Chapter 3

The Science of Plant Tissue Culture as a Catalyst for Agricultural and Industrial Development in an Emerging Economy

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1. Introduction

In the last few decades, the flow of biological discovery has swelled from a trickle into a torrent, driven by a number of new methodologies developed in plant tissue culture, recombinant DNA technology, monoclonal antibodies and micro chemical instrumentation [1] Biological research has been transformed from a collection of single discipline endeavors into an interactive science with bridges between numbers of traditional disciplines. This synergy has made biology the "sunrise field" of the new millennium. The whole gamut of new discoveries in biology and allied sciences can be grouped together under a single umbrella term of "Biotechnology".

Biotechnology has been defined as "any technique that uses living organisms, or substances from these organisms, to make or modify a product, to improve plants or animals, or to develop microorganisms for specific uses" [2]. No society has advanced without deploying appropriate technology in place to set the pace for addressing its major problems. Public investment in relevant technology, the application to industries and capturing of the benefit accrue to it is what sets developed nations apart. Previous reports have shown that there is no National economic growth without proper investment in a right technology which is applied in a Nation. Real solutions to priority on national problems like job creation and poverty alleviation is investment in appropriate technology. This is evident in countries that embraced and adopted biotechnology in past technological revolutions and are practicing on an unprecedented scale. Such countries like India, Cuba and South Africa. The application of biotechnology has greater opportunities for developing countries than previous technologies i.e. greater comparative advantages[3]. Agricultural biotechnology addresses issues such as the production of disease resistant, high yielding and very

profitable agricultural ventures in both plants and animals. The world population has grown tremendously over the past two thousand years. In 1999, the world population passed the six billion mark. Latest official current world population estimate, for mid-year 2011, is estimated at 7 billion [4]. The population increase in developing countries constitutes 97% of the global increase [5], and it is estimated that by 2050, 90% of the planet's population will reside in the developing countries of the southern hemisphere. The challenge for the future, therefore, lies in global food security that necessitates a doubling of food production in the next 50 years to meet the needs of the population [6]. Most developing countries yet to fulfill their food production potentials; are especially vulnerable in terms of food security. Plant biotechnology plays a key role in complementing other factors necessary for the improvement of crop production such as the use of agrochemicals, irrigation, plant breeding and farm management to address food security. Plant biotechnology, has three broad fields of study. They include plant tissue culture, genetic engineering and plant molecular markers. These applications range from the simple to the sophisticated and in many cases have been appropriate for use in developing countries [2]. For example, biotechnology techniques such as plant tissue culture have been utilized appropriately for many agronomic and food crops to provide more food and planting materials for farmers.

Micropropagation, popularly known for large-scale clonal propagation, is the first major and widely accepted practical application of plant biotechnology. It is described as the *in vitro* initiation of plant culture, propagation, and rooting under controlled environmental conditions for *ex vitro* establishment in the soil. New contributions to *in vitro* techniques for plant propagation in the last decade have simplified micropropagation technology [7]. This covers a wide range of plants including Agronomic species, economic and forest trees.

In Cassava *(Manihot esceulenta) for example,* Tissue culture has made possible the mass production of disease-free and uniform plants. The techniques thus bring farmers the great benefit *of* high- quality planting materials [8].Production of planting materials is indispensable in the overall structure of research for conservation of variety purity and supply of high yielding cassava cultivars to stem multipliers and producers[9]. The possibility of using screen house to maintain *in vitro* cultures and rapidly propagate important vegetative crops with less contamination at a reduced cost was investigated A[10]. *In vitro* propagation has been used to regenerate, establish and conserve both economic trees and forest species through organogenesis and somatic embryogenesis. For instance, *Khayagrandifoliola* is an important species native to West Africa [11]. Khaya wood, African mahoganyas it is commercially called is a high priced wood often used for furniture and construction purposes. With its threatened conservation status [12], micropropagation has been auseful tool for mass propagation of superior stock plants as well as genetic improvement and conservation. For the purpose of conservation and multiplication, a reliable plant regeneration protocol from matured seed embryo of *Khayagrandifoliola was developed* [13]. Another species of economic importance is*Plukenetiaconophora*Mull.Arg. (Family: Euphorbiaceae), formerly known as *Tetracarpidium conophorum* and popularly called African walnut. This tree speciesis a perennial climber of economic importance, an edible species and is used medicinally [14]. A prolific shoot multiplication system (protocol)

for *Plukenetia conophora* has been reported [15]. Furthermore, the need to conserve and regenerate recalcitrant species has led to the development of *in vitro* protocols for many recalcitrant species including vegetables. *Telfaria Occidentalis*(fluted pumpkin) is a tropical vegetable grown in West African and widely consumed in tropical regions mainly for its richness in protein [16]. Due to problems associated with the sex of the plant regarding reproduction, the female plant in preferred with respect to leaf and fruit production [17]. *In vitro* culture of *T. occidentalis* under different cytokinins and auxins combination was studied [18]. The commercial use of micropropagation is mainly limited to crops with high value and the commercial utility of this technique for the most important crop species is limited as a result of the large numbers needed annually to start up new farms in addition to high production costs. In order to overcome the problems of conventional micropropagation, protocols have been proposed using bioreactors and liquid medium [19]. Bioreactor system which incorporates a number of features in its design has been used to simplify operation and reduce production costs. Automation, using a bioreactor, is one of the most effective ways to reduce the costs of micropropagation [20].

The Temporary Immersion Bioreactors (TIBs) has been shown to reduce some problems usually encountered in permanent liquid cultures such as hyper-hydricity, poor quality of propagules, andnecessity of transplanting on a solid medium in the elongation and/or rooting stage. In comparison with conventional micropropagation on semisolid medium, TIBs provides a superior mass balance. Indeed in the latter comparison the proliferation rate is higher, labor efficiency is improved and, as a consequence, the cost is reduced [21].

This chapter attempts to give an insight on how methods and applications of *in vitro* technology can serve as a catalyst for both agricultural and industrial development in an emerging economy.

2. Methodology

2.1. In vitro propagation of Cassava plantlet *(Manihot esceulenta)*

This study aims at the possibility of using screen house to maintain *in vitro* cultures and rapidly propagate important vegetative crops with less contamination at a reduced cost.

