IN VITRO REGENERATION OF *TRECULIA AFRICANA* DECNE. FROM EMBRYO EXPLANTS ON THE MEDIA OF MURASHIGE AND SKOOG (MS) AND GAMBORG *ET AL*. (B5)

BY

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CERTIFICATION

Isaac, Ugonna Gloria, a postgraduate student of the Department of Plant Science and Biotechnology, University of Nigeria, Nsukka, with registration number: PG/ M.Sc./13/65674 have satisfactorily completed the requirements for the course and research work for the degree of Master of Science (M.Sc.) in the Department of Plant Science and Biotechnology, University of Nigeria, Nsukka. The work contained in the project report is original and has not been submitted in part or whole for any diploma or degree of this or in any other university.

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EXTERNAL EXAMINER

TITLE PAGE

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DEDICATION

This project report is dedicated to God Almighty for his immeasurable love.

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ABSTRACT

The effect of three different concentrations of sucrose namely 2, 3 and 4% were investigated on the *in vitro* regeneration of embryo explants of *Treculia africana* Decne. on the media of Murashige and Skoog (MS) (1962) and Gamborg *et al*. (B5) (1968) respectively without any growth regulator. The experimental design was a 2 x 5 factorial in a completely randomized design with each treatment consisting of ten replicates. Results showed that while both media including control supported the *in vitro* regeneration of *T. africana* embryo explants, B5 medium was found to be significantly superior ($P \leq 0.05$) to MS medium in all the growth parameters studied. B5 medium at 4% sucrose elicited the best response in all the growth parameters determined while control gave the least response. The protocol reported here can be used for large scale propagation of true-to-type *T. africana* plants within a short time for the purpose of improvement through genetic transformation (mutagenesis) and the development of a viable conservation programme.

CHAPTER ONE

INTRODUCTION

There are approximately 400,000 species of higher plants, of which 250,000 have been identified or described (FAO 1996). Among them, some could be described as endangered, rare, vulnerable or endemic and all these are of plant genetic resource (PGR) conservation concern to the country considering the unprecedented rates of occurrence in comparison with normal natural history rate (Ogbu *et* a*l.,* 2010). . The causes of the loss of our plant bio resources in general have been identified to include overexploitation, massive deforestation, and desertification, paucity of institutional frame work to engage in deliberate conservation of PGR relevant to food and agriculture/forestry, inadvertent emphasis on more exotic crop species/ varieties to the neglect of our useful indigenous plants etc. (Ogbu *et* a*l.,* 2010). Given the combined effort of these deleterious factors, genetic erosion becomes inevitable where no deliberate effort is made to forestall it (Petters, 2000).

Treculia africana Decne. (African bread fruit, Wild jack fruit, or African boxwood), is an important multipurpose indigenous tree species in Africa belonging to the taxonomic family Moraceae. The tree serves for the provision of nutritive food for the local population. Analysis of the hexane extract of the seeds indicates that it contains a stearine solid fat fraction, resembling that of palm kernel oil and an aleine fraction with a composition similar to that of cotton seed oil; carbohydrate content 40-50% starch, 3-8% glucose and a good protein content with its lysine 50% higher than that of soya beans and methionine content 1.65% as in soya beans Nutrecul Agroforestry Company (N A C, 2013),. Lyseine is the most limiting amino acid of all vegetable protein; therefore, *T. africana* seems to have an important role in those regions that have a shortage of good protein sources and especially where kwashiorkor appears.

Despite the dietary and economic importance of African breadfruit, it has remained an under-utilized species till now and its potentials remain under-exploited. This problem is capable of causing serious threat of erosion of its genetic resources as well as extinction threat. *T. africana* is currently included in the list of endangered species of Southern Nigeria (Meregini, 2005) and this is quite worrisome. The underutilization has been caused by a number of reasons. Firstly, increase in population pressure especially in the urban places due to rural-urban migration. The increased population is resulting in the conversion of more forests to agricultural lands, industrial estates and homes in order to satisfy the basic needs (food, clothing and shelter) of the teeming population. Currently, erstwhile village fruit tree forests which served as sanctuaries for *in situ* and some degree of *ex situ* conservation of choice plants in several communities are now being shared out to community members for the purpose of agricultural production and even being sold or leased out to companies. The thinking of the village heads is that the establishment of such companies in their domain will provide jobs for their subjects as well as bestow development on their communities. This is however to the contrary often-times as rather than assisting to actualize the development dream, most of these companies end up exploring and over-exploiting the natural resources in these areas and degrade the land through unsustainable exploitation (resulting in grave consequences) which leave the people highly vulnerable to environmental shocks (Nuga and Ofodile, 2010). The Niger Delta region of southern Nigeria is currently having this experience. FAO (2005) reported that Nigeria has the worldós highest deforestation rate of primary forests and a further report, FAO (2006) revealed that the country has lost a staggering 79% of its old-growth forests between 1990 and 2005.

Added to that, the fully grown trees have such a large size that minority of the people would want to leave it in their compound for food, ornamental or aesthetic purposes. The height of the tree and the fruit on the tree of the unimproved varieties such that the fruit cannot be

plucked rather falls downs on its own instills fear in the mind of many. This is because falling down of the fruit head on an individual may cause death or serious injury. South easterners believe that the fruit of the species have some mystical powers which cause the death of persons the fruit falls on accidentally (Nuga and Ofodile, 2010). It is viewed as a ñtabooò for the fruit to fall on a person. The resultant effect of this belief is the felling of African breadfruit trees found in home gardens in spite of the increasing demand for its products. Also, the long gestation period of ten (10) or more years of the species has not helped matters either. This leads to the second reason which is non-improvement and limited cultivation of the species.

Thirdly, there is high rate of deforestation due to industrial, construction and agricultural purposes. The heartwood is yellow with very narrow pale sapwood; very dense, fairly elastic and flexible with fine, even structure. This component makes it suitable for paper industries, building, construction companies, carpentry and joinery (N A C, 2013). All these above reasons call for the need of conservation of this species to avoid genetic erosion. Therefore, there is an urgent need for application of a reliable and efficient *in vitro* system that results in efficient differentiation, shoot development, and whole plant regeneration for the improvement of *T. africana* through genetic transformation or mutagenesis.

Conservation of plants in the field is often costly and carries high risk of loss due to disease infections, demographic and environmental variations, habitat loss, genetic drift, human disturbances etc. Therefore, the strategies and procedures employed to establish and maintain field collections need to be practical, rational and economic in addition to being scientifically sound (Ogbu *et al*., 2010).Most times, experience in field collections lies with individual curators and may not be readily available to guide others. Furthermore, there are methods of using *in vitro* methods (biotechnological approach) for the conservation of crops normally

conserved in the field (Reed *et al*., 2004; Singh, 2006). *In vitro* cell and tissue culture methodology is envisaged as a means for germplasm conservation to ensure survival of endangered plant species, rapid mass propagation for large-scale re-vegetation and for genetic manipulation studies.

Therefore, the aim and objectives of this study are:

- To develop a protocol for regeneration of *Treculia africana* from embryonic axis of seed as a prerequisite for improvement through genetic transformation.
- To compare the effect of two different media i.e. Murashige and Skoog (1962) and Gamborg *et al.*(1968)on the growth of embryonic axis of *T. africana* seed
- To assess the effects of different levels of sucrose on the germination of zygotic embryo of *T. africana*.

