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Full Length Research Paper

Regeneration of the East African greenheart, *Warburgia ugandensis* (Sprague) through tissue culture

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***Warburgia ugandensis* is a medicinal plant in the family Canellaceae. There has been a very high demand for *Warburgia* products for medicinal purposes leading to overexploitation. *Warburgia* also produces recalcitrant seeds, a fact that has hindered the natural regeneration of this species. In this study, clonal propagation was postulated to be an alternative to propagation through seeds. Shoot tip explant materials were obtained from glasshouse maintained seedlings and these were surface sterilized using both ethanol and sodium hypochlorite. Shoot proliferation and elongation was achieved on shoot tips cultured on full strength Murashige and Skoog (MS) medium containing 3% sucrose, 1.13 mg L^{-1} benzylamine purine (BAP) and 0.11 mg L^{-1} of kinetin (KIN). A combination of BAP and kinetin resulted in a significant ($p< 0.01$) shoot elongation (3.0 cm) and the number of shoots (4 shoots per explants) after 44 days. The best *in vitro* rooting (50%) was induced through 1 mg L^{-1} naphthalene acetic acid (NAA) on a half strength Gamborg's woody plant medium (WPM) containing 3% sucrose. These results indicate that *W. ugandensis* can be regenerated via *in vitro* culture using a combined BAP and kinetin to induce multiple shoots and subsequently rooting them under 1 mg/L NAA. The study has therefore developed a protocol for mass clonal propagation of this important medicinal tree.**

Key words: *In vitro*, organogenesis, plant shoots, *Warburgia ugandensis*.

INTRODUCTION

Warburgia ugandensis is a medicinal plant belonging to the family Canellaceae. It is a spreading evergreen tree growing to a height of 25 to 40 m. The bark is rough, rich brown with a short bole that is clear of branches for the lower 0.3 cm. The leaves are alternate and simple with dotted glands. The apex and the base are tapering with an entire margin. The margin is glossy dark-green above and pale dark green below. Flowers are solitary or in a small three to four flowered cymes and they can be axillary, regular and bisexual. The fruits are green and ellipsoidal and later turn purplish and sub-spherical with leathery glandular skin. Seeds are compressed more or less cordate; yellow to brown in color (Verdcourt, 1956; Ecocrop, 2012).

W. ugandensis is a slow-growing tree species native to Kenya. There has been a very high demand for the tree because of its high medicinal value both to the local people and herbal traders (FAO, 1986). The bark is highly overharvested for antifungal and antibacterial properties (Akwatulina et al., 2011). Overexploitation has resulted into a constant destruction of large trees in their natural habitat, and only remnants tree are left in its endemic habitats. Besides over-exploitation through debarking, the tree produces recalcitrant seeds (Omondi et al., 2004), a fact that has hindered natural regeneration of this species. Seed propagation is difficult because they are often attacked by fungus and propagation though cuttings is also difficult (Kowalski and Staden, 2001). However, development of suitable procedures for plant regeneration through either organogenesis or somatic embryogenesis is one of the main prerequisites for the potential applications of clonal propagation, genetic transformation and *in vitro* conservation of many woody plants (Handley, 1995; Park et al., 1998; Minocha and Jain, 2000). Conventionally, the species reproduces through seeds. Propagation through stem cuttings has

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been reported (Kiriba, 2006), but only a few propagules could be produced through this method. Therefore, development of an efficient method for rapid mass propagation is an essential goal for production of clonal planting stocks of *W. ugandensis*. Clonal propagation is desirable to obtain a uniform and high-quality plantation without unwanted genetic variation (Vila et al., 2003). This study endeavoured to develop suitable micro-propagation techniques for mass production of planting materials for *W. ugandensis*.