Two genotypes of cassava TMS *188/00106* and TMS *083/00125* were obtained from the Inte~na1ionaf Institute of Tropical Agriculture (IITA) while live different medium were prepared using medium [22] with minor adjustments. as follows:

Treatment 1 (T_1) Liquid only
Treatment 2 (T_2) - liquid with 50% normal agar (2 g/l).
Treatment 3 (T_3) - liquid media with filter paper embedded.
Treatment 4 (T_4) - Media with normal agar (4 g/l).
Treatment 5 (T_5) - liquid media with filter paper projecting out.

The pH was taken and dispensing was done at the rate of 3 ml before autoclaving. The sub culturing was done the following day. One hundred and twenty test tubes were used for

each variety with 12 test tubes per treatment. A complete set of 60 test tubes With 5 treatments of 12 replicates was placed in the laboratory while the second set was placed in the screen house at the same day for TMS *188/00106;* the same procedure was adopted the following day for TMS *083/00125.* Data was recorded weekly for 5 weeks before sub culturing. The second generation was observed for only two weeks to ensure the sustainability of the observation made during the first generation. The observation on explants survival was scored on a scale of 0 - 3 as follows:

0 - Dead
1 - Alive but not growing
2 - Growing slowly
3 - Growing very well

There were six parameters recorded during the investigation. These include survival (ate, shoot development, root growth. nodal increase, leave development and increases in height. Survival rate was observed for two weeks only white the other five parameters were scored continuously for the rest three weeks consecutively. Only the screen house explants were subculture after 5 weeks to ensure the sustainability of the findings. The subculture materials from the screen house explants' were also placed in both screen house and culture room (laboratory). The same set of observation was carried out on the responses of the explants to the culture medium and environment as in the first generation explants. The summary in Table 1 indicates that out of the six parameters studied, F-probability on survival is significantly different for all the media used. Observation shows no significant difference on the five treatment for shoot root node, , leaves and height development Although there were some effects on the survival of the explants, the laboratory plantlets grows better in liquid and liquid with filter paper embedded media than when placed in the screen house. This might be due to high temperature recorded at the time of placement (32° - 36°C compared to 22 - 25°C in the laboratory), which indicates an interaction between treatment and environment (Table 1).

SIN	Survival		Shoot		Root		Node		Leaves		Helaht	
	SH	LAB	SH	LAB	SH	LAB	SH	LAB	SH	LAB	SH	LAB
I	1.77	2.41	1.92	1.93	0.73	1.55	2.00	1.93	1.92	1.93	1.92	1.96
11	1.67	1.44	1.56	1.42	1.67	1.33	1.86	1.63	1.67	1.46	1.61	1.33
III	1.02	1.81	1.21	2.04	0.46	1.42	1.46	2.17	1.29	2.00	121	2.04
IV	1.73	1.27	1.83	1.00	1.00	0.75	2.04	1.29	2.00	0.96	1.79	1.00
V	1.73	1.27	1.88	1.13	1.63	1.04	2.13	1.29	1.79	1.13	1.92	1.17
CV	42%		49%		89%		48%		51%		48%	
Lsd	0.38.4		0.4470		0.2987		0.4889		0.470		0.439	
Std	0.193		0.2266		0.5892		0.2479		0.2383		0.2228	

SH - Screen House. LAB - Culture room in the Laboratory.

Table 1. Summary of environmental effect on the *in vitro* growth rate of cassava tissue culture.

Obviously, the laboratory' supports the survival of explants in the liquid medium and liquid medium with embedded filter paper. On the other hand, survival is lowest in the screen house with liquid medium containing embedded filter paper. This suggests that before the

explants can be transferred to the screen house, there is need to ensure their survival in the laboratory. For T 2 (liquid with 50% normal agar), T4 (media with normal agar, 4 *g/l)* and T5 (liquid media with filter paper projecting out), the survival was significantly higher in the screen house than in the laboratory. On the other hand TMS 083/00/25 survived better in the liquid media with embedded filter than TMS 188/00106. The survival rate of TMS 188100106 was also better in liquid medium with 50% normal agar (2*g/l),* media with normal agar and liquid media with filter paper projecting out than TMS 083/00/25. This suggests that for long storage before sub culturing, laboratory may be ideal while for short time storage and immediate rapid mass propagation screen house may be adopted.

Table 2 shows that TMS 188/00106 survived better in liquid media than TMS 083/00125. No significant different between the two genotypes in most of the media except on survival in liquid media only. Figure 1 Shows that screen house plantlet grow relatively uniform for all the five treatments while Figure 2 indicates that plantlets in T1 grew faster than others in the culture room.

It can therefore be concluded that when the need arises, in vitro plantlets of cassava can be raised adequately in the screen house and even be raised faster than the laboratory as long as the temperature does not exceed

SIN	Survival		Shoot		Root		Node		Leaves		Height	
	01	02	01	02	01	02	01	G2	01	02	01	G2
I	2.37	1.51	2.25	1.34	1.29	0.77	2.29	1.39	2.29	1.30	2.21	1.46
11	1.67	1.37	1.46	1.53	1.79	1.05	1.67	1.84	1.54	1.60	1.46	1.47
III	1.13	1.71	1.21	2.04	0.71	1.17	1.25	2.38	1.13	1.17	1.29	1.96
IV	1.54	1.46	1.33	1.50	1.00	0.75	1.79	1.54	1.54	1.42	1.42	1.38
V	1.54	1.46	1.58	1.42	1.00	1.67	1.63	1.79	1.42	1.5	1.54	1.54
CV	43%		50%		92%		48%		52%		51%	
Lsd	0.3898		0.4494		0.5889		0.4791		0.4684		0.4600	
Sed	0.1976		0.2278		0.2985		0.2429		0.2375		0.2332	

G_1=Genotype1=TMS*88100106*. G_2= Genotype 2 = TMS *083/00125*

Table 2. Summary of genotypic effect on the *in vitro* growth rate of cassava tissue culture.