CHAPTER TWO

LITERATURE REVIEW

2.1 BOTANY OF *TRECULIAAFRICANA*

Treculia africana is widely grown in southern Nigeria for its seeds and it is known by various tribal names in the country. Such names include: ñafonò (Yoruba), ñbarafutaò (Hausa), ñizeò (Bini), ñeyoò (Igala), ñediangò (Efik) and ñukwaò (Igbo) [Onweluzo and Odume, 2008].The scientific classification is as follows:

Kingdom: Plantae
Phylum: Tracheophyta
Class: Magnoliopsida
Order: Rosales
Family: Moraceae
Genus: *Treculia*
Species: T. *africana*

Treculia africana is a large, evergreen forest tree growing up to 30m high with a girth of 4-6m. It has a dense spreading crown and fluted trunk. The bark is grey, smooth and thick, exuding copious, cream latex which later turns rusty red when cut (Agbogidi and Onomeregbor, 2008). The leaves are simple, alternate, very large, dark green, smooth above, tough and paler below with some hairs on the 10th ï 18th pairs of clear veins. It has pointed tips and a short stalk (WAC, 2004). African breadfruit is a monoecious dicotyledonous plant with flowers crowded into compact heads. The flowers of both sexes are surrounded by specialized bracts between which stamens or stigmas project above the surface of the flower head (Ugwoke *et al.*, 2003). These bracts sometimes terminate in a flat disc or they may develop into bristles. They are persistent and surrounding the developing ovaries and producing a compound fruit often of considerable size and usually found on the trunk or older branches. The fruit is round in shape, big, weighs 8-30kg, greenish yellow in colour and spongy in texture when ripe and contains numerous seeds which are embedded at various depths in the fleshy pulp (Enibe, 2007). The seeds are brown in colour with fibrous coating

(Okafor,1990). There are three varieties of African breadfruit which include: *T. africana* var. *Africana*, *T. africana* var. *Inversa* and *T. africana* var. *Mollis* (Okafor, 1983). Their taxonomic differences are based mainly on the size of the fruit head (infructence) and the hairiness of the branchlets and leaves (N A C, 2013).

Plate 1a: *T. africana* tree (x0.1)

Plate1b: Fluted nature of *T.africana* trunk (x 0.1)

2.2 GEOGRAPHICAL DISTRIBUTION OF *TRECULIA AFRICANA*

Treculia africana is found in Senegal, southern Sudan, Angola, Nigeria, the Islands of Principe and Sao-Tome and throughout the tropics including West Africa and Central Africa sub-region (Okafor, 1985). It is a fruit tree of riverine forest in tropical Africa and it is usually found near streams or swampy areas in forests. It is shade tolerant and grows in a wide variety of soils and climatic conditions (Nuga and Ofodile, 2010).

2.3 USES OF *TRECULIA AFRICANA*

AS FOOD- The seeds are mainly used as food: fried, roasted or cooked. The seeds can also be used for breadfruit flour (after it has been macerated and ground) which has high potential usage for pastries (Keay, 1989). The husks are used in feeding animals. Ejiofor(1988) prepared a non-alcoholic drink from seeds of the species which was found acceptable when taken without milk and sugar therefore; giving the beverage obtained from the species an obvious advantage over cocoa derived beverage in view of the expensiveness of milk and sugar in rural areas of developing countries.

AS MEDICINE- In Ghana, a root decoction of the species is used as an anthelmintic and febrifuge, and the caustic sap (from the male species) is applied on carious teeth. Also, a bark decoction is used as cure for cough and whooping cough while the ground bark mixed with oil and other plant parts is used as cure for swellings (WAC, 2004). Liquid extract of the boiled leaves is taken as cure for rashes in the Eastern part of Nigeria. It was also gathered that the water extracted from the boiled seeds helps to cleanse or purify the stomach when taken.

AS FUEL- *Treculia africana* is a suitable source of firewood and charcoal.

ENVIRONMENTAL FUNCTIONS- African breadfruit grows on a wide range of sites and Is found at altitudes ranging between 0 to 1500m (N A C, 2013). The species makes good use of marginal areas where other species may not be able to grow. The species therefore, helps to control erosion and also in soil conservation as the tree is a good source of mulch.

WOOD PRODUCTS- The wood of the species is put into various uses. It is suitable for roofing, carving, furniture making, pulp and paper as well as fibre-board production as pointed out by Agbogidi and Onomeregbor (2008) and WAC (2004).

2.4 CONVENTIONAL PROPAGATION OF *TRECULIA AFRICANA*

Propagation of *Treculia africana* is mostly by seed. It can also be propagated through cuttings, budding and shift grafting. With juvenile cutting, one can obtain 69% rooting. In normal circumstances, the duration of the germination of the seeds is 2 weeks with 70-90% germination (N A C, 2013); therefore it is a fast-growing tree. Study conducted by Efemena (2013) shows that seed storage has a deleterious effect on the germination of the species, thus implying that storing the seeds beyond two weeks could impede viability.

2.5 *IN VITRO* PLANT REGENERATION

Totipotency is the ability of a cell if given a favourable environment to regenerate into whole plant. Totipotency forms the basis upon which plant regeneration occurs *in vitro* in tissue culture studies. Plant tissue culture is the science (or art) of growing plant cells, tissues or organs isolated from ó motherô plant on artificial media. It could also be defined as the *in vitro* cultivation of plant cells, tissues or organs on solid or liquid artificial media under aseptic conditions (Kyesmu *et al.,* 2004). Efficient, repeatable, and rapid *in vitro* regeneration systems are a prerequisite for using recent advances in biotechnology to improve crop plants.

In vitro plant regeneration has allowed for mass propagation of plants using any part, controlled manipulation of plants at the cellular level, germination of recalcitrant and dormant seeds and more importantly in embryo rescue. Interspecific hybridization is an important tool for introducing valuable tools from wild species into the gene pool of cultivated plant species. A major difficulty in obtaining hybrids appears to be in some cases, the failure of seed development, since fertilization occurs but embryos are aborted (due to embryo/endosperm incompatibility). By extracting such embryos before they abort and culturing them *in vitro*, plant breeders can have access to rare and desirable genetic combinations (Kyesmu *et al.*, 2004).The process of extracting such embryo is referred to as embryo rescue.

In vitro meristem culture technique offers the possibility of eliminating viruses and thus, exchanges of virus-free germplasm. *In vitro* slow/normal growth techniques offer up to medium-term storage option, avoiding risk of losses of germplasm on field genebank due to insects, nematodes, disease attacks and natural disasters (Ogbu *et al.*, 2010). It is commonly used for vegetatively propagated species, non-orthodox seeded species and wild species which produce little or no seeds. Additionally, it also opens new avenues for producing novel planting materials via genetic engineering because tissue culture is the means for regeneration of tissues transformed with genes or traits of interest. The process of tissue culture from the explant to the final stage of plantlet requires four basic stages, namely:

Stage 0 selection and maintenance of stock plants for culture initiation

Stage 1 preparation and establishment of explants on suitable culture media

Stage2 multiplication of shoot or somatic embryo on defined culture media

Stage3 rooting of regenerated shoot or somatic embryo *in vitro*

Stage4 transfer of plantlets to soil for hardening under green house (Kyemsu *et al.*, 2004)

Different works on plants have been done using *in vitro* regeneration methods. *In vitro* culture of embryonic axis of different cultivars of *Phaseolus vulgaris* showed successful regeneration using the media of Murashige and Skoog supplemented with 100 mg l^{-1}myo-inositol, 1 mg l^{-1} thiamine, 30 g l^{-1} sucrose, BAP(0,5 and 10 mg l^{-1}) and 8 g 1^{-1} agar (Jenny *et al.,* 2010). Prabhat *et al.* (2009) reported that *Rauwolfia serpentina* L. an endangered species, was also regenerated using the juvenile leaf explants on the media of Murashige and Skoog supplemented with various combinations of growth regulators.

Some factors have been identified as affecting *in vitro* regeneration and these include: temperature, light, pH, plant growth regulators and orientation of the explants on the medium. The physical status of the plant and the genotype also has roles to play in the regeneration process. Continous light period was found optimum for endosperm proliferation of *Ricinus communis.* The optimum temperature for the growth of its endosperm was reported to be 25^{o}C and pH was 5.0 (Srivastava and Johri, 1973). Ripley and Preece (1986) working on *Euphorbia lathyris* found that minimal wounding and vertical orientation of the explants during inoculation are vital for the shoot tip rise for which 6-Benzyadenine (BA) is not required whereas for callousing and adventitious root formation, 6-Benzyadenine was necessary.

