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Full Length Research paper

Micropropagation of *Allanblackia stuhlmannii*: Amenability to tissue culture technique

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***Allanblackia stuhlmannii* is an endangered forest tree valued for its edible nut oil. Its limited regenerative potential in the wild hinders the sustainable utilization of its products. To achieve mass production of *A. stuhlmannii*, its amenability to micropropagation technique was examined. Explants were best surface sterilized at 8% sodium hypochlorite for 10 min and rinsed using sterile distilled water. Of eight basal nutrient media tested, Lloyd and McCown Woody plant medium (WPM) was the most suitable (88.89% explants survival). Microshoots were induced from apical meristems cultured on WPM supplemented with different concentrations of 6-benzyladenine (BAP), kinetin (KN), Dichlorophenoacetic acid (2, 4 - D), Naphthalene acetic acid (NAA) and Thidiazuron (TDZ), ($P < 0.05$). All responding explants produced a single microshoot irrespective of the type and concentration of PGRs used. 1.2 mg/lBAP and 1.2 mg/lKIN exhibited the most rapid and consistent shoot length increase ($P < 0.05$). Prolonged culture or sub culturing did not promote further shoot proliferation. Callus was induced from leaf explants cultured on WPM fortified with Gamborg's vitamins, 3% sucrose, 1 mg/lKIN combined with 1.2 mg/l 2, 4 - D. No somatic embryos emerged from the callus. The success in explant sterilization and induction of microshoot and callus in this study is a milestone step in the regeneration of *A. stuhlmannii*.**

Key words: Sterilization, media plant growth regulators, shoot proliferation, callus induction.

INTRODUCTION

Allanblackia stuhlmannii Eng. (Clusiaceae) locally known as 'Msambu' is a forest tree with high market potential and grows naturally in East and West Usambara forests, Nguru and Uluguru forest mountains in Tanzania (El Tahir and Mlowe, 2002; Meshack, 2004, Van, 2005). During World War I, German soldiers in Tanzania used fat extracted from *A. stuhlmannii* nuts as an alternative edible fat to butter (Saka, 1995). The communities living around the Eastern ArcMountains, particularly farmers, use the oil extracted from *A. stuhlmannii* nuts for food and soap production (Lovett, 1983; Monela et al., 2001; Osemeobo, 2005, Pye-Smith, 2009). They also use dry leaves of this tree as medicinal tea to treat chest pain and smear heated seed oil on aching joints, rashes and wounds (Meshack, 2004). Phytochemical analysis of *A. stuhlmannnii* crude extracts showed that Guttiferone F, a prenylatedbenzophenone, a compound related to a group of compounds that have been studied for their anti-HIV property, was present (Fuller et al., 2003). This tree species also has a great commercial potential for margarine production from its edible seed oil whose extraction requires less chemical processing and refraction than palm oil (Atangana et al., 2006). Already, the oil from *A. stuhlmannii* has received the approval of the European Union (EU) Novel Food Regulations that certify safe usage as a foodstuff (Hermann, 2009; Ramni et al., 2010).

Regeneration of *A. stuhlmannii* via seed is however

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slow and low (low seed fecundity/viability). Germination typically takes 1 to 7 months to begin and a minimum of 18 months to complete after sowing (Mwaura and Munjuga, 2011). Rooting of cuttings is poor while survival rate of grafted materials is dismal (Mwaura and Munjuga, 2011). Under natural conditions, the trees begin to bloom at the age of 12 years. The fruits take over a year to mature and become ripe between November and March and between August and October (Mathayo et al., 2009). Rodents and monkeys eat the fruits, and hence, providing the only mode of seed dispersal (Glynn and Ritzl, 2000). The limited regenerative potential and dispersal powers of *A. stuhlmannii* in the wild are likely to be a vulnerable element of the local biodiversity (Amanor et al., 2003; Attipoe et al., 2006). Loss of *A. stuhlmannii* biodiversity is likely to result to decline in the quality of harvested products (Amanor et al., 2003; Cordeiro et al., 2007; Egyir, 2007). Sustainable development of *A.stuhlmannii* industry will greatly dependent on mass production of *A. stuhlmannii.* A micropropagation protocol with high multiplication rates will greatly contribute in the domestication and hence, conserve this economically endangered tree species.