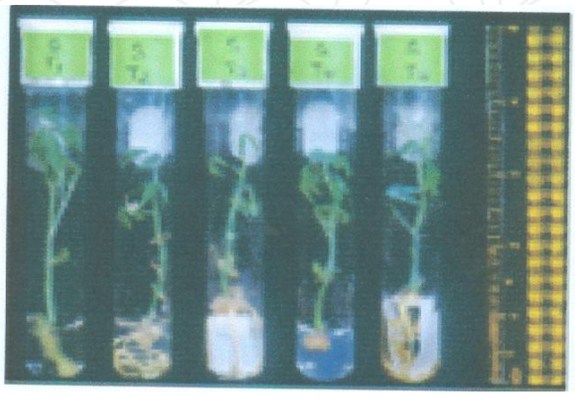


Figure 1. Screen house performance of the 5 treatments of cassava tissue culture. T_1 - Liquid only; T_2• liquid with 50% normal agar (2 g/l); T_3 - liquid media with filter paper embedded; T_4 - media with normal agar (4 g/l); and T_5 - liquid media with filter paper projecting out.

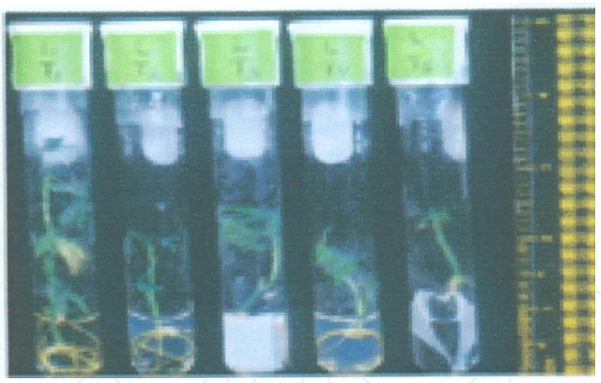


Figure 2. Laboratory performance of the 5 treatments of cassava tissue culture. T_1 - Liquid *only;* T_2 - liquid with 50"10 normal agar (2 gll); T_3 . liquid media with filter paper embedded; T_4 - media with normal agar (4 g/I); and T_5 - liquid media with filter paper projecting out.

2.2. *In vitro* propagation of an endangered medicinal timber species *Khaya grandifoliola* C. Dc.

Considering the fact that this forest tree species seeds are recalcitrant in nature and producing adequate number of seedlings for any meaningful plantation establishment programme from seeds stored for long time is very difficult, this present work aims to describe a reliable plant regeneration protocol from matured seed embryo.

Seeds of *K. grandifoliola* used were collected from the Genebank of National Center for Genetic Resources and Biotechnology (NACGRAB), Ibadan, Nigeria (07°23.048'N 003°50.431'E). Ninety (90) seeds used for this experiment were washed with mild liquid detergent (Tween-20) under running tap water for 10 min. This is followed by surface sterilization in 70% ethanol for 5 min and 0.1% mercuric chloride for 10 min followed by 3 rinses in sterile distilled water. The embryos were carefully excised with ease together with some endosperm attached and then cultured on basal medium supplemented with 3% w/v sucrose, 0.1 g inositol and gelled with 0.7% w/v agar at various concentration of cytokinins and auxin in a 17 ml test tube. The cytokinins used were benzylaminopurine (BAP) and kinetin (KIN), while naphthalene acetic acid (NAA) was the auxin used. All growth regulators were added before autoclaving. The pH was adjusted to 5.7 ± 0.2 and autoclaving was done at 121°C for 15 min. The cultures were incubated in a growth room at 26 ± 2°C under a 16 h photoperiod with cool-white fluorescent light. There were nine treatments, and ten explants were cultured per treatment and later arranged randomly on the shelves in the growth room. After four weeks, the cultures were evaluated for shoot length, root length, number of nodes and number of roots. The data taken were subjected to statistical analysis using SAS/PC version 9.1. The observed means of the characters were subjected to Least Significant Difference (LSD) to show the mean separation.

Data in Table 3 revealed that different concentrations of the cytokinin BAP and the auxin NAA tested in this study had a significant effect on the regeneration of plantlets. The longest shoot length (7.4 mm) was exhibited for explants cultured on MS-medium supplemented with 0.075 mg/L (Kin) + 0.01 mg/L (NAA) and this value is 3 fold higher than

that found for embryo cultured on 0.10 mg/L (Kin) + 0.01 mg/L (NAA) whose average shoot length was 2.7 mm. These results showed that the most adequate culture medium for obtaining the longest aver-age root length (7.53cm) per culture after four weeks was MS-medium supplemented with of BAP at 1.0 mg/L plus NAA at 0.1 mg/L, while the shortest root length (1.47 cm) was exhibited by MS-medium supplemented with 0.15 mg/L (BAP) + 0.01 mg/L (NAA), this indicates that increasing the level of auxin (NAA) increases the length of roots and vice-versa. However, the highest number of nodes (4.0) was observed on plantlets cultured on MS-medium supplemented with 1.0 mg/L (KIN) + 0.01 mg/L (NAA).

S/N	Media	Shoot length	Root length	Number of nodes	Number of roots
1	0.125 mg/l (BAP) + 0.01 mg/l (NAA)	4.20	1.53	3.00	1.00
2	0.15 mg/l (BAP) + 0.01 mg/l (NAA)	4.67	1.47	2.00	1.00
3	0.05 mg/l (KIN) + 0.01 mg/l (NAA)	3.7	4.53	2.00	1.00
4	0.075 mg/l (KIN) + 0.01 mg/l (NAA)	7.4	4.20	3.00	1.00
5	0.10 mg/l (KIN) + 0.01 mg/l (NAA)	2.7	4.03	2.00	3.00
6	0.125 mg/l (KIN) + 0.01 mg/l (NAA)	4.92	5.53	3.00	1.00
7	1 mg/l (BAP) + 0.1 mg/l (NAA) + 10 mg/l (adenine sulphate)	5.78	3.50	3.00	3.00
8	1 mg/l (KIN) + 0.01mg/L (NAA) + 10 mg/l (adenine sulphate)	4.82	4.50	4.00	1.00
9	1 mg/l (BAP) + 0.1 mg/l (NAA)	5.87	7.53	3.00	2.00
	LSD	0.14	0.13	0.00	0.00

