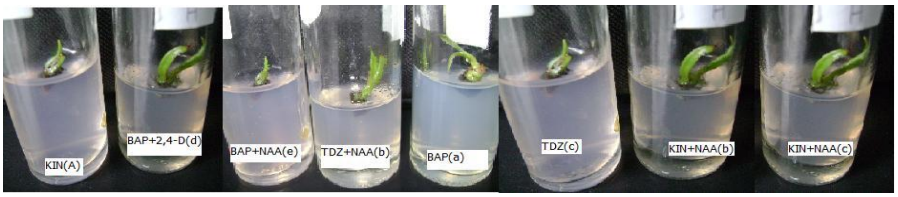

Plate 7: Direct shoot induction from *A. stuhlmannii* explants cultured on WPM supplemented with KIN, BAP and TDZ alone or in combination with NAA or 2,4-D.

4.4 Induction of Callus

The percentage mean of callus induced from *in vitro* leaf discs of *A. stuhlmannii* (as seen in **Plate 8**) differed significantly between different treatments of PGRs when compared using Duncan's multiple range test (**Table 8**). *A. stuhlmannii* leaf discs cultured on treatment $1mgl^{-1}$KIN + $1.25mgl^{-1}$2,4-D exhibited the highest callus induction of 32.22% (**Table 8**). Explants cultured in treatment $2mgl^{-1}$KIN + $2mgl^{-1}$2,4-D and control did not induce callus after four weeks. In week 8 and week 12, no further callus induction was observed for treatment $1mgl^{-1}$KIN + $1.25mgl^{-1}$2,4-D while treatment $2mgl^{-1}$KIN + $2mgl^{-1}$2,4-D was not significantly different from the control when compared using Duncan's multiple range test at $P \geq 0.05$ (**Table 8**). Induction of somatic embryos on the other hand was not successful.

Table 8 Callus induction (mean %) from leaf disk cultured on WPM supplemented with KIN+2, 4-D at various concentration *(n=135)*

	Callus induction (%)		
Treatment (mg/l)	**week4**	**week8**	**week12**
Ctrl	0.00a	0.00a	0.00a
2KIN+2 2,4-D	0.000a	0.11a	0.11a
0.5KIN+1 2,4-D	8.33b	8.33b	8.33b
2KIN+1.5 2,4-D	10.56b	10.56b	10.56b
1KIN+1.25 2,4-D	32.22c	32.22c	32.22c
$L.S.D_{0.05}$	13.581	13.582	13.582

Mean values within a column followed by the same letter are not significantly different by Duncan's multiple range test (*P≥0.05*)

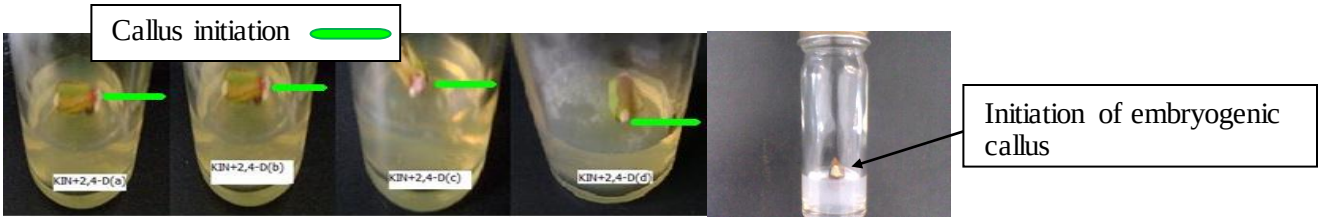

Plate 8: Callus and somatic embryo induction from leaf explants

4.5 Root induction

No explants rooted in the two rooting experimental designs.

CHAPTER FIVE

5.0 DISCUSSION

5.1 Sterilization Protocol

Surface sterilization of explants is a prerequisite to successful establishment of clean cultures for manipulation. Of the two sterilants used, sodium hypochlorite was more effective in the eradication of bacterial and partially fungal contamination than formaldehyde (**Table 2** of results) and hence selected for subsequent sterilization experiments. Although there was a significant reduction of fungal and bacterial contamination when the concentration and exposure time of sodium hypochlorite were increased ($P<0.05$), explant mortality level on the other hand increased significantly ($P<0.05$) thereby rendering such concentration levels and exposure time unsuitable for adoption (**Table 1**and **3**).