Micropropagation offers a rapid means of producing large quantity of clonal planting stocks and propagation of some commercial crops and also tree species that are difficult to establish conventionally (Bonga, 1987; Merkle and Dean, 2000; Thorpe et al., 1990). Micropropagation of a wide range of tree species have been successfully achieved (Pankaj and Toshiyuki, 2001). However, numerous recalcitrant forest trees of economic value are still difficult to establishment *in vitro* (Anna et al., 2010). There are no documented studies on the micropropagation of *A. stuhlmannii* or any member of this genus. The use of explants from mature plants is not frequently accomplished, mainly due to the high level of contamination (Drew, 1988), reduced or absence of morphogenetic ability (Bonga, 2010) and poor rooting of the regenerated shoots. The selection of suitable explants and establishment of shoot cultures are two critical factors for cloning of trees while maintaining clonal fidelity and purity (Maynard, 1988). Induction of cellular differentiation *in vitro* however, depends on genetic totipotency, culture medium formulation, and incubation conditions (Gasper et al., 1996). During the *in vitro* culture process, undesirable or inhibitory compounds such as excess phenolic metabolites (Carlberg et al., 1983), ethylene (Mensuali-Sodi et al., 1996) and 5-hydroxy methyl furfural?, an inhibitory by-product of autoclaving sucrose, can be produced during medium preparation. These compounds hinder successful *in vitro* induction of cellular differentiation. As a result, there is need for preliminary experiments for selecting or modifying the known basal media that will be suitable for micropropagation of the plant species (Preece and Compton, 1991). In this paper, we reported on the adoptable explant sterilization protocol, direct organogenesis and callus induction from leaf explants of *A. stuhlmannii*.

MATERIALS AND METHODS

Collection and management of plant material

Plant materials used in this study were collected in October, 2009 at Amani Nature Reserve (ANR) located in the Southern part of the East Usambara Mountains (4°48 to 5°13'S, 38°32 to 48°E) in Tanzania. The plant materials consisted of mature seeds, seedlings and cuttings from coppices. They were thoroughly washed at sampling site and then stored in cool boxes and later transported to Kenya Forestry Research Institute (KEFRI) at Muguga in Kenya. On arrival, the seedlings were transplanted in potting bags containing well mixed loam soil. The seeds were germinated on growth trays half of which contained sawdust mixed with sand (1:1) and the other half contained sawdust mixed with decomposing manure. Both transplantation and germination were done in a glasshouse at KEFRI. The cuttings were dipped vertically in sawdust mixed with sand (1:1) in a nursery at KEFRI. They were all watered twice a week using a sprinkler. Seeds that germinated were transplanted into similar potting bags. Well adapted seedlings were used as stock plants in subsequent experiments.

Explants preparation and establishment of sterilization protocol

All the glassware and metallic equipment used for this section were sterilized by autoclaving at 121°C at 1.06 kg cm^{-2} pressure for 15 min before use. Young emerging leaves shoot apices and slender branches were harvested from stock plants and used as explants. The explants were placed in a glass jar containing 500 ml of water into which three drops of Tween® 20 and five drops of Dettol® soap detergent had been added. The jar was swirled gently for 15 min before washing the explants with running tap water for 10 min. Under a clean lamina flow hood, half the number of harvested explants was subjected to sodium hypochlorite and the other half subjected to formaldehyde (sterilants) at varying concentrations and exposure times using the following experimental designs: In experiment 1; explants were separately exposed to the two sterilants at four concentration levels that is, 0 10, 15 and 20% with each concentration level having two exposure times (10 and 20 min). In experiment 2; explants were exposed to 2% Redomil® solution for 15 min prior subjection to sodium hypochlorite at three concentration levels (6, 8 and 10%) each subjected to three exposure times (6, 8 and 10 min). In experiments (1 and 2), explants were rinsed three times using sterile distilled water and placed on sterile blotting paper. The shoots were trimmed to a length of 1 cm and leaves cut into squares of 1 cm dimension before culturing in full strength MS medium. All cultures were daily monitored and assessed for fungal and bacterial infection. The number of explants infected by either fungi or bacteria or both (using morphological descriptors) and those that died due to bleaching were recorded after one week for two consecutive weeks (whereby score 1 represented presence of bacteria and fungi and score 0 represented absence of bacteria and fungi).

Effect of different basal media on explants survival

Eight nutrient media namely; Murashige and Skoog medium, Gamborg (B_5) medium, Lloyd and McCown's Woody Plant medium (WPM), White's medium, Preece Hybrid medium, Driver and Kiniyuki walnut (DKW)medium, Anderson medium, and Quorin and

Table 1. Comparison of element levels in the various media used to assess explants survival rate.