Table 3. Effect of plant growth regulators on shoot length, root length, number of nodes, and number of roots regeneration from embryo culture of *K. grandifoliola.*

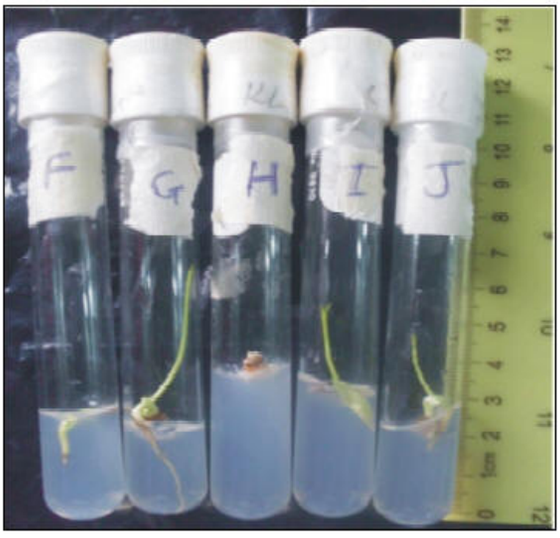


Figure 3. The growth stages of *K. grandifoliola* through embryo culture *in vitro* (two weeks after culture).

These findings are in agreement with those reported earlier [23] on Cacti (*Pelecyphora aselliformis*) and *Nealolydia lophophoroides*; the work on *Aloe barbebsi* [24] and *Turbinicapus laui* [25] indicate that using a high concentration of BAP and NAA in different concentrations was a limiting factor for shoot formation and increases root formation. The result of this study showed that the optimum medium for regeneration of *K. grandifoliola* MS-medium

supplemented with 1.0 mg/L (BAP) + 0.1 mg/L (NAA) + 10 mg/L adenine sulphate because the values obtained for all the parameter measure was moderately high and optimum. The fact that the number of roots increased to 3 in medium 5 and 7 could be due to the increase in the concentration of NAA to 0.1 mg/L. It has been established that auxins like NAA increases the root formation in the presence of low cytokinins [26].

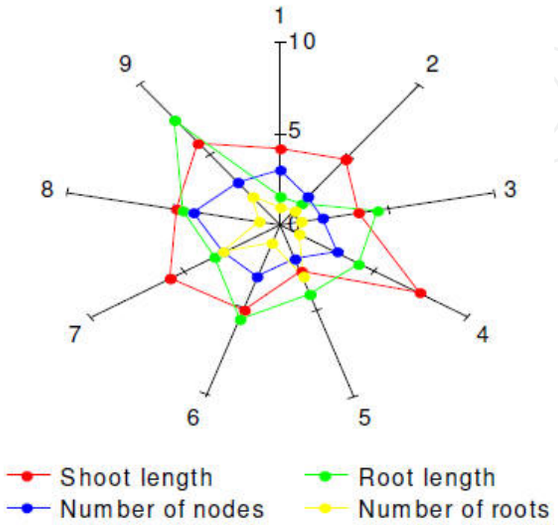


Figure 4. The effect of different levels of kinetin and BAP on the performance of *K. grandifoliola*.

2.3. *In vitro* culture of *Telfairia occidentalis* under different cytokinins and auxin combinations

The aim of this work was to investigate the *in vitro* regeneration potential of *Telfairia occidentalis* under different hormonal combination. The nodal cuttings collected from four weeks old seedlings raised in the screen house at NACGRAB were used as explant. The explants were surface-sterilized in 15% NaOCl + 2 drops of Tween 20 per 100 ml for 25 min. They were then cut with a sharp sterile knife into single node cuttings. Three or four explants from a seedling were cultured on the prepared medium to which either naphthalene acetic acid (NAA) or benzylaminopurine (BAP), indolebutyric acid (IBA), indole-3-acetic acid (IAA) and kinetin had been added. Different concentrations were investigated for each of the auxins and cytokinins. The basal medium used comprised of Murashige and Skoog macro and micro-elements, vitamins 3% sucrose, 10 mg/L ascorbic acid, 0.1 g/L myo-inositol, and 0.02 g/L cysteine. Cultures were incubated in the dark at 25 ± 2oC for duration of six weeks for shooting and rooting induction. The number of roots and nodes were counted and recorded on the sixth week.

Among all the growth hormones used, IBA (0.05 mg/L) + BAP (0.01 mg/L) combination gave the best result for both rooting and shooting while the highest number of nodes was observed in BAP (0.05 mg/L) + NAA (0.01 mg/L). The application of kinetin both in combination with NAA and alone resulted in premature senescence with lower number of nodes. This is in agreement to the findings of [27] who showed that kinetin is not a suitable

hormone for regeneration of *Telfairia* especially if it will be kept *in vitro* for a long time. However BAP (0.05 mg/L) + IAA (0.01 mg/L) combination resulted in lowest number of nodes and MS alone produced callus without regenerating into a plantlet. The result shows that *in vitro* growth of *T. occidentalis* is hormone specific.

2.4. In vitro micro-propagation of *Plukenetia conophora* Mull.Arg

This study describes a reliable and prolific shoot multiplication system (protocol) for *Plukenetia conophora.* Excised embryos and nodal cuttings from growing seedlings served as the major explants used for the study. Matured fruits were collected from the field gene bank of NACGRAB, Ibadan.

2.4.1. Dis-infection of explants

Nodal cuttings: The nodal segments were obtained from the stems of actively growing seedlings, washed with liquid detergent under running tap water and disinfected using standard disinfection procedures before culturing. For *embryos,* cotyledons obtained from the nuts were reduced into small size, washed with liquid detergent under running tap water and then disinfected appropriately.

2.4.2. Culture conditions

The culture media consisted of MS basal medium supplemented with vitamins, myo-inositol, sucrose, casein hydroxylate and growth regulators. The pH of the medium was adjusted before autoclaving. All the cultures were kept at 24±2 ^{0}C under cool light fluorescent lamp for a photoperiod of 16 hours.