The use of sodium hypochlorite alone did not sufficiently eradicate fungal contamination. To eliminate the fungal menace, explants were subjected to Redomil® (fungicide) solution prior exposure to Sodium hypochlorite (Perez *et al.*, 2009). It was evident from the analyzed data of the final sterilization experiment that, subjection of *A. stuhlmannii* explants to 2% Redomil® solution for 15 minutes eliminated most fungal contamination (approximately 90%). Subsequent immersion of explants in 8% (v/v) sodium hypochlorite concentration at exposure time of 10 minutes resulted in a large number of live and bacteria-free explants (**Table 3**). This was adopted as the best sterilization protocol for *A. stuhlmannii* seedlings grown in the glasshouse.

For explants materials obtained straight from the field, the level of contamination was high. The relatively higher contamination level of field materials could be attributed to the presence of systemic microorganisms in the tissues (Drew, 1988). This is in agreement with the work reported by Karkonen *et al.* (1999) on *Melaleuca alternifolia* that contamination rates are often higher for explants harvested from field trees than those obtained from greenhouse. Nevertheless, the high level of contamination of explants harvested from field trees was significantly reduced by exposing the explants to systemic fungicide, 4 % Redomil®, and 0.1 % boric acid for two hours before subjecting them to the sodium hypochlorite (Perez *et al.*, 2009).

4.2 Medium Selection

Data on media selection show that, McCown's woody Plant medium is the best nutrient medium which resulted in 88.89% explant survival chance, for tissue culture of *A. stuhlmannii* (**Table 4**). Browning of *in vitro* explants and their eventual death on various media such as MS medium, Anderson's medium, and Quorin and Lepoivre medium was due to production of phenolic compounds and possibly due to unsuitability of constituents of individual medium (Preece and Compton, 1991). Explant browning is usually ascribed to phenolic accumulation which reduces cell competence and leads to its eventual loss of totipotency (Benson, 2000). This is a major unsolved problem in the initiation of tissue cultures, especially for woody

plants (Pinto *et al.*, 2008). There is a general tendency of attributing the occurrence of shoot-tip necrosis and subsequent death of *in vitro* explants to the high salt concentration found in some medium e.g. MS medium (Bairu *et al.*, 2009).

Alterations of NH_4/NO_3 ratio and sulphur content have been shown to significantly reduce shoot-tip necrosis (Laskshmi and Raghava 1993). For instance, NH_4/NO_3 ratio for WPM is 1.03:1 while that of Anderson's medium and Gamborg medium are 0.83:1 and 0.05:1 respectively (**Appendix 1**). The sulphur content of WPM is 1192.325mgl^{-1} while that of Anderson's medium and Gamborg medium are 192.025mgl^{-1} and 255.835mgl^{-1} respectively (**Appendix 1**).

Some constituent elements of the various media tested play critical roles in cell division and differentiation and hence affect growth and development of cultured tissues and organs (Marschner, 1995). For instance, Calcium is a relatively large essential divalent cation found in high concentration in plant tissues especially cell walls (Hirschi, 2004). All the tested media had different amounts of Calcium ion in different constituent compounds (**Appendix 1**). Due to its versatility and specificity, Calcium plays major structural and functional roles in plants (Hepler, 2005). Plants rely on the unique properties of Calcium for their structural, enzymatic and signaling functions and also its role in physiological processes such as cell elongation and cell division (Hirschi *et al.*, 2004).

Boron requirements on the other hand differ widely among plant species and is known to have a narrow range between deficiency and toxicity levels when compared to other mineral nutrients (Abdulnour *et al.*, 2000). In addition, what is

optimum for one species could be toxic for another (Barghchi and Alderson 1996) and as a result there was need to determine the appropriate amount of Boron for *A. stuhlmannii*. Modified McCown's woody Plant medium was preferred for subsequent experiments.

4.3 Direct shoot and root induction

The achievement of uniform and continuous *in vitro* shoot growth is highly problematic for most woody tree species especially those with strong episodic growth characteristic (McCown, 2000), such is the case in *A. stuhlmannii*. Experiments comparing different cytokinin regimes showed that, explants produced between 90-100% responsive (organogenic) explants (**Plate 7**).