Concentration (mg/l) Macro elements	Anderson's medium	DKW medium	Lloyd and McCown's woody plant medium	Quorin and Lepoivre medium	Murashige and Skoog medium	White's medium	Gamborg (B5) medium	Preece hybrid medium
NH_4NO_3	400	1416	400	400	1650	0	0	908
KNO_3	480	0	0	1800	1900	80	2500	0
K_2SO_4	0	1559	990	0	0	0	0	1275
$MgSO_4.7H_2O$	180.7	361.49	180.7	175.8	180.5	720	121.6	271.1
KH_2PO_4	0	265	170	270	170	0	170	217.5
$CaCl_2$	332.2	112.5	72.5	0	332	0	113.2	92.5
$Ca(NO_3)_2.4H_2O$	0	1367	386	833.8	0	300	0	876.5
$Na(H_2PO_4).H_2O$	330	0	0	0	0	16.5	130.4	0
$(NH_4)_2SO_4$	0	0	0	0	0	0	134	0
Micro elements								
$CoCl_2.6H_2O$	0.025	0	0	0.025	0.025	0	0.025	0
$CuSO_4.5H_2O$	0.025	0.25	0.25	0.025	0.025	0	0.025	0.25
$FeNa_2EDTA$	36.7	33.8	36.7	36.7	36.7	3.47	36.7	30.83
H_3BO_3	3	4.8	6.2	6.2	6.2	1.5	3	5.5
KI	0.75	0	0	0.08	0.83	0.75	0.75	0
$MnSO_4.H_2O$	10	33.5	22.3	0.76	16.9	5.31	10	27.9
$Na_2MoO_4.2H_2O$	0.25	0	0.25	0.25	0.25	0	0.25	0.32
$ZnSO_4.7H_20$	2	17	8.6	8.6	8.6	2.67	2	4.3
$NiSO_4$	0	0.005	0	0	0	0	0	0
$Zn(NO_3)_2$	0	17	0	0	0	0	0	8.5
Vitamins								
Glycine	0	0	2	0	2	0	0	1
Nicotinic acid	0	0	0.5	0	0.5	0	1	0.25
Pyridoxine	0	0	0.5	0	0.5	0	1	0.25
Thiamine	0.4	0	0	0	0.2	0	10	0
Myo-inositol	100	0	0.5	0	100	0	100	0
Adenine hemisulphate	80	0	0	0	0	0	0	0

Lepoivre medium were tested for their suitability in micropropagation of *A. stuhlmannii*. Table 1 presents the ion composition of these media. All the media were supplemented with 30 gl^{-1}sucrose and 0.8 g/l agar. The pH value of each media was adjusted to 5.75 using 0.1M HCl or 0.1M NaOH and dispensed in 150 mm (height) by 25 mm (diameter) culture tubes (10 ml of medium per tube). The media were then sterilized in an autoclave at 121˚C at 1.06 kg cm^{-2}pressure for 15 min before use. Cultures were grown at 25 ± 1°C and a 16 h photoperiod provided by white fluorescent Philips light bulbs (40 W) in the growth chamber.

Effect of plant growth regulators and their concentrations on proliferation stage

Modified Lloyd and McCown's Woody plant medium (modification: 76 mg/l $CaCl_2$ and 6.0 mg/l H_3BO_3) was used to test the effect of different PGRs at varying concentrations on shoot proliferation. In the single plant growth regulator applications, 1.2 mg/l, 2.4 mg/l and 3.6 mg/l separately for BAP and KIN and 1.2 mg/land 2.4 mg/l for TDZ and a control (media without PGRs) totaling to nine treatments were set. In the combination sets, three different concentrations of BAP (1.2, 2.4 and 3.6) mg/land two concentrations of NAA (0.2 and 0.4) mg/l were combined in six different treatments. In another combination, three different concentrations of KIN (1.2, 2.4 and 3.6) mg/land two concentrations of NAA that is, 0.2

Table 2. Fungal and bacterial contamination levels and explants mortality when exposed to different concentrations of Sodium hypochlorite and Formaldehyde at varying exposure times (Rep = 9).

