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Effective decontamination and multiplication of *Croton membranaceus* in vitro

IRENE AGBLESI ADUKONU, KWADWO OWUSU AYEH, ELIZABETH ACHEAMPONG♥
Department of Botany, University of Ghana. Legon Boundary, Accra, Ghana. Tel.: +233-27-4619, ♥email: e.acheampong@foe-ghana.org

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Abstract. *Adukonu IA, Ayeh KO, Acheampong E. 2022. Effective decontamination and multiplication of* Croton membranaceus in vitro. *Cell Biol Dev 6: 68-81. Croton membranaceus* Müll. Arg. is a useful herb with medicinal properties, and its leaves, roots, and bark are used to treat diverse ailments. However, the harvesting method by traditional medical practitioners without replacement exposes it to extinction. Therefore, the only means of propagating is by relatively slow use of seeds, and an alternative propagation method is needed for field establishment and nursery. Therefore, this study aims at determining an effective propagation by sterilization regime and subsequent in vitro regeneration using different explants; intact seeds, isolated embryos, coatless seeds, and nodal-cutting. The explants were decontaminated using double sterilization; the best was achieved by pre-treatment with 70% ethanol for 3 min before immersion in sodium hypochlorite (NaOCl). Intact seeds were effectively decontaminated by immersion in 15% NaOCl solution for 20 min, then by 10% NaOCl for 15 min. Conversely, coatless seeds were effectively decontaminated when isolated from intact seeds immersed in 20% NaOCl solution for 20 min and 15% NaOCl for 15 min. Further, embryos isolated from intact seeds were effectively decontaminated in 20% NaOCl for 15 min, then by 15% NaOCl for 10 min sequentially. These sterilization regimes successfully decontaminated 86% intact seeds, 100% isolated embryos, and 80% coatless seeds. Nodal-cutting explants were best decontaminated by immersion in 20% NaOCl solution for 15 min, followed by 15% NaOCl for 10 min sequentially without ethanol pre-treatment. This sterilization regime successfully decontaminated 100% of the nodal-cutting explants. However, the development of shoot explants varied in response to sterilization. Intact seeds did not develop into shoots, while isolated embryos, coatless seeds, and nodal-cutting explants developed into shoots independent of the sterilization regime. Shoot development was highest with the medium's shoot-tip explants added with BAP, NAA, and GA3. Shoot multiplication was best achieved on an MS basal medium with 5.0 μM BAP, 0.5 μM NAA, and 5.0 μM GA3 amendment.

Keywords: *Croton membranaceus*, decontamination, in vitro, multiplication

INTRODUCTION

Croton membranaceus Müll. Arg. is a singly used plant by almost herbal hospitals to manage benign prostatic hyperplasia (BPH) and other prostate-related diseases (Obu 2015). The extracts of the roots exhibit general cytotoxicity and growth inhibition against three cancer cell lines; MCF-7(breast), DLD-1(colon), and M14(melanoma) (Bayor et al. 2007; Salatino et al. 2007; Bayor 2008; Nath et al. 2013). Root and stem extracts are used to relieve symptoms of BPH (Afriyie et al. 2014b; Sarkodie et al. 2014). Also, the aqueous root extract is used to treat measles (Schmelzer and Gurib-Fakim 2008), while it is non-toxic yet exhibits cardio-protective, anti-atherogenic, and anti-ischemic potentials (Afriyie et al. 2013). Furthermore, the ethanolic and aqueous root extracts have improve glycemic index in diabetic patients and reduce glucose levels (Afriyie et al. 2013; Sarkodie et al. 2014; Asare et al. 2015a); it demonstrates their potential to treat type I diabetes (diabetes mellitus), which justifies the use of leaf extract by Ghana traditional healers (Afriyie et al. 2013; Sarkodie et al. 2014; Asare et al. 2015a). The leaf and root extracts are used to aromatize tobacco in the Bahamas and improve digestion in Nigeria. While, the bark oil is essential in treating fever, flatulence, cough, diarrhea, and nausea (Asare et al. 2011; 2015b).

The *C. membranaceus* is an economically important medicinal plant derived from the wide range of the plant's leaves, stems, and roots of phytochemicals. The root bark contains julocrotine (a glutarimide alkaloid), scopoletin, and calcium oxalate crystals (Schmelzer and Gurib-Fakim 2008) which are potent in treating cancer, tumors, BPH, and diabetes (Aboagye 1997; Bayor et al. 2007; Sarkodie et al. 2014). The methanolic root extracts contain cajucarinolide to induce apoptosis (programmed cell death) in cancer cells (Block et al. 2005), and recent findings by Bayor et al. (2009) revealed antimicrobial activity, antihyperglycaemic properties, and cytotoxic effects against human prostate cells. The ethanolic and aqueous root extracts contain a compound, N[N-(2-methylbutanoyl) gluta minoyl]-2-phenylethya mine, to improve glycemic index in diabetic (type I diabetes, i.e, diabetes mellitus) and reduce glucose levels (Afriyie et al. 2013; Sarkodie et al. 2014; Asare et al. 2015a). Despite its rich phytochemicals, the plant still grows without propagating commercially in the wild.

The propagation of croton species is by asexual and sexual means; Asexually, techniques such as cutting, division, budding, layering, and grafting (Ingram and Yeager 2003; Relf and Ball 2009; Lott and Lindgren 2012; Devi and Shanthi 2013). Besides, a few species of the genus (*Croton antisyphyliticus, Croton sublyratus,*

Codiaeum variegatum, *Croton urucurana*, and *Croton bonpladinum*) have been cultured and multiplied successfully in vitro (Nasib et al. 2008; Kondamudi et al. 2009; Ashish and Sharma 2011; Oliveira et al. 2011). The *C. membranaceus* is propagated only sexually on seeds by dehiscence and dispersed (Aboagye 1997; Lott and Lindgren 2012). Several Center for Plant Medicine Research attempts by asexual means yielded low results, specifically stem cutting and air layering.

Ghana's report to the Food and Agriculture Organization (FAO) (1996) indicated that *C. membranaceus* is a rare species that would become extinct due to the high demand required in treating and managing diseases such as BPH, diabetes, cough, fever, flatulence, diarrhea, measles, and nausea (Mshana et al. 2000; Bayor et al. 2009; Asare et al. 2011; Devi and Shanthi 2013;). Therefore, its barks and roots are prone to unsustainable harvesting (Abbiw et al. 2002) and bad environmental such as drought and bush fire. As a result, several large-scale propagation attempts using seeds or stem cuttings have been unsuccessful (Aboagye 1997).

The *C. membranaceus* is in rising demand in Ghana and the other tropics (Asare et al. 2011; Afriyie et al. 2013), even though endangered in the wild because of exposure to adverse environmental conditions, mainly drought and bushfires and over-exploitation. In contrast with the increased demand (Abbiw et al. 2002; Afriyie et al. 2014a; Sarkodie et al. 2014), no alternative propagation methods have been established yet. The increasing demand may wipe out its existence if measures are not taken, hence the need to study propagation using in vitro techniques; Apart from using seeds, no report has been published yet. However, it is possible to work on the multiplication using tissue culture techniques on some croton species (Shibata et al. 1996; Ashish and Sharma 2011; Salamma and Rao 2013; Silva et al. 2013).

MATERIALS AND METHODS

Research site

The current research was undertaken in the Tissue culture section of the Department of Botany, University of Ghana, Legon, Ghana, with funding from the United Nations University Institute for Natural Resources in Africa (UNU/INRA).

Plant materials

Seeds and seedlings of *C. membranaceus* were obtained from the Center for Plant Medicine Research (CPMR), Mampong-Akuapem, Ghana. Mature fruits of *C. membranaceus* were obtained randomLy from field-grown plants. Four-week-old seedlings received from CPMR were kept outside the screen house under ambient conditions in the Department for four months to allow the development of shoots to be used as explants. Explants used for the research were dry-mature seeds with seed coats (intact seeds), seeds without seed coats (coatless seeds), embryos isolated from seeds (isolated embryos), and cuttings (nodal cuttings) from young shoots of *C. membranaceus.*

Culture medium

Murashige and Skoog (MS) (1962), produced by Sigma-Aldrich Company, Spain, used the basal medium for the experiment. Stock solutions of macronutrients, micronutrients, vitamins, and growth hormones were prepared based on the required composition and quantity of nutrients. All stock solutions were kept in a refrigerator and stored at 4^{o}C. Media were prepared from stock solutions and used for culturing explants. The pH of the medium was adjusted to 5.7. Agar was added as a gelling agent to harden the medium and dispensed into test tubes, after which they were sterilized in an autoclave at a temperature of 121^{o}C and a pressure 102.97KPa for 15 min. The medium was allowed to cool after autoclaving before being used.