2.5.1 *IN VITRO* REGENERATION IN MORACEAE FAMILY

Successful cases of plantlet regeneration *in vitro* from apical/auxiliary shoot buds and nodal explants either with or without encapsulation have been reported for a number of species of mulberry, a member of the Moraceae family. Best shoot induction was observed on MS basal media supplemented with 0.5 mg/l BAP and 0.1 mg/l IAA, in which 78% of the explants produced 16 shoots per culture (Sayeed-Hassan *et al.*, 2009). Also, Judy and Julian (2000) established that *Artocarpus altilis* shooted more than 50% in Murashige and Skoog supplemented with zeatin at 2.2um and also rooted on hormone free media with success rate

of about 60%. Studies on *Artocarpus heterophyllus* (Ashrafuzzaaman *et al.,* 2012*)* showed that regeneration of roots increased comparatively better when MS medium was enriched with 2 mg/l of BAP (6-benzyladenine).

2.5.2 *IN VITRO* REGENERATION OF *TRECULIA AFRICANA*

To abate total loss or extinction of *Treculia africana,* this tree species must be cultivated intensely. Significant to achieving this is the improved propagation of the concerned variety of this species either by seed or stem cuttings. *In vitro* regeneration seems to be a suitable option for this mass propagation since few empirical studies (Baiyeri 2003; Ugwunze, 2003) using fresh seeds for planting reported less than 80% seedling emergence. Besides, practical observation showed that the seeds must be planted when fairly fresh as they lose viability in few weeks (Osuji and Owei, 2010). This viability problem due to long storage of seeds can also be overcome by *in vitro* regeneration but few works have been done on this specie necessitating the need for this study. Attah and Okezie (2015) worked on *Treculia africana* and discovered growth of the embryo explants on MS , Gamborg and Hilderbrant media.

2.6 EMBRYO CULTURE

Embryo culture involves the use of sexually produced embryo (zygotic embryogenesis) for *in vitro* culture. Embryo culture has been used in overcoming embryo inviability and seed dormancy; reduction of germination time and production of interspecific and intergeneric hybrids. Embryo culture techniques can eliminate the constraint to seed germination caused by the seed coat and endosperm and provide a long-term storage of germplasm in a disease-and-insect free form (Okezie and Okonkwo, 1992). In embryo culture, either immature or matured embryo can be used. In culture of immature embryos, isolated embryos originated from unripe or hybrid seeds which failed to germinate are used while in that of matured embryo, mature embryos are excised from ripe seeds and cultured mainly to avoid inhibition

of germination. Many species of terrestrial orchids are very difficult to germinate from fully mature seeds therefore the use of immature embryos can circumvent this problem e.g. in *Cypripedium* where isolated embryos that originate from unripe or hybrid seeds which failed to germinate were grown in embryo culture. Teixeira *et al.* (1993) have established that culture of immature zygotic embryo (ize) at various stages of development could lead to unique responses in comparison with mature zygotic embryos (mze).

The most useful and popular application of embryo culture, has been to raise rare hybrids by rescuing embryos of incompatible crosses. Also, due to their juvenile nature, embryos have a high potential for regeneration and hence may be used for *in vitro* clonal propagation (Razdan, 2003. Embryo culture has been successful in solving problem of low seed set, seed dormancy, slow seed dormancy, inducing embryo growth in the absence of a symbiotic partner (Raghavan, 1980, 1994; Zenkteler, 1990).

2.7 SUCROSE AS A CARBON SOURCE

Sugar is a very important part of nutrient medium as energy source, since most plant cultures are unable to photosynthesize effectively owing to inadequate cellular and tissue development, lack of chlorophyll, limited gas exchange and carbon dioxide in tissue culture vessels. Hence, they lack autotrophic ability and need external carbon for energy. Sugars have important role acting in a hormone-like manner to affect photosynthesis, respiration and metabolic activity (Rolland *et al.*, 2002). They affect the growth of some tissues; physiology differentiation of tissues in increasing growth and organ induction of tissues (Aloni, 1980; De Faria *et al*. 2004).

Sucrose at a concentration of 2-5% is the most frequently used and preferred carbon source *in vitro*. This is because during autoclaving, sucrose is easily hydrolyzed into more efficiently utilizable sugars such as glucose and fructose (Saad and Elshahed, 2012).

Glucose is utilized by cell in the beginning, followed by fructose later making sucrose better than either glucose or fructose alone. Beside sucrose, other carbohydrates are also used which include lactose, galactose, maltose and starch, but they were reported to be less effective than either sucrose or glucose. Fructose, if autoclaved is toxic. Sucrose contains no free aldehyde or ketone group making it less reactive as it travels along the plant. Also, sucrose has high solubility in water hence, to transport molecule in plant system and to apparent lack of inhibitory effect on the various biochemical mechanism (Smith, 1993). It was frequently demonstrated that autoclaved sucrose was better for growth than filter sterilized sucrose (Saad and Elshahed, 2012). This is because autoclaving seems to hydrolyze sucrose into more efficiently utilizable sugars such as fructose. Sucrose was reported to act as morphogenetic trigger in the formation of auxiliary buds and branching of adventitious roots (Vinterhalter and Vinterhalter, 1997). Carbohydrates also provide osmoticum and hence in anther culture higher concentration of sucrose (6-12%) is used.

2.8 **NUTRIENT MEDIA**

The type and composition of culture media strongly govern the growth and morphogenesis of plant tissues. The choice of tissue culture medium largely depends upon the species to be cultured. Some species are sensitive to high salts or have different requirements for PGRs (Plant Growth Regulators). Some tissues show better response on solid medium while others prefer a liquid medium. Therefore, development of culture medium formulations for a species is a result of systematic trial and experimentation considering specific requirements of a particular culture system.

The earliest nutrient media used for growing plant tissues *in vitro* were based on the nutrient formulations for whole plants, for which they were many(White,1963) but Knopôs solution and that of Uspenski and Uspenskia were used the most, and provided less than 200 mg/l of

total salt (Thorpe, 2005). Based on studies with carrot and virginia creeper tissues, the concentration of salts was increased twofold (Heller**,** 1953), and was further increased to 4 g/L based on work with Jerusalem artichoke (Nitsch and Nitsch, 1956). However, these changes did not provide optimum growth for tissues, and complex addenda, such as yeast extract, protein hydrolysates, and coconut water, were frequently required. In a different approach, based on an examination of the ash of tobacco callus Murashige and Skoog (1962) developed a new medium. The concentration of some salts was 25 times that of Knopôs solution. In particular, the levels of $NO_3^{ï}$ and NH_4^+ were very high and the arrays of micronutrients were increased. MS formulation allowed for a further increase in the number of plant species that could be cultured, many of them using only a defined medium consisting of macro- and micronutrients, a carbon source, reduced N, B vitamins, and growth regulators (Gamborg *et al*., 1976). The MS salt formulation is now the most suitable and widely used nutrient medium in plant tissue culture. It is a high salt medium due to its content of potassium and nitrogen salts. In bambaranut regeneration, Kone *et al.* found out that among the five culture media tested, the highest size of the seedling (10.48 cm) and the most important root length (21.51 cm) were observed with the plants developed from seed without coat on MS basal medium. Also, better plant growth from embryonic axis was observed on MS medium compared to other basal media in *Juglans regia* L. (Sánchez-Zamora, *et al*., 2006).

CHAPTER THREE
MATERIALS AND METHODS

3. 1 SITE OF THE EXPERIMENT

This study was conducted at the South- East Zonal Biotechnology Central Laboratory, University of Nigeria, Nsukka.

3.2 SOURCE OF EXPLANTS

Treculia africana seeds were obtained from fresh fruit heads (after 3days of fall of the fruit heads) at a farm in Nsukka, in Nsukka Local Government area of Enugu State. They were later identified in the Herbarium of Department of Plant Science and Biotechnology, University of Nigeria, Nsukka. The explants were obtained by excising the seeds (separating the cotyledons since the plant is dicotyledonous in nature). The embryos measured between 0.7 and 0.9cm.

Plate 2a: Fruits of *Treculia africana*(x 0.2)

Plate 2b: Seeds of *Treculia africana* (x 0.5)

3.3 **PREPARATION AND STERILIZATION OF EXPLANTS**

Seeds were washed with running tap water and soaked for a minute in 70% ethanol while being stirred. This was followed by placing the seeds in 20% (v/v) sodium hypochlorite with two drops of Tween twenty for 20 minutes and rinsed in three changes of sterile distilled water. The seed coat along with the two cotyledons was separated from the embryo using

sterile forceps and scalpels and then the embryo was used for the *in vitro* culture on the growth media. Embryo excision and culture was done in the laminar air flow chamber that was previously exposed to ultraviolet radiation for 30 minutes in order to avoid contamination.

