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Effects of Sterilization and Phytohormones on shoot Tip Culture of *Telfairia Occidentalis*

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Abstract
Telfairia occidentalis is highly heterozygous with continuous out-crossing. Therefore, conservation of its genetic resources will require the culture of explants from vegetative tissue rather than seeds, in order to maintain improved or superior genotypes. Experiments were carried out to establish a procedure for the sterilization of shoot tip explants from field grown seedlings and to induce organogenesis using different treatment combinations of phytohormones. Shoot tip explants obtained from 4 to 5 weeks old seedlings were surface sterilized using 3 concentrations of sodium hypochlorites (NaOCl) (0.5%, 0.75% and 1%) at 3 exposure time (10minutes, 15minutes and 20minutes). The percentage clean cultures were recorded after 4, 8 and 12 days. The lowest frequency of clean culture (0.33) was obtained when shoot tip explants were treated with 0.5% of NaOCl for 20minutes. Sterilization was most effective when shoot tip explants were treated with 1% NaOCl for 15minutes and 20minutes, after 4 and 12 days. Ninety three percent clean and viable cultures were obtained. Surface sterilized shoot tip explants were inoculated on ½ N6 media supplemented with different concentrations and combination of BAP, NAA, IAA and 2,4D. Shoot tip explants in ½ N6 media supplemented with 0.05mg/l BAP showed the highest shoot length of 1.46 cm and highest shoot number of 1.60. In all concentrations and combination of BAP and NAA, there was callus formation. In all concentrations and combination of BAP and 2,4D, calluses were formed to prevent shoot bud induction while there was induction of multiple shoot in treatment combination of 0.5mg/l BAP and 0.1mg/l 2,4D. In all concentrations and combination of BAP and IAA, there were formation of calluses, but not formed in the treatments containing 0.5mg/l BAP and 0.1mg/l IAA, while BAP 2.0mg/l and IAA 1.0mg/l induced tiny shoots.
Keywords: Conservation, *Telfairia occidentalis*, Surface sterilization, Explants, Organogenesis

1. Introduction

Telfairia occidentalis (fluted pumpkin) is a tropical vegetable grown in West Africa countries and widely consumed in tropical regions (Fagbemi *et* al., 2005). There are threats to its conservation and utilization. Localized and narrow natural genetic diversity is exacerbated by the systemic neglect of the species in scientific research. Seeds of *T. occidentalis* are recalcitrant due to their germination capacity which decline when seed moisture is less than 40%, and are completely lost below 30% (Balogun *et al.,* 2002). There is no known method of handling both seeds and fruits in a way of keeping the seeds alive and retain vigor during the off-season period when seeds must be stored.

In vitro culture techniques can be the alternative for the continuous provision of plantlet stocks for large scale field cultivation. Many plant species are now propagated via *in vitro* culture techniques (Tiwari *et al*., 2000; Santarém and Astarita, 2003; Malik *et al*., 2005; Mei, 2005). Plant tissue culture techniques have been used in propagated plants. Moreover, plant regeneration is essential and pre-requisite for crop improvement (Barakat and El-Sammak, 2011). Recently, *In vitro* culture and plant regeneration from shoot tip and nodal cuttings explants of *Telfairia occidentalis* have been reported (Ajayi *et al*., 2006; Balogun *et al.,* 2007; Sanusi *et al.,* 2008).

Contamination with microorganisms such as viruses, bacteria, yeast and fungi is considered to be the most important reason for losses during *in vitro* culture of plants (Omamor *et al.,* 2007). These microbes compete adversely with plant tissue cultures for nutrients and their presence usually cause increased culture mortality which can also result in variable growth, tissue necrosis, reduced shoot proliferation and reduced rooting (Kane, 2003). Thus sterilization of explants is employed prior to culture initiation, since in vitro condition provide bacteria and fungi with an optimal growth environment. The use of field grown plants directly as source of explants material for the production of clean in vitro plantlet may pose a lot of challenges. However, sterilization should guarantee sterility of the explants and regeneration capacity which are known to be affected by disinfectant concentration and sterilization period. Ajayi *et al*. (2006) reported the behavior of excised embryonic axes and shoot tips of fluted pumpkin under *in vitro* conditions when the systemic infection of seeds from field led to frequent and high microbial contamination in culture.