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

and 0.4 mg/l were combined in six different treatments. In yet another combination, two concentrations of TDZ (1.2 and 2.4) mg/land, two concentrations of NAA (0.2 and 0.4) mg/l were combined in four different treatments. All the media were supplemented with 30 mg/l sucrose and 8 mg/l agar while medium sterilization and culture conditions were effected as described earlier. Induction of microshoots and their length was evaluated at an interval of 4 weeks after initiation.

Effect of plant growth regulators and their concentrations on callus induction stage

Lloyd and McCown's Woody plant medium with two modifications (76 mg/l $CaCl_2$ and 6.0 mg/l H_3BO_3) supplemented with Gamborg's vitamins (McCown and Sellmer, 1987 and Gamborg et al., 1974) was used to test the effect of different plant growth regulators at varying concentrations on induction of callus from leaf discs. Three different concentrations of KIN (0.5, 1 and 2) mg/l and four concentrations of 2, 4-D (1, 1.25, 1.5 and 2) mg/l were combined in five different treatments including a control (media without PGRs). All the media were supplemented with 30 mg/l sucrose and 8 mg/l agar and medium sterilization and culture conditions as described earlier. The percentage proportion of callus induction around the leaf discs was evaluated at an interval of 4 weeks after initiation.

Effect of plant growth regulators and their concentrations on rooting of shoots

In the first experiment, half strength MS and WPM media in which half of each media contained 30 mg/l and the other half contained 1 mg/l sucrose were prepared. All the media were fortified with IBA, IAA and NAA at various concentrations (0.0, 0.01, 0.05, 0.1 and 2.5) mg/l separately and 8 mg/l agar added into each treatment. In the second experiment, a two-step procedure (Bennett et al., 1994; Gasper and Coumans, 1987) was used. Stable shoot were initially cultured in half-strength MS medium (containing 30 mg/l sucrose and 8 mg/l agar) fortified with IBA, IAA and NAA at 0, 0.01, 0.03, 0.05 and 0.1 mg/l concentration levels separately. The shootlets were then transferred into the same MS medium but without PGRs after three weeks and regularly checked for any sign of rooting.

Data collection and analysis

Data was collected in MS Excel spreadsheets and analysed using Statistical Analysis System (SAS) and Genstat 12th Edition, statistical softwares. Mean number of explants contaminated by bacteria and fungi and those that died as a result of bleaching / scorching was determined. The best sterilant suitable concentration and the preferred time of exposure were deduced from the analyzed means, hence, adopted as the sterilization protocol. The medium with the highest explants survival rate was also determined. ANOVA tests showing the effects of variations and interactions of the various plant growth regulators used and duration (weeks) on induction of microshoots, callus and roots were compared at $P > 0.05$ (Turkey's test).

RESULTS

Sterilization protocol

Results on the effectiveness of sodium hypochlorite and formaldehyde in the sterilization of *A. stulhmannii* explants show that, increase in concentration and time of exposure, resulted in high explant mortality rate and decrease in bacterial and fungal (although, inconsistent) contamination levels, $P < 0.05$, (Table 2) with sodium hypochlorite being the preferred sterilant. The trend observed in the outcome of experiment 1 was used to design experiment 2 in which the concentration level and exposure time of sodium hypochlorite were optimized with regard to reduction in bacterial and fungal contamination levels and explant mortality rate.

To adequately control fungal contamination, explants

Table 3. Fungal and bacterial contamination levels and mortality of explants exposed to varying concentrations at different exposure durations of Sodium hypochlorite (%), Rep = 9.

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

Table 4. Explants survival rate on various nutrient media.

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

were subjected to 2% Redomil® solution (Perez et al., 2009) prior exposure to sodium hypochlorite in the sterilization experiment 2 (Table 3). It was observed that, *A. stuhlmannii* explants were best surface sterilized when exposed to 2% Redomil® solution for 15 min followed by 10 min exposure to 8% sodium hypochlorite and finally rinsed three times using sterile distilled water (Table 3).

Media selection

When explant survival rates on the selected media were compared using $LSD_{0.05}$ (Turkey's test), significant differences were observed (Table 4). The medium with high explants survival rate (Lloyd and McCown's Woody plant medium with 88.89% explants survival rate) was preferred for subsequent experiments. The modification introduced in the selected medium significantly reduced *in vitro* shoot-tip necrosis and phenol exudation by explants.

Direct shoot induction

When the response (mean shoot length of *in vitro* explants) of PGRs treatments were compared using Turkey's test, significant differences were observed.