Sterilization of labware

All dissecting kits (scalpel, forceps), and glassware (petri dishes, covered bottles, etc.), were sterilized in an autoclave at a temperature of 121^{o}C and a pressure of 102.97KPa for 15 min. Distilled water was also sterilized under the same condition.

Experiment 1: Decontamination of explants

A decontamination experiment was done using seeds and nodal cuttings to determine the optimal sterilization regime for Croton explants. In addition, the effects of ethanol and various concentrations of sodium hypochlorite were determined. Seeds of an average length of 4mm were selected, and young shoots of the potted plants were used for the various experiments. Intact seeds were double sterilized by immersing in 15% NaOCl for 20 min, followed by 10% NaOCl for 15 min, without ethanol pre-treatment (A0) or with ethanol pre-treatment (A1), 20% NaOCl for 15 min, followed by 15% for 10 min, without ethanol pre-treatment (B0) or with ethanol pre-treatment (B1) and 20% NaOCl for 20 min followed by 15% NaOCl for 15 min, without ethanol pre-treatment (C0) or with ethanol pre-treatment (C1). Nodal cuttings were first washed under running tap water containing a few drops of household liquid detergent (Klene lene) containing sodium Laureth sulfate for 5 min and rinsed with sterile distilled water, trimmed to a length of 1.5 cm with two nodes and sterilized as described for intact seeds.

Seeds and nodal cuttings were placed in separate bottles, completely immersed in NaOCl, and carefully agitated by hand. Four drops of household liquid detergent (Klene lene) were added to every 100 mL NaOCl, then thoroughly washed with sterile distilled water to remove traces of the sterilant. Coatless seed and embryo explants were aseptically isolated from sterilized intact seeds previously soaked for 48 hours. The nodal cuttings were trimmed to 1 cm. Sterilized explants were inoculated on MS basal medium supplemented with 5.0 µM BAP and 5.0 µM NAA. One explant was inoculated in a test tube containing 10 mL of the medium. The cultures were incubated in 16 hours of light and 8 hours of darkness at a temperature of 26±1^{o}C. Each explant was replicated five times, and the experiment was repeated three times. The

number of decontaminated explants and the number of explants that developed shoots or plantlets were recorded.

Experiment 2: Initiation of cultures

Initiation of cultures from seeds

Intact seeds were sterilized using the best sterilization regime from the decontamination experiment and cultured on MS basal medium supplemented with 100 mg/L *myo*-inositol, 0.1 mg/L thiamine HCl and varying concentrations of BAP, NAA, and GA3 (Table 1). Coatless seeds and embryos were obtained from sterilized intact seeds and cultured on MS medium as described for intact seeds.

One explant was inoculated per test tube containing 10mL of the medium. One explant in each test tube formed an experimental unit and was replicated ten times. Data on the number of explants that developed shoots, the number of shoots per explant, and the height of shoots were recorded four weeks after culture.

Initiation of cultures from nodal-cutting and shoot-tip explants

Sterilized nodal-cutting and shoot-tip explants bearing two buds were cultured on MS basal medium amended with 100 mg/L *myo*-inositol, 0.1 mg/L thiamine HCl, 5.0 µM BAP only, 5.0 µM BAP and 0.5 µM NAA, 5.0 µM BAP and 5.0 µM NAA, 5.0 µM BAP and 5.0 µM GA3, 5.0 µM

BAP, 0.5 µM NAA and 5.0 µM GA3 or 5.0 µM BAP, 5.0 µM NAA and 5.0 µM GA3. One explant was cultured per test tube containing 10mL of the medium. The medium was sterilized as described in section 3.3. One explant in each test tube formed an experimental unit and was replicated ten times. The number of explants that developed shoots, the number of shoots per explant, and the number of roots per explant were counted. In addition, the height of shoots was measured with a meter rule. All data were recorded four weeks after culture.

Experiment 3: Comparison of nodal-cutting and shoot-tip explants for shoot initiation in *Croton membranaceus*

This experiment was carried out to investigate the most responsive part of nodal explants for culture initiation, using nodal cuttings and shoot-tips. First, the explants were washed in running tap water containing four drops of household liquid detergent by Klene Lene, Ghana, which contains the active ingredient, sodium Laureth sulfate, per 100mL water for five min and rinsed thoroughly with sterile distilled water. That was followed by double sterilizing with 15% sodium hypochlorite (NaOCl) for 20 min and 10% NaOCl solution for 15 min sequentially. Finally, the nodal explants were rinsed thoroughly with sterile distilled water to remove any remaining sterilant, then aseptically trimmed to obtain cuttings of 1cm long with two buds each. The sterilized explants were cultured on solid MS basal medium amended with 100 mg/L *myo*-inositol, 0.1 mg/L thiamine HCl, 30 g/L sucrose without growth hormone, 5.0 µM BAP and 5.0 µMNAA. One nodal explant was cultured in one test tube containing 10mL of MS medium and replicated ten times. The cultures were incubated in the growth room in light for 16 hours and darkness for 8 hours at a temperature of 26 ± 1^0C for four weeks. The number of shoots and leaves formed by each explant was counted, and the height of the shoot was measured with a meter rule.

Multiplication of *Croton membranaceus*

Two experiments were conducted to determine an optimum medium for the multiplication; the first experiment was in vitro shoots, which were subcultured on MS basal medium containing 100 mg/L *myo*-inositol, 0.1 mg/L thiamine HCl, 30 g/L sucrose without growth hormone, with 5.0 µM BAP only or in combination with 0.5 µM or 5.0 µM NAA, 5.0 µM GA3, 0.5 µM NAA, and 5.0 µM GA3 or 5.0 µM NAA and 5.0 µM GA3; Other was cultured in a test tube, and this was replicated ten times. All cultures were incubated under growth room conditions of 25 ± 1^0C and light intensity of 3000 Lux for four weeks; the number of cultures that developed shoots were recorded. Also, the number of shoots and leaves per culture was counted. Finally, the shoot height was measured with a meter rule.

The second experiment investigated the response of in vitro nodal cuttings of *C. membranaceus* on an MS basal medium amended with BAP, NAA, and varying concentrations of GA3. In vitro shoots were subcultured on MS basal medium amended with 5.0 µM BAP and 0.5 µM NAA (control) and in combination with 5.0 µM GA3 or 50 µM GA3. One nodal-cutting was cultured in a test tube and replicated ten times. All cultures were incubated as described above. The number of shoots per explant, the number of leaves per shoot, and the height of shoots were recorded after four weeks.

Design and data analysis

The experiments were laid out in a Completely Randomized Block Design. Data collected were quantified and analyzed statistically by ANOVA or Two-Sample T-Test, using the minitab software version 17 where necessary. Means were separated using the Fisher Least Significant Difference (LSD) method at α level of 0.05.

Table 1. Concentrations of BAP, NAA, and GA3 in MS basal medium used for the initiation experiments

Growth hormones (µM)		
BAP	**NAA**	**GA3**
0.0	0.0	0.0
5.0	0.0	0.0
5.0	0.5	0.0
5.0	5.0	0.0
5.0	0.0	5.0
5.0	0.5	5.0
5.0	5.0	5.0

RESULTS AND DISCUSSION

Effect of double sterilization with sodium hypochlorite (NaOCl) on decontamination of explants

The decontamination of explants using the double sterilization method with or without immersion in ethanol is shown in Table 2.

When intact seeds were double sterilized with 15% NaOCl for 20 min and 10% NaOCl solution for 15 min sequentially, the least explant decontamination was 33% and was obtained without pre-treatment with ethanol (A0), while the highest explant decontamination was 86.7% and was obtained when intact seeds were immersed in 70% ethanol for 3 min before immersion in NaOCl (A1). When intact seeds were sterilized with treatment B, the least explant decontamination was 60% and obtained when intact seeds were double sterilized with 20% NaOCl for 15 min and 15% NaOCl for 10 min sequentially without pre-treatment with ethanol (B0) while the highest explant decontamination was 80% and was obtained when intact seeds were immersed in 70% ethanol for 3 min followed by double sterilization with 20% NaOCl solution for 15 min and 15% NaOCl solution for 10 min sequentially (B1). With treatment C, the least explant decontamination was 46.7% and obtained when intact seeds were double sterilized with 20% NaOCl for 15 min and 15% NaOCl for 10 min sequentially without pre-treatment with ethanol (C0), while the highest explant decontamination was 80% and was obtained when intact seeds were immersed in 70% ethanol for 3 min followed by double sterilization with 20% NaOCl for 20 min and 15% NaOCl for 15 min sequentially (C1). None of the intact seeds developed shoots or roots when sterilized using all the sterilization regimes (Table 2).