3.4 PREPARATION OF STOCK SOLUTIONS

Stock solution is a concentrated solution of known or accurate concentration to be diluted for future laboratory use. It is used to save time, conserve materials, reduce storage space and improve accuracy with which working lower concentrations solutions are prepared.

In this study, series of stock solution of the media were made. The media comprised of macro salts, micro salts, iron compounds and organics (myo-inositol, thiamine-HCl, nicotinic acid, pyridoxine and glycine). Appropriate amount of these inorganic salts and vitamins were measured out using a weighing balance (Sartorius BS 323s) (Appendix 1). One litre of sterile distilled water was added to dissolve the macro and micro nutrients differently using a magnetic stirrer while 100 ml of sterile distilled water was used to dissolve iron compounds, $CaCl_2.H_2O$, myo-inositol and other vitamins. Macro salts were then considered as stock solution A, iron compounds as stock solution B (stored in an amber bottle to protect it from light) and calcium chloride dehydrate ($CaCl_2.H_2O$) as stock solution C. $CaCl_2.H_2O$ even though a macro salt was dissolved separately to avoid precipitation. Similarly, micro salts were considered stock solution D, vitamins as stock solution E and then myo- inositol as stock solution F. They were dissolved using a magnetic stirring bar on a magnetic stirrer hotplate to ensure homogeneity. They were properly labeled and stored at 4^0C.

3.5 MEDIA AND CULTURE CONDITIONS

The media comprising of macro and minor elements according to Murashige and Skoog and then Gamborg *et al.* supplemented with myo-inositol (100 mg/l), Thiamine HCl (1 mg/l), pyridoxine(5 mg/l), Nicotinic acid(5 mg/l) and sucrose were employed as basal media in this

experiment. Cultures with only agar and water were maintained as control. Twenty, 30 and 40 g of sucrose were weighed out in two sets (each set for a media) and each put in 1000 ml bottom conical flask to which 900 ml of sterile distilled water was added to respectively. These were dissolved using a magnetic stirrer. Fifty ml of stock solution A, 5 ml each of B, C and D and 1 ml each of E and F were added to each conical flask. The pH of the medium was adjusted to 5.8 with 1M NaOH. Seven grams of Fluka agar was added to each conical flask and made up to 1 litre prior to autoclaving by steam sterilization at 103 KN M^{-2} pressure and 121^0C for 15 mins. Ten ml of the media were later dispensed into the test tubes accordingly and left to solidify. The embryos were cultured singly in Pyrex test tubes at 27 ± 2^0C under 16-h light/8-h dark photoperiod at a photon flux density of 60 µmol m^{-2} S^{-1} provided by cool white fluorescent tubes. All operations starting from the preparation of explants to establishment of cultures were carried out in a laminar air flow hood chamber previously kept sterile by swabbing with alcohol and exposure to ultraviolet light for 30 mins. The cultured embryos were left to grow for four weeks after which they were scored for the requisite growth parameters.

3.6 EXPERIMENTAL DESIGN

In this study, experiment was carried out in a 2x5 factorial in a Completely Randomized Design (CRD). Experimental design consisted of nine treatments with ten replications in each treatment.

3.7 GROWTH PARAMETERS ASSESSED

The growth and development of embryos of *Treculia africana* were monitored at a two-day interval from the day of inoculation. On the thirtieth day, regenerated plantlets under each treatment were scored for the following parameters: number of adventitious roots, length of roots, length of shoots, leaf area, sprout rate and sprout percentage.

The numbers of adventitious roots was determined by counting while the length of shoots and roots were measured with a rope later superimposed on a metre rule.

For the leaf area, each of the plantlets was detached and the length and width of the leaves measured with a metre rule. The value obtained was multiplied with a constant, 0.75 (Francis *et al*., 1969). Leaf area was determined using:

Leaf area (L A) = L x W x 0.75

The percentage and rate of germination were estimated using a modified Timson Index of Germination Velocity (Khan and Ungar, 1984).

Percentage of germination at 2-day interval = G/T X 100/1

Where,

G = Germinated number of embryos

T = Total number of embryos

Also, rate of germination = $\sum G/t$

Where,

G = Percentage germination at 2-day interval

t = total germination period

Sprout rate of was calculated as the reciprocal of the number of days on which 50% sprouting was achieved.

3.7 **STATISTICAL ANALYSIS**

SPSS software was used to carry out data analysis. One-way analysis of variance (ANOVA) followed by Duncanôs New Multiple Range Test (DNMRT) was used to test for significance ($P \leq 0.05$) and compare mean values respectively.

CHAPTER FOUR

RESULTS

4.1 *IN VITRO* REGENERATION OF EMBRYO EXPLANTS

The embryos of *Treculia africana* cultured on both MS and B5 media began to show visible changes by the second day in culture. The white or milkish embryo that had a large size of about 0.7-0.9cm (**Plate 3)** had begun expansion on that day. By the third day, the embryos had all turned greened (**Plate 4**). There was emergence of the radicle and plumule (sprouting) from of the embryo between the fourth and the fifth day (**Plate 5**) and by the sixth day, all the embryos had sprouted except some in the control. The plumule and radicle finally resulted into shoot and root respectively (**Plate 6**). Embryo explants cultured on only 0.7 per cent agar and water alone also showed changes by the second day and maintained a healthy growth till the last day of the culture. These visible growths in all the treatments enhanced the comparison of growth parameters among them. There were no changes in the growth parameters between the third and the fourth week.

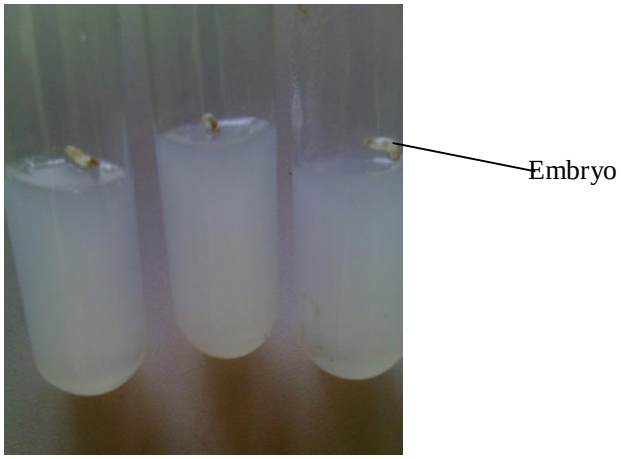

Plate 3: Embryos of *Treculia africana* (x 1)

On first day

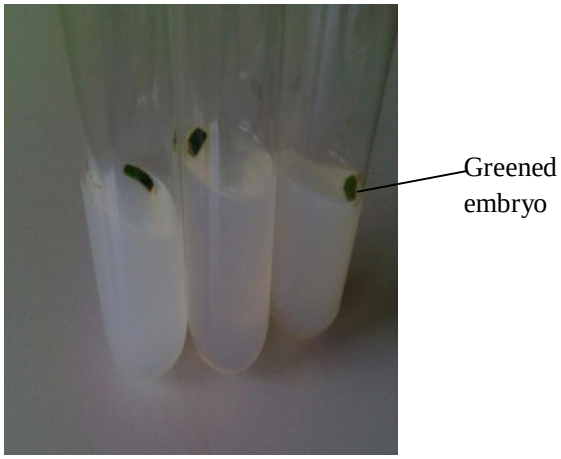

Plate 4: Embryos of *Treculia africana* showing greening (x0.5) on third day

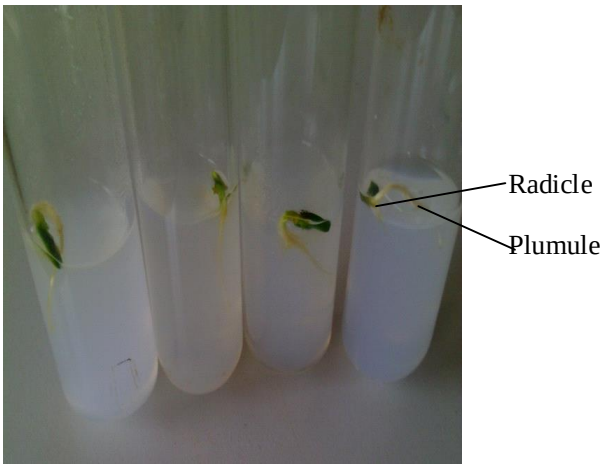

Plate 5: Embryo of *T. africana* on fourth day showing embryo explants showing sprouting (x1)

