

Full Length Research Paper

***In vitro* regeneration of *Treculia africana* Decne. from embryo explants on different nutrients and sucrose conditions**

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The study is essential in reducing germination time of *Treculia africana* var. *inversa*. The effect of three different concentrations of sucrose namely 2, 3 and 4% were investigated on the *in vitro* regeneration of embryo explants of *T. africana* Decne. on the media of Murashige and Skoog (MS) and Gamborg et al. (B5) respectively without any growth regulator. The experimental design was a 2 × 5 factorial in a completely randomized design with each treatment consisting of ten replicates. Results showed that while both media including control (contains agar only) 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.

Key words: *Treculia africana*, Murashige and Skoog (MS) medium and Gamborg et al. (B5) medium, embryo explant.

INTRODUCTION

Treculia africana Decne. (commonly known as African bread fruit, Wild jack fruit, or African boxwood), is an important multipurpose indigenous tree species in West Africa belonging to the family Moraceae (Nutreco Agroforestry Company (NAC), 2013). It is a monoecious dicotyledonous plant with flowers crowded into compact heads (Ugwoke et al., 2003). *T. africana* is a large, evergreen tree growing in the forest up to 30 m high with a girth of 4-6 m (Agbogidi and Onomerebor, 2008). It

has a dense spreading crown and fluted trunk. 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 (NAC, 2013).

T. africana serves as a nutritive food for the local population in its distribution area. Analysis of the hexane

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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 (NAC, 2013). Therefore, *T. africana* seems to have an important role in those regions that have a shortage of good protein sources due to its high protein content.

Appiah et al. (2014) affirmed that despite the dietary and economic importance of African breadfruit, it has remained an underutilized species and its potentials remain under-exploited. The underutilization is due to a number of reasons. Firstly, the long gestation period of ten or more years of the species has not helped in cultivar improvement leading to limited cultivation of the species (Nuga and Ofodile, 2010). Furthermore, high rate of deforestation especially in urban places due to industrial, construction and agricultural purposes call for the need of conservation of this species to avoid genetic erosion (NAC, 2013). 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 (NAC, 2013). In addition, cell and tissue culture techniques have been used to obtain biotic/abiotic tolerant plants employing two *in vitro* culture approaches including selection of mutant cell line (somaclones) and to introduce novel strands of interest (Arzani and Mirodagh, 1999; Arzani, 2008; Arzani and Ashraf, 2016; Kyesmu et al., 2004). Some factors have been identified as affecting *in vitro* regeneration and these include: temperature, light, pH, plant growth regulators and orientation of the explant on the medium (Arzani and Mirodagh, 1999; Srivastava and Johri, 1973). The physical status of the plant and the genotypes also has a role to play in regeneration process (Srivastava and Johri, 1973). The choice of tissue culture media largely depends upon the species and even genotype of the same species to be cultured (Arzani Darvey, 2001). Therefore, there is an urgent need for application of a reliable and efficient *in vitro* system that result in efficient differentiation, shoot development and whole plant regeneration for the improvement of *T. africana* through genetic transformation or mutagenesis (NAC, 2013).

Different studies on plants other than *T. africana* have been done using *in vitro* regeneration methods. *In vitro* culture of embryonic axis of different cultivars of *Phaseolus vulgaris*, Motta-Aldana et al. (2010) showed successful regeneration using the MS (Murashige and Skoog, 1962) medium supplemented with 100 mg l⁻¹ myoinositol, 1 mg l⁻¹ thiamine, 30 g l⁻¹ sucrose, BAP (0, 5 and 10 mg l⁻¹) and 8 g l⁻¹ agar. Prabhat et al. (2009) reported that *Rauwolfia serpentina* L. an endangered species, was also regenerated using the juvenile leaf

explants on the MS medium supplemented with various combinations of growth regulators. In addition, a study on *Artocarpus heterophyllus* (Ashrafuzzaaman et al., 2012) showed that regeneration of roots increased comparatively better when MS medium was enriched with 2 mg l⁻¹ of BAP (6 benzyladenine). However, few works have been done on *T. africana* zygotic embryos. For example, Okafor et al. (2016) compared the strengths of MS medium on *T. africana* zygotic embryos while Attah and Okezie (2015) compared the efficacy of three basal medium on *T. Africana*, namely: MS, Gamborg (B5) and Hilderbrant basal media. No study have determined the effect of sucrose conditions on zygotic embryos of this plant thus, necessitating the need for this study. This study is aimed at developing a protocol for regeneration of *T. africana* from embryonic axis of seed as a prerequisite for improvement through genetic transformation and to assess the effects of different levels of sucrose on the germination and growth of zygotic embryo of *T. africana* in two different media (MS and B5).