Coatless seeds were least decontaminated (13.7%) when sterilized with treatment A0 (15% NaOCl for 20 min, 10% NaOCl for 15 min). Still, the decontamination rate increased to 66.67% when coatless seeds were treated with A1 (70% ethanol for 3 min, 15% NaOCl for 20 min, and 10% NaOCl for 15 min) (Table 2). With treatment B, 66.67% of coatless seeds were decontaminated when sterilized with B0 (20% NaOCl for 15 min, 15% NaOCl for 10 min), while 80% of the coatless seeds were decontaminated with B1 (70% ethanol for 3 min, 20% NaOCl for 15 min, 15% NaOCl for 10 min). With treatment C0 (20% NaOCl for 20 min, 15% NaOCl for 15 min), 73.33% coatless seeds were decontaminated, whereas C1 (70% ethanol for 3 min, 20% NaOCl for 20 min, 15% NaOCl for 15 min), 80% decontamination was recorded. There was no development of shoot or root when coatless seeds were treated with A1; however, when coatless seeds were treated with A0, 6.7% developed shoots and roots. Shoot increased (13.33%) with treatment B0 though this was lower than 26.67% shoot development when coatless seeds were decontaminated with treatment C1. The optimum number of 46.7% coatless seeds developed shoots and roots when treated with B1 and C0.

Embryos isolated from intact seeds sterilized with treatment A0 (15% NaOCl for 20 min, 10% NaOCl for 15 min) were least decontaminated 73.3%, while with Treatment A1 (70% ethanol for 3 min, 15% NaOCl for 20 min, 10% NaOCl for 15 min), 93.33% isolated embryos were decontaminated. When the percentage of NaOCl was increased as in B0 (20% NaOCl for 15 min, 15% NaOCl for 10 min), the number of decontaminated embryos observed was 93.33%, but 100% decontamination was observed when explants were pretreated with ethanol before immersion in the same concentration of NaOCl for the same time, B1 (70% ethanol for 3 min, 20% NaOCl for 15 min, 15% NaOCl for 10 min). With treatment C, 80% of isolated embryos were decontaminated when ethanol was not used C0 (20% NaOCl for 20 min, 15% NaOCl for 15 min). In comparison, an optimum of 100% isolated embryos was decontaminated when sterilized treatment C1 (70% ethanol for 3 min, 20% NaOCl for 20 min, 15% NaOCl for 15 min). The Embryos decontaminated with the various sterilization regimes developed shoots and roots (Table 2). The least number of embryos that developed shoots with roots was 33.3%, which was observed in embryos decontaminated with treatment A0. The number increased to 40% for embryos treated with A1 and B0. A further increase of 60% shoot development was observed for embryos treated with C0 and C1, while an optimum of 80% of embryos developed into shoots with roots when decontaminated with Treatment B1, as described earlier.

Table 2. Effect of ethanol and various concentrations of sodium hypochlorite (NaOCl) on decontamination of explants. Explants were cultured on MS basal medium amended with 5 μM BAP and 5 μM NAA

Treatment	No. of cultures	Intact seeds		Coatless seeds		Isolated embryos		Nodal explants	
		Decontaminated (%)	Plantlets (%)	Decontaminated (%)	Plantlets (%)	Decontaminated (%)	Plantlets (%)	Decontaminated (%)	Plantlets (%)
A0	15	33.33	0.00	13.33	6.67	73.33	33.33	80.00	93.33
A1	15	86.67	0.00	66.67	0.00	93.33	40.00	93.33	80.00
B0	15	60.00	0.00	66.67	13.33	93.33	40.00	100.00	66.67
B1	15	80.00	0.00	80.00	46.70	100.00	80.00	93.33	66.67
C0	15	46.67	0.00	73.33	46.70	80.00	60.00	93.33	73.33
C1	15	80.00	0.00	80.00	26.67	100.00	60.00	100.00	66.67

Note: A0-15% NaOCl for 20 min, 10%NaOCl for 15 min; A1-70% ethanol for 3 min, 15% NaOCl for 20 min, 10% NaOCl for 15 min; B0-20% NaOCl for 15 min, 15% NaOCl for 10 min; B1-70% ethanol for 3 min, 20% NaOCl for 15 min, 15% NaOCl for 10 min; C0-20% NaOCl for 20 min, 15% NaOCl for 15 min; C1-70% ethanol for 3 min, 20% NaOCl for 20 min, 15% NaOCl for 15 min

Nodal-cutting explants were least decontaminated (80%) when sterilized with treatment A0 (15% NaOCl for 20 min, 10% NaOCl for 15 min). The decontamination rate increased (93.33%) when nodal-cutting explants were sterilized with treatment A1 (70% ethanol for 3 min, 15% NaOCl for 20 min, 10% NaOCl for 15 min), B1 (70% ethanol for 3 min, 20% NaOCl for 15 min, 15% NaOCl for 10 min) and C0 (20% NaOCl for 20 min, 15% NaOCl for 15 min). An optimum rate of 100% decontamination was observed when nodal-cutting explants were sterilized with treatments B0 (20% NaOCl for 15 min, 15% NaOCl for 10 min) and C1 (70% ethanol for 3 min, 20% NaOCl for 20 min, 15% NaOCl for 15 min). Nodal-cutting explants developed shoots with roots when decontaminated using all the sterilization regimes (Table 2). However, nodal-cutting explants decontaminated with treatments B0, B1, and C1 had the least rate of plantlet regeneration. With these treatments, 66.7% of nodal-cutting explants developed shoots with roots. The rate of shoot development increased to 73.33% and 80% when nodal-cutting explants were sterilized with treatments C0 and A1, respectively. An optimum number of 93.3% of nodal explants developed shoots and roots when nodal explants were sterilized with treatment A0.

Response of explants to BAP, NAA, and GA3 in the culture medium

The effect of BAP, NAA, and GA3 in MS basal medium on shoot development is shown in Tables 3 to 5.

Intact seeds failed to develop into shoots when cultured on MS basal medium amended with BAP alone, BAP with NAA or BAP, NAA, and GA3. However, only one intact seed developed radicle on MS basal medium amended with 5.0 µM BAP and 5.0 µM NAA after twelve weeks of culture (Figure 1). Coatless seed explants developed into shoots nine days after culture on MS basal medium with or without hormone (the control). However, the number of shoots developed depended on the combination of growth hormones (Table 3).

All explants cultured on MS basal medium with 5.0 µM BAP only, did not develop shoots. Similarly, all explants cultured on MS basal medium amended with either 0.5 or 5.0 µM NAA and GA3 also did not develop shoots. These explants were swollen by the second week of culture but turned brown at four weeks of culture. Shoots were only developed on MS basal medium without hormone (the control) and MS basal medium amended with BAP and NAA only or BAP with GA3 only (Table 3). The mean number of shoots per explant was 0.10, independent of the hormone manipulation and combination. However, the number of leaves and roots per explant and the height of the shoots varied. The mean number of leaves was 0.2 on MS medium without hormone and MS basal medium fortified with 5.0 µM BAP and 5.0 µM NAA or GA3. The mean number of leaves increased (0.5) when an MS basal medium was amended with 5.0 µM BAP and 0.5 µM NAA. Statistically, the differences observed were not significant ($P \leq 0.712$).

The mean number of roots per explant on MS basal medium without hormone treatment was 0.4. That was higher than the mean number (0.1) observed on MS basal medium amended with 5.0 µM BAP and 5.0 µM NAA. The mean number of roots increased to 0.5 per explant when an MS basal medium was amended with 5.0 µM BAP and 5.0 µM GA3 with an optimum of 0.7 roots per shoot on an MS basal medium amended with 5.0 µM BAP and 0.5 µM NAA. Statistical analysis showed that the observed differences were insignificant ($P \leq 0.700$). The mean height of the shoot was 0.12cm on MS basal medium without hormone, which was higher than 0.10cm and 0.11cm as observed on MS basal medium amended with 5.0 µM BAP and 5.0 µM NAA or 5.0 µM GA3, respectively. The highest shoot (0.38cm) was obtained on an MS basal medium amended with 5.0 µM BAP and 0.5 µM NAA (Figure 2). However, the differences in shoot height observed were not statistically significant ($P \leq 0.649$).

Isolated embryos developed into shoots five days after culture. However, the shoots' survival rate and development depended on a combination of growth hormones in the MS medium (Table 4).