The use of different PGRs either alone or in combinations, significantly affected shoot length of the cultured nodal explants (Nehra *et al.*, 1994 and Zaer and Mapes, 1982). Treatments 2,4-D+BAP, BAP+NAA, KIN+NAA and KIN+2,4-D were significantly different ($p<0.05$) for all the time intervals while treatment TDZ+NAA appeared to affect shoot length as time progressed ($p<0.05$), **Appendix 4**. All the explants produced single small white protuberance after four weeks of culture. In all the treatments, the protuberance elongated at different rates and directly differentiated into well-defined shoot without any intervening callus (**Table 5** and **6**). Similar mode of organogenesis was reported by Meena *et al.*, (2010) on adventitious shoot induction of *Malaxis acuminata* from cultured internodal explants.

Among the PGRs applications (either single or in combinations) tested, 1.2mgl^{-1}BAP and 1.2mgl^{-1}KIN had explants that exhibited the highest and consistent increase in shoot length for both *in vitro* shoot tip and nodal explants a cross the three time intervals (**Table 7**). This finding is in agreement with those of Werner *et al.,* (2001) who concluded that the growth of leaves *in vitro* in cytokinin-deficient tobacco plants not only required cytokinins, but also the fine adjustment of natural cytokinin levels.

When a cytokinin (BAP or KIN or TDZ) was combined with an auxin (2, 4-D or NAA), increase in microshoot length was also observed (**Table 5** and **6**). In the combined PGRs applications, treatments containing 2,4-D greatly increased shoot length compared to those of NAA with exception of 2.4mgl^{-1}KIN+0.2mgl^{-1}NAA in the shoot tip experiments (**Table 7**). Existing reports suggest that, when auxins at lower concentrations are combined with cytokinins, they have a critical role in plant regeneration in several systems like *Petasites hybridus* (Wldi *et al.,* 1998), *Eucalyptus grandis* (Luis *et al.,* 1999), *Hybanthus enneaspermus* (Prakash *et al.,* 1999), *Coleus forskohlii* (Sairam Reddy *et al.,* 2001) and *Eleusine indica* (Yemets *et al.,* 2003). Treatments 6mgl^{-1}BAP + 1mgl^{-1}2,4-D and 6mgl^{-1}BAP + 1mgl^{-1}2,4-D were however the most effective PGRs combinations (**Table 7**). Superiority of BAP in shoot induction has been reported to be due to the ability of plant tissue to metabolize natural hormones more readily than artificial growth regulators or due to the ability of BAP to induce production of natural hormones such as zeatin in the tissue and thus work through natural hormone system (Sharma and Wakhlu, 2003).

Further elongation of the solitary shoots did not occur after the 12th week in the same medium but instead they began to turn brown. Extended culture on the same medium or subculture on a fresh medium with the same composition did not improve shoot yield (increase in shoot length). The shoots of explants cultured on media with high PGR concentrations, mostly suffered hyperhydricity (vitrification) and hence leaf fall and browning of the shoot apexes (Kataeva *et al.*, 1994). Subsequent subculture of explants to either fresh media or media without PGRs resulted in massive loss of explants. All experimental attempts to induce roots on stabilized *in vitro* shoots also failed.

4.4 Induction of Callus

Callus was successfully induced from leaf discs (particularly from the midrib) cultured on McCown's basal medium supplemented with Gamborg's vitamins, 3% (w/v) sucrose, 8mgl^{-1}agar and fortified with KIN + 2,4-D treatments (**Table 8**). The induced callus turned brown and eventually died after 12 weeks in culture. Similar results were reported in *Eryngium foetidum* (Martin, 2004). Browning of the callus, which has been reported previously, may be due to the activation of secondary metabolite synthesis (Ignacimuthu *et al.*, 1999).

Somatic embryogenesis was however not achieved. For expression of embryogenic competence, the plant growth regulators supplied to the culture medium and the endogenous hormones in the tissue of the primary explant must be in a proper balance (Jimenez 2005). The available literatures indicate that the concentration of

endogenous cytokinins in the zygotic embryo is species-specific and that it changes during seed development (Van Staden 1983). An excess of a cytokinin is known to inhibit SE induction, as has been shown in legumes by Murthy *et al.,* (1995) and more recently by Pintos *et al.,* (2002). In assessing the effect of exogenous growth regulators on SE induction, the regulatory role of auxins and cytokinins on the expression of somatic embryogenesis receptor kinases (SERKs) also needs to be taken into account (Nolan *et al.,* 2003). These enzymes are differentially expressed during zygotic embryo development, and this is believed to be directly related to the SE competence of particular embryo cells (Hecht *et al.,* 2001).