MATERIALS AND METHODS

Site of the experiment

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

Source of explants

T. africana tree (Figure 1) at a farm in Nsukka, in Nsukka Local Government area of Enugu State was used for this study. Fresh fruit heads (after 3 days of fall of the fruit heads) (Figure 2a) was processed to liberate seeds for the experiment. Five hundred seeds of *T. africana* (Figure 2b) were obtained from fresh fruit heads, and were later taxonomically 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 0.9 cm.

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 min 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 min in order to avoid contamination.

Preparation of stock solutions

In this study, series of stock solution of the media were made. The media comprised macro salts, micro salts, iron compounds and



Figure 1. *Treculia africana* tree.



Figure 2. (a) Fruits and (b) Seeds of *Treculia africana*.

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). 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, $\text{CaCl}_2 \cdot \text{H}_2\text{O}$, 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 ($\text{CaCl}_2 \cdot \text{H}_2\text{O}$) as stock solution C. $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ 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 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°C .

Media and culture conditions

The media comprising macro and minor elements according to

Murashige and Skoog (1962) and then Gamborg et al. (1968) supplemented with myo-inositol (100 mg l^{-1}), Thiamine HCL (mg l^{-1}), pyridoxine (5 mg l^{-1}), Nicotinic acid (5 mg l^{-1}) and sucrose were employed as basal media in this experiment. Cultures with only agar and water were maintained as control. 20, 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. 50 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 1 M NaOH. 7 g of Fluka agar was added to each conical flask and made up to 1 L prior to autoclaving by steam sterilization at 103 KN M^{-2} pressure and 121°C for 15 min. Ten millilitres 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 $27 \pm 2^\circ\text{C}$ under 16-h light/8-h dark photoperiod at a photon flux density of $60 \mu\text{mol m}^{-2} \text{ s}^{-1}$ 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 min. The cultured embryos were left to grow

for four weeks, after which they were scored for the requisite growth parameters.

Experimental design and statistical analysis

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

Growth parameters assessed after four weeks in culture

The growth and development of embryos of *T. africana* were monitored at a two-day interval from the day of inoculation. On the 13th 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 were 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). Sprout rate was calculated as the reciprocal of the number of days on which 50% sprouting was achieved.

RESULTS

Percent sprouting

The embryos (Figure 3) of *T. africana* cultured on both MS and B5 media began to show visible changes by the 2nd day in culture. The white or milkish embryo that had a large size of about 0.7 - 0.9 cm had begun expansion on the 2nd day. By the 3rd day, all embryos turned green. The radicle and plumule (sprouting) emerge from the embryo between the 4th and the 5th day (Figure 4) and by the 6th day, all the embryos had sprouted except some in the control. The plumule and radicle finally resulted into shoot and root respectively. Embryo explants cultured on only 0.7 agar and water alone also showed changes by the 2nd day and maintained a healthy growth till the 28th 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 3rd and the 4th week in MS (Figure 5a-d) and B5 basal media (Figure 6a-d).

Analysis of variance showed that there was no significant difference in percentage sprouting among the treatment means at $P \geq 0.05$. 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 also showed that there was a significant difference among the treatment means ($P \leq 0.05$) in terms of sprout rate. Comparison test using



Figure 3. Embryos of *T. africana* on 1st day.



Figure 4. Embryo of *T. africana* on 4th day showing sprouting.

Duncan's New Multiple Range Test (DNMRT) (Table 1) showed that all the treatment means were significantly higher than control in terms of sprout rate. It further shows that seven of the nine treatments had the same and highest sprout rate of 0.25 ± 0.00 , followed by MS 0 (0.23 ± 0.02) and then control (0.20 ± 0.00) as the least.