Figure 1. Intact seed explant developing root on MS basal medium amended with 5.0 µM BAP and 5.0 µM NAA after twelve weeks of culture

Table 3. Response of coatless seeds to MS basal medium amended with BAP and NAA. Data were collected four weeks after culture

Growth hormone (µM)	Number of explants	Surviving explants	Shoot per explant	Leaf per explant	Root per explant	Shoot height (cm)
0	10	0.10±0.32[a]	0.10±0.32[a]	0.20±0.63[a]	0.40±1.27[a]	0.12±0.38[a]
5.0 BAP	10	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]
5.0 BAP + 0.5 NAA	10	0.10±0.32[a]	0.10±0.32[a]	0.50±1.58[a]	0.70±2.20[a]	0.38±1.20[a]
5.0 BAP + 5.0 NAA	10	0.10±0.32[a]	0.10±0.32[a]	0.20±0.63[a]	0.10±0.32[a]	0.10±0.32[a]
5.0 BAP + 5.0 GA3	10	0.10±0.32[a]	0.10±0.32[a]	0.20±0.63[a]	0.50±1.58[a]	0.11±0.35[a]
5.0 BAP + 0.5 NAA + 5.0 GA3	10	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]
5.0 BAP + 5.0 NAA + 5.0 GA3	10	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]

Note: Each value in the table is the mean and standard deviation of ten replicates. Values that do not share a letter in a column differ significantly by LSD ($\alpha = 0.05$)

Table 4. The response of isolated embryos to MS basal medium was amended with BAP, NAA, and GA3. Data were collected four weeks after culture

Growth hormone (µM)	Number of explants	Surviving explants	Shoot per explant	Leaf per explant	Root per explant	Shoot height (cm)
0	10	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]
5.0 BAP	10	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]
5.0 BAP + 0.5 NAA	10	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]
5.0 BAP + 5.0 NAA	10	0.20±0.42[a]	0.20±0.42[a]	0.70±1.89[a]	0.80±2.53[a]	0.28±0.89[a]
5.0 BAP + 5.0 GA3	10	0.10±0.32[a]	0.10±0.32[a]	0.70±2.21[a]	0.20±0.63[a]	0.25±0.79[a]
5.0 BAP + 0.5 NAA + 5.0 GA3	10	0.10±0.32[a]	0.10±0.32[a]	0.20±0.63[a]	0.00±0.00[a]	0.08±0.25[a]
5.0 BAP + 5.0 NAA + 5.0 GA3	10	0.10±0.32[a]	0.10±0.32[a]	0.10±0.32[a]	0.40±1.27[a]	0.10±0.32[a]

Note: Each value in the table is the mean and standard deviation of ten replicates. Values that do not share a letter in a column differ significantly by LSD (α = 0.05)

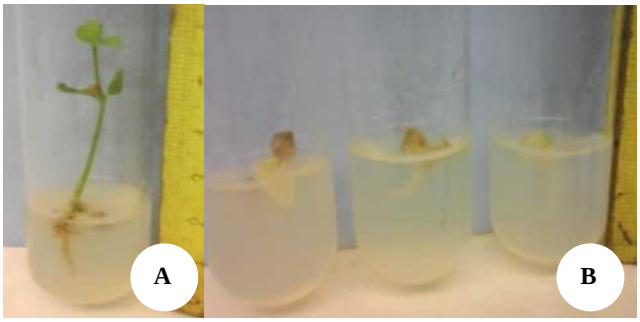


Figure 2. Coatless seed explants developing shoots on MS basal medium amended with A. 5.0 µM BAP and 0.5 µM NAA and B. 5.0 µM BAP and 5.0 µM NAA four weeks after culture

All isolated embryos cultured on MS basal medium without any growth hormone failed to develop shoots. Also, all explants cultured on MS basal medium with 5.0 µM BAP only or 0.5 µM NAA did not develop shoots. Shoot development occurred when the concentration of NAA in the culture medium was increased or when GA3 was added to the medium. Isolated embryos cultured on MS basal medium amended with 5.0 µM BAP and 5.0 µM GA3, 5.0 µM BAP, 0.5 µM NAA, and 5.0 µM GA3 or 5.0 µM BAP, 5.0 µM NAA and 5.0 µM GA3 had a mean of 0.1 surviving over four weeks period. However, this was not statistically different from the highest survival rate of 0.2 when an MS basal medium was amended with 5.0 µM BAP and 5.0 µM NAA (P≤0.546). Similarly, shoot development was lower with a mean of 0.1 shoots per explant on an MS basal medium amended with 5 µM BAP and 5 µM GA3 or with 0.5 or 5.0 µM NAA. In contrast, an optimum of 0.2 shoots per explant was observed on an MS basal medium amended with 5.0 µM BAP and 0.5 µM NAA but not statistically different (P≤0.546).

Leaf development was least (0.1) when an MS basal medium was amended with 5.0 µM BAP, 5.0 µM GA3, and 5.0 µM NAA, but the number increased to 0.2 when NAA was reduced to 0.5 µM. An optimum mean (0.7) was observed when an MS basal medium was amended with 5.0 µM BAP and 5.0 µM NAA or 5.0 µM GA3. Statistically, the differences observed for leaf development were not significant (P≤0.587).

Root development was not evident for shoots on MS basal medium amended with 5.0 µM BAP and 0.5 µM NAA and 5 µM GA3. When an MS basal medium was amended with 5.0 µM BAP and 5.0 µM GA3, a mean of 0.2 roots per explant was observed. That increased to 0.4 roots per explant when an MS basal medium was amended with 5.0 µM BAP and 5.0 µM NAA and 5.0 µM GA3, while the highest mean of 0.8 roots per shoot was observed on an MS basal medium amended with 5.0 µM BAP and 5.0 µM NAA (Table 4). However, the differences observed were not statistically significant (P≤0.590).

The height of the shoot was the least (0.08cm) on MS basal medium amended with 5.0 µM BAP, 5.0 µM GA3, and 0.5 µM NAA but increased to 0.1cm when NAA was increased to 5.0 µM. The height of the shoot increased from 0.25cm when an MS medium was amended with 5.0 µM BAP and 5.0 µM GA3 to 0.28cm when an MS medium was amended with 5.0 µM BAP and 5.0 µM NAA. However, the differences observed in the height of the shoot were not statistically significant (P≤ 0.757).

Thus, only a medium amended with 5.0 µM BAP in combination with 5.0 µM NAA, 5.0 µM GA3, and 5.0 µM BAP in combination with both NAA and GA3 irrespective of NAA concentration, developed into shoots (Figure 3).

Nodal-cutting and shoot-tip explants

Of all the explants used, nodal-cutting explants had the best shoot development as all the media combinations developed shoots (Table 5).

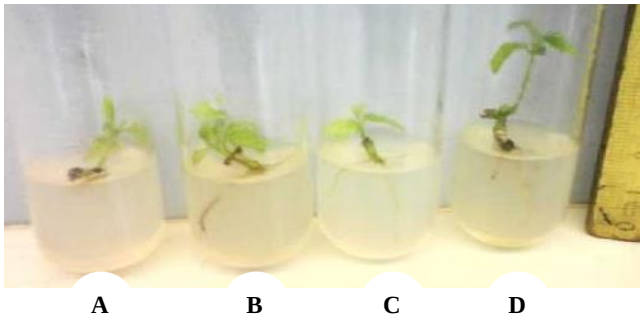


Figure 3. Response of isolated embryos to BAP, NAA, and GA3 treatments; A. 5.0 µM BAP, 0.5 µM NAA and 5.0 µM GA3, B. 5.0 µM BAP and 5.0 µM GA3, C. 5.0 µM BAP, 5.0 µM NAA and 5.0 µM GA3 and D. 5.0 µM BAP and 5.0 µM NAA. Photos were taken four weeks after culture

Explants cultured on MS basal medium without hormone (the control) had a survival rate of 0.3 (Table 5). That was higher than 0.10 as observed for explants cultured on an MS basal medium amended with 5.0 µM BAP and 5.0 µM NAA or 5.0 µM BAP, 0.5 µM NAA and 5.0 µM GA3. When an MS basal medium was amended with 5.0 µM BAP and 0.5 µM NAA, the survival rate increased to 0.2 explants. A further increase (0.4) was observed when an MS basal medium was amended with 5.0 µM BAP only or combined with 5.0 µM GA3. The survival rate was optimum (0.6) when an MS basal medium was amended with 5.0 µM BAP, 5.0 µM NAA, and 5.0 µM GA3. Statistically, the differences observed in the explants' survival were insignificant ($P \leq 0.147$).