Induction of callus in *A. stuhlmannii* leaf discs is however a possible indication of the potential of this tree species to produce primary somatic embryos that can be made to undergo secondary somatic embryogenesis (Raemakers *et al*., 1995). Some cultures are able to retain their competence for SSE for many years, and thus provide useful material for various studies, as described for *Vitis rupestris* (Martinelli *et al.,* 2001). Since new embryos are continually formed from existing embryos, SSE has the potential to produce many plants and once initiated, may continue to produce embryos over a long period of time (Pinto *et al.,* 2002). Therefore, in plants with long life cycles, such as dicotyledonous woody plants like *A. stuhlmannii*, preserving embryogenic lines can be a cost-effective maintenance while those line are tested in field (Handley, 1995).

CHAPTER SIX

6.0 CONCLUSION

This is research work successfully established a sterilization protocol for *A. stuhlmannii* explants for tissue culture. The established protocol consisted of subjection of explants to 2% Redomil® for 15 minutes followed by immersion into 8% sodium hypochlorite for 10 minutes and then rinsing them three times using sterile distilled water. The findings of this research work also showed that choice of nutrient medium (Mccown's woody plant medium, with modification of Calcium (76mg/l) and Boron (6.0mg/l)) for tissue culture of *A. stuhlmannii* is a requirement for successful establishment of viable *in vitro* explants for manipulation. Of all plant growth regulators used in shoot induction, it was established that fortification of the modified Woody plant medium with $1.2mgl^{-1}$KIN or $1.2mgl^{-1}$ BAP enhance shoot induction (axillary shoot production). Callus was also successfully induced from leaf discs cultured on McCown's basal medium supplemented with Gamborg's vitamins, 3% (w/v) sucrose, $8mgl^{-1}$agar and $1mgl^{-1}$ KIN + 1.25mg/l 2,4-D. Establishment of sterilization protocol and induction of shoots from shoot tips and nodal explants and also callus induction from leaf discs clearly shows that *Allanblackia stulhmannii* is amenable to micropropagation technique.

6.1 RECOMMENDATIONS

Successful establishment of sterilization protocol and identification of suitable medium and plant growth regulators for shoot and callus induction provides ground for exploring the *in vitro* regenerative potential of *Allanblackia stuhlmannii*. There is however need for more studies to determine the effects of using other types of polyamines such as 6-γ-γ-Dimethylaminopurine (2iP), putrescine and spermidines at varied concentrations in optimizing shoot multiplication and root induction for this particular tree species. There is also need to explore other documented scientific procedures (micropropagation protocols) and different type of explants (cotyledons, hypocotyls and roots) to induce somatic embryos for this particular tree species. Microscopic study on the induced callus is highly recommended to determine the various forms and stages of growth and development made by *A. stuhlmannii*. Further research work to determine the cause of death of *in vitro* explants at 12^{th} week will help stabilizes shoot proliferation stage and hence avail more explants for rooting experiments. Finally, there is need to attempt other *in vitro* rooting techniques and also use other plant growth regulators (not used in this study) to induce root in *A. stuhlmannii.*