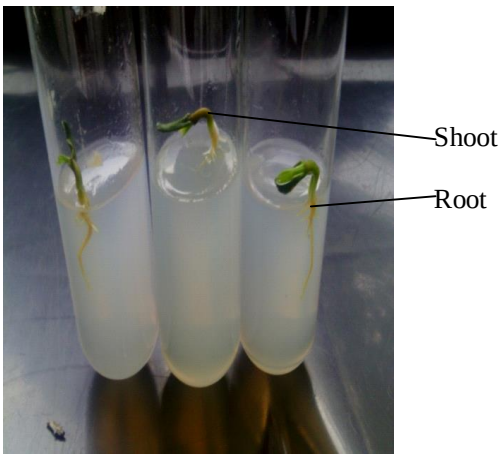

Plate 6: Plantlets arising from shoot and root (x1)

4.2 EFFECTS OF SUCROSE CONCENTRATIONS AND BASAL MEDIA ON PERCENT SPROUTING AND SPROUT RATE OF EMBRYO EXPLANTS OF *T. AFRICANA*

Analysis of variance on (**Appendix 2)** showed that there was no significant difference in per cent sprouting among the treatment means since P≥0.05. Maximum sprouting percentage was achieved on 4% sucrose in B5 (91.10 ± 8.90) while the least was control (68.87± 15.55) (Table 1).

Analysis of variance (**Appendix2**) also showed that there was a significant difference among the treatment means (P ≤ 0.05) in terms of sprout rate. Comparison test using Duncan's New Multiple Range Test (DNMRT) **(Table1)** showed that all the treatment means were significantly higher than control in terms of sprout rate. It further shows that seven out of the nine treatments had same and highest sprout rate of 0.25± 0.00, followed by MS 0 and then control (0.20 ± 0.00) as the least.

Table 1: Sprout rate of embryo explants of *T. africana* as affected by sucrose concentrations and basal media.

Basal Media	Sucrose Concentration %	Sprout Rate	Per cent sprouting
MS	0	0.23± 0.02^{b}	76.90±11.80
	2	0.25± 0.00^{b}	88.90±11.10
	3	0.25± 0.00^{b}	88.87±4.43
	4	0.25± 0.00^{b}	88.87±4.43
B5	0	0.25± 0.00^{b}	82.20±11.10
	2	0.25± 0.00^{b}	86.67±13.33
	3	0.25± 0.00^{b}	84.48±8.87
	4	0.25± 0.00^{b}	91.10±8.90
CONTROL	0	0.20±0.00^{a}	68.87±15.55

Values represent Mean± SE. Mean values within a column followed by different letters are significantly different from each other by DNMRT (P≤0.05)

4.3 EFFECT OF BASAL MEDIA AND CONCENTRATIONS OFSUCROSE ON ROOT AND SHOOT LENGTH OF PLANTLETS ARISING FROM THE EMBRYO.

The highest length of root was recorded at 4% sucrose in B5 (5.02±0.3) and this was significantly different from all others while the least was recorded for control (2.44±0.21) (Table 2)

The 4% sucrose in B5 media promoted maximum growth of *T. africana* shoot but did not differ significantly from 3% and 4% sucrose in B5 and MS respectively while differing significantly from others. ANOVA (**Appendix 3)** showed that there was a significant difference in basal medium and the interaction between the sucrose levels and basal media in terms of root length and shoot length since $p \leq 0.05$.

Table 2: Effect of basal media and sucrose concentrations on root and shoot length of *T. africana* plantlets.

Basal Media	Sucrose Concentration %	Mean no of Root length (cm)	Mean no of Shoot length (cm)
MS	0	2.54 ± 0.12^{a}	1.80 ± 0.10^{ab}
	2	2.63 ± 0.15^{a}	2.11 ± 0.20^{abc}
	3	2.95 ± 0.30^{a}	2.87 ± 0.38^{bcd}
	4	3.94 ± 0.42^{bc}	4.41 ± 0.66^{ef}
B5	0	2.54 ± 0.19^{a}	1.81 ± 0.25^{ab}
	2	3.15 ± 0.19^{ab}	3.33 ± 0.35^{cde}
	3	4.74 ± 0.53^{cd}	3.62 ± 0.25^{def}
	4	5.02 ± 0.32^{d}	4.77 ± 0.78^{f}
CONTROL		2.44 ± 0.21^{a}	1.50 ± 0.17^{a}

Values represent Mean ± SE. Mean values within a column followed by different letters are significantly different from each other by DNMRT ($P \leq 0.05$)

4.4 EFFECT OF BASAL MEDIA AND CONCENTRATIONS OF SUCROSE ON NUMBER OF ADVENTITIOUS ROOT AND NUMBER OF LEAVES

Result showed that for the both media, the number of adventitious root increased as the sucrose level increased. The same table also showed that there was a significant difference among the treatment means in terms of leaf area (Table 3).

The 4% sucrose in B5 had the highest effect on number of adventitious root (41.0±2.40) and differed significantly from others while control (7.20±0.93) recorded the least. Results also showed that for leaf area, 4% sucrose in B5 medium had highest growth (2.94±0.43) but did not differ significantly from 4% sucrose in MS while differing from others. Control also recorded the least. It could be seen from the table that Gamborg media (B5) had better effect on leaf area at each level of sucrose concentration (Table 3). Analysis of variance on **Appendix 4** indicated that there were significant differences between the basal media in terms of number of adventitious roots since $p \leq 0.05$ and this was due to sugar-media interaction

Table 3: Effect of basal media and sucrose concentrations on number of adventitious root and leaf area of *T. africana* plantlets.

Basal Media	Sucrose Concentration %	Mean no of Adventitious Root	Leaf Area (cm^2)
MS	0	9.56 ± 1.07^{ab}	0.47 ± 0.11^{a}
	2	14.40 ± 1.64^{b}	0.81 ± 0.26^{a}
	3	28.10 ± 1.97^{c}	1.25 ± 0.25^{ab}
	4	31.30 ± 3.42^{c}	2.30 ± 0.25^{cd}
B5	0	7.80 ± 0.93^{a}	0.37 ± 0.08^{a}
	2	25.80 ± 2.25^{c}	1.12 ± 0.27^{ab}
	3	30.10 ± 3.00^{c}	1.75 ± 0.44^{bc}
	4	41.00 ± 2.40^{d}	2.94 ± 0.43^{d}
CONTROL		7.20 ± 0.93^{a}	0.39 ± 0.07^{a}

Values represent Mean ± SE. Mean values within a column followed by different letters are significantlydifferent from each other by DNMRT ($P \leq 0.05$)

TWO WEEK OLD PLANTLETS

Leaf

Leaf

Stem

Stem

Root

Root

Plate 7: 2 week old *Treculiaafricana* plantlets arising from embryo explants inMS medium containing 2% sucrose (x 1)

Plate 8: 2 week old *Treculiaafricana* plantlets arising from embryo explants in MS medium containing 3% sucrose (x 0.5)

Plate 9: 2 week old *Treculiaafricana* plantlets arising from embryo explants in B5 medium containing 2% sucrose (x 1)

Plate 10: 2 week old *Treculiaafricana* plantlets arising from embryo explants in B5 medium containing 3% sucrose (x0.2)

Plate 11: 2 week old *Treculiaafricana* plantlets arising from embryo explants in MS medium containing 4% sucrose (x 0.5)

Plate 12: 2 week old *Treculiaafricana* plantlets arising from embryo explants in B5 medium containing 4% sucrose (x1)

Plate 13: 2 week old *Treculia africana* plantlets arising from embryo explants in B5 medium containing no sucrose (x 0.2)

Plate 14: 2 week old *Treculia africana* plantlets arising from embryo explants in MS medium containing no sucrose (x0.1)