MATERIALS AND METHODS

Explant materials were collected from glasshouse seedlings raised from both stem cuttings and seeds. They were surface-sterilized using 6% sodium hypochlorite containing Tween ® 20 for 10 min followed by a quick immersion in 70% ethanol for 1 min and finally rinsed three to four times using sterile distilled water. A control treatment was included. They were reduced to 1 cm in length and cultured on Murashige and Skoog (1962) growth medium. The medium was supplemented with cytokinins such as benzylamine purine (BAP) and kinetin both singly or in combination at a concentration range of 0 to 0.5 mg L^{-1} and combined with either of the auxin; indoleacetic acid (IAA) or indole butyric acid (IBA) at a concentration range of 0.01 to 0.5 mgL^{-1} for shoot multiplication. Half strength Gamborg's woody plant medium (WPM) was supplemented with the auxin naphthalene acetic acid (NAA) at a concentration range of 0 to10 mgL^{-1} to induce *in vitro* rooting. The WPM medium contained mineral salts, sucrose and vitamins. The pH was adjusted to 5.75 using 1 M NaOH or 1 M HCl prior to the addition of 0.8% agar and the media were autoclaved at 121°C and 1 bar pressure for 15 min.

Explants were aseptically placed into the culture bottles each containing 15 ml of respective growth medium and the cultures transferred to a growth chamber at 25 ± 5°C and 16 h photoperiod provided by cool-white fluorescent lamps at 45 µmol m^{-2} s^{-1}. After four weeks of initiation, shoots showing growth were sub-cultured to a fresh medium for further growth. Multiple shoots proliferating from the initial explants were excised and transferred to a fresh medium to continue the multiplication cycle. Data on shoot length and number were collected every two weeks.

Moreover, a two-step procedure was adopted for root initiation. In the first-step, shoots for root induction were selected from the multiplication jars and inoculated on half strength WPM containing the various concentrations of NAA. The shoots were sub-cultured thrice after every two weeks on the rooting medium in the second step. Data on percent rooting, root number and basal callus were collected every two weeks. Well-rooted shoots were removed from the culture bottles after 42 days. They were washed thoroughly using sterile distilled water to remove adhering medium and transferred to potted sterile vermiculite. White polythene bags were inverted over each plantlet to maintain high humidity. Plants were watered with half strength WPM on alternate days to harden them. The pots were maintained in a growth cabinet at the same conditions as *in vitro* cultures.

Statistical analysis

The experiments were laid out in a completely randomized design (CRD). Each treatment had three replications of nine explants. Data were analysed using Genstat computer statistical package Version 10, 2007, Lawes Agricultural Trust (Rothamsted Experimental Station). Analysis of variance (ANOVA) was carried out to determine the significant differences among the means of shoot elongation and rooting. Least significant differences $P < 0.05$ among the mean values were estimated using Fisher's LSD test. Regression analysis was used to analyse the data on shoot multiplication.

RESULTS

As regard the sterilization of explants materials, Tables 1 to 3 respectively show the result of the effects of 70% ethanol on surface sterilization of *W. ugandensis* explant materials, the effects of combined 6% sodium hypochlorite and 70% ethanol on surface sterilization and the effects of full MS medium supplemented with combined BAP and KIN on mean shoot length and number of shoots per explant in *W. ugandensis*.

Concerning the shoot multiplication and elongation, Tables 4 and 5 respectively show the effects of full MS medium supplemented with combined BAP and KIN, and full MS medium supplemented with combined BAP and IBA on mean shoot length and number of shoots per explant in *W. ugandensis.* While for the results on *in vitro* rooting, Tables 6 and 7 show the effects of IAA, IBA and NAA on the number of *in vitro* rooted shoots of *W. ugandensis*, as well as the effects of NAA on percentage rooting and root numbers of *in vitro* shoots of *W. ugandensis*, respectively. From the results, all treatments tested induced rooting apart from the controls. At least one or two shoots developed the roots however, NAA at 1 mg L^{-1} was found the best concentration, with 50% of the shoots forming roots and hence an average of four roots per shoot (Table 5, Figure 1).

DISCUSSION

During surface sterilization, all controls treatments were contaminated. However contamination rate decreased with increased duration of plant materials in 6% NaOCl and 70% ethanol (Tables 1 and 2). The best sterilization was achieved by immersing the explants for 10 min in 6% NaOCl followed by 1 min immersion in 70% ethanol (Table 3). The incubation period for 6% NaOCl was significantly different ($p < 0.05$), whereas there were no significant differences ($p = 0.05$) for 70% ethanol (Table 3). Moreover, increased death rate was observed in the explants that were immersed for more than 10 min in 6% NaOCl and 1 min in a 70% ethanol due to the bleaching effects (Table 3). The durations for both 6% NaOCl and 70% ethanol significantly influenced the death rate of the plant materials.

When cytokinins were used singly, benzylamine purine significantly influenced shoot length ($P < 0.05$) and accounted for 69.5% of the total variability among the various concentrations as compared to kinetin, which accounted for 43.3% of the total variability in shoot length. Overall, benzylamine purine and kinetin accounted for 65 and 36% of the total variability in shoot

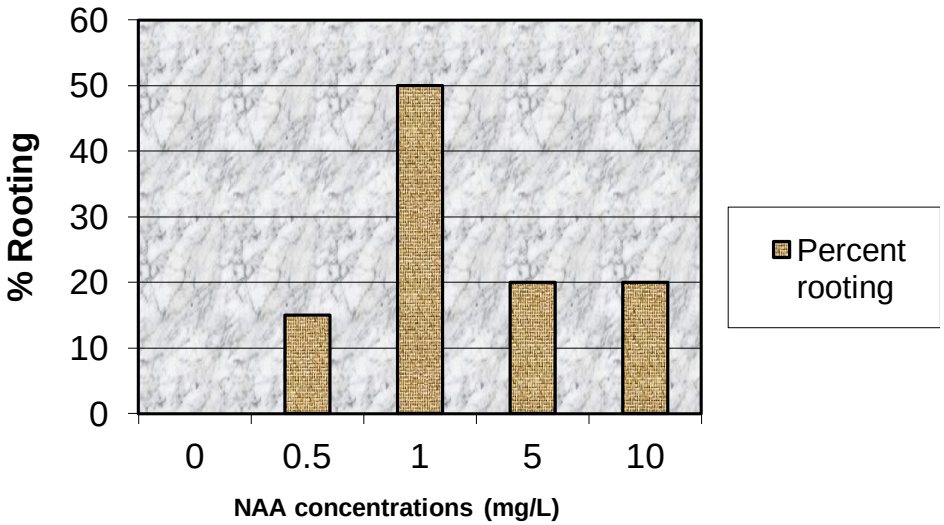

Figure 1. *In vitro* rooting of *W. ugandensis* on half strength WPM with NAA at various concentrations.

Table 1. Effects of 6% sodium hypochlorite on surface sterilization of *W. ugandensis* explant materials.

Parameter	Time of exposure (min) in 6% NaOCl			SED
	0	10	15	
% Survival	94	39	22	13
Death from bleaching	83	67	0	7

Table 2. Effects of 70% ethanol on surface sterilization of *W. ugandensis* explant materials.

Parameter	Time of exposure (min) in ethanol			SED
	0	1	2	
% Explant survival	67	56	28	7

Table 3. Effects of combined 6% sodium hypochlorite (vertical) and 70% ethanol (horizontal) on surface sterilization of *W. ugandensis* explant materials.

Time of exposure (min) against % explant survival	0	1	2	SED
0	100	67	83	13
10	100	100	0	13
15	0	0	0	13

number. The superiority of benzylamine purine in shoot multiplication has been reported in *Maytenus emarginata* (Rathore et al., 1992) and *Tecomella undulata* (Rathore et al., 1991). The effectiveness of cytokinins in promoting *in vitro* axillary bud development in forest trees is also well documented (McCown and Selmer, 1987; Zaer and Mapes, 1982). For combined cytokinins, results from analysis of variance revealed a significant differences ($P < 0.05$) in shoot length and number among the various concentrations of combined benzylamine purine and kinetin. An increased mean shoot length of 3.04 cm and 4 multiple shoots per explant material were obtained on the medium supplemented with 1.13 mg L^{-1} benzylamine purine and 0.11 mg L^{-1} kinetin (Table 4, Figure 2a and b). However, the shoots suffered hyperhydricity (vitrification), leaf fall, browning of leaves and shoot tips. These conditions have been previously attributed to high salt contents, particularly chloride and ammonium ions in the

Table 4. Effects of full MS medium supplemented with combined BAP and KIN on mean shoot length and number of shoots per explant in *Warburgia ugandensis.*

Treatment	Concentration (mg/L)		Shoot Length (cm)		Shoot number
	KIN	BAP	20 days	44 days	
Control	0		2.04[a]	2.14[a]	0
Treat 2	0	0.11	2.25[a]	2.50[b]	1.50 ± 0.58[a]
	0	0.56	2.35[a]	2.26[a]	1.00 ± 0.67[a]
	0	1.13	1.67[a]	2.14[a]	2.50 ± 0.75[ab]
	0	2.25	2.22[a]	1.86[a]	1.00 ± 0.39[a]
Treat 3	0.11	0	2.18[a]	2.24[a]	1.00 ± 0.67[a]
	0.54	0	2.54[a]	2.82[b]	1.57 ± 0.32[a]
	1.18	0	2.50[a]	2.69[b]	2.00 ± 0.67[a]
	2.15	0	2.54[a]	2.68[b]	2.60 ± 0.48[ab]
Treat 4	0.11	0.11	2.22[a]	2.28[a]	0
	0.11	0.56	2.16[a]	2.36[a]	1.67 ± 0.50[a]
	0.11	1.13	2.83[b]	3.04	4.00 ± 0.67[a]
	0.11	2.25	2.92[b]	2.82[b]	1.00 ± 0.39[a]
Treat 5	0.54	0.11	2.30[a]	2.36[a]	1.00 ± 0.39[a]
	0.54	0.56	3.06[c]	2.99[b]	1.67 ± 0.50[a]
	0.54	1.13	2.40[a]	2.02[a]	1.00 ± 0.47[a]
	0.54	2.25	1.84[a]	2.26[a]	1.50 ± 0.58[a]
Treat 6	1.18	0.11	2.50[a]	2.46[a]	2.00 ± 0.67[a]
	1.18	0.56	2.48[a]	2.62[b]	1.50 ± 0.58[a]
	1.18	1.13	2.58[b]	2.87[b]	1.20 ± 0.33[a]
	1.18	2.25	2.20[a]	2.24[a]	1.00 ± 0.39[a]
Treat 7	2.15	0.11	2.52[b]	2.52[b]	2.00 ± 0.67[a]
	2.15	0.56	2.00[a]	2.56[b]	2.50 ± 0.53[ab]
	2.15	1.13	1.82[a]	1.88[a]	3.00 ± 1.15[b]
	2.15	2.25	2.82[b]	2.36[a]	2.00 ± 0.47[a]
SE			0.164	0.223	0.444
LSD (5%)			0.297	0.627	
F- test probability			$p < 0.001$	$p = 0.009$	$p = 0.020$

Means within the column followed by the same letters are not significantly ($p < 0.05$) different as determined by F-LSD test.

nutrient media (Quirin and Lepoivre, 1977; Vieitez et al., 1985) or the use of growth regulators especially cytokinins along with the high relative humidity in the culture vessels (Kataeva et al., 1991). In *Populus maximowiczii* such conditions were reduced when shoots were multiplied on a modified MS medium with half concentration of NH_4NO_3 (10.3 mM) or when shoot of *Prunus nipponica* were transferred to a medium with reduced concentration of cytokinins (Kärkönen et al., 1999).

The addition of indole butyric acid at a concentration range of 0.01 to 0.5 mg L^{-1} in the medium containing benzylamine purine promoted shoot elongation ($p < 0.05$), whereas there were no significant differences ($p > 0.05$) in shoot number among the various treatments (Table 5). However, a slight reduction in shoot number per explants resulted from this combination as compared to the combinations of benzylamine purine and kinetin (Table 4). Among the auxins tested, indole butyric acid was more effective for both increased shoot length and multiplication. The results conformed to the work on woody species that resulted to an increased number of

Table 5. Effects of full MS medium supplemented with combined BAP and IBA on mean shoot length and number of shoots per explant in *Warburgia ugandensis.*

Treatment	Concentration (mg/L)		Shoot Length (cm)			Shoot number
	IBA	BAP	14 days	42 days	84 days	Mean ± SE
Control		0	2.78[a]	2.84[b]	3.04[c]	1.00 ± 0.71
Treat 1	0	0.1	2.70[a]	2.74[b]	3.19[c]	1.67 ± 0.75
	0	0.5	2.78[a]	2.94[c]	3,16[c]	1±.78 ± 0.44
	0	1	2.66[a]	2.62[a]	2.89[b]	2.33 ± 0.62
Treat 2	0.05	0	2.66[a]	2.68[b]	3.12[c]	1.17 ± 0.44
	0.25	0	2.48[a]	2.48[b]	2.84[b]	1.00 ± 0.71
	0.5	0	2.59[a]	2.42[b]	2.01[a]	1.67 ± 0.75
Treat 3	0.05	0.1	2.64[a]	2.70[b]	2.94[c]	1.50 ± 0.87
	0.05	0.5	2.88[a]	2.94[c]	3.22[c]	2.14 ± 0.55
	0.05	1	2.74[a]	2.74[b]	2.84[b]	2.54 ± 0.44
Treat 4	0.25	0.1	2.82[a]	2.92[c]	3.31[c]	1.50 ± 0.87
	0.25	0.5	2.70[a]	2.88[b]	3.32[c]	2.00 ± 0.54
	0.25	1	2.58[a]	2.69[b]	2.62[b]	2.67 ± 0.67
Treat 5	0.5	0.1	2.56[a]	2.80[b]	2.74[b]	1.40 ± 0.51
	0.5	0.5	2.58[a]	2.56[b]	3.24[c]	1.63 ± 0.45
	0.5	1	2.58[a]	1.74[a]	3.12c	3.10 ± 0.56
SE			0.097	0.103	0.078	0.617
LSD (5%)			0.273	0.292	0.393	
F-test probability			p=0.249	p<0.001	p<0.001	p=0.062

Means within the column followed by the same letters are not significantly ($p < 0.05$) different as determined by F-LSD test.

Table 6. Effects of IAA, IBA and NAA on number of *in vitro* rooted shoots of *W. ugandensis*.

Auxins concentration (mg/L)	Rooted shoot		
	IAA	IBA	NAA
0.1	0	0	0
0.5	0	0	6
1.0	0	0	10
5.0	0	0	4
10.0	0	0	3
Probability (P)	**	**	**

SED = 0.3; ** Significant at $p < 0.01$.

shoots when indole butyric acid was combined with cytokinins (Barakat and El-Lakany, 1992). The addition of low levels of auxins along with cytokinins has been known to increase the percent establishment as well as shoot numbers in forest trees (Rathore et al., 1991), as well as shoot elongation in others plants like *Gmelina arborea* (Tiwari et al., 1997) and *Sapium sebiferum* (Siril and Dhar, 1997).

Rooting and acclimatization in plantlets is one of the major obstacles in *in-vitro* propagation. It is a pre-transplanting stage during which the plantlets must be conditioned to withstand the shock of transplantation. *In*

Table 7. Effects of NAA on percentage rooting and root numbers of *in vitro* shoots of *Warburgia ugandensis*.