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APPENDICES

1. Nutrient media

	Anderson's Basal Salt mixture	DKW Basal Salt Mixture	Lloyd &McCown's Woody Plant Salts	Quoirin & Lepoivre Basal Salt Mixture	Murashige &Scoog	White's media	Gamborg (B5)	Preece Hybrid
MacroElements	**Mg/l**	**Mg/l**	**Mg/l**	**Mg/l**	**Mg/l**	**Mg/l**	**Mg/l**	**Mg/l**
NH_4NO_3	400.0	1416.0	400.0	400.0	1650	0.0	0.0	908.0
KNO_3	480	0.00	0.00	1800	1900	80	2500	0.00
K_2SO_4	0.0	1559.0	990	0.00	0.00	0.00	0.00	1274.5
$MgSO_4.7H_2O$	180.7	361.49	180.7	175.79	180.54	720	121.56	271.09
KH_2PO_4	0.0	265	170	270	170	0.00	170	217.5
Anhydrous $CaCl_2$	332.2	112.5	72.5	0.00	332.02	0.00	113.23	92.5
$Ca(NO_3)_2.4H_2O$	0.00	1367	386.0	833.77	0.00	300	0.00	876.5
$NaH_2PO_4).H_2O$	330.0	0.00	0.00	0.00	0.00	16.5	130.44	0.00
$(NH_4)_2SO_4$	0.00	0.00	0.00	0.00	0.00	0.00	134	0.00
MicroElements	**Mg/l**	**Mg/l**	**Mg/l**	**Mg/l**	**Mg/l**	**Mg/l**	**Mg/l**	**Mg/l**
$CoCl_2.6H_2O$	0.025	0.00	0.00	0.025	0.025	0.00	0.025	0.00
$CuSO_4.5H_2O$	0.025	0.025	0.025	0.025	0.025	0.001	0.025	0.025
FeNaEDTA	36.7	33.8	36.7	36.7	36.7	3.47	36.7	30.83
H_3BO_3	3	4.8	6.2	6.2	6.2	1.5	3	5.5
KI	0.75	0.00	0.00	0.08	0.83	0.75	0.75	0.00
$MnSO_4.H_2O$	10	33.5	22.3	0.76	16.9	5.31	10	27.9
$Na_2MoO_4.2H_2O$	0.25	0.00	0.25	0.25	0.25	0.001	0.25	0.32
$ZnSO_4.7H_2O$	2	17.0	8.6	8.6	8.6	2.67	2.0	4.3
$NiSO_4$	0.00	17.0	0.00	0.00	0.00	0.00	0.00	8.5
Vitamins	**Mg/l**	**Mg/l**	**Mg/l**	**Mg/l**	**Mg/l**	**Mg/l**	**Mg/l**	**Mg/l**
Glycine	0.00	0.00	2.0	0.00	2.00	0.00	0.00	1.0
Nicotinic acid	0.00	0.00	0.5	0.00	0.5	0.00	1.00	0.25
Pyridoxine	0.00	0.00	0.5	0.00	0.5	0.00	1.00	0.25
Thiamine	0.40	0.00	0.00	0.00	0.20	0.00	10.0	0.00
Myo-inositol	100.0	0.00	0.5	0.00	100.0	0.00	100.0	0.00
Adenine hemisulphate	80.0	0.00	0.00	0.00	0.00	0.00	0.00	0.00

2. Plant growth Regulators

Hormone	Product Name	Function in Plant Tissue Culture
Auxins	Indole-3-Acetic Acid Indole-3-Butyric Acid Indole-3-Butyric Acid, Potassium Salt α-Naphthaleneacetic Acid 2,4-Dichlorophenoxyacetic Acid p-Chlorophenoxyacetic acid Picloram Dicamba	Adventitious root formation (high concentration) Adventitious shoot formation (low concentration) Induction of somatic embryos Cell Division Callus formation and growth Inhibition of axillary buds Inhibition of root elongation
Cytokinins	6-Benzylaminopurine 6-γ,γ-Dimethylaminopurine (2iP) Kinetin Thidiazuron (TDZ) N-(2-chloro-4-pyridyl)-N'Phenylurea Zeatin Zeatin Riboside	Adventitious shoot formation Inhibition of root formation Promotes cell division Modulates callus initiation and growth Stimulation of axillary's bud breaking and growth Inhibition of shoot elongation Inhibition of leaf senescence
Gibberellins	Gibberellic Acid	Stimulates shoot elongation Release seeds, embryos, and apical buds from dormancy Inhibits adventitious root formation Paclobutrazol and ancymidol inhibit gibberellin synthesis thus resulting in shorter shoots, and promoting tuber, corm, and bulb formation
Abscisic Acid	Abscisic Acid	Stimulates bulb and tubes formation Stimulates the maturation of embryos Promotes the start of dormancy

3. Analysis of sterilization protocol

Table a. Fungal and Bacterial contamination rates and mortality of explants when exposed to different concentrations of Sodium hypochlorite at different times of exposure (means %), *n=90*.