The number of shoots per explant was 0.5 when nodal-cutting and shoot-tip explants were grown on MS basal medium without hormone (control). When an MS basal medium was amended with 5.0 µM BAP and 5.0 µM NAA or 5.0 µM BAP, 0.5 µM NAA, and 5.0 µM GA3, the number of shoots per explant reduced to 0.1. The number of shoots per explant was observed (0.2) when an MS basal medium was amended with 5.0 µM BAP, and 0.5 µM NAA was still lower than the control medium. An increase of 0.6 shoots per explant was observed when explants were cultured on an MS basal medium amended with 5.0 µM BAP and 5.0 µM GA3. When an MS basal medium was amended with 5.0 µM BAP, 5.0 µM NAA, and 5.0 µM GA3, 0.7 shoots per explants were observed. The shoot number was optimum (0.8) when nodal-cutting and shoot-tip explants were cultured on MS basal medium amended only with 5.0 µM BAP. Statistically, the differences in shoot development observed from nodal-cutting and shoot-tip explants were insignificant ($P \leq 0.165$).

Leaf development was 2.2 leaves per shoot, as observed when an MS medium was not amended with hormones (control) (Table 5). The number of leaves per shoot was reduced to 0.2 when an MS basal medium was amended with 5.0 µM BAP, 0.5 µM NAA, and 5.0 µM GA3. An increase of 0.4 leaves per explant was observed when an MS basal medium was amended with 5.0 µM BAP and 5.0 µM NAA. That was followed by 0.8 leaves per explant when an MS basal medium was amended with 5.0 µM BAP and 0.5 µM NAA. Leaf development increased to 2.3 per shoot when an MS basal medium was amended with 5.0 µM BAP and 5.0 µM GA3. The optimum number of leaves per explant (3.2) was observed when nodal-cutting explants were cultured on an MS basal medium amended with either 5.0 µM BAP only or 5.0 µM BAP, 5.0 µM NAA and 5.0 µM GA3. However, the differences observed in leaf development were not statistically significant ($P \leq 0.183$).

The shoot height observed on the MS basal medium without hormone was 0.61cm (Table 5). That was higher than the height of the shoot observed on four treatments with growth hormones. When an MS basal medium was amended with 5.0 µM BAP, 0.5 µM NAA and 5.0 µM GA3, 5.0 µM BAP and 0.5 µM NAA, 5.0 µM BAP and 5.0 µM NAA and 5.0 µM BAP only, the height of shoot observed were 0.10cm, 0.18cm, 0.23cm, and 0.59cm respectively. Shoot height was enhanced when an MS basal medium was amended with 5.0 µM BAP and 5.0 µM GA3 (Figure 4C). On this medium, nodal-cutting and shoot-tip explants grew to a height of 0.64cm. The optimum shoot height (0.80cm) was observed when an MS basal medium was amended with 5.0 µM BAP, 5.0 µM NAA, and 5.0 µM GA3 (Figure 4E). Statistically, the differences observed were not significant ($P \leq 0.270$). However, the overall response of nodal-cutting explants to the development of shoot leaves, roots, and the height of shoots was optimum on MS basal medium amended with 5.0 µM BAP, 5.0 µM NAA, and 5.0 µM GA3 (Figure 4).

Comparison of nodal-cutting and shoot-tip explants

The finding that an MS basal medium amended with a combination of the growth hormones 5.0 µM BAP, 5.0 µM NAA, and 5.0 µM GA3, was effective in the response of nodal-cutting and shoot-tip explants (Table 5) led to the investigation of the comparison between the use of nodal-cutting and shoot-tips explants in micropropagation of *C. membranaceus*. Thus, in this experiment, nodal-cutting and shoot-tip explants were cultured on MS basal medium amended with 5.0 µM BAP, 5.0 µM NAA, and 5.0 µM GA3, to compare their regeneration potential. Shoot-tip explants developed into shoots earlier and faster than nodal-cutting explants. It was observed that shoot tips started sprouting 5 days after culture, whereas it took ten days for nodal cuttings to start sprouting. There was a significant effect of shoot-tip and nodal-cutting explants on shoot regeneration from *C. membranaceus.* The effect was observed in the number of surviving explants, shoot per explant, leaves per explant, and shoot height (Table 6).

Table 5. Effect of MS basal medium amended with BAP, NAA, and GA3 on nodal-cutting and shoot-tip explants. Data were collected four weeks after culture

Growth hormone (µM)	Number of explants	Surviving explants	No. of shoot per explant	No. of leaves per explant	Shoot height (cm)
0	10	0.30 ± 0.48^{ab}	0.50 ± 0.85^{ab}	2.20 ± 3.85^{ab}	0.61 ± 1.03^{ab}
5.0 BAP	10	0.40 ± 0.52^{ab}	0.80 ± 1.14^{a}	3.20 ± 4.83^{a}	0.59 ± 0.93^{ab}
5.0 BAP + 0.5 NAA	10	0.20 ± 0.42^{ab}	0.20 ± 0.42^{ab}	0.80 ± 2.20^{ab}	0.18 ± 0.42^{ab}
5.0 BAP + 5.0 NAA	10	0.10 ± 0.32^{b}	0.10 ± 0.32^{b}	0.40 ± 1.27^{ab}	0.23 ± 0.73^{ab}
5.0 BAP + 5.0 GA3	10	0.40 ± 0.52^{ab}	0.60 ± 0.97^{ab}	2.30 ± 3.83^{ab}	0.64 ± 0.94^{ab}
5.0 BAP + 0.5 NAA + 5.0 GA3	10	0.10 ± 0.32^{b}	0.10 ± 0.32^{b}	0.20 ± 0.63^{b}	0.05 ± 0.16^{b}
5.0 BAP + 5.0 NAA + 5.0 GA3	10	0.60 ± 0.52^{a}	0.70 ± 0.68^{ab}	3.20 ± 3.08^{a}	0.80 ± 0.91^{a}

Note: Each value in the table is the mean and standard deviation of ten replicates. Values that do not share a letter in a column differ significantly by LSD ($\alpha = 0.05$)

Table 6. Comparison of nodal-cutting and shoot-tip explants on shoot regeneration of *Croton membranaceus*. Explants were cultured on MS basal medium amended with 5.0 µM BAP, 5.0 µM NAA, and 5.0 µM GA3. Data were collected four weeks after culture

Explant	Number of explants	Surviving explants	No. of shoot per explant	No. of leaves per explant	Shoot height (cm)
Nodal cutting	10	0.50±0.53[a]	0.50±0.53[a]	1.30±1.57[a]	0.30±0.41[b]
Shoot-tip	10	0.80±0.42[a]	1.20±0.79[b]	3.00±2.58[a]	1.29±0.83[a]

Note: Each value in the table is the mean and standard deviation of ten replicates. Values that do not share a letter in a column differ significantly by LSD ($\alpha = 0.05$)

A B C D E F G

Figure 4. Response of nodal explants to BAP, NAA, and GA3 treatments; A. Control, B. 5.0 µM BAP only, C. 5.0 µM BAP and 5.0 µM GA3, D. 5.0 µM BAP and 5.0 µM NAA, E. 5.0 µM BAP, 5.0 µM NAA and 5.0 µM GA3, F. 5.0 µM BAP and 0.5 µM NAA and G. 5.0 µM BAP, 0.5 µM NAA and 5.0 µM GA3. Photos were taken four weeks after culture

The number of surviving explants from nodal-cutting explants was not significantly lower (0.50) ($P \leq 0.177$) than shoot-tip explants (0.80). Similarly, shoot development in nodal-cutting explants was lower (0.50) shoots per explant. On the other hand, the number of shoot-tip explants that developed into shoots was more than twice (1.20) that of nodal-cutting explants. Statistically, there is evidence of a significant difference ($P \leq 0.031$) in the development of shoots from nodal-cutting and shoot-tip explants.

Leaf development was also less in nodal-cutting explants than in shoot-tip explants. A mean of 1.30 leaves per explant was observed when nodal-cutting explants were used, whereas a mean of 3.00 leaves per explant was observed for shoot-tip explants. Although the difference observed was more than twice, there was no statistical evidence of a significant difference in leaf development for nodal-cutting and shoot-tip explants ($P \leq 0.092$).

The mean height of shoots observed in nodal-cutting explants was 0.30, while that of shoot-tip explants was 1.29. Statistically, there was evidence of a significant difference ($P \leq 0.003$) in the shoot height for nodal-cutting and shoot-tip explants.

Thus, the comparison between the response of nodal-cutting and shoot-tip explants showed that shoot-tip explants enhanced the regeneration of shoots better (Figure 5).

Effect of BAP, NAA, and GA3 on in vitro nodal-cutting explants

This experiment was conducted to investigate the effect of BAP, NAA, and GA3 on shoot development using in vitro nodal cuttings as explants. Shoot development was observed on MS basal medium without growth hormone and MS basal medium amended with BAP, NAA, and GA3 irrespective of the concentration or combination (Table 7).