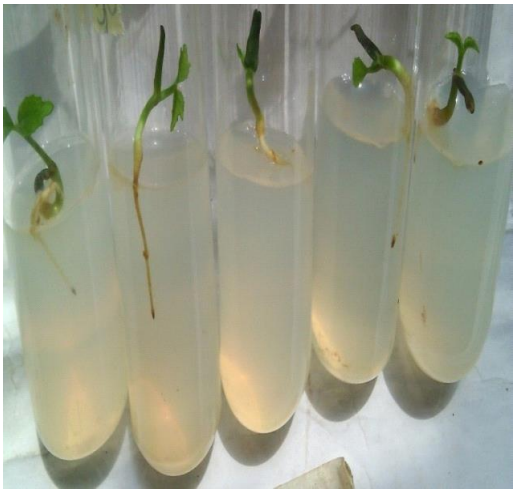

Plate 15: Control (x1)

THREE WEEK OLD PLANTLETS

Plate 16: 3 week old *Treculiaafricana* plantlets arising from embryo explants in MS medium containing 2% sucrose (x 1)

Plate 17: 3 week old *Treculiaafricana* plantlets arising from embryo explants in MS medium containing 3% sucrose (x1.5)

Plate 18: 3 week old *Treculiaafricana* plantlets arising from embryo explants in B5 medium containing 2% sucrose (x 0.5)

Plate 19: 3 week old *Treculiaafricana* plantlets arising from embryo explants in B5 medium containing 3% sucrose (x1)

Plate 20:3week old *Treculiaafricana* plantletsarising from embryo explants in B5 medium containing 4% sucrose (x 1)

Plate 21:3 week old *Treculiaafricana* plantlets arising from embryo explants in B5 medium containing 4% sucrose (x 1)

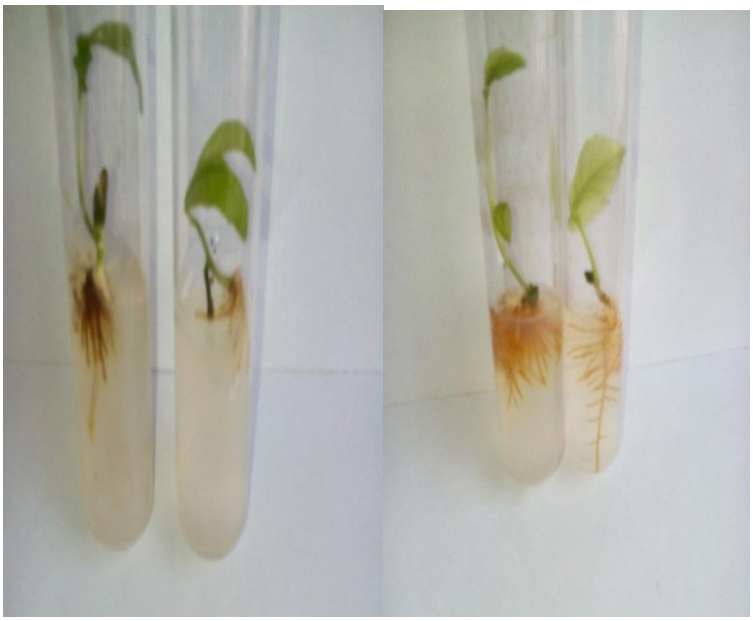

Plate 22:3 week old *Treculiaafricana* plantlets arising from embryo explants in MS medium containing 4%sucrose (x 1)

Plate 23:3 week old *Treculiaafricana* plantletsarising from embryo explants inMS medium containing 4% sucrose (x 1)

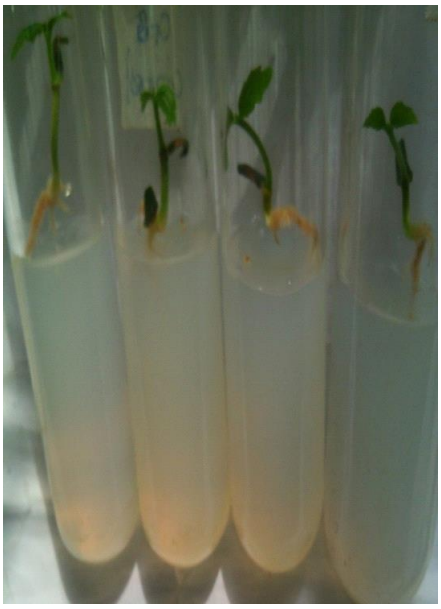

Plate 24: control at 3 weeks (x1)

Plate 25: 3 week old *Treculia africana* plantlets arising from embryo explants in MS medium containing no sucrose (x 1.5)

Plate 26: 3 week old *Treculia africana* plantlets arising from embryo explants in B5 medium containing no sucrose (x 1)

CHAPTER FIVE

DISCUSSION

The expansion and greening of the embryo by the second and third day respectively showed the viability of the embryos and the readiness for sprouting. The change of embryo colour from milk to green indicated that photosynthesis has taken place and this is as a result of transition from semi autotrophy to full autotrophy characteristics of *in vitro* systems. Sprouting followed later with the emergence of radicle and plumule. The green pigment involved in photosynthesis is the chlorophyll which aids in collecting and transmitting energy.

The initiation of radicle either occurred when the osmotic potentials of the cells in the radicle became more negative due to the metabolism of storage reserves or cell walls were more flexible to allow expansion. (Hartmann *et al.,* 2007). Growth did not involve callus formation nor proliferation of shoots either as a result of the type of explants (which maybe shoot tip, root tip, leaf etc) used or the absence of growth regulators. In the work by Singh *et. al* (2009) using juvenile leaf explants, among the various combinations of BAP(1.0-3.0) and BAA(0.1-0.5), the intensity of callus induction was highest at 77.77% in BAP 91.00 + IAA(0.5) mg/l. In the work on bambaranut, the shortest time for germination (4-5 days) was observed with the embryo axis (EA) followed by the seeds without seed coat (SWtC) which germinated in 8-9 days while the seeds with coat (SWC) have taken between 10 and 14 days to germinate. Compared to the embryonic axis, the time taken by the water to cross the barrier of the integument and to hydrate the cotyledons to initiate the physiological process of germination could explain the delay in germination observed with seeds with or without seed coat (Kone *et. a*l, 2015).

The three various sucrose levels in MS and B5 media respectively supported maximum growth of *T. africana* indicating that energy source and media were essential for growth *in vitro*. For the growth of plant tissues, the carbon source serves as the energy and osmotic agent (Lipavska and Konradova, 2004) for various energy requiring processes that can occur at the expense of available metabolic substrates for the growth and root initiation (Thorpe, 1983). Control had the least growth which may be due to lack of basic nutrients, since the zygotic embryo has been removed of its food storage tissue. On the other hand, significant growth of the control showed it has internally stored carbohydrate and nutrients for initial growth. This is because the study of the seed revealed it contains much carbohydrate, about 40-50% starch aside 3-8% glucose (N A C, 2013). Other *in vitro* works also supports the fact that growth of explants especially embryo is possible only on water and agar alone depending on food reserve. Louis and Okezie (2015) found out that embryo explants of Ricinus communis planted on water and agar gave a shoot length of 2.1300±0.22. Kone *et al.* (2015) experimented that there was lack of significant difference between basal media and the control containing only agar suggesting that macro and microelements were not necessary for germination in Bambara groundnut and, thus, the success of seed germination was mainly related to water availability.

The growth observed on all the treatments without plant growth regulators support the work of George,(2008) which states that ñmatured embryos are hormone autonomousò meaning that matured embryos posses a high level of endogenous hormone compared to immature embryos which always require growth regulators. Mohammed *et al.*, (1992) recorded that adventitious roots or a single shoot with roots formed on the explants of common and tepary beans cultured on media without plant growth regulators. This is in contrast to the work of Pierik (1997) who reported that *in vitro* culture is often impossible without growth hormone.