Conc (v/v)	Time(min)	Fungi	Bacteria	mortality
0	10	100.0±0.0	96.0±0.10	0.00±0.0
0	20	100.0±0.0	92.1±0.12	0.00±0.0
0	30	100.0±0.0	76.0±0.11	0.00±0.0
10	10	16.7±0.17	33.3±0.21	33.3±0.21
10	20	33.3±0.21	33.3±0.21	89.0±0.17
10	30	16.7±-0.16	0.0±0.00	92.0±0.01
15	10	33.3±21	16.7±0.16	100.0±0.00
15	20	0.0±0.00	0.0±0.00	100.0±0.00
15	30	0.0±0.00	0.0±0.00	100.0±0.00
20	10	16.7±0.17	16.7±0.17	100.0±0.00
20	20	0.0±0.00	0.0±0.00	100.0±0.00
20	30	0.0±0.00	0.0±0.00	100.0±0.00
25	10	0.0±0.00	0.0±0.00	100.0±0.00
25	20	0.0±0.00	0.0±0.00	100.0±0.00
25	30	0.0±0.00	0.0±0.00	100.0±0.00

Table b.Fungal and Bacterial contamination rates and mortality of explants when exposed to different concentrations and different times of exposure of Sodium hypochlorite (means %), *n=90*.

		Contamination (%)		
Treatment		**Fungi**	**Bacteria**	**Mortality**
Conc. (%)	0	100.0±0.00	88.0±0.11	0.0±0.00
	10	22.2±0.10	22.2±0.10	77.8±0.10
	15	11.1±0.08	5.6±0.06	100.0±0.15
	20	5.6±0.06	5.6±0.06	100.0±0.15
	25	0.0±1.01	0.0±0.01	100.0±0.15
Time (Minutes)	10	26.7±0.12	33.3±0.13	66.7±0.13
	20	20.0±0.11	20.0±0.11	80.0±0.11
	30	20.0±0.11	13.3±0.09	80.0±0.11
L.S.D$_{0.05}$		0.4262	0.5043	0.2695

Table c: Fungal and Bacterial contamination rates and mortality of explants when exposed to different concentrations and different times of exposure of Formaldehyde (means %), *n=90*.

		Contamination (%)		
Treatment		**Fungi**	**Bacteria**	**Mortality**
Conc. (%)	0	100.0±0.00	100.0±0.00	0.0±0.00
	10	44.4±0.99	44.4±0.10	77.8±0.10
	15	11.1±0.99	22.2±0.10	100.0±0.50
	20	11.1±0.99	11.1±0.10	100.0±0.50
	25	0.00±0.98	0.0±0.00	100.0±0.50
Time (Minutes)	10	40.0±0.13	40.0±0.12	66.7±0.13
	20	33.3±0.13	40.0±0.12	80.0±0.10
	30	26.7±0.11	26.7±0.10	80.0±0.10
$L.S.D_{0.05}$		0.4262	0.4243	0.2695

Table d: Fungal and Bacterial contamination rates and mortality of explants when exposed to different concentrations and different times of exposure (means %), *n=180*.

	Time(Minutes)			Conc (v/v)%			
	10	20	30	0	10	15	20
Fungi	36.7	26.7	20	94	27.8	11	5.6
Bacteria	36.7	33.3	23.3	100	33.3	11.1	11.1
Mortality	66.7	80	80	0	77.8	100	100

Table e: Effect of concentration and exposure time on mortality rate (means %), *n=180*

Conc(v/v)%	Time (Minutes)		
	10	20	30
0	0	0	0
10	33.3	100	100
15	100	100	100
20	100	100	100
25	100	100	100

Table f: Effectiveness of the two sterilants in the sterilization (mean %), *n=180*

Variate	Sterilant	
	Formalin	Sodium Hypochlorite
Fungi	66.7	77.2
Bacteria	64.4	73.3
Mortality	24.4	24.4

Table g. Fungal and Bacterial contamination rates and mortality of explants when exposed to different concentrations of Sodium hypochlorite at different times of exposure (means %), *n=54*.

		Contamination (%)		
Conc (v/v)%	**Time(min)**	**Fungi**	**Bacteria**	**mortality**
10	10	33.0±0.33	33.0±0.33	0.0±0.00
10	12	33.0±0.33	33.0±-.33	0.0±0.00
10	15	0.0±0.00	33.0±0.33	33.0±0.33
11	10	0.0±0.00	33.0±0.33	33.0±0.33
11	12	33.0±0.33	0.0±0.00	33.0±0.33
11	15	0.0±0.00	0.0±0.00	67.0±-.33
12	10	33.0±0.33	33.0±0.33	67.0±0.33
12	12	0.0±0.00	33.0±0.33	67.0±0.33
12	15	0.0±0.00	0.0±0.00	100.0±0.00

Table h. Fungal and Bacterial contamination rates and mortality of explants when exposed to different concentrations and different times of exposure of Sodium hypochlorite (means %), *n=54*.