2.4.3. Shoot induction from matured embryos

Matured excised embryos were cultured on shoot induction medium supplemented with different concentrations of KIN (0.0 – 0.50) mg/l with NAA /0.05 mg and BAP (0.0 – 0.50) mg/l with NAA 0.05mg,whileexcised nodal segment from actively growing stem were cultured for direct organogenesis on MS medium supplemented with 0.0-0.45mg/l BAP/0.05mg NAA and 0.0-0.45mg/l KIN/0.05mg NAA for shoot proliferation.

The studies showed that the excised embryos regenerated *in vitro* after12 weeks of culture had a healthy appearance. *In vitro* regeneration was achieved on MS basal medium without growth regulators. On MS basal media fortified with growth regulators, the best mean result of shoot length was recorded on medium supplemented with 0.3mg KIN and 0.01mg NAA. The medium augemented with 0.3mg KIN and 0.05mg NAA gives the longest root length. These concentrations induced a higher percentage of explants with shoots and shoot number per explant than the hormone-free treatment. Therefore, the introduction of growth regulators led to the increase in shoot length and number of nodes. However, an increase in the concentrations of NAA from 0.01mg to 0.05 mg resulted in a decline in the number of shoots formed and an increase in the root length (Table 6).

In the *in vitro* regeneration of nodal cuttings, nodal culture on MS basal (the hormone-free treatment) showed no significant growth until supplemented with cytokinin, hence the need for supplementing the MS basal medium with cytokinin. MS medium containing 0.30mg BAP and 0.05mg NAA gave the best mean shoot length, number of shoots, and node number. An increase in the concentration of BAP above 0.30mg/l led to a decline in shoot length. The addition of Casein hydroxylate improved the shoot response as observed in the difference in growth response of nodal cuttings on MS containing 0.2mg/l BAP and casein hydroxylate and 0.2mg/l BAP without casein hydroxylate as there were differences in their shoot response.

Media	Shoot length (cm)	Root length (cm)	No. of nodes
MS only	4.97±0.03	3.90±0.05	2.00±0.00
MS + 0.30mg KIN + 0.01mg NAA	3.10±0.00	2.00±0.24	3.00±0.00
MS + 0.30mg KIN + 0.05mg NAA	2.00±0.00	5.50±0.24	2.00±0.00
MS + 0.40mg BAP + 0.01mg NAA	2.00±0.00	5.50±0.24	2.00±0.00

Mean result ±standard error.

Table 4. Effect of KIN, BAP and NAA on *in vitro* regeneration of *Plukenetia conophora* embryos after 12 weeks.

Media	Shoot length (cm)	No. of shoots	No. of nodes
MS only	No significant growth		
MS + 0.20mg BAP (no casein hydroxylate)	0.57±0.03	1.00±0.00	1.00±0.00
MS + 0.20mg BAP + 0.05mg NAA	0.70±0.08	1.00±0.00	1.00±0.00
MS + 0.30mg BAP + 0.05mg NAA	2.13±0.05	2.00±0.00	2.00±0.00
MS + 0.35mg BAP + 0.05mg NAA	1.10±0.05	2.00±0.00	2.00±0.00
MS + 0.40mg BAP + 0.05mg NAA	0.83±0.07	1.50±0.29	1.67±0.27
MS + 0.45mg BAP + 0.05mg NAA	0.63±0.13	1.30±0.27	1.25±0.25
MS + 0.30mg KIN + 0.05mg NAA	0.53±0.02	1.00 ±0.00	1.00 ±0.00

Table 5. Effect of BAP, KIN and NAA on *in vitro* regeneration of *Plukenetia conophora* nodal explants.

3. The Potential of Temporary Immersion Bioreactors (TIBs) in scaling up crop production, to meet agricultural demand in developing countries

Temporary Immersion Bioreactor system (TIBs) is a relatively recent micropropagation procedure that employs the use of automated gadgets to control rapid multiplication of plant cultures under adequate conditions. TIBs provide a more precise control of the adequate conditions (gaseous exchange, illumination etc.) required by plants for growth, development and survival than the conventional culture vessels. This bioreactor system incorporates a number of features specifically designed to simplify its operation and reduce production costs.

TIBs consist of three main phases: Multiplication, Elongation and rooting phase. Plantlets propagated in TIBs have better performance than those propagated by conventional

methods of micropropagation. This is as a result of a better handling of the *in vitro* atmosphere and the nutrition. The system also provides a rapid and efficient plant propagation system for many agricultural and forestry species, utilizing liquid media to avoid intensive manual handling. In addition to diminishing production costs regarding labour force, Temporary Immersion Bioreactors save energy, augment micropropagation productivity and efficiency.

3.1. Use of bioreactor technology?

Bioreactors provide a rapid and efficient plant propagation system for many agricultural and forestry species, utilizing liquid media to avoid intensive manual handling. Several authors have reported the use of bioreactors for plants propagation [28],[29]. To reduce the intensive labour requirement along with the production cost during plant propagation by tissue culture technique, there is an immense need of developing scale-up systems and automation [30]. This method for large scale production of plants is promising at industrial level. Employing bioreactors with liquid medium for micropropagation is advantageous due to the ease of scaling-up [31]. Large-scale plant propagation using bioreactor can also be beneficial in terms of year round production of the propagules of useful plants resulting in comparatively less labour cost and time [32]. The major advantages of using bioreactor culture system for micropropagation of economically important plants includes the potential for scaling-up in lesser time limit; Reduction in the production cost as well as an automated control of physical and chemical environments during growth phase of the plant cultures.

3.2. Importance of TIBs technology to agricultural development in emerging countries

Modern biotechnology has put the micropropagation industry on the verge of exciting new breakthroughs. It offers improvements in virtually every area of crop production and utilization, with potential benefits to agriculture, the food industry, consumers and the environment. As the world's population continues to grow, it is anticipated that there could be many mouths to feed in the next few decades. The advances made possible through micropropagation (TIBs) will be essential to meet global food needs by increasing the yield, quality and quantity of crops available to farmers. TIBs offer further benefits in form of non-food crops. Through mass propagation of specific economic species, it will be possible to arrest desertification, soil erosion in affected areas and also increase industrial crop production as renewable sources of medicines, industrial chemicals, fuels etc. They offer potential benefits to the commercial farmers, industries, public, research scientists and students. The potential benefits of TIBs are summarized below.