Treatment 1.2 mg/l KIN exhibited the most significant increase in shoot elongation compared to any other treatment while 6 mg/lBAP combined with 2 mg/l of 2, 4 - D was the most effective combined PGRs application treatment (Plate 1).

Callus induction

The mean (%) of callus induced from leaf discs of *A. stuhlmannii* differed significantly between the PGRs treatments used (Tables 5 to 6). Callus was induced from leaf disks (especially from the midrib) cultured on modified WPM supplemented with Kinetin combined with 2, 4 – D (Plate 2).

Root induction

No explants rooted in the two-step experimental setup are described in the methodology.

DISCUSSION

Explant sterilization

Surface sterilization of explants is a prerequisite to successful establishment of clean cultures for manipulation. In this study, although, there was a

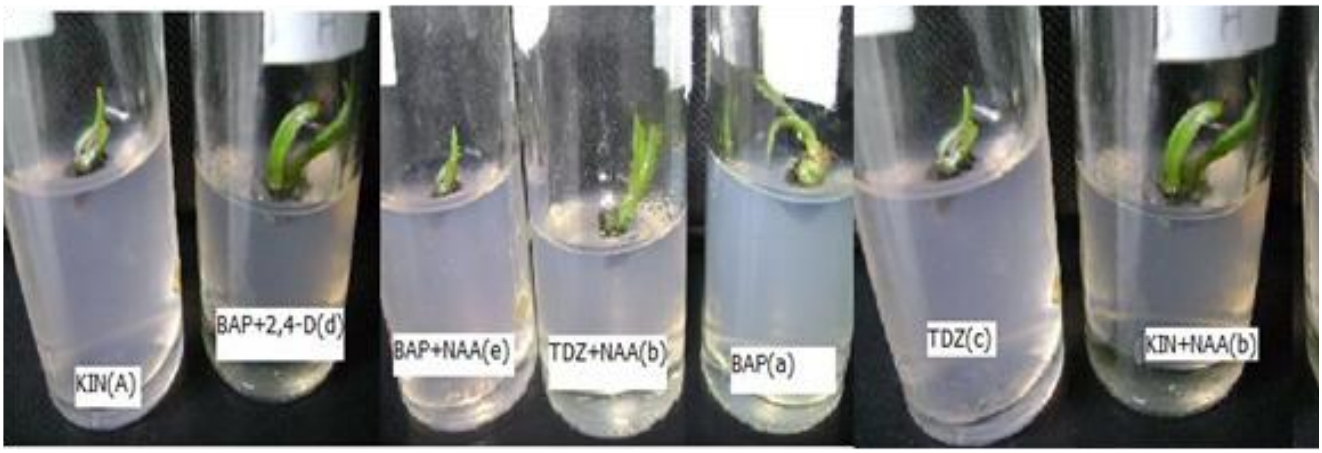

Plate 1. Direct shoot formation from nodal explants cultured on WPM supplemented with KIN, BAP and TDZ alone or in combination with NAA or 2, 4-D. Key :i) KIN(A)……..1.2KIN ii) BAP+2,4-D(d)…….2,2,4-D+2BAP; iii) BAP+NAA(e)……..3.6BAP+0.4NAA; iv) TDZ+NAA(b)…….1.2TDZ+0.2NAA; v) BAP(a)……..……2.4BAP; vi) TDZ(c)……..…..2.4TDZ+0.4NAA; vii) KIN+NAA(b)…….2.4KIN+0.2NAA; viii) KIN+NAA(c)……..3.6KIN+0.4NAA..

Table 5. The mean shoot length of microshoots induced from nodal explants cultured on WPM supplemented with KIN,BAP, TDZ alone or in combination with NAA and 2,4-D , Rep = 9.