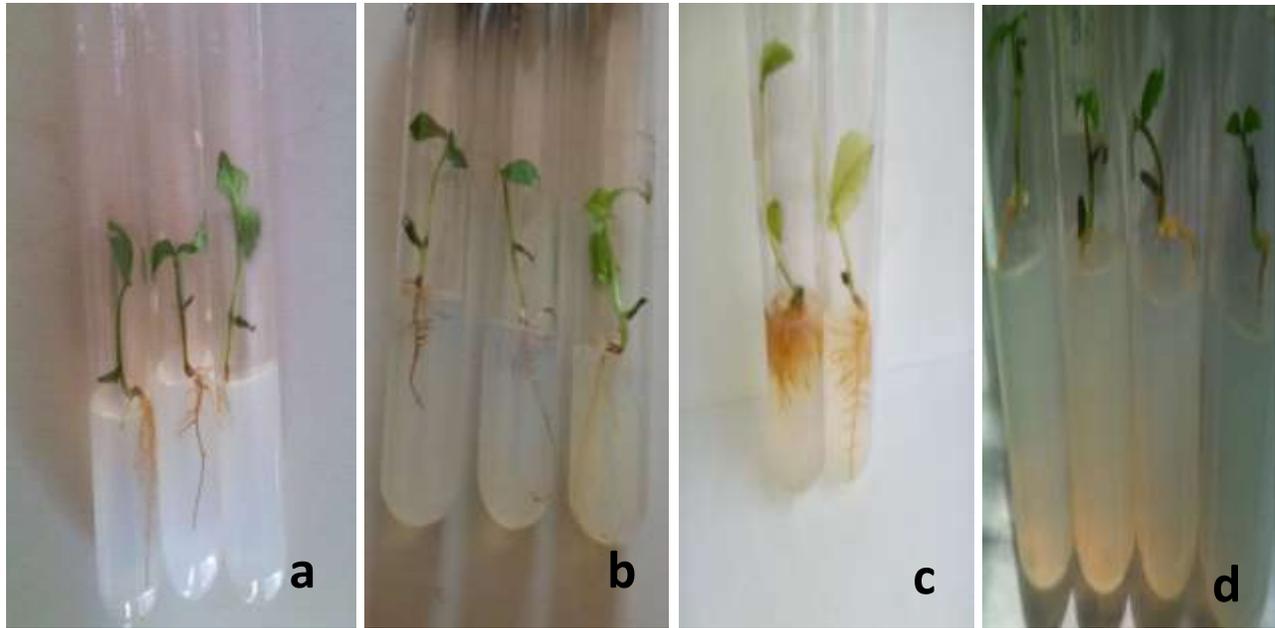


Figure 5. 3 weeks old *Treculia africana* plantlets arising from embryo explants in MS medium containing (a) 2% sucrose (b) 3% sucrose (c) 4% sucrose (d) no sucrose.