- Mass propagation of agronomic food crops to enhance food security. (i.e All year round production and supply of planting materials to farmers).
- Scaling up of the production of specific crops for industrial use (A step towards commercialization) e.g pineapple juice.

- Mass propagation of economic tree species (e.g *Eucalyptus spp., Adzadiracta indica, Accacia sp*) for addressing environmental problems like desertification and erosion.
- Job creation.
- Inspire collaborations among institutions on specific economic and ecological projects.

3.3. The use of TIBs in the mass propagation of Plant Genetic Resources (Using Pineapple, plantain, sugarcane and *Eucalyptus sp* as a case study)

The objectives of this work were:

- To produce high planting density of crops through an efficient and rapid production system to meet conservation and large scale farming production demands.
- To produce homogenous plantlets for research and development purposes.

Four major stages are recommended for effective mass propagation of plant cultures using temporary immersion systems, these include:

3.3.1. Stage 1: Collection and Establishment of the mother explants on agar gel medium.

The establishment of truly aseptic cultures usually involves the following sequential steps:

Step 1. Pre-propagation step or selection and pre-treatment of suitable plants.

The mother plants are selected and screened before transporting to the green house environment. The health status of the donor mother plant and of the plants multiplied from it are among the most critical factors, which determine the success of a tissue culture operation. Hence, indexing of the mother plants for freedom from viral, bacterial, and fungal diseases is a normal procedure before undertaking propagation in large-scale plant propagation through tissue culture [33]. This step is crucial as it tends to reduce the microbial load present at the time of collection and which may hinder or interfere with the *in vitro* processes.

Step 2. Initiation of explants - surface sterilization, establishment of mother explants.

This involves the sequential disinfection of the mother plant under aseptic conditions, culture initiation and establishment on a suitable growth media. The process requires excision of tiny plant pieces and their surface sterilization with chemicals such as ethyl alcohol, sodium hypochlorite and repeated washing with sterile distilled water before and after treatment with chemicals. The appropriate growth media for each crop was prepared. The pH was adjusted to 5.7 ± 0.2 before autoclaving at 121°C for 15 min and culture initiation was carried out under the laminar flow hood.

The initiated cultures were then transferred to the growth room and incubated at 26 ± 2°C under a 16 h photoperiod with cool-white fluorescent light.

Step 3. Subculture of explants on agar gelled media for multiplication and proliferation.

This involves the subculture of established explants on agar gelled media with a specific auxin/cytokinins combination to induce proliferation. In this step, explants were cultured on

the appropriate media for multiplication of shoots. The primary goal was to achieve propagation without losing the genetic stability. Repeated culture of axillary and adventitious shoots, cutting with nodes, somatic embryos and other organs from Stage I led to multiplication of propagules in large numbers. The propagules produced at this stage were further used for multiplication by their repeated culture.

3.3.2. Stage 2: Zero shelving of plants to liquid medium.

Sometimes it is necessary to subculture the *in vitro* derived shoots onto different media for elongation and rooting for *ex vitro* transfer. However, if cultures must be mass propagated in Temporary Immersion System, they must be allowed to pass through the zero phase upon establishment and when they have gained a proliferation capacity/potential on the agar gelled multiplication media. This is usually done in order to prepare the explants for proper adaptation, survival and desired *in vitro* response in the next phase which utilizes only liquid medium.

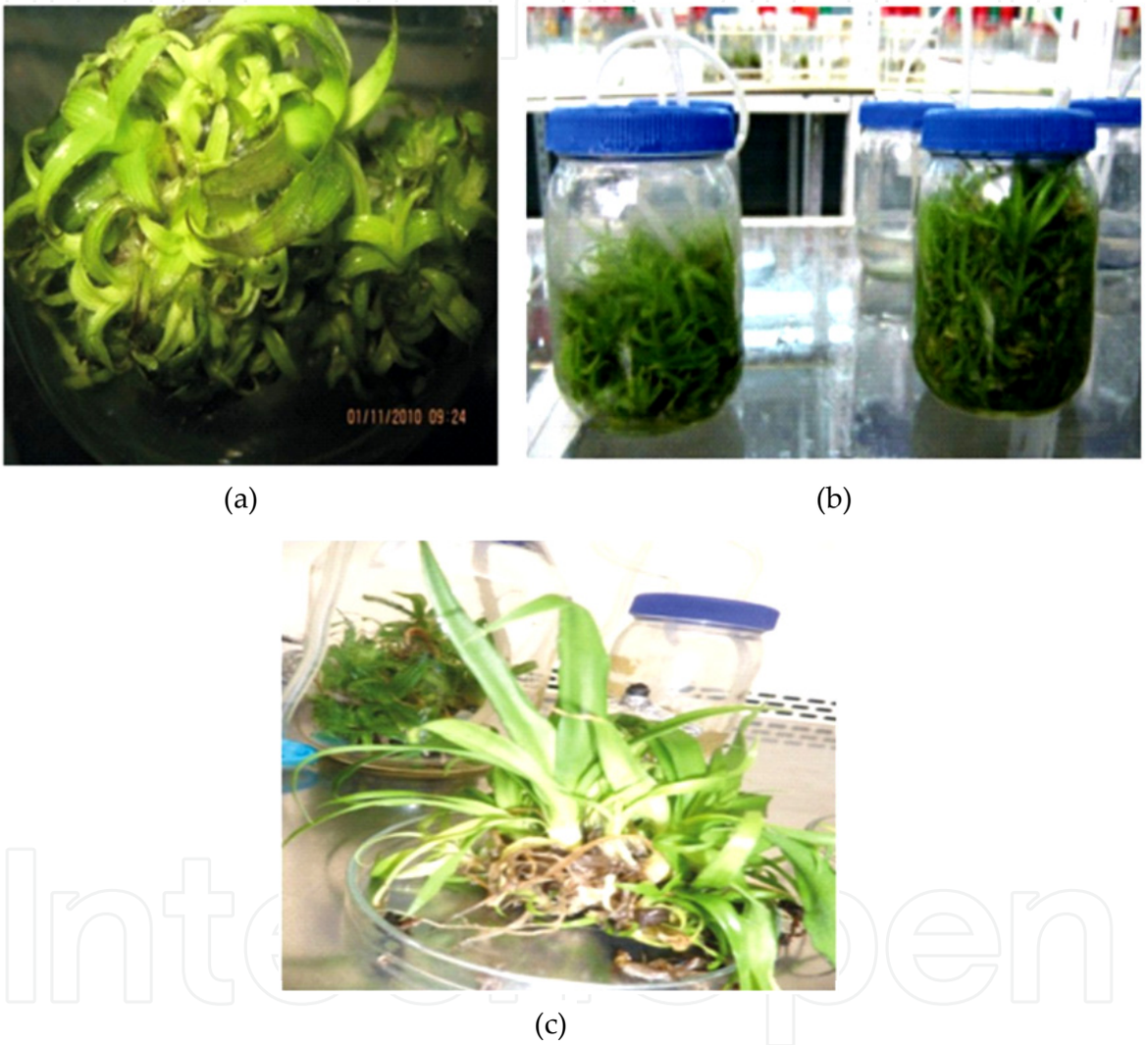
3.3.3. Stage 3: Introduction and cultivation of ex-palnts into Temporary Immersion Bioreactors.