Concentration (mg/L)	Percent rooting	Root number
0.5	15	0
1	50	4
5	20	1
10	20	1
Control	0	0
Probability (P)	**	**

** Significant at $p < 0.01$.

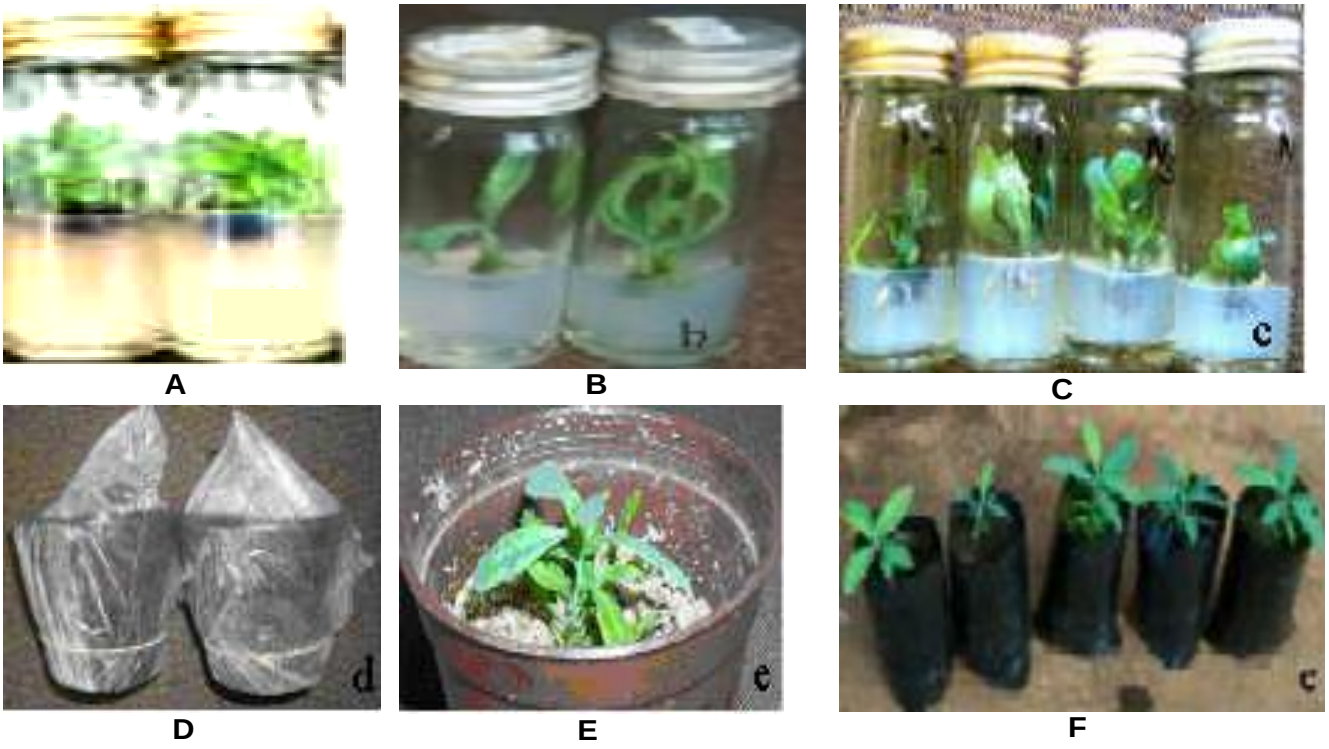

Figure 2. Direct organogenesis and plant regeneration from cultured shoot tips of *W. ugandensis*. A and B, Shoot proliferation and elongation on full MS medium containing combined BAP and KIN; C, *in vitro* rooting of regenerated shoots on half strength WPM containing NAA; D and E, acclimatization of plantlets under growth cabinet conditions; F, established seedlings on forest soil substrate in the glasshouse.

vitro rooting is influenced by factors such as growth regulators, macronutrients, micronutrients, organic supplements, support medium light and temperature (Bonga and Durzan, 1987; Torres, 1989).

In *W. ugandensis*, rooting was achieved when shoots were transferred into half strength woody plant medium supplemented with a higher auxin levels for root induction and transferred to auxin-free medium for root development (Tables 6 and 7; Figure 1). The shoots were sub-cultured thrice after every two weeks in the root induction medium. Root primordia were observed seven days after transferring the shoots to auxin-free medium and well-rooted plantlets were observed at 1 mg L^{-1} naphthalene acetic acid within 14 days (Table 7 and Figure 2c).

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