Treatment (mg/l)	Shootlength (cm)		
	Week 4	Week 8	Week 12
Control	**1.04 ± 0.010^{a}**	**1.11 ± 0.006^{a}**	**1.15 ± 0.030^{a}**
3.6KIN + 0.4NAA	1.13 ± 0.021^{ab}	1.21 ± 0.014^{abcd}	1.21 ± 0.014^{ab}
1.2BAP + 0.4NAA	1.14 ± 0.015^{abc}	1.21 ± 0.023^{abcd}	1.21± 0.019^{ab}
2.4TDZ + 0.2NAA	1.15 ± 0.015^{abc}	1.36 ± 0.055^{cde}	1.21 ± 0.013^{ab}
1.2KIN + 0.4NAA	1.15 ± 0.017^{bc}	1.21 ± 0.023^{abcd}	1.22 ± 0.018^{abc}
2,2,4-D+4BAP	1.15 ± 0.016^{bcd}	1.22 ± 0.016^{abcd}	1.25 ± 0.020^{abc}
3.6BAP + 0.4NAA	1.15 ± 0.013^{bcd}	1.21 ± 0.013^{abc}	1.21 ± 0.015^{ab}
1.2BAP +0 .2NAA	1.15 ± 0.015^{bcd}	1.22 ± 0.013^{abcd}	1.25 ± 0.013^{abc}
1.2TDZ + 0.4NAA	1.16 ± 0.012^{bcde}	1.42 ± 0.067^{e}	1.26 ± 0.018^{abcd}
2,2,4-D + 2BAP	1.16 ± 0.014^{bcde}	1.21 ± 0.014^{abc}	1.24 ± 0.010^{abc}
1.2KIN + 0.2NAA	1.16 ± 0.015^{bcde}	1.23 ± 0.016^{abcde}	1.28 ± 0.009^{abcde}
2.4BAP + 0.4NAA	1.19 ± 0.014^{bcdef}	1.24 ± 0.017^{abcde}	1.23 ± 0.016^{abc}
2.4KIN + 0.4NAA	1.19 ± 0.014^{bcdef}	1.24 ± 0.017^{abcde}	1.23 ± 0.019^{abc}
3.6BAP + 0.2NAA	1.19 ± 0.018^{bcdefg}	1.24 ± 0.025^{abcde}	1.25 ± 0.027^{abc}
1,2,4-D + 2BAP	1.19 ± 0.020^{bcdefgh}	1.25 ± 0.021^{abcde}	1.33 ± 0.025^{bcde}
1,2,4-D + 4BAP	1.19 ± 0.024^{bcdefgh}	1.26 ± 0.030^{abcde}	1.30 ± 0.027^{abcde}
1.2KIN	1.20 ± 0.021^{bcdefgh}	1.39 ± 0.071^{cde}	1.44 ± 0.066^{e}
1.2BAP	1.20 ± 0.021^{bcdefgh}	1.40 ± 0.070^{de}	1.43 ± 0.077^{de}
3.6KIN + 0.2NAA	1.20 ± 0.021^{bcdefgh}	1.24 ± 0.025^{abcde}	1.28 ± 0.016^{abcde}
2.4TDZ	1.20 ± 0.028^{bcdefgh}	1.17 ± 0.015^{ab}	1.35 ± 0.030^{bcde}
2.4KIN	1.20 ± 0.024^{bcdefgh}	1.35 ± 0.058^{bcde}	1.38 ± 0.055^{cde}
2.4BAP	1.21 ± 0.026^{bcdefgh}	1.37 ± 0.058^{cde}	1.36 ± 0.052^{bcde}
1.2TDZ	1.21 ± 0.022^{bcdefgh}	1.24 ± 0.017^{abcde}	1.20 ± 0.022^{ab}
1,2,4-D + 6BAP	1.25 ± 0.021^{cdefgh}	1.34 ± 0.026^{bcde}	1.37 ± 0.025^{bcde}
3.6BAP	1.26 ± 0.026^{defgh}	1.36 ± 0.031^{cde}	1.38 ± 0.035^{cde}
1.2TDZ + 0.2NAA	1.27 ± 0.027^{efgh}	1.11 ± 0.006^{a}	1.27 ± 0.018^{abcde}
3.6KIN	1.28 ± 0.027^{fgh}	1.36 ± 0.013^{cde}	1.38 ± 0.036^{cde}
2.4TDZ + 0.4NAA	1.28 ± 0.024^{fgh}	1.36 ± 0.032^{cde}	1.36 ± 0.052^{a}
2.4KIN + 0.2NAA	1.30 ± 0.021^{fgh}	1.36 ± 0.030^{cde}	1.35 ± 0.030^{bcde}
2.4BAP + 0.2NAA	1.30 ± 0.026^{gh}	1.36 ± 0.034^{cde}	1.34 ± 0.032^{bcde}
2,2,4-D + 6BAP	1.30 ± 0.026^{h}	1.36 ± 0.028^{cde}	1.35 ± 0.041^{bcde}
$L.S.D_{0.05}$	0.058	0.096	0.089

Mean values within a column followed by the same letter are not significantly different by Turkey's test (*P≥0.05*). Letters are assigned in ascending order to treatments with higher mean values within the column.