The mean number of explants that survived on the MS basal medium without hormonal modification (the control) (0.5) was less than the number of surviving explants on the MS basal medium amended with growth hormones. When an MS basal medium was amended with 5.0 µM BAP only or combined with 5.0 µM GA3, the mean number of surviving explants increased to 0.8. The mean number of surviving explants further increased to 0.9 when the MS medium was amended with 5.0 µM BAP and 5.0 µM GA3 combined with either 0.5 µM or 5.0 µM NAA. The highest mean of surviving explant was 1.0, and this was observed on an MS basal medium amended with either 5.0 µM BAP and 0.5 µM NAA or 5.0 µM BAP and 5.0 µM NAA. However, there was no statistical evidence of a significant difference ($P \leq 0.187$) in the number of surviving explants.

The mean number of shoots per explant observed on an MS basal medium without hormone (0.6) was the least, which increased to 0.9 when the MS medium was amended with 5.0 µM BAP, 5.0 µM NAA, and 5.0 µM GA3. A further increase (1.0) was observed when an MS medium was amended with 5.0 µM BAP and 5.0 µM GA3 or (1.2) when an MS medium was amended with 5.0 µM BAP, 0.5 µM NAA, and 5.0 µM GA3. Finally, the highest mean number of shoots per explant (1.4) was observed on an MS medium amended with 5.0 µM BAP only and 5.0 µM BAP combined with either 0.5 µM or 5 µM NAA. Statistically, the differences observed were not significant ($P \leq 0.121$).

Leaf development was the least (1.4) on the MS medium without hormones. When 5.0 µM BAP and 5.0 µM GA3 were added to an MS medium, leaf development increased to 2.3 leaves per shoot. When an MS basal medium was amended with 5.0 µM BAP, 5.0 µM NAA, and 5.0 µM GA3, or 5.0 µM BAP and 5.0 µM NAA, the mean number of leaves per shoot observed were 3.0 and 3.1 respectively. The number of leaves per shoot increased to 3.7 when 5.0 µM BAP only was added to an MS medium, 3.8 when 5.0 µM BAP and 0.5 µM NAA were added to an MS basal medium. The highest mean number

of leaves per explant (3.9) was observed when an MS basal medium was amended with 5.0 µM BAP, 0.5 µM NAA, and 5.0 µM GA3. However, there was no statistical evidence of a significant difference (P≤ 0.193) in the number of leaves per explant on each MS medium.

The height of the shoot observed when an MS basal medium was not manipulated with growth hormone (0.51cm) was less than when an MS basal medium was amended with growth hormones. A mean height of 0.63cm was observed when an MS basal medium was amended with 5.0 µM BAP and 5.0 µM GA3. The shoot height increased to 0.70cm on an MS basal medium amended with 5.0 µM BAP, 5.0 µM NAA, and 5.0 µM GA3. A further increase in shoot height (0.80cm) was observed when an MS basal medium was amended with 5.0 µM BAP and 0.5 µM NAA, while a mean height of 0.84cm was observed when an MS basal medium was amended with 5.0 µM BAP only. A mean shoot height of 0.92cm was observed on an MS basal medium amended with 5.0 µM BAP, 0.5 µM NAA, and 5.0 µMGA3 with an optimum mean height of 1.03cm observed when an MS basal medium was amended with 5.0 µM BAP and 5.0 µM NAA. Statistically, the differences observed were not significant (P≤0.156).

Effect of different GA3 concentrations on shoot multiplication of in vitro nodal cuttings

Modification of an MS basal medium amended with 5.0 µM BAP and 0.5 µM NAA with either 5.0 µM GA3 or 50 µM GA3 had different effects on shoot development of in vitro nodal-cutting explants (Table 8).

The mean number of surviving explants on an MS basal medium amended with 5.0 µM BAP and 0.5 µM NAA without GA3 (control) was 0.8, and the same was observed when an MS basal medium was amended with 5.0 µM BAP, 0.5 µM NAA and 50.0 µM GA3 (Table 8). Explant survival was enhanced when an MS basal medium was amended with 5.0 µM BAP, 0.5 µM NAA, and 5.0 µM GA3. The number of surviving explants increased when the concentration of GA3 increased from 0 to 5.0 µM and then decreased as the hormone concentration increased to 50.0 µM, suggesting that GA3 was phytotoxic at this concentration. However, there was no statistical evidence of a significant difference (P≤0.354) in the survival rate.

The number of shoots per explant observed when an MS basal medium was amended with 5.0 µM BAP and 0.5 µM NAA without GA3 (the control) was 1.8. That increased to 2.0 shoots per explant when GA3 was increased to 50.0 µM. An optimum number of shoots per explant (2.7) was observed when GA3 was reduced to 5.0 µM. However, there was no evidence of statistical significance (P≤0.169) in the differences observed.

Leaf development on MS basal medium amended with 5.0 µM BAP and 0.5 µM NAA without GA3 was 7.0 leaves per explant, which was higher than 6.8 leaves per explant observed when an MS basal medium was amended with 5.0 µM BAP, 0.5 µM NAA and 50.0 µM GA3. An increase to 9.3 leaves per explant was observed when an MS basal medium was amended with 5.0 µM BAP, 0.5 µM NAA, and 5.0 µM GA3. Statistically, the differences observed for leaf development were insignificant (P≤0.281).

The height of the shoot followed a similar trend as leaf development. Shoot height was 1.3cm on an MS basal medium that was amended with 5.0 µM BAP and 0.5 µM NAA without GA3. Shoot height increased to 1.4cm on an MS basal medium amended with 5.0 µM BAP, 0.5 µM NAA, and 5.0 µM GA3, while it reduced to 1.2cm when an MS basal medium amended with 5.0 µM BAP, 0.5 µM NAA and 50.0 µM GA3. Although the differences observed in the height of the shoot were not statistically significant (P≤ 0.643), shoot development was vigorous on MS basal medium amended with 5.0 µM BAP, 0.5 µM NAA, and 5.0 µM GA3 (Figure 6).

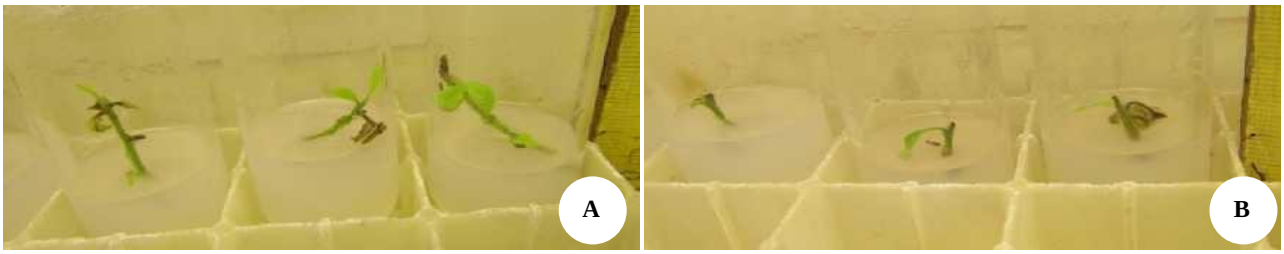
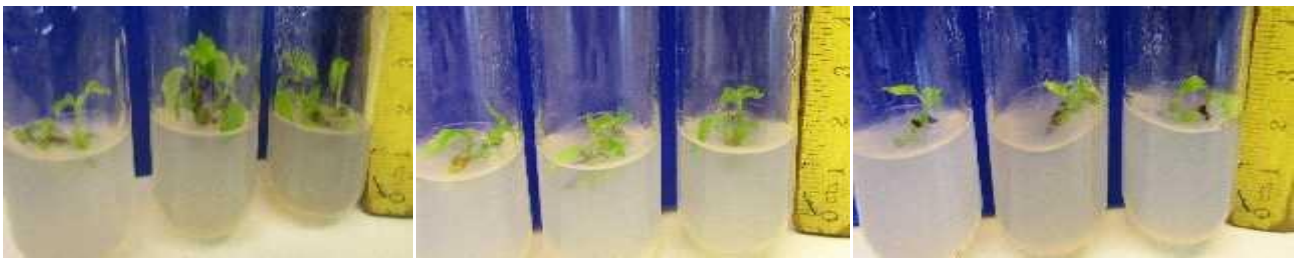


Figure 5. Shoot development from A) shoot-tip and B) nodal-cutting explants at four weeks of culture on MS basal medium amended with 5.0 µM BAP, 5.0 µM NAA, and 5.0 µM GA3



A **B** **C**

Figure 6. Effect of different GA3 concentrations on shoot multiplication of in vitro nodal cuttings; A. 5.0 µM BAP, 0.5 µM NAA and 5.0 µM GA3, B. 5.0 µM BAP and 0.5 µM NAA and C. 5.0 µM BAP, 0.5 µM NAA and 50 µM GA3