A number of researches have been carried out on the *in vitro* propagation of *T. occidentalis* ranging from studying the effect of plant growth regulators to the behavior of different shoot tip explants (Balogun *et al*., 2007;

Sanusi *et al*., 2008).
However, there is limited information on the genetic conservation of *T. occidentalis* using the technique of *in vitro* culture of explants from vegetative tissues of field grown seedlings. Therefore, this present study aimed at:
Investigating the effectiveness of NaOCl sterilant on shoot tip explants of *T. occidentalis* and the effect of different hormonal combination on the shoot induction of *T. occidentalis*

2. Methodology

2.1 Experimental Location and Research Design

Research was carried out in the tissue culture laboratory of National Centre for Genetics and Biotechnology (NACGRAB), Moor Plantation, Ibadan, Nigeria. Experiments were laid up in a completely randomized design under factorial arrangement. Three concentrations of NaOCl (0.5%, 0.75% and 1.0%) for 10, 15 and 20 minutes and then rinsed thrice with sterile water.

2.2 Source of T. occidentalis explants and Surface sterilization

Matured fruits of T. occidentalis were purchased from the market at Ojoo in Ibadan, Nigeria and brought to the laboratory, which were maintained under laboratory conditions. Seed of T. Occidentalis were planted in an open field, shoot tip explants were collected from 4 to 5 weeks old of T. occidentalis from the field and used as explants for induction of multiple shoot. Explants were washed thoroughly under running tap water for 15 minutes and then surface sterilized by submerging in 70% ethanol for 5minutes which was done under a laminar flow. Different sterilization treatment were carried out on the shoot tips prior to culture in order to determine the most effective treatment concentration and time of sterilization of the shoot tips. For regeneration experiments at least ten test tubes were used for each treatment.

2.3 Culture Establishment

The basic nutrients solution of 1/2N6 was used as basal medium with the addition of 30g sucrose, 0.025g myoinositol, 7g agar per liter in nine different combinations of plant growth hormone and a control medium, that is, having no growth hormones. The different combinations of hormones are as follows;

- Benzylaminopurine (BAP) 0.05mg/litre
- Benzylaminopurine (BAP) 0.1mg/litre
- Benzylaminopurine (BAP) 0.5mg/litre
- Benzylaminopurine (BAP) 1.0mg/litre
- Benzylaminopurine (BAP) 2.0mg/litre
- Benzylaminopurine (BAP) 0.05mg/litre + Naphthalene acetic acid (NAA) 0.01mg/litre
- Benzylaminopurine (BAP) 0.1mg/litre + Naphthalene acetic acid (NAA) 0.01mg/litre
- Benzylaminopurine (BAP) 0.5mg/litre + 2, 4-dichlorophenoxy acetic acid (2,4-D) 0.1mg/litre
- Benzylaminopurine (BAP) 1.0mg/litre + 2, 4-dichlorophenoxy acetic acid (2,4-D) 0.5mg/litre
- Benzylaminopurine (BAP) 2.0mg/litre + 2, 4-dichlorophenoxy acetic acid (2,4-D) 1.0mg/litre
- Benzylaminopurine (BAP) 0.5mg/litre + indole-3-acetic acid (IAA) 0.1mg/litre
- Benzylaminopurine (BAP) 1.0mg/litre + indole-3-acetic acid (IAA) 0.5mg/litre
- Benzylaminopurine (BAP) 2.0mg/litre + + indole-3-acetic acid (IAA) 1.0mg/litre
- Control; no plant growth hormones.