Table 6. Callus induction (mean %) from leaf disk cultured on WPM supplemented with KIN+2, 4-D at various concentration (Re p= 9).

Callus induction (%) Treatment (mg/l)	Week 4	Week 8	Week 12
Control	**0.00[a]**	**0.00[a]**	**0.00[a]**
2KIN + 2 2,4-D[(a)]	0.000[a]	0.11[a]	0.11[a]
0.5KIN + 1 2,4-D[(b)]	8.33[b]	8.33[b]	8.33[b]
2KIN + 1.5 2,4-D[(c)]	10.56[b]	10.56[b]	10.56[b]
1KIN + 1.25 2,4-D[(d)]	32.22[c]	32.22[c]	32.22[c]
$L.S.D_{0.05}$	13.581	13.582	13.582

Mean values within a column followed by the same letter are not significantly different by Turkey's test ($P < 0.05$).

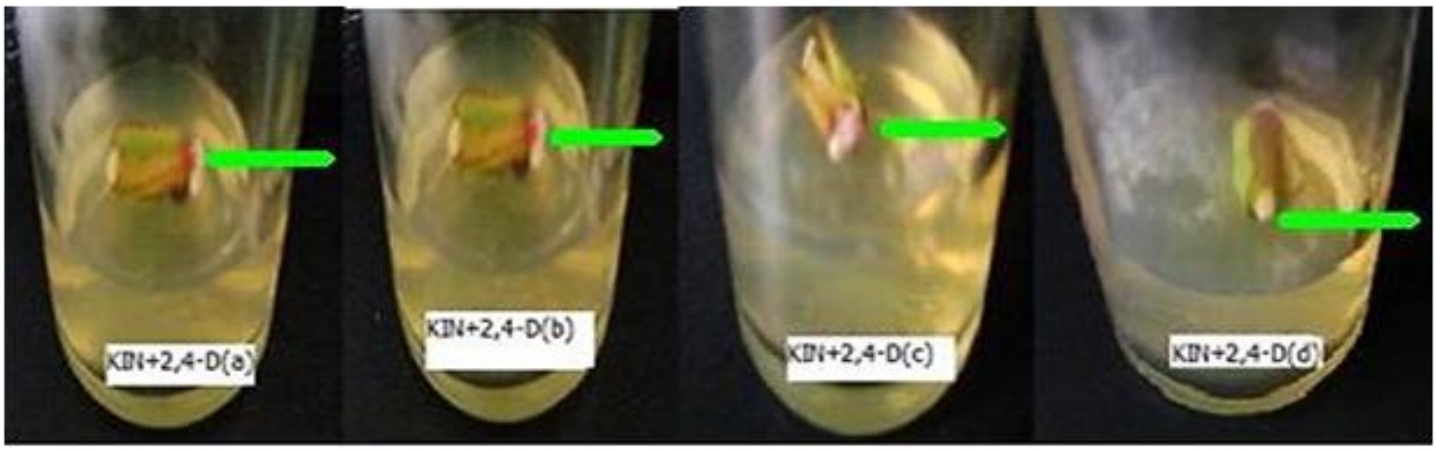

Plate 2. Callus induction from leaf explants (green line indicate the pinkish callus).

significant reduction of fungal and bacterial contamination when the concentrations of the sterilant and exposure time were increased ($P < 0.05$), the mortality rate increased significantly ($P < 0.05$) thereby rendering higher concentration levels and exposure time unsuitable for adoption. The final sterilization experiment showed that, subjection of *A. stuhlmannii* explants to 2% Redomil® solution for 15 min prior to immersion in 8% (ml) sodium hypochlorite at exposure time of 10 min results in clean and live explants than any other concentration level and exposure time used in the experiments (Tables 2 and 3). This is in agreement with Karkonen et al. (1999) findings on *Melaleuca alternifolia.* This was adopted in this research work as the best sterilization protocol for *A. stuhlmannii* seedlings grown in the glasshouse.