This Phase refers to Plant cell/tissue growth and development in liquid medium under the control of Temporary immersion systems. It utilizes the advantages of liquid medium coupled with automated control of culture conditions to rapidly multiply explants thereby increasing exponentially the multiplication coefficient of the explants. Only healthy *in vitro* derived shoots that successfully passed through the zero shelving were introduced in TIBs set up. For the set up at NACGRAB, a total of 6 temporal immersions by pneumatic driven medium transfer were made daily. The immersion frequency was 3 minutes at 3 hours interval with a pre-immersion and post immersion period of 10 minutes respectively. This stage involves 3 major phases **(Figure 5a, b & c).**

In vitro response of plantlets in each of the 3 phases of TIBs is highly dependent on certain factors including hormonal combination, duration of each feeding cycle and the overall timing/duration to which it is subjected to. eg. higher cytokinins (BAP) concentration to auxins in the multiplication media, Gibberillic acid (GA_3) for elongation and auxins (IAA, NAA and IBA) for rooting.

i. *Multiplication phase-* Plantlets were cultured on MS liquid medium void of agar with the appropriate di-hormonal combination depending on the plant species. Pineapple and plantain were transferred to MS liquid medium + 4.0mg/l –BAP and 1.8mg/l –NAA, while Eucalyptus and sugarcane were transferred to MS liquid medium + 0.5mg/l-BAP and 0.1mg/l –NAA, for a period of 8 -12 weeks respectively.
ii. *Elongation phase-* This phase aims at developing proliferating buds into plantlets that are lengthy, strong and robust enough to stand acclimatization and withstand adverse conditions during *ex vitro* transfer. To achieve this, Plantlets were cultured in MS liquid medium void of agar with 1.0g/l of Gibberellic acid. After 21 days, plantlets were removed and placed in a rooting medium.

iii. *Rooting and harvest-* In order to induce rooting, elongated plantlets were cultured in a liquid MS media containing Auxin treatments of (A) 0.5mg/l- IBA, (B) 1.0mg/l-IBA (C) 0.5mg/l- NAA (D)1.0mg/l –NAA (E) A combination of 0.5 mg/l NAA and 0.5 mg/l IBA and (F) A combination of 1.0mg/l- IBA+ and 1.0mg/l-NAA for 4 weeks respectively. All media had equal volume in the same culture vessel. At the end of 4 weeks, plantlets were harvested by an initial disinfection of the mouth of culture bottle with 1% sodium hypochlorite. Bottle was opened, plantlets carefully collected.



Source: Lyam *et al*., 2012.

Figure 5. (a) Multiplication (proliferation in clusters), (b) Elongation (Stem growth elongation), (c) Rooting (well developed root system) of pineapple

The timing to achieve the desired goal at each phase varies depending on the individual species ability to respond to each phase accordingly. The common feature to all the phases is the use of liquid media (void of agar) to aid nutrient uptake and automation. Transfer of plantlets from one step to the next is carried out aseptically under the laminar flow hood.

Clumps of shoots derived were separated after rooting and not before as this usually cause tissue wounding and stimulate the exudation of phenolic compound which interferes with the physicochemical factors that trigger root formation. In this way, multiplied plantlets were elongated and rooted to produce complete plants and harvested. Harvest is carried out by an initial disinfection of the mouth of culture bottle with 1% Sodium hypochlorite. Bottle was opened and plantlets carefully collected.

3.3.4. Stage 4: Acclimatization and Ex vitro transfer

This is the final stage of the tissue culture operation including the use of bioreactor after which the micro propagated plantlets are ready for transfer to the greenhouse. Steps are taken to grow individual plantlets capable of carrying out photosynthesis. Collected plantlets were sorted and prepared for acclimatization based on their sizes and rooting capacity. *In vitro* micro propagated plants are weaned and hardened. The hardening of the bioreactor propagated plantlets is done gradually from high to low humidity and from low light intensity to high intensity conditions. Rooted plants were washed with tap water and acclimatized *ex vitro* on a medium composed of Coconut fibre, Top Soil and Stone dust mixed in the ratio 7:2:1 which can be left in shade for 3 to 6 days where diffused natural light conditions them to the new environment. The plants were transferred to an appropriate substrate for gradual hardening Figure 15 -20.

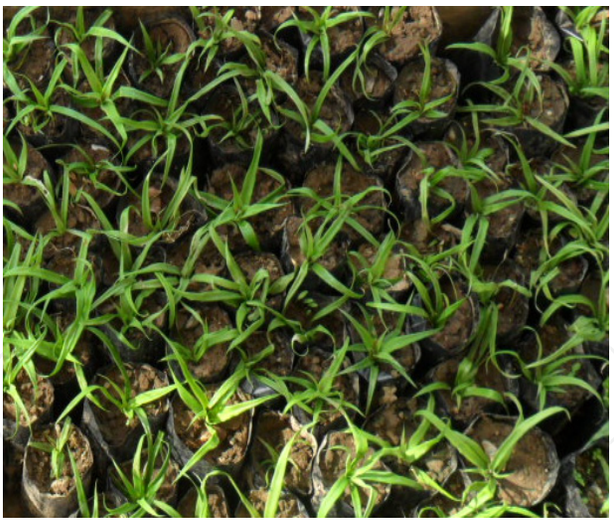


Figure 6. Potted and acclimatized plants in the screen house at NACGRAB.