The medium was made up from commercially prepared stock solutions of macronutrients, micronutrients and vitamins. The pH of the medium used was adjusted to 5.7 using a pH meter with sodium hydroxide and hydrochloric acid. Proper dissolution of agar in media was done in glass jars placed in microwave oven before dispensing into test-tubes for autoclaving. The media in the test-tubes were autoclaved at 121^0C for 15 minutes

2.4 Data collection and Statistical analysis

Presence of callus, shoot length and number of shoots formed per explants were recorded. Number of viable shoot tip explants was after 4 days, 8days and 12days of culture establishment. Results were based on average mean of five explants. The data generated was analyzed using Analysis of variance (ANOVA) while mean separation was done using Duncan Multiple Range Test (DMRT) at $p<0.05$

3.1 Result

3.1 Sterilization of shoot tips at different concentrations of NaOCl over different exposure time intervals

Table 1 shows that the concentrations of NaOCl employed in sterilization differed significantly in the sterility rates ($p<0.05$).The mean values of the different concentrations of NaOCl show that the effectiveness of the sterilization procedure increases with concentration. 1% NaOCl recorded the highest mean (0.93) of shoot tip explants free from contamination at 15 and 20minutes on the fourth day after culture establishment. However, the concentrations had no significant effect at 10minute. Again, there was no significant effect after 8 and 14days regardless of the exposure time. 0.5% NaOCl showed the least effect on the shoot tip explant sterilization which

recorded a mean of 0.33 clean cultures.

3.2 Effect of BAP Concentrations on Shoot tip Culture

For shoot initiation, elongation and multiplication, ½N6 medium supplemented with different plant growth regulator (PGR) of different concentrations were used. Not all the media formulations induced shoots. Amongst the combinations that induced multiple shoot, BAP 0.05mg/l of 1.60 was the best compared to other treatments. The control medium induced shoots and roots, but did not develop multiple shoots (Plate 1). The results showed that concentrations of BAP at 0.05mg/l in ½ N6 medium had significant effect ($p>0.05$) on the number of shoot per explants in *T. occidentalis* (Table 2). The number of shoots ranged from 0.80-1.60. Maximum number of 1.60 shoots per explants was on ½N6 medium containing 0.05mg/l BAP, while reduced number of 0.80 shoot per explants was recorded on other concentrations of BAP.

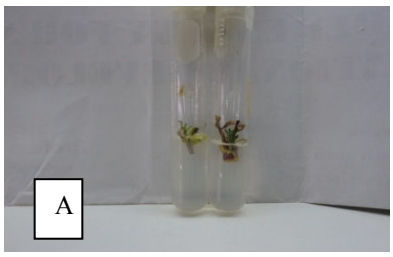
3.3 Effect of BAP Concentrations with NAA, IAA and 2, 4 D on Shoot tips Culture

The results showed that various concentrations of BAP and auxins (NAA, 2,4D, and IAA) had significant effect on the number of shoots per explants in *T. occidentalis* (Table 3). Adventitious buds were initiated from meristematic region of the shoot tip explants after three weeks of inoculation in the treatment combination containing BAP and NAA at concentrations of 0.1mg/l and 0.01mg/l respectively. In other concentrations and combination of BAP and NAA, there was callus formation, also in all concentrations and combination of BAP and 2,4D, calluses were formed thus, preventing shoot bud induction. In treatment combination of BAP and 2,4D of 0.5mg/l and 0.1mg/l respectively, there were induction of multiple shoots. In all concentrations and combinations of BAP and IAA, there was presence of calluses. In the treatments containing BAP and IAA of concentrations 0.5mg/l and 0.1mg/l and 2.0mg/l and 1.0mg/l respectively induced tiny shoots.

Table 1: Effect of sterilization on shoot tips at different concentrations of sodium hypochlorides solution over different exposure period of time

	Treatment (Mean ± SD)		
	0.5%	0.75%	1.0%
After 4 days			
10mins	0.60±0.00	0.47±0.31	0.67±0.23
15mins	$47±0.12^{b}$	$0.53±0.31^{b}$	$0.93±0.12^{a}$
20mins	$0.40±0.20^{b}$	$0.73±0.31^{ab}$	$0.93±0.12^{a}$
After 8 days			
10mins	0.80±0.17	0.93±0.12	0.93±0.12
15mins	0.83±0.29	0.90±0.17	0.73±0.31
20mins	0.10±0.17	0.77±0.25	0.53±0.50
After 14 days			
10mins	1.00±0.00	0.83±0.29	0.67±0.35
15mins	0.67±0.29	0.77±0.25	0.77±0.40
20mins	0.33±0.58	1.00±0.00	0.77±0.40

Note: Mean ± S.D. with the different superscripts are significantly different. Mean separation done with Duncan Multiple Range test with $p>0.05$



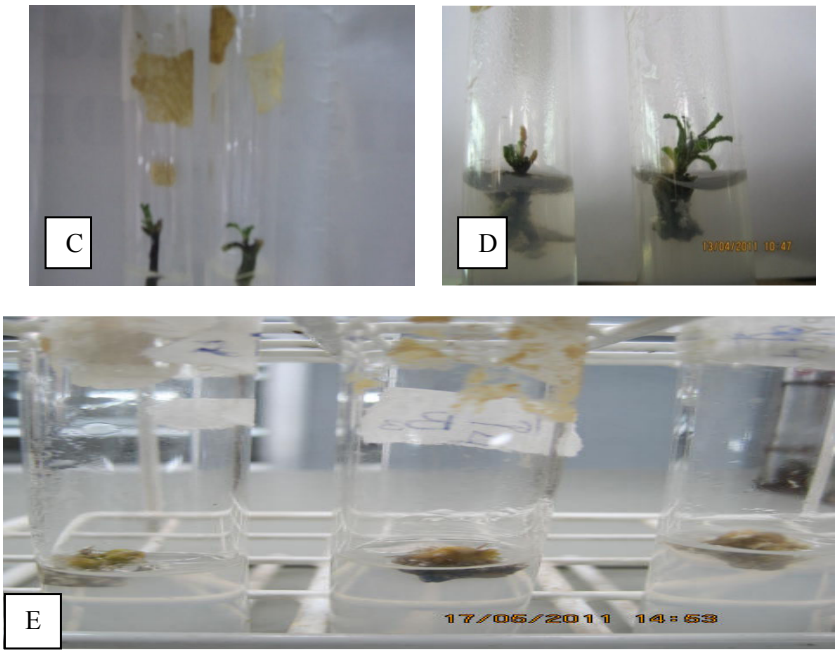


Plate1: Regeneration of Telfairia occidentalis in Shoot tip Culture.
(A) Shoot induction (B) Shoot elongation (C) Explants in control medium
(D) Shoot multiplication (E) Callus formed

Table 2. Effect of BAP Concentrations on Shoot tip Culture

Treatment (Mean ± SD)	Control	0.05mg/l BAP	0.1mg/l BAP	0.5mg/l BAP	1.0mg/l BAP	2.0mg/l BAP
Shoot Length	1.00 ± 0.12^{b}	1.46 ± 0.17^{a}	0.80 ± 0.47^{b}	0.72 ± 0.41^{b}	0.68 ± 0.40^{b}	0.26 ± 0.18^{c}
Shoot No.	1.00 ± 0.00^{ab}	1.60 ± 0.89^{a}	0.80 ± 0.45^{b}	0.80 ± 0.45^{b}	0.80 ± 0.45^{b}	0.80 ± 0.45^{b}

Note: Mean ± S.D. with the different superscripts are significantly different. Mean separation done with Duncan Multiple Range test at p>0.05

Table 3. Effect of BAP Concentrations with NAA, IAA and 2, 4 D on Shoot tips Culture

	Treatment (Mean ± SD)								
	0.05mg/l BAP+0.01 mg/l NAA	0.1mg/l BAP+0.01 mg/l NAA	0.5mg/l BAP+0.1 mg/l 2,4 D	1.0mg/l BAP+0.5 mg/l 2,4 D	2.0mg/l BAP+1.0 mg/l 2,4 D	0.5mg/l BAP+0.1 mg/l IAA	1.0mg/l BAP+0.5mg/l IAA	2.0mg/l BAP +1.0mg/l IAA	control
Shoot Length	1.02 ± 0.93^{a}	0.50 ± 0.29^{ab}	0.16 ± 0.15^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.54 ± 0.51^{ab}	0.00 ± 0.00^{b}	0.16 ± 0.15^{b}	1.00 ± 0.12^{a}
Shoot Number.	1.40 ± 1.34^{a}	0.80 ± 0.45^{ab}	0.60 ± 0.55^{ab}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.60 ± 0.55^{ab}	0.00 ± 0.00^{b}	0.60 ± 0.55^{ab}	1.00 ± 0.00^{a}

Note: Mean ± S.D. with the different superscripts are significantly different. Mean separation done with Duncan Multiple Range test at p>0.05

KEY
BAP: Benzylaminopurine
NAA: Naphthalene acetic acid
IAA: Indole-3-acetic acid
2,4 D: 2, 4-dichlorophenoxy acetic acid

Table 3. Effect of BAP Concentrations with NAA, IAA and 2, 4 D on Shoot tips Culture

Treatment (Mean ± SD)	Control	0.05mg/l BAP	0.1mg/l BAP	0.5mg/l BAP	1.0mg/l BAP	2.0mg/l BAP
Shoot Length	1.00 ± 0.12^{b}	1.46 ± 0.17^{a}	0.80 ± 0.47^{b}	0.72 ± 0.41^{b}	0.68 ± 0.40^{b}	0.26 ± 0.18^{c}
Shoot No.	1.00 ± 0.00^{ab}	1.60 ± 0.89^{a}	0.80 ± 0.45^{b}	0.80 ± 0.45^{b}	0.80 ± 0.45^{b}	0.80 ± 0.45^{b}

Note: Mean ± S.D. with the different superscripts are significantly different. Mean separation done with Duncan Multiple Range test at p>0.05

4. Discussion and Conclusion

The results show that the procedure for sterilization of shoot tip explants from field grown seedlings and the effect of using different concentrations of sodium hypochlorites (NaOCl) at different time interval agree with Ajayi *et al*.,(2006). However, the highest frequency of clean culture which was recorded when shoot tips were sterilized with the highest concentration of NaOCl (1%) for 15 and 20 minutes is not in line with the report of Nguyen, (2005).

The findings of some researchers on *in vitro* culture of various plant species indicates that even different cultivar of the same species had a diverse response and growth patterns under *in vitro* or *ex vitro* condition (Norton and Norton 1985; Simpson and Bell 1989). In a significant development, it was observed that multiple shoot bud originated from shoot tip explants when ½ N6 was supplemented with different concentrations of hormones. The shoot tips showed swelling at the base of the explants prior to the emergence of shoot buds from the pre existing materials. The importance of cytokinin for organogenesis has been studied in many plant species (Pereira *et al*., 2000; Guo *et al*., 2005; Zhang *et al*., 2008), and was considered as an exclusive element for shoot formation . It was noted that BAP singly showed little or no effect in callus induction. There was significant effect in the number of shoot and in shoot length having the average mean of 1.46 and 1.60 respectively which is similar to report of Xu *et al*. (2008), who reported that BAP containing medium had significant effect on shoot regeneration on *in vitro* regeneration of leafy spurge (*Euphorbia esula*). In this study, it was found that all combinations and concentrations of BAP and auxins used induced callus, which is in line with the report of Balogun *et al*., (2007), that stated that a high degree of calluse formation was reported in single node explants of fluted pumpkin, cultured in medium containing both a cytokine (Kinetin (Kn) and an auxin (NAA). Considerable number of shoot tip explants died even in the suitable media, probably due to dissection injury. About 25-40% death rate was described as a phenomenon in most of the published protocols (Alam *et al.,* 2010).

In conclusion, the present study shows how genetic resources of *Telfairia occidentalis* can be conserved to avoid potent threats to its existence, requiring the culture of explants from vegetative tissues rather than seeds, in order to maintain improved or superior genotypes. Sterilization should therefore guarantee the explants sterility and regeneration capacity which are known to be affected by disinfectant concentration and sterilization period. The *in vitro* protocol reported in this study can be used for sterilization of explants from vegetative tissues, rapid multiplication of disease free plants, sustainability of genetic modification and transformations.

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