EFFECTIVE DECONTAMINATION AND MULTIPLICATION OF *Croton membranaceus* Müll. Arg. *IN VITRO*

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THIS THESIS IS SUBMITTED TO THE UNIVERSITY OF GHANA, LEGON IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF MPHIL BOTANY DEGREE

DEPARTMENT OF BOTANY

OCTOBER, 2017

DECLARATION

I hereby declare that work reported in this thesis is my original work except for references to other peoples work which have been duly cited and that the work has neither in whole nor part been presented to this university or elsewhere for the award of any degree.

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DEDICATION

To God be the glory.

ACKNOWLEDGEMENT

I am very grateful to the following persons and institution for their immerse contributions towards the success of this work:

- Dr. Elizabeth Acheampong, (principal supervisor) whose initial ideas led to the choice of the research area. Her personal interest in my work, her guidance and assistance from the beginning to the end of this research cannot be overlooked.
- Dr. Kwadwo Owusu Ayeh (supervisor) who was always available to assist me to organize and present my findings in the best order.
- Prof. L. Enu-Kwesi and Dr. Carol Markwei, whose advise, criticism, and moral support brought out the confidence in me.
- Mrs. Bernice Asante, Mr. Baku, Mr. Akwetey and all the Technical Staff in the Department who willingly assisted me in carrying out the laboratory work smoothly.
- Mad. Gladys Adotey, my mother who sponsored the course.
- Center for Plant Medicine Research, the source of plant materials for the research.

LIST OF ABBREVIATIONS

IAA	Indole-3-acetic acid
BA	Benzyl adenine
BAP	6-Benzylaminopurine
BPH	Benign prostatic hyperplasia
B5	Gamborg medium
CPMR	Center for Plant Medicine Research
GA_3	Gibberellic acid
$HgCl_2$	Mercuric chloride
H_2O_2	Hydrogen peroxide
IBA	Indole-3-butyric acid
MS	Murashige and Skoog
NAA	α-Naphthalene acetic acid
NaOCl	Sodium hypochlorite
WPM	Woody plant medium

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ABSTRACT

Croton membranaceus is a herb with useful medicinal properties. The leaves, bark and roots are used for the treatment of diverse ailments. The harvesting of the plