MICROPROPAGATION OF AN ENDANGERED COMMIPHORA SPECIES (*COMMIPHORA EMINII SSP. ZIMMERMANNII)*

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Abstract
Commiphora eminii ssp. zimmermannii is an endangered forest tree which has been kept under Data Deficient category. It is found in the Eastern Africa and North Eastern Zambia. Fruits are used for the treatment of typhoid fever, stomach problems, constipation, fever, snakebites and toothaches. It is valued for resin and is traditionally used as a yam and banana support. Its propagation is quite difficult because seeds are rarely found in nature. Long maturity and over exploitation has depleted it. The main objective was to carry out mass propagation and specifically to determine optimum sterilization protocol, multiple shoot and root production. Explants used were cotyledonary node and leaf discs. Series of sterilization and micro propagation experiments were conducted on explants collected from Mt. Kenya and coastal hill forests. Sodium hypochlorite and formaldehyde were used in sterilization protocol. Explants were best surface sterilized after subjection to 70% alcohol for 1second and exposure to 10% sodium hypochlorite solution for 10 minutes. Four basal media were tested for their suitability in micro propagation of *C. eminii ssp zimmermannii.* Multiple micro-shoots were obtained on Murashige and Skoog (MS) medium supplemented with 1.25mgl-1 α-Naphthalene acetic acid (NAA) and 1.25mgl-1 6-Benzylamino purine (BAP). Micro-shoot was significantly observed on the 0.5mgl-1 Indole-3-butyric acid (IBA) and 1.25mgl-1 BAP supplemented MS medium. Callus was induced from leaf discs cultured on MS basal medium supplemented with 0.1mgl-1 TDZ. However no somatic embryogenesis was achieved from callus. Rooting was not successful on pretreated micro-shoot (1.25mgl-1 IBA for 24 hours) followed by transfer to white's medium without plant growth regulators (PGR). Rooting should further be tested using high concentration of activated charcoal. Success in shoot proliferation and callus induction is a step in regenerating C. eminii ssp zimmermannii clonal plantlets.

Keywords: Sterilization, callus induction, shoot regeneration,endangered medicinal plant,Commiphora *eminni ssp. Zimmermannii*

Abbreviations
MS -Murashige and Skoog
BAP -benzyladenine
NAA -Naphthaleneacetic acid
IBA- Indole-3-butyric acid
2,4-D 2,4-Dichlorophenoxyacetic acid
Kn- Kinetin
TDZ- thidiazuron-1 phenyl-3-(1,2,3-thiadiazolon-5-yl)
LS_Linsmair and Skoog
IBA_ indole-3-butyric acid
IAA- indole-3-acetic acid
PGR- Plant growth regulators

1.0 Introduction

Commiphora eminni ssp. Zimmermannii Eng. (Burseraceae) locally known as 'Mpondo' is a forest tree with high market potential and grows naturally in Eastern Africa and North Eastern Zambia. In Kenya it's distributed from the Coastal hill forests to Central region, but now very rare due to overexploitation.*C. eminii ssp. zimmermannii* provides firewood, timber, furniture, utensils (bowls), medicine (roots, leaves and bark), fodder, shade, soil conservation, live fence, boundary marking, and yam support. The tree has no adverse effects on food crops due to its small root system and canopy. Traditionally used by the Kikuyu as a quick growing hedge and as a support for yams. Fruits are used for the treatment of typhoid fever and as a remedy for stomach problems, constipation, fever, snakebites and toothaches. The powdered bark is mixed with porridge to cure malaria. The resin has medicinal uses including sealing and disinfecting wounds. It is applied as a plaster and used for spasms. It is even

used in embalming. The fumes of burnt resin are used as an insecticide and an aphrodisiac. Leaves are browsed by goats, especially at the end of the dry season when young leaves appear. The nutritive value of the leaves is about 8 to 14% crude protein.

Wood of *C. eminii ssp zimmermanii* has a reputation of being termite resistant although soft. Because it is easy to work with it is used in the construction of local houses, tool handles, spoons, water trough musical instruments and furniture. *C. zimmermanni* is used to make hives because the wood is hard and durable. It yields a light straight-grained timber which takes a dark polish and propagated as a quick growing live fence for boundary marking and for banana and yam supports As the Kikuyu phrase goes 'gikwa na mukungugu' express the ultimate love affair between the two trees.. Can be intercropped with annual crops, and provide agroforestry benefits such as improved productivity, diversity of products (such as fodder), or erosion control. The wood is also used for fence posts. Roots, leaves and fruits are edible. Dried sap and bark are used as incense. Extracted oils are used in perfumes and religious ceremonies. It's used as a fodder grass for supplementary dry grass. Traditionally, cuttings of this tree were used to mark boundaries within farms. According to the studies of FRLHT (2006), *Commiphora* has been listed as critically endangered species in Madhya Pradesh and it also has been kept under Data Deficient category (IUCN, 2010).Regeneration of *Commiphora eminiissp. zimmermannii* via seed is however slow and low. This is because it only produces two seeds that take long to germinate. Cuttings used for its germination have poor survival rate. Today, its existence is threatened because of low seed production in an adverse natural condition and recent environmental changes due to change in rainfall pattern and increasing level of atmospheric pollution. Excessive and unscientific tapping for its gum resin by the pharmaceutical industries and religious prophets has also led it to diminish in the natural environment. In reference to its limited regenerative potential and dispersal powers, sustainable harvesting of *C. eminii ssp. zimmermannii* from natural habitat to meet market demand is not feasible. 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).

Table 1: Comparison of element levels in the various media used to culture C. eminnii spp. zimmermannii

	Lloyd &McCown's	**Murashige &Scoog**	**Gamborg (B5)**	**Linsmaier and Skoog**
MacroElements	**Mg/l**	**Mg/l**	**Mg/l**	**Mg/l**
NH_4NO_3	400.0	1650	0.0	1650.0
KNO_3	0.00	1900	2500	1900.0
K_2SO_4	990	0.00	0.00	0.00
$MgSO_4.7H_2O$	180.54	180.54	121.56	180.54
KH_2PO_4	170	170	170	170.0
$CaCl_2$	72.50	332.02	113.23	0.00
$Ca(NO_3)_2.4H_2O$	386.80	0.00	0.00	332.02
$NaH_2PO_4).H_2O$	0.00	0.00	130.44	0.00
$(NH_4)_2SO_4$	0.00	0.00	134	0.00
MicroElements	**Mg/l**	**Mg/l**	**Mg/l**	**Mg/l**
$CoCl_2.6H_2O$	0.00	0.025	0.025	0.25
$CuSO_4.5H_2O$	0.025	0.025	0.025	0.025
FeNaEDTA	36.7	36.7	36.7	36.70

H_3BO_3	6.20	6.20	3.00	6.20
KI	0.00	0.83	0.75	0.83
$MnSO_4.H_2O$	22.3	16.9	10.00	16.90
$Na_2MoO_4.2H_2O$	0.25	0.25	0.25	0.25
$ZnSO_4.7H_2O$	8.60	8.60	2.00	8.60
Vitamins	**Mg/l**	**Mg/l**	**Mg/l**	**Mg/l**
Glycine	2.00	2.00	0.00	1.00
Nicotinic acid	0.50	0.50	1.00	0.00
Pyridoxine	0.50	0.50	1.00	0.00
Thiamine	0.00	0.20	10.00	0.40
Myo-inositol	100.00	100.0	100.0	100.0

2.0 Materials and Method

2.1 Plant Material

C. eminii ssp. zimmermannii cuttings were collected from marked, visibly healthy trees growing in the natural population in Shimba hills and Mt. Kenya forest. The cuttings were planted in sand, loam and manure mixed well and watered regularly at Kenya Forestry Research Institute (KEFRI) Muguga. Axillary buds and leaves were used as explants.

2.2 Autoclaving and Growth Room Conditions

All media were adjusted at pH 5.8 and followed by autoclaving at 121 °C and 20 psi (137,900 pa) pressure for 15 minutes. All the cultures were aseptically inoculated and manipulated under a sterile laminar flow hood and incubated in tissue culture racks in an aseptic culture room having a temperature of 26 ± 2°C, 16 h/d photoperiod and 1600 lux intensity light (via white cool florescent tubes).

2.3 Surface Sterilization

Influence of sodium hypochlorite and formaldehyde and their concentrations on axillary bud cultures were studied by washing explants under running and then put in a glass jar containing 500ml of water with 3 drops of Tween 20 and swirled gently for 15 minutes before washing the explants with running water for 10 minutes. Explants had 30-second dip in 70% ethanol then separately subjected to formaldehyde and sodium hypochlorite at five different concentration levels (0, 5, 10, 15 and 20 mg/l) each subjected to four different exposure times (0, 10, 20 and 30 minutes). All the explants were rinsed three times using sterile distilled water and put on sterile blotting paper. The shoots were trimmed to a length of 1cm and cultured in MS medium then placed in culture growth cabinets. The observations were recorded weekly for the non-growing cultures, infected cultures and healthy cultures (whereby score 1 represented presence of bacteria and fungi and score 0 represented absence of bacteria and fungi).

2.4 Effect of Growth Regulators and their Concentrations on Shoot Proliferation

Influence of cytokinins, their concentrations and combinations, on axillary bud cultures and leaves was studied using BAP (0.5 - 2.0 mg/l), Kn (0.5 - 2.0 mg/l) and TDZ (0.5 - 2.0 mg/l). In the combination sets, BAP (0.5 - 2.0 mg/l) and NAA (0.2- 0.6 mg/l) were combined. TDZ (0.5 - 2.0 mg/l) and NAA (0.2- 0.4mgl) formed another combination set. MS and LS and McCown's Woody plant medium were used. Number of new shoots arising and their length were recorded.

2.5 Effect of Growth Regulators and Their Concentrations on Callus Induction

Effect of growth regulators and their concentrations on callus induction was studied using McCown's Woody plant media and Gamborg's supplemented with KIN (0.5-2 mg/l), TDZ (0.05-0.2mg/l) and 2.4-D (0.5-2 mg/l) were used. Callus induction was evaluated weekly for 5 weeks.

2.6 Effect of PGRs and their Concentrations on Induction of Somatic Embryogenesis

To induce somatic embryogenesis, LS media and MS medium supplemented with 0.5, - 2.0 mg/l 2.4-D, NAA and Kn and TDZ at 0.01- 0.1mg/l were used singly. The cultures were maintained in the dark. Data on the percentage of explants percentage embryogenic cultures were taken.

2.7 Effect of PGRs and their Concentrations on Rooting Of Shoots

Half strength MS and WPM was used in rooting supplemented with IBA, IAA and NAA at various concentrations (0.5- 2.0) mgl^{-1} and also a two-step procedure (Gasper and Coumans, 1987) was performed. Stable shoot were initially cultured in half-strength MS medium containing 30mgl sucrose and 8mgl agar and separately supplemented with IBA, IAA and NAA 0.03, 0.05 and 0.1mgl^{-1} concentration levels. Pretreated micro-shoots (1.25mgl-1 IBA for 24 hours) were transferred to White's medium without Plant Growth Regulators (PGR).

2.8 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)

3.0 Results

3.1 Media Selection

Different media were selected to show the best media for the explants survival. When explants survival rates on the selected media were compared. Significant differences were observed (Table 2). The medium with high explants survival rate plant medium with 88.89% explants survival rate) was preferred for subsequent experiments.

Table 2. Explants survival rate on various nutrient media

Type of media	No. of cultured explants	No. of live explants	Explant survival (%)
Murashige and Skoog	45	40	88.89
Linsmaier and Skoog	45	29	64.44
Lloyd and McCown's (WPM)	45	19	42.22
Gamborg (B5)	45	3	6.67

3.2 Sterilization Protocal

Results on the effectiveness of sterilants shows that increase in concentration and time of exposure resulted in high explant mortality rate and decrease in bacterial and fungal (Table 1).

Table 3: Fungal and Bacterial contamination rates and mortality of explants when exposed to different concentrations and different times of exposure of Sterilant (means %)

	Contamination (%)		
Treatment	**Fungi**	**Bacteria**	**Mortality**
Conc. (%) 0	100.0±0.04	100.0±0.05	50.8±0.05
5	43.3±0.05	33.33±0.06	76.7±0.06
10	33.3±0.05	16.7±0.06	66.7±0.06
15	6.7±0.05	0.00±0.06	100.0±0.06
20	100.0±0.05	0.00±0.06	100.0±0.06
Time (Min.)10	24.7±0.10	33.33±0.13	66.7±0.13
20	19.0±0.11	20.0±0.11	84.0±0.12
30	20.0±0.11	15.3±0.09	86.0±0.11

Values indicate Mean ± SE.

Table 4: Summary of Analysis of variance (ANOVA) table showing the effects of the Sterilants on fungi, bacteria and mortality

Source of variation	**d.f.**	**Fungi**	**Bacteria**	**Mortality**
Sterilant	1	0.082	0.187	1
Conc(v/v)%	4	0. 001	0.10	0. 001
Time(min)	2	0.102	0.24	0. 001
Sterilant * Conc.	4	0.067	0.096	1
Sterilant * Time	2	0.883	0.895	1
Conc * Time	8	0.851	0.647	0. 001
Sterilant * Conc * Xtime	8	0.647	0.979	1

3.4 Effect of Growth Regulators and their Concentrations on Shoot Proliferation

Auxillary buds inoculated on MS media supplemented with lower concentrations of cytokinin (BAP and Kn) and with combination of auxin (NAA) (Table 5). Data collected included the number of shoots and shoot length (cm). Shoots were clearly seen in most cultures.

Table 5: Effect of different cytokinin on shoot proliferation cultured in MS media

PGRs concentrations ((mg/l) Response (%) Mean no. of shoots Mean shoot length (cm)

PGRs concentrations ((mg/l)	Response (%)	Mean no. of shoots	Mean shoot length (cm)
1.25 BAP+1.25 NAA	84.6	$1.19^{a}\pm0.1$	$0.43^{c}\pm0.0$
1.25 BAP+1.50 NAA	73.1	$1.56^{ab}\pm0.1$	$0.38^{abc}\pm0.0$
1.00 BAP+1.50 NAA	38.5	$1.61^{b}\pm0.1$	$0.46^{c}\pm0.1$
1.25 BAP+1.25 Kn	53.8	$2.22^{a}\pm0.1$	$0.23^{a}\pm0.0$
1.25 BAP+1.50Kn	34.6	$1.74^{bc}\pm0.1$	$0.38^{abc}\pm0.0$
1.00 BAP+1.50Kn	19.2	$1.80^{b}\pm0.1$	$0.39^{bc}\pm0.1$
1.25 BAP	65.4	$1.93^{bc}\pm0.2$	$0.30^{ab}\pm0.0$
1.00 BAP	53.8	$1.87^{bc}\pm0.1$	$0.28^{ab}\pm0.0$
1.25 Kn	23.1	$1.73^{b}\pm0.1$	$0.28^{ab}\pm0.0$
1.25 Kn	20.8	$1.69^{b}\pm0.1$	$0.28^{ab}\pm0.0$

Figure 1: Sterilized explants after 5 weeks showing the best plantlet of NaOCl chemical with 10mg/l for 10 minutes, explants shooting in early stages, the best callus formed in MS media supplemented with TDZ 0.1mg/l and the best explants cultured in MS media supplemented with 1.25 BAP+ 1.25 NAA mg/l.

4.0 Discussion

4.1 Sterilization

It was observed that, for explants materials obtained straight from the field, the level of contamination was high. The relatively higher contamination level of field materials could be attributed to the presence of systemic microorganisms in the tissues (Drew, 1988). This is in agreement with the work reported by Karkonen *et al.* (1999) on Melaleuca alternifolia that contamination rates are often higher for explants harvested from field trees than those obtained from greenhouse. C. eminnii spp. zimmermannii explants were best surface sterilized when exposed to Bavistin (3g/l) for 10 minutes followed by 10 min exposure to 10% sodium hypochlorite and finally

rinsed three times using sterile distilled water. Explants soaked in Bavistin (3g/l) for 10 minutes adequately reduced fungal infection significantly. Results on the effectiveness of sterilants shows that increase in concentration and time of exposure resulted in high explants mortality rate and decrease in bacterial and fungal (Table 3). Formaldehyde caused high mortality of plants compared to sodium hypochlorite. Formaldehyde is a fixative and bleaches explants. High concentration of sodium hypochlorite however bleaches plants.