Treatment		**Fungi**	**Bacteria**	**Mortality**
Conc. (%)	10	22.0±0.15	33.0±0.17	11.1±0.11
	11	11.1±0.11	11.1±0.11	44.0±0.18
	12	11.1±0.11	22.0±0.11	78.0±0.15
Time (Minutes)	10	22.0±0.15	33.0±0.17	33.0±0.17
	12	22.0±0.15	22.0±0.15	44.0±0.18
	15	0.0±0.00	11.1±0.11	56.0±0.18

Table i. Fungal and Bacterial contamination rates and mortality of explants when exposed to different concentrations of Sodium hypochlorite at different times of exposure (means %), *n=54*.

Conc (v/v)%	Time(min)	Fungi	Bacteria	mortality
6	6	66.7±0.33	66.7±0.33	0.0±0.00
6	8	100.0±0.00	66.7±0.33	0.0±0.00
6	10	100.0±0.00	66.7±0.33	0.0±0.0
8	6	66.7±0.33	33.3±0.33	0.0±0.00
8	8	66.7±0.33	33.3±0.33	0.0±0.00
8	10	0.0±0.00	33.3±0.33	0.0±0.00
10	6	66.7±0.33	33.3±0.33	33.3±0.33
10	8	33.3±0.33	0.0±0.00	66.7±0.33
10	10	0.0±0.00	0.0±0.00	100.0±0.33

Table j: Fungal and Bacterial contamination rates and mortality of explants when exposed to different concentrations and different times of exposure of Sodium hypochlorite (means %), *n=54*.

		Contamination(%)		
Treatment		Fungi	Bacteria	Mortality
Conc. (%)	6	88.9±0.23	66.7±0.24	0.0±0.00
	8	44.4±0.20	33.3±0.24	0.0±0.0
	10	33.3±0.20	11.1±0.24	66.7±0.0
Time (Minutes)	6	66.7±0.17	44.4±0.18	22.2±0.15
	8	66.7±0.17	33.3±0.17	11.1±0.11
	10	33.3±0.17	33.3±0.17	33.3±0.17

Table k: Summary of Analysis of variance (ANOVA) table showing the effects of the Sterilants on fungi, Bacteria and Mortality

Source of variation	d.f.	Fungi	Bacteria	Mortality
Sterilant	1	0.082	0.187	1
Conc(v/v)%	4	<. 001	<. 001	<. 001
Time(min)	2	0.102	0.244	<. 001
Sterilant * Conc.	4	0.449	0.618	1
Sterilant * Time	2	0.883	0.895	1
Conc * Time	8	0.851	0.647	<. 001
Sterilant * Conc * Xtime	8	0.647	0.979	1

4. Analysis of variance for Shoot and callus induction

Table 1Analysis of variance Variate: shoot_length (treatment:KIN*NAA)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	9	4.34042	0.48227	11.83	<.001
Week	2	0.14056	0.07028	1.72	0.181
Treatment *Week	18	0.44293	0.02461	0.60	0.895
Residual	239	9.74594	0.04078		
Total	268	14.66986			

Table 2 Analysis of variance Variate: shoot_length(treatment:BAP*NAA)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	9	4.38884	0.48765	12.03	<.001
Week	2	0.14136	0.07068	1.74	0.177
Treatment * Week	18	0.49058	0.02725	0.67	0.837
Residual	239	9.68704	0.04053		
Total	268	14.70783			

Table 3 Analysis of variance Variate: shoot_length(treatment:2,4-D*BAP

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	6	1.07621	0.17937	7.58	<.001
Week	2	0.06826	0.03413	1.44	0.239
Treatment * Week	12	0.12607	0.01051	0.44	0.943
Residual	167	3.95037	0.02365		
Total	187	5.22090			

Table4 Analysis of variance Variate: shoot_length(treatment:TDZ*NAA)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	6	0.35330	0.05888	1.31	0.254
Week	2	0.03942	0.01971	0.44	0.645
Treatment * Week	12	3.31828	0.27652	6.17	<.001
Residual	167	7.48901	0.04484		
Total	187	11.20001			

Table 5 Analysis of variance Variate: callus% (Treatment: KIN*2,4-D;explant;leaf)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treat	4	18663.7	4665.9	22.77	<.001
Week	2	0.0	0.0	0.00	1.000
Treat *Week	8	0.1	0.0	0.00	1.000
Residual	119	24385.1	204.9		
Total	133	43048.8			