In this work, increase in growth parameters including number of adventitious root resulted with increase in sugar concentration until an optimum was reached. For example, for shoot length, it increased from 2.54±0.19 in 0% sucrose to 5.02±0.32 in 4% sucrose both in B5 media. For most observed work, optimum is usually 4 - 5% sucrose, while levels higher than that led to a decline in growth parameters and regeneration frequency. This is in line with the work of Manikyam *et al*. (2014) that used different sucrose levels and different carbon sources on *in vitro* regeneration of *Solanum viarum* (Dunal). They found that as the sucrose concentration increased from 1-4%, the mean shoot length also increased from 2.32±0.53 to 4.81±0.37 while at 6%, it declined to 2.23±0.11. Lu *et al*., (1983) found that culture media with a higher concentration of sucrose improved plant regeneration in *Zea mays*. In the work on *Centella asiatica*, Panathula *et al*. (2014) found out also that for sucrose, the maximum shoot length was obtained in 4% (w/v) (13.6±0.21cm) while the least was in 1% at 7.02±0.42. Gurel and Gulsen(1998) who worked with Almond (*Amygdalus cmmunis* L.) recorded that Shoot growth capacity, expressed as the growth rate of the developing shoots showed a steady increase with increasing concentrations, with 5 and 6% sucrose concentrations producing more vigorous shoots than the lower concentrations. This may be mainly due to the fact that high sugar levels available in the culture medium may speed up cell division thus leading to an increase in the volume and weight of tissues cultured, as suggested by other researchers (Chong and Taper, 1972). In the case of adventitious roots, sucrose was reported to act as morphogenetic trigger in the formation of auxillary buds and branching of adventitious root (Vinterhalter and Vinterhalter, 1997).

In this study, two media (MS and B5) were compared. Both of them supported the *in vitro* regeneration of *Treculia africana* with B5 being superior to MS in all the growth parameters. The differences may be due to different compositions of each media and the quantity of the various salts since they were all subjected to the same environmental conditions. The

differences between the media could also be the result of the quantity of ions in the basal media since a survey shows that the main difference in the composition of a range of commonly used tissue culture media is based on the quantity of various salts and ions; that the active factor in the medium is the ion of different types rather than the compound(Bhojwani and Razdan, 1996). Ions and the forms in which they are supplied are important in eliciting particular *in vitro* responses (Murashige and Skoog, 1962). Murashige and Skoog (1962) also noted that nutritional requirement for optimal growth of a tissue *in vitro* may vary with the species and that tissues from different parts of same plant may even have different requirement for maximum growth; therefore it is believed that no single medium is entirely satisfactory for all plant types, tissues and organs. George (2008) considered two important factors useful in finding out media formulations suitable for different plant species and different culture types which include: total concentration of nitrogen and the ratio of nitrate and ammonium ions in the medium. B5 medium has low quantity of ammonium ions than MS and therefore it is possible that this contributed to its greater yield since Gamborg *et al*. (1968) recorded that ammonium ions depressed the growth of soya beans cells when the concentration exceeded 2mM. The results of this study agrees with the work on *Jatropha curcas* by Amaefule (2014) who found out that B5 was better than MS media in most growth parameter assessed. However, this may be in contrast with some works since it is obvious that Murashige and Skoog (1962) medium is very popular because most plants react favourably to it. In bambaranut regeneration, Kone *et al.* found out that among the five culture media tested, the highest size of the seedling (10.48 cm) and the most important root length (21.51 cm) were observed with the plants developed from seed without coat on MS basal medium. Also, better plant growth from embryonic axis was observed on MS medium compared to other basal media in *Juglans regia* L. (Sánchez-Zamora, *et al*., 2006).

The hundred per cent germination recorded for most treatments in this study may be attributed to the maturity of the embryo used and this is in line with Warakogda and Subasinghe (2009) who reported that the stage of maturity of *Jatropha curcas* seeds and the basal media had a significant effect on seed germination and subsequent growth of seedlings.

CONCLUSION

This study has described a protocol that will be relevant for the mass propagation of *Treculia africana* plantlets. High levels of sucrose (3-4%) showed maximum increase in the various growth parameters therefore, it can be established that high sucrose levels are required for better yield. Also, use of B5 media instead of MS will be most suitable for *in vitro* culture of this plant embryo explants. The plantlets after acclimation would be raised *in situ* (*ex vitro*) to ensure a steady supply of its protein to man and animals; for various pharmaceutical uses and researches.

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APPENDICES

Appendix 1: COMPOSITION OF THE MEDIA

MEDIUM COMPONENTS ($mg.l^{-1}$)	MS	B5
MACRO NUTRIENTS		
NH_4NO_3	1650.0	-
KNO_3	1900.0	2500.0
$CaCl_2.2H_2O$	440.0	150.0
$MgSO_4.7H_2O$	370.0	250.0
KH_2PO_4	170.0	-
$(NH4)_2SO_4$	_	134.0
$NaH_2PO_4.H_2O$	_	150.0
MICRO NUTRIENTS		
KI	0.83	0.75
H_3BO_3	6.20	3.0
$MnSO_4.4H_2O$	22.30	_
$MnSO_4.H_2O$	_	10.0
$ZnSO_4.7H_2O$	8.6	2.0
$Na_2MoO_4.2H_2O$	0.25	0.25
$CuSO_4.5H_2O$	0.025	0.025
$CoCl_2.6H_2O$	0.025	0.025
$Na_2EDTA.2H_2O$	37.3	37.3

$FeSO_4.7H_2O$	27.8	27.8
VITAMINS AND OTHER SUPPLEMENTS		
Myo-inositol	100.0	100.0
Glycine	2.0	2.0
Thiamine HCl	0.1	10.0
Pyridoxine	0.5	_
Nicotinic acid	0.5	_

Source: Murashige and Skoog (MS) (1962) and Gamborg *et al.* (B5) (1968)

Appendix 2: Analysis of variance showing the effects of sucrose concentrations and basal media on per cent sprouting and sprout rate of embryo explants of *T. africana*

Source of variation		Sum of Squares	df	Mean Square	F	Sig.
Sprout Rate	Between Groups	.007	8	.001	9.250	.000***
	Within Group	.002	18	.000		
	Total	.009	26			
Per cent Sprouting	Between Groups	1235.053	8	154.382	.462	.867
	Within Groups	6013.373	18	334.076		

Appendix 2: Analysis of variance showing the effects of sucrose concentrations and basal media on per cent sprouting and sprout rate of embryo explants of *T. africana*

Source of variation		Sum of Squares	df	Mean Square	F	Sig.
Sprout Rate	Between Groups	.007	8	.001	9.250	.000***
	Within Group	.002	18	.000		
	Total	.009	26			
Per cent Sprouting	Between Groups	1235.053	8	154.382	.462	.867
	Within Groups	6013.373	18	334.076		
	Total	7248.427	26			

* Significantly different at PÒ0.0

Appendix 3: Analysis of variance of the effect of basal media and different sucrose concentrations on root and shoot length of *T. africana*

Source of variation	Sum of Squares	df	Mean Square	F	Sig.

LENGHT OF ROOT	Between Groups	78.365	8	9.796	11.021	.000***
	Within Groups	71.105	80	.889		
	Total	149.470	88			
	Between Groups	113.365	8	14.171	8.411	.000***
LENGHT OF SHOOT	Within Groups	134.786	80	1.685		
	Total	248.151	88			

***** Significantly different at PÒ0.05

Appendix 4: Analysis of variance of the effect of basal media and different sucrose concentrations on number of adventitious root and leaf area of *T. africana*

Source of variation		Sum of squares	df	Mean square	F	Sig
ADVENTITIOUS ROOTS	Between Groups	11823.150	8	1477.894	32.239	.000***
	Within Groups	3667.322	80	45.842		
	Total	15490.472	88			
	Between Groups	64.761	8	8.095	10.733	.000***

LEAF AREA				
	Within groups	60.336	80	.754
	Total	125.097	88	

* Significantly different at PÒ0.05

Appendix 5

One way
LR LS NL FW AR LA BY MEDIA /STATISTICS DESCRIPTIVES /MISSING ANALYSIS /POSTHOC=DUNCAN ALPHA(0.05).