Table 7. Response of in vitro nodal-cutting explants to BAP, NAA, and GA3. Data were collected four weeks after the culture

Growth hormone (µM)	Number of explants	Surviving explants	No. of shoot per explant	No. of leaves per explant	Shoot height (cm)
0	10	0.50±0.53^{b}	0.60±0.70^{b}	1.40±1.71^{b}	0.51±0.62^{c}
5.0 BAP	10	0.80±0.42^{ab}	1.40±0.97^{a}	3.70±2.45^{a}	0.84±0.46^{abc}
5.0 BAP +0.5 NAA	10	1.00±0.00^{a}	1.40±0.70^{a}	3.80±3.16^{a}	0.80±0.29^{abc}
5.0 BAP +5.0 NAA	10	1.00±0.00^{a}	1.40±0.52^{a}	3.10±1.10^{ab}	1.03±0.30^{a}
5.0 BAP +5.0 GA3	10	0.80±0.42^{ab}	1.00±0.67^{ab}	2.30±1.42^{ab}	0.63±0.37^{bc}
5.0 BAP +0.5 NAA +5.0 GA3	10	0.90±0.32^{ab}	1.20±0.79^{ab}	3.90±3.67^{a}	0.92±0.49^{ab}
5.0 BAP +5.0 NAA +5.0 GA3	10	0.90±0.32^{ab}	0.90±0.57^{ab}	3.00±1.83^{ab}	0.70±0.62^{abc}

Note: Each value in the table is the mean and standard deviation of ten replicates. Values that do not share a letter in a column differ significantly by LSD ($\alpha = 0.05$)

Table 8. Response of in vitro nodal-cutting explants to different GA3 concentrations on an MS basal medium supplemented with BAP and NAA. Data were collected four weeks after the culture

GA3 concentration (µM)	Number of explants	Surviving explants	No. of shoots per explant	No. of leaves per explant	Shoot height (cm)
0	10	0.80±0.42^{a}	1.80±1.14^{a}	7.00±3.92^{a}	1.33±0.77^{a}
5.0	10	1.00±0.00^{a}	2.70±0.82^{a}	9.30±3.40^{a}	1.44±0.40^{a}
50.0	10	0.80±0.42^{a}	2.00±1.25^{a}	6.80±4.08^{a}	1.19±0.67^{a}

Note: Each value in the table is the mean and standard deviation of ten replicates. Values that do not share a letter in a column are different from each other significantly by LSD ($\alpha = 0.05$)

Discussion

Decontamination of explants

Reports on in vitro propagation of *C. membranaceus* are scanty, with few propagation methods using seeds as explants with low germination rates due to the sterility (Aboagye 1997). However, there are reports on in vitro multiplication of many species belonging to the same genus which have been successfully cultured in vitro (Shibata et al. 1996; Orlikowska et al. 2000; Nasib et al. 2008; Ashish and Sharma 2011; Salamma and Rao 2013). Therefore, the present study was conducted to establish an effective sterilization regime and determine an appropriate totipotent tissue for the rapid multiplication of *C. membranaceus*.

An appropriate sterilization scheme may greatly affect the efficiency of subsequent explants regeneration (Sen et al. 2013; Evtushenko et al. 2016). In addition, the exposure duration to the sterilants may be vital in producing viable explants in vitro (Badoni and Chauhan 2010). These treatments tend to remove pathogens in seeds, nodal cuttings, and shoot tips (Srivastava et al. 2010; Donnaruma et al. 2011; Garg et al. 2011). The explants' contamination depends on plant species, donor age, source, and weather (Srivastava et al. 2010); single or double sterilization regimes may be used depending on the source. Before culture, this study used double sterilization with NaOCl to effectively sterilize explants, including seeds obtained from the field under ambient conditions, which may be highly susceptible to contaminations. Even though NaOCl was used, double sterilization of field-grown *Aloe vera* was effectively decontaminated before culture using 0.1% mercuric chloride and 0.04% streptomycin in 5-6 min (Singh and Sood 2009). Coatless seeds and embryos were not immersed in the sterilant after isolation since they are well protected in intact double sterilized seeds. With double sterilization of explants, 86.7%, 80%, and 100% of intact, coatless seeds and isolated embryos were decontaminated, respectively. The frequency of decontamination was high when seed explants were immersed in ethanol before sterilizing in NaOCl solution. While a lower percentage of NaOCl and more time was optimum for decontaminating intact seeds, a higher percentage of NaOCl, irrespective of time, was optimum for decontaminating seeds before the seed coat removal and isolation of embryos. Thus, the increase in the concentration of sterilant and timing did not improve the decontamination of intact seeds but was necessary when the seed coat ought to be removed. Using 15% NaOCl solution for 20 min, followed by 10% NaOCl, given the optimal decontamination frequency and did not improve when the concentration and time were increased. Pre-treatment with ethanol and double sterilization may have accounted for the high decontamination rates at a lower sterilant concentration. For each treatment A, B, and C, using ethanol in the sterilization regime (A1, B1, and C1) resulted in higher numbers of decontaminated intact seeds than when ethanol was not used before sterilization (A0, B0, and C0). Although the frequency of decontaminated coatless seeds and embryos was higher when isolated from seeds immersed in ethanol before sterilizing in NaOCl solution, the frequency improved with increased NaOCl solution (20%) while the time was either maintained (20 min) or reduced (15 min). A detailed review of work on other croton species revealed that only single sterilization was employed. Salamma and Rao (2013) reported that seeds of *Croton scabiosus* were best sterilized when immersed in 70% ethanol for 60 seconds after sterilizing with 30% Sodium hypochlorite solution for 15 min. Again, effective double sterilization of *Croton variegatum* (Croton) leaf explants, using ethanol pre-treatment and followed by 25% NaOCl and $HgCl_2$, has been reported by Radice (2010). That may support the generally high numbers of decontaminated seeds, coatless seeds, and

isolated embryos obtained when ethanol precedes double sterilization in this study, even though $HgCl_2$ was used as the second sterilization agent by Radice (2010).

The current study also tested the effect of sterilization on explants regeneration. None of the decontaminated intact seeds developed shoots; this failure could be due to factors other than the sterilant. One factor may be the hard seed coat limiting the water uptake to initiate germination and the embryo's size. According to Koornnef et al. (2002), seed germination is influenced by the embryo's growth potential and the limitation caused by the surrounding tissues. The underdeveloped but differentiated embryo size and the presence of a seed coat are reported as the most limiting germination (Finch-Savage and Leubner-Metzger 2006).

Coatless seeds and isolated embryos formed plantlets irrespective of the use or non-use of ethanol and the concentration of NaOCl in the sterilization regime used. Since plantlet formation was not limited by ethanol pre-treatment and decontamination was highest with ethanol pre-treatment for both coatless seeds and isolated embryos, an established sterilization regime for effective decontamination of both explants should include ethanol pre-treatment.

Nodal-cutting explants were decontaminated with double sterilization, irrespective of ethanol as pre-treatment. There was a 100% rate of decontamination with or without ethanol pre-treatment, which established the effectiveness of double sterilization. With double sterilization, 20% NaOCl for 15 or 20 min, followed by 15% NaOCl for 10 or 15 min, was optimum for decontaminating nodal explants. The ineffectiveness of single sterilization with NaOCl was reported by Salamma and Rao (2013). They found that a single application of 5-20% NaOCl was ineffective for surface sterilization of nodal explant of *C. scabiosus* from the wild. A comparison of the three sterilization regimes showed that the higher percentage of 20% NaOCl (treatments B and C) resulted in an optimum number of decontaminated nodal explants (Table 2). However, shoot development from nodal-cutting explants was higher (73.3- 93.3%) when explants were not pretreated with ethanol than (67-80%) pretreated. The low rate of shoot development explants pretreated with ethanol and sterilized with high concentrations of NaOCl could be delicate meristematic tissues that may have been scorched by ethanol and higher concentrations of NaOCl (Sen et al. 2013).

Initiation of cultures from seeds

The successful multiplication of plants in vitro is determined by the type of explant used (Gitonga et al. 2010; Alagumanian et al. 2004; Ali and Mirza 2006; Kumar et al. 2011), the growth regulators (Kumar et al. 2011) in the culture medium and the different levels of endogenous phytohormones present in the plant tissues (Kumar and Reddy 2011). Gitonga et al. (2010) observed varying responses of different explants in culture media with only nodal segments and cotyledons of *Macadamia* spp. form shoots while leaf explants dried up within the first week of culture. This thesis used four explants to initiate in vitro multiplication for Croton. These were intact seeds, coatless seeds, isolated embryos, and nodal cuttings. The explants were grown on media amended with various concentrations and combinations of BAP, NAA, and GA3 and compared with MS basal medium without growth hormone (the control).