4.2 Media Selection

When explant survival rates on the selected media were compared using LSD0.05 (Turkey's test), significant differences were observed (Table 4). MS medium exhibited high explants survival rate of 88.89% hence was preferred for subsequent experiments. Different element constitution of MS media contributed to the results.

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. High concentration of nitrate in potassium nitrate and ammonium provides weakly buffering to the media.(Abdulnour *et al*., 2000).Browning of in vitro explants and their eventual death on various media may be due to production of phenolic compounds and possibly due to unsuitability of constituents of individual medium (Preece and Compton, 1991). Explant browning is usually ascribed to phenolic accumulation which reduces cell competence and leads to its eventual loss of totipotency (Benson, 2000). This is a major unsolved problem in the initiation of tissue cultures, especially for woody plants (Pinto et al., 2008). Polyvynylpyrrolidone (0.02) an antiphenol eliminated any phenolic compound.
Direct shoot induction

Different media supplemented with different plant growth regulators indicate that C. eminnii spp zimmermanniiresponds to BAP and Kn either used singly or in combinations. When the response (mean shoot length of in vitro explants) of PGRs treatments were compared using Turkey's test, significant differences were observed. Treatment 1.25 mg/l BAP exhibited the most significant increase in shoot elongation compared to any other treatment. 1.00 mg/l BAP+ 1.50 mg/l NAA exhibited the most significant increase in shoot length (Table 5) while 1.25 mg/l BAP+ 1.25 mg/l NAA was the most effective combined PGRs application treatment (figure 1). Leaves did not yield any shoot and therefore auxillary bud used as the expalnts in the experiment.

4.3 Callus Induction

Only TDZ exhibited callus formation in *C. eminnii spp zimmermannii*. High concentration of TDZ resulted to increase in length of callus however very low concentration of TDZ exhibited callus formation. Hence low TDZ concentration was the best to avoid wastage of chemical. Parma and Kant (2012) reported A whitish callus was exhibited in Commiphora wightii. However somatic embryogenesis was not successful. So far rooting is not successful and more experiments are being carried out.

5.0 Conclusion

C. eminnii spp zimmermannii is an important, endangered and sought after species due to its valuable oleo-gum-resin having tremendous medicinal importance. Sterilization protocol for *C. eminnii spp zimmermannii* was successfully established. The ability to induce shoots and callus from axillary bud clearly indicates that this tree species is amenable to micropropagation technique. In vitro raised plants can now be raised of *C. eminnii spp zimmermannii* through axillary bud proliferation. Multiplication rate of micro-shoots is still low.

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APPENDIX

Plant growth Regulators

Hormone	Product Name	Function in Plant Tissue Culture
Auxins	Indole-3-Acetic Acid Indole-3-Butyric Acid Indole-3-Butyric Acid, Potassium Salt α-Naphthaleneacetic Acid 2,4-Dichlorophenoxyacetic Acid p-Chlorophenoxyacetic acid Picloram Dicamba	Adventitious root formation (high concentration) Adventitious shoot formation (low concentration) Induction of somatic embryos Cell Division Callus formation and growth Inhibition of axillary buds Inhibition of root elongation
Cytokinins	6-Benzylaminopurine 6-γ,γ-Dimethylaminopurine (2iP) Kinetin Thidiazuron (TDZ) N-(2-chloro-4-pyridyl)-N'Phenylurea Zeatin Zeatin Riboside	Adventitious shoot formation Inhibition of root formation Promotes cell division Modulates callus initiation and growth Stimulation of axillary's bud breaking and growth Inhibition of shoot elongation Inhibition of leaf senescence
Gibberellins	Gibberellic Acid	Stimulates shoot elongation Release seeds, embryos, and apical buds from dormancy Inhibits adventitious root formation Paclobutrazol and ancymidol inhibit gibberellin synthesis thus resulting in shorter shoots, and promoting tuber, corm, and bulb formation
Abscisic Acid	Abscisic Acid	Stimulates bulb and tubes formation Stimulates the maturation of embryos Promotes the start of dormancy