[DataSet1]

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interva Mean	
						Lower Bound	Upper Bo
LENGHT OF ROOT	0% GAMBORG	10	2.5400	.59852	.18927	2.1118	2.9
	2% GAMBORG	10	3.9400	1.31757	.41665	2.9975	4.8
	3% GAMBORG	10	4.7400	1.67013	.52814	3.5453	5.9
	4% GAMBORG	10	5.0200	1.00421	.31756	4.3016	5.7
	0% MS	10	2.5400	.39215	.12401	2.2595	2.8
	S	10	2.6300	.47152	.14911	2.2927	2.9
	3% MS	10	2.9500	.96292	.30450	2.2612	3.6
	4% MS	10	3.1500	.59861	.18930	2.7218	3.5
	NO MEDIA, NO SUCROSE	9	2.4444	.62472	.20824	1.9642	2.9
	Total	89	3.3382	1.30327	.13815	3.0637	3.6
LENGHT OF SHOOT	0% GAMBORG	10	1.5000	.54772	.17321	1.1082	1.8
	2% GAMBORG	10	4.7700	2.47389	.78231	3.0003	6.5

	3% GAMBORG	10	4.4100	2.07549	.65633	2.9253	5.8
	4% GAMBORG	10	3.6200	.78003	.24667	3.0620	4.
	0% MS	10	1.8000	.32998	.10435	1.5639	2.0
	2% MS	10	2.1100	.64023	.20246	1.6520	2.5
	3% MS	10	2.8700	1.18701	.37537	2.0209	3.
	4% MS	10	3.3300	1.09752	.34707	2.5449	4.
	NO MEDIA, NO SUCROSE	9	1.8111	.75572	.25191	1.2302	2.
	Total	89	2.9258	1.67925	.17800	2.5721	3.
ADVENTITI ooooouu0oooo ooooooooouuo ouuoous OUS ROOTS	0% GAMBORG	10	7.8000	2.93636	.92856	5.6995	9.
	2% GAMBORG	10	28.1000	6.24411	1.97456	23.6332	32.
	3% GAMBORG	10	31.3000	10.80175	3.41581	23.5729	39.
	4% GAMBORG	10	41.0000	7.60117	2.40370	35.5625	46.
	0% MS	10	7.2000	2.93636	.92856	5.0995	9.
	2% MS	10	14.4000	5.18973	1.64114	10.6875	18.
	3% MS	10	25.8000	7.11493	2.24994	20.7103	30.
	4% MS	10	30.1000	9.49210	3.00167	23.3098	36.
	NO MEDIA, NO SUCROSE	9	9.5556	3.20590	1.06863	7.0913	12.
	Total	89	21.8315	13.26756	1.40636	19.0366	24.
LEAF AREA	0% GAMBORG	10	.372830	.2436584	.0770516	.198527	.54
	2% GAMBORG	10	1.117220	.8580879	.2713512	.503381	1.73
	3% GAMBORG	10	1.749250	1.3991815	.4424600	.748336	2.75
	4% GAMBORG	10	2.944600	1.3608821	.4303487	1.971084	3.91
	0% MS	10	4.630860	13.1349346	4.1536310	-4.765306	14.02
	2% MS	10	.810570	.8304337	.2626062	.216514	1.40
	3% MS	10	1.252620	.7925836	.2506370	.685640	1.81
	4% MS	10	2.304210	.7846212	.2481190	1.742926	2.86
	NO MEDIA, NO SUCROSE	9	.389300	.2118627	.0706209	.226448	.55
	Total	89	1.745228	4.4762170	.4744780	.802303	2.68

Post Hoc Tests

Homogeneous Subsets

LENGTH OF ROOT

Duncan[a,b]

MEDIA	N	Subset for alpha = 0.05			
		1	2	3	4
NO MEDIA, NO SUCROSE	9	2.4444			
0% GAMBORG	10	2.5400			
0% MS	10	2.5400			
2% MS	10	2.6300			
3% MS	10	2.9500			
2% GAMBORG	10	3.1500	3.1500		
4% MS	10		3.9400	3.9400	
3% GAMBORG	10			4.7400	4.7400
4% GAMBORG	10				5.0200
Sig.		.151	.066	.063	.511

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 9.878.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

LENGTH OF SHOOT

Duncan [a,b]

MEDIA	N	Subset for alpha = 0.05					
		1	2	3	4	5	6
0% GAMBORG	10	1.8100					
0% MS	10	1.8000	1.8000				
NO MEDIA, NO SUCROSE	9	1.8111	1.8111				
2% MS	10	2.1100	2.1100	2.1100			
3% MS	10		2.8700	2.8700	2.8700		
2% GAMBORG	10			3.3300	3.3300	3.3300	
3% GAMBORG	10				3.6200	3.6200	3.6200
4% MS	10					4.4100	4.4100
4% GAMBORG	10						4.7700
Sig.		.349	.098	.051	.231	.084	.065

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 9.878.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

ADVENTITIOUS ROOTS

Duncan a,b

		Subset for alpha = 0.05			
MEDIA	N	1	2	3	4
NO MEDIA, NO SUCROSE	10	7.2000			
0% GAMBORG	10	7.8000			
0% MS	9	9.5556	9.5556		
2% MS	10		14.4000		
2% GAMBORG	10			25.8000	
3% MS	10			28.1000	
3% GAMBORG	10			30.1000	
4% MS	10			31.3000	
4% GAMBORG	10				41.0000
Sig.		.471	.116	.103	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 9.878.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are guaranteed.

LEAF AREA

Duncan [a,b]

MEDIA	N	Subset for alpha = 0.05			
		1	2	3	4
0% GAMBORG	10	.372830			
NO MEDIA, NO SUCROSE	9	.389300			
0% MS	10	.472860			
2% MS	10	.810570			
2% GAMBORG	10	1.117220	1.117220		
3% MS	10	1.252620	1.252620		
3% GAMBORG	10		1.749250	1.749250	
4% MS	10			2.304210	2.30
4% GAMBORG	10				2.94
Sig.		.050	.131	.159	

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 9.878.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are guaranteed.

Homogeneous Subsets

LENGTH OF ROOT

Duncan[a,b]

MEDIA	N	Subset for alpha = 0.05			
		1	2	3	4
NO MEDIA, NO SUCROSE	9	2.4444			
0% GAMBORG	10	2.5400			
0% MS	10	2.5400			
2% MS	10	2.6300			
3% MS	10	2.9500			
2% GAMBORG	10	3.1500	3.1500		
4% MS	10		3.9400	3.9400	
3% GAMBORG	10			4.7400	4.7400

4% GAMBORG	10				5.0200
Sig.		.151	.066	.063	.511

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 9.878.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

LENGTH OF SHOOT

Duncan [a,b]

MEDIA	N	Subset for alpha = 0.05					
		1	2	3	4	5	6
0% GAMBORG	10	1.8100					
0% MS	10	1.8000	1.8000				
NO MEDIA, NO SUCROSE	9	1.8111	1.8111				
2% MS	10	2.1100	2.1100	2.1100			
3% MS	10		2.8700	2.8700	2.8700		
2% GAMBORG	10			3.3300	3.3300	3.3300	
3% GAMBORG	10				3.6200	3.6200	3.6200
4% MS	10					4.4100	4.4100
4% GAMBORG	10						4.7700
Sig.		.349	.098	.051	.231	.084	.065

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 9.878.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

ADVENTITIOUS ROOTS

Duncan a,b

MEDIA	N	Subset for alpha = 0.05			
		1	2	3	4
NO MEDIA, NO SUCROSE	10	7.2000			
0% GAMBORG	10	7.8000			
0% MS	9	9.5556	9.5556		
2% MS	10		14.4000		
2% GAMBORG	10			25.8000	
3% MS	10			28.1000	
3% GAMBORG	10			30.1000	
4% MS	10			31.3000	
4% GAMBORG	10				41.0000
Sig.		.471	.116	.103	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 9.878.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

LEAF AREA

Duncan [a,b]

MEDIA	N	Subset for alpha = 0.05			
		1	2	3	4
0% GAMBORG	10	.372830			
NO MEDIA, NO SUCROSE	9	.389300			
0% MS	10	.472860			
2% MS	10	.810570			
2% GAMBORG	10	1.117220	1.117220		
3% MS	10	1.252620	1.252620		
3% GAMBORG	10		1.749250	1.749250	
4% MS	10			2.304210	2.304210
4% GAMBORG	10				2.944600
Sig.		.050	.131	.159	.105

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 9.878.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.