Media selection

It is evident that, Lloyd and McCown's Woody plant medium (with 88.89% explants survival) is the best nutrient medium that can used in tissue culture of *A. stuhlmannii* (Table 4).The eventual death of *in vitro* explants on various media such as MS medium, Anderson's medium, and Quorin and Lepoivre medium was due to production of phenolic compounds and possibly as a result of unsuitability of constituents of individual medium (Preece and Compton, 1991). There is a general tendency of attributing the occurrence of shoot-tip necrosis and subsequent death of *in vitro* explants to the high salt concentration found in some medium for example, MS medium (Bairu et al., 2009). The aforementioned phenomena reduced cell competence and led to eventual loss of their totipotency (Bairu et al., 2009). Alterations of NH_4/NO_3 ratio and sulphur content have been shown to significantly reduce shoot-tip necrosis (Laskshmi and Raghava, 1993). The amounts of calcium and boron in the media also play a critical role in plant tissue culture. Due to its versatility and specificity, calcium plays major structural and functional roles in plants (Hepler, 2005). Plants rely on the unique properties of calcium for their structural, enzymatic and signaling functions and also its role in physiological processes such as cell elongation and cell division (Hirschi et al., 2004). Boron requirements on the other hand differ widely among plant species and are known to have a narrow range between deficiency and toxicity levels when compared to other mineral nutrients (Abdulnour et al., 2000). McCown's woody plant medium

was the medium selected for subsequent experiments.

Direct shoot induction

The achievement of uniform and consistent *in vitro* shoot growth is highly problematic for most woody tree species especially those with strong episodic growth characteristic (Gupta et al., 1981; McCown, 2000), such as *A. stuhlmannii.* For many other tree species, *in vitro* clonal propagation is either not possible or the frequency of plant regeneration is too low to be of commercial use (Bonga et al., 2010). Experiments comparing different cytokinin regimes showed that, explants produced between 90 to 100% of responsive (organogenic) explants (Werner et al., 2001). The use of different plant growth regulators either alone or in combinations, significantly affected shoot length of the cultured nodal explants (Zaer, 1982; Nehra et al., 1994; Perez-Tornero and Burgos, 2009). Explants cultured in treatment 1.2 mg/lKIN exhibited significant increase in shoot elongation as compared to any other treatments. The shoot of explants cultured on media with high PGR concentrations, mostly suffered hyperhydricity (vitrification) and hence leaf fall and browning of the shoot apexes (Kataeva et al., 1994). Treatment 6 mg/lBAP combined with 2 mg/ 2, 4- D was the most effective PGRs combination in inducing shoot elongation. Existing reports suggests that, when auxins at lower concentrations are combined with cytokinins, they have critical role in plant regeneration in several systems like *Petasiteshybridus* (Wldi et al., 1998), *Eucalyptus grandis* (Luis et al., 1999), *Hybanthusenneaspermus* (Prakash et al., 1999), *Coleus forskohlii* (Sairam et al., 2001) and *Eleusine indica* (Yemets et al., 2003). Subsequent subculture of explants to either fresh media or media without PGRs resulted in massive loss of explants. All the attempts to induce roots on stabilized *in vitro* shoots failed.

Callus induction

Induction of somatic embryogenesis is a complex phenomenon, which is regulated by numerous factors. In most cases, treatments with exogenous PGRs are required to manipulate cell differentiation (Carman, 1990). Callus was successfully induced from leaf discs (particularly from the midrib) cultured on WPM supplemented with Gamborg's vitamins, 3% (w/v) sucrose and 8 mg/l, 1 mg/l Kinetin combined with 1.2 mg/l 2, 4 - D. Somatic embryogenesis was however not achieved. Induction of callus in *A. stuhlmannii* leaf discs is a possible indication of the potential of this tree species to produce primary somatic embryos that can be made to undergo secondary somatic embryogenesis (SSE). SSE is a phenomenon whereby new somatic embryos are initiated from pre-existing somatic embryos (Raemakers et al., 1995). Some cultures are able to retain their competence for SSE for many years, and thus provide material for various studies, as described for *Vitisrupestris* (Martinelli et al., 2001). Since new embryos are continually formed from existing embryos, SSE has the potential to produce many plants and once initiated, may continue to produce embryos over a long period of time (Pinto et al., 2002). Therefore, in plants with long life cycles, such as dicotyledonous woody plants for example, *A. stuhlmannii* preserving embryogenic lines can be a cost-effective maintenance while those line are tested in field (Handley, 1995).

Conclusion

Sterilization protocol for *A. stuhlmannii* was successfully established. The ability to induce shoots from nodal explants and callus from leaf discs clearly indicates that this tree species is amenable to micropropagation technique. There is however need to study the effects of using other polyamines such as putrescine and spermidines in optimization of shoot multiplication and root induction for this particular tree species.

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