Intact seeds did not show any shoot development at four weeks independent of the media composition, except that only one seed developed radicle on an MS medium amended with 5.0 µM BAP and 5.0 µM NAA after twelve weeks. Rather than growth hormones, the hard seed coat may be the limiting factor to shoot development from intact seeds as inadequate water is absorbed, and the comparatively small embryos cannot break through the seed coat. Further, the intact seeds did not develop shoots in this study, which suggests that the media formulated for *C. membranaceus* may require further additives such as peptone to aid shoot development. Even though peptone on seed germination was not investigated, peptone has been reported to support in vitro germination of *Dendrobium lasianthera* (Utami et al. 2017) and *Phalaenopsis* hybrid (Shekarriz et al. 2014).

Development of coatless seeds into plantlets was observed on MS basal medium (the control) and MS basal medium amended with BAP combined with NAA or GA3; those several plantlets depended on hormonal treatment. A high BAP to low NAA concentration ratio gave the highest plantlet formation (Table 4). A similar result on high BAP to low NAA combination has been reported by Vijendra et al. (2017) for *Mentha piperitau*, though shoot-tip and nodal explants were used. Further, a combination of BAP and NAA in MS basal medium has been reported in inducing shoots from different sources of explants, such as embryos in *C. scabiosus* (Salamma and Rao 2013) and callus induction and shoot regeneration of *Lantara camara*, a medicinal plant (Veraplakron 2016). On the other hand, a combination of BAP, NAA, and GA3 did not support plantlet formation from coatless seeds. Similar results were obtained by Masekesa et al. (2016). They reported a near similar observation that 0.5 mg/L (2.7 µM) NAA + 1 mg/L (4.4 µM) BAP and 10 mg/L (28.9 µM) GA3 did not support the induction of shoot from cultured meristems of sweet potato. Therefore, it seems that the presence of GA3 may have an inhibitory effect on NAA for producing shoots from coatless seeds.

Unlike coatless seeds, plantlet development from isolated embryos was highest (Table 6) when cultured on an MS basal medium amended with 5.0 µM BAP and 5.0 µM NAA. Contrary to the present findings, Karami (2016) has reported that the addition of 3mg/L (17.1 µM) IAA+ 0.5 mg/L (2.2µM) BAP, a high auxin to cytokinin ratio enhanced the development of embryos in *C. scabiosus*.

The high regeneration rate of isolated embryos in comparison with coatless seed and intact seed explants may be explained by the fact that embryos have a fully differentiated morphogenetic structure with potential shoots and roots that may undergo full regeneration into matured plants on a medium containing BAP and GA3 (Freitas et al. 2016) as compared to coatless seeds and intact seeds with no well-defined morphogenetic structure.

Further, the use of embryo culture for the regeneration of plantlets from recalcitrant plant species has been reported by Moura et al. (2009) while working with macaw palm (*Arocomia aculeata*).

Initiation of cultures from nodal cuttings and shoot-tips

Considering the type of explants for shoot induction in the current study, explants from nodal segments showed the best regeneration of shoots regarding the number of shoots, leaves per explant, and height. These observations were similar to findings in *Croton bonplandinum*, as reported by Ashish and Sharma (2011). Nodal-cutting explants showed the best response and developed into shoots independent of the concentration of growth hormones in the culture medium. However, the development of multiple shoots was highest, with a mean of 0.8 shoots per explant on an MS basal medium amended with 5.0 µM BAP alone, followed by 0.7 shoots per explant when an MS basal medium was amended with 5.0 µM BAP, 5.0 µM NAA, and 5.0 µM GA3. Multiple shoot induction from nodal cuttings of *C. bonplandinum* was obtained on MS basal medium amended with 0.5mg/L (2.22 µM) BAP alone (Ashish and Sharma 2011). 5.0 µM BAP alone or in combination with 5.0 µM NAA and 5.0 µM GA3 equally promoted leaf development but shoot height was optimum with all three hormones at the same concentration. The addition of GA3 was more effective in the formation of shoot from nodal explants of *C. membranaceus* than BAP alone or BAP combined with NAA as GA3 enhanced the effect of BAP and NAA, resulting in shoot elongation, thereby making the nodes distinct for subculturing. It was therefore established that the combined effects of BAP, NAA, and GA3 on shoot development were the best for nodal-cutting explants of *C. membranaceus*.

However, this is not in line with shoot regeneration from other croton species, as Nasib et al. (2008) reported. They observed that the formation of shoot from nodal cuttings of *Co. variegatum* produced axillary buds on MS basal medium amended with 0.5mg/L (2.22 µM) BAP alone. That may have resulted from the differences in concentration of endogenous hormones in the plant (Schween and Schwenkel 2003) and the type of plant growth hormones being cultured in the medium plant (Thompson et al. 2008). Apart from growth hormones, shoot regeneration depended on the part of the stem segment used. Shoot-tip explants showed faster bud break and rapid shoot growth over nodal cuttings, more leaves, and longer internodes from shoot-tip explants. The response of shoot-tip explants over nodal segments in shoot induction has been observed in *Spilanthes mauritiana* (Sharma et al. 2009) and *Coleus blumei* (Rani et al. 2006) while in *Simarouba glauca*, nodal cuttings showed higher frequencies of axillary bud initiation than shoot-tip explants (Shukla and Padmaja 2014), which may suggest that the response of nodal cuttings and shoot-tips would be species-specific.

Shoot multiplication from in vitro nodal cuttings

The investigation of the effect of various combinations and concentrations of BAP, NAA, and GA3 on the multiplication of in vitro nodal explants showed that multiple shoots were established on MS basal medium with and without growth hormones. An MS basal medium amended with 5.0 µM BAP alone or in combination with 0.5 µM NAA or 5.0 µM NAA resulted in the highest number of shoots per explant. That is comparable to optimum multiple shoot formation on MS medium amended with 5.0 µM BAP and 0.5 µM IAA for shoot multiplication of *Celastrus paniculatus* (De Silva and Senarath 2009). On the contrary, Thangavel et al. (2016), working on the medicinal plant *Janakia arayalpathra*, reported that adding NAA and BAP in shoot multiplication medium induced basal callus formation but suppressed the growth of adventitious shoots. The different responses reported may be related to the genotype, and the level of hormones already existing in the plant part used (Li et al. 2002; Schween and Schwenkel 2003, and Thompson et al. 2008).

In the current study, adding 5.0 µM GA3 to 5.0 µM BAP and 0.5 µM NAA resulted in the highest number of leaves per explant. Further experimentation with two different concentrations (5.0 µM and 50 µM) of GA3 combined with 5.0 µM BAP and 0.5 µM NAA to investigate their effect on the multiplication of *C. membranaceus* in vitro showed the highest shoot regeneration when 5.0 µM GA3 was added and the least when 50 µM GA3 was added. Generally, in culture, GA3 is added in the medium to prevent rosette growth as it enhances shoot elongation and the growth of meristems and buds (Kumar and Reddy 2011). However, the current study observed that the concentration of GA3, in combination with BAP and NAA, had a profound effect on shoot development. This situation conforms to the fact that interaction and ratio between growth hormones in the medium can affect the growth and development of plants in vitro (Kumar and Reddy 2011).

Adding 5.0 µM GA3 to 5.0 µM BAP and 0.5 µM NAA greatly enhanced the number of shoots and leaves per explant and the height of shoots. This study showed that a ten-fold increase in the concentration of GA3 from 5.0 µM to 50 µM was inhibitory to shoot development in *C. membranaceus*. Even though in all reports on the multiplication of other croton species, GA3 was not used, similar findings were observed and reported by Fotopoulos and Sotiropoulos (2004) that the addition of 0.028 to 0.28 µM GA3 enhanced vigorous shoot formation while a ten-fold increase from 0.28 to 2.8 µM GA3 in combination with various concentrations of BAP and NAA resulted in a reduction of the rate of shoot proliferation in *Prunus persica*, indicating that a higher concentration of GA3 may be inhibitory to shoot development.

In conclusion, the current research has established a protocol for effective decontamination of explants for initiation and multiplication of culture of *C. membranaceus* in vitro. Decontamination was achieved using 70% ethanol for 3 min and double sterilization with NaOCl solution.

Different explants responded differently to the double sterilization regimes used. Sterilized intact seeds did not develop into shoots which may be due to inhibitory effects associated with the seed. Explants from nodal sources showed better shoot formation over seed explants, with shoot-tip explants as the best for shoot induction and rapid shoot development. The culture medium for rapid multiplication is an MS medium amended with 5.0 µM BAP, 0.5 µM NAA, and 5.0 µM GA3. The various hormone combinations and concentrations used did not promote the root development of *C. membranaceus*.

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