These stages are universally applicable in large-scale multiplication of plants. The individual plant species, varieties and clones require specific modification of the growth media, weaning and hardening conditions. A rule of the thumb is to propagate plants under conditions as natural or similar to those in which the plants will be ultimately grown *ex-vitro*. Micropropagated plants must be subjected to an adequate duration of time required for their proper hardening.

Figure 7. TIBs set up at the NACGRAB, Ibadan, Nigeria

3.4. Challenges of temporary immersion bioreactor systems

The use of liquid cultures in bioreactor for plant propagation imposes several problems such as leakage of endogenous growth factors, the need for an initial high concentration of the inoculum, lack of protocols and production procedures, increased hyperhydricity and malformation, foam development, shearing and oxidative stress, release of growth inhibiting compounds by the cultures and contamination. Unfortunately culture contamination which is a major problem in conventional commercial micropropagation is even more acute in bioreactors [34]. In conventional micropropagation, discarding a small number of the contaminated vessels is an acceptable loss; in bioreactors, even a single contaminated unit is a huge loss. However, despite these difficulties, a number of commercial laboratories have developed effective procedures to control contamination in bioreactors. Highlighted below are some of the challenges [35], [36], [37].

3.4.1. Inadequate protocols and production procedures

Protocols for proliferation on semi solid media are not always efficient when used in bioreactors. However, as no one protocol is utilized for all species, it becomes quite difficult to achieve success at a goal. Development of protocol for scaling up cultures in bioreactors entails extensive research and development in all phases of TIBs (multiplication, elongation and rooting). It is possible to record success at one phase and not overcome the challenges at the next phase. For the efficient scaling up of cultures in temporary immersion bioreactors for commercialization, protocol for multiplication, elongation and rooting must be developed.

3.4.2. Increased hyperhydricity and malformation

The major disadvantage encountered when plants are cultured in liquid media is the problem of shoot malformation. Plants tend to accumulate excess of water in their tissue resulting to anomalous morphogenesis, a phenomenon known as Hyperhydricity. The plants that develop in liquid media are fragile, have a glassy appearance, with succulent leaves or shoots and a poor root system [38]. Hyperhydricity in micropropagation has been reported in previous studies [39], [40].

3.4.3. Problems of foaming, shear and oxidative stress

Growth and proliferation of the biomass in bioreactors depends on airflow supply for the aeration and mixing, and for the prevention of the plant biomass sedimentation. In many plants cultivated in bioreactors, continuous aeration, mixing, and circulation cause shearing damage, cell wall breakdown, and accumulation of cell debris, which is made up mainly of polysaccharides.

The problem of foaming and shear damage of tissues including their potential solutions in bioreactors has been reported [41], [40].

3.4.4. Release of growth inhibiting compounds by the cultures

This is also known as the *in vitro* Phenolic browning or oxidation. The presence of phenolic compounds which cause death of explants has been another important problem of micropropagation especially in woody perennials, in addition to various bacterial and fungal infection. Some of these exudates appear as a reaction to injury and/or infection. In tissue culture they appear after tissue excision and are many times aggravated by growth media constituents [42]. The release of growth inhibiting compounds by *in vitro* cultures has been reported [43], [44]

Some of the solutions to this problem as suggested are as follows:

- Addition of activated charcoal (0.2-3.0% w/v) to the medium ,
- Addition of polymeric polyvinylpyrrolidone (PVP) or polyvinylpolypyrrolidone (PVPP) to the medium. These absorb phenols through hydrogen bonding.
- Additions of anti-oxidants or reducing agents like citric and ascorbic acids, thiourea glutathione and L-cysteine in the medium or before surface sterilization. These reduce the redox potential of explants and stop the oxidation reactions (Marks and Simpson, 1990) [45].
- Addition of diethyl-dithiocarbonate (DIECA) (2g.l-1) in the rinses after surface sterilization and as droplets at the time of micro grafting.
- Addition of amino acids like glutamine, arginine and asparagine to the media.
- Reduction of salt concentration in the growth media. Others may include:
- Frequent subcultures onto fresh media.
- Use of liquid medium for easier and quicker dilution of toxic products.

- Reduction of wounded tissues to decrease exudation.
- Soaking of explants in water before culturing to reduce browning.
- Incubation of fresh cultures in darkness for the first few days of culture.

The suggestions above have provided solution to phenolic oxidation in micropropagation and are widely employed in most laboratories across the globe.

3.4.5. Microbial contamination

After three decades of research and development in plant tissue culture, microbial contamination by yeasts, fungi, bacteria, viruses, mites and thrips are still the major problem that has hampered the establishment of truly aseptic plants and their successful Micro - propagation in bioreactors. The influence of bacteria on shoot growth can range from total inhibition to no apparent effect. The contaminating bacteria and fungi may be endophytic or epiphytic, pathogenic or saprophytic [46]. Another type of hazard for plant tissue and cell cultures is caused by 'latent' bacteria and viruses that do not produce any symptoms on the plant or any visible growth on the medium for long periods of time *in vitro* even after several subculture cycles; microbial contamination in culture has been reported [47].

3.4.6. Control of contamination

Prevention of contamination in bioreactors requires a proper handling of the plant material, equipment and cultures during transfers and production. Only the surface sterilized explants, multiplied in small vessels and indexed for freedom from diseases are used to initiate cultures in bioreactors. If the bioreactor is small, it is sterilized in an autoclavable plastic bag, sealed with a cotton wool plug, and opened only under the laminar flow cabinet. Despite the precautions taken in initiating cultures, bioreactors can become contaminated from the environment or from latent microbes in the culture. The contamination can be controlled with one or a combination of anti-microbial compounds, acidification of the media, and micro-filtration of the medium [48]. While most of the fungal and bacterial diseases are eliminated during surface sterilization and culture, viruses and viroids survive through successive multiplication if the mother plant is infected [49].

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