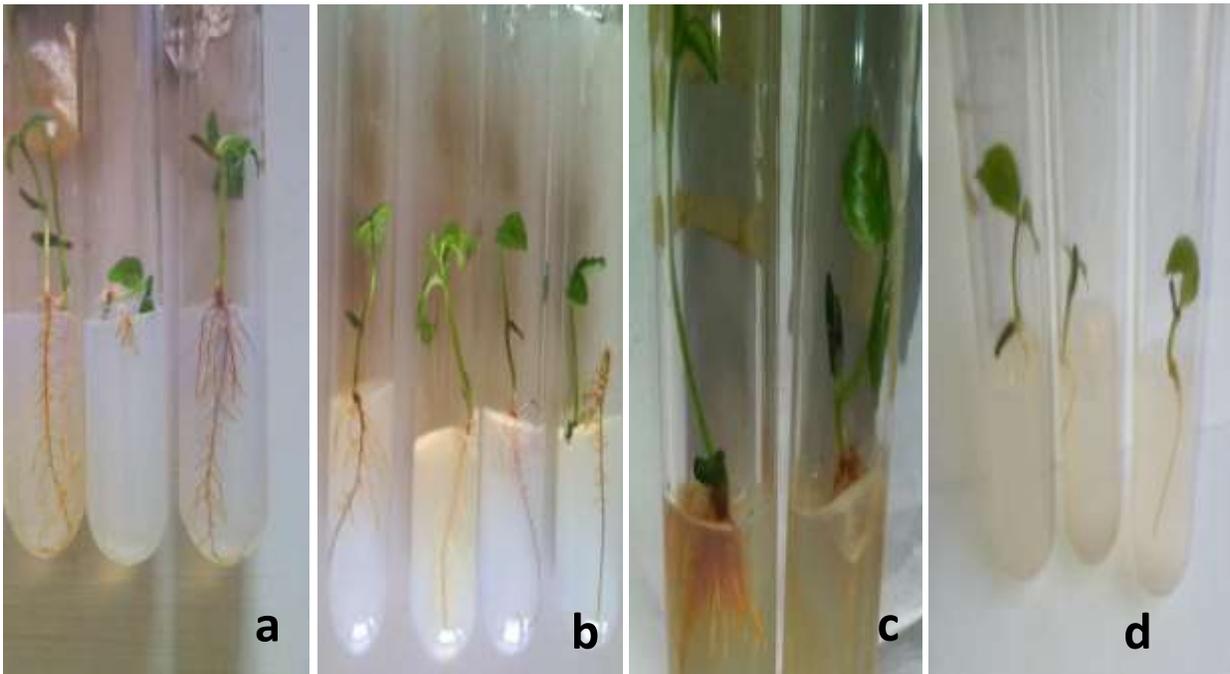


Figure 6. 3 weeks old *Treculia africana* plantlets arising from embryo explants in B5 medium containing (a) 2% sucrose (b) 3% sucrose (c) 4% sucrose (d) no sucrose.

Root and shoot length of plantlets

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 growth of *T. africana* shoot but did not differ significantly from 3 and 4% sucrose in B5 and MS while

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).

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

Basal media	Sucrose concentration (%)	Root length (cm)	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≤0.05).

differing significantly from others. ANOVA 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 at p≤0.05.

Number of adventitious root and leaf area of plantlets

Result showed that for both media, the number of adventitious root increased as the sucrose level increased (Table 3). The same table also showed that there was a significant difference among the treatment means in terms of leaf area. 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 B5 had better effect on leaf area at each level of sucrose concentration (Table 3). Analysis of mean indicated that there were significant differences between the basal media in terms of number of adventitious roots at p≤0.05 and this was due to sugar-media interaction.

DISCUSSION

Effects of sucrose on growth parameters

In this study, increase in growth parameters including number of adventitious root resulted with an 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 studies, optimum is usually 4 - 5% sucrose, while levels higher than that led to a decline

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 significantly different from each other by DNMRT (P≤0.05).

in growth parameters and regeneration frequency. This is in line with the results 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*. Gurel and Gulsen (1998) who worked with Almond (*Amygdalus communis* 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 lower concentrations. This may be 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 study (Chong and Taper, 1972). In the case of adventitious roots, sucrose was reported to act as morphogenetic trigger in the formation of axillary buds and branching of adventitious root (Vinterhalter and Vinterhalter, 1997).

Effects of basal medium

In this study, two media (MS and B5) were compared. Both of them supported the *in vitro* regeneration of *T. 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 Bhojwani and Razdan (1996) showed 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. 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 a lower 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 2 mM. 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 most studies since it is obvious that MS medium is very popular because most plants react favourably to it. The hundred percent 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 *J. curcas* seeds and the basal media had a significant effect on seed germination and subsequent growth of seedlings.

Morphogenesis of *T. africana* embryos

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 white to green indicated that photosynthesis has taken place which is a result of transition from semi-autotrophy to full autotrophy that is a feature of *in vitro* systems. Sprouting followed later with the emergence of radicle and plumule. 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 or 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 on bambara nut, the shortest time for germination (4-5 days) was observed with the embryo axis followed by the seeds without seed coat which germinated in 8-9 days while the seeds with coat 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 in seeds with or without seed coat (Kone et al., 2015).

Synergistic effects of sucrose and media

The three various sucrose levels in MS and B5 media respectively supported 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 deprived 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 *T. africana* revealed it contains much carbohydrate, about 40-50% starch aside 3-8% glucose (NAC, 2013). Other *in vitro* works also support the fact that growth of explants especially embryo is possible only on water and agar alone depending on the level of food reserve. Kone et al. (2015) noted that there was a 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 finding of George (2008), which states that "matured embryos are hormone autonomous", meaning that matured embryos possess a high level of endogenous hormones 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.

Conclusion

This study has described a protocol that will be relevant for the mass propagation of *T. 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. For future research, there is also an urgent need to employ this protocol in producing modified *T. africana* with shorter gestation period to encourage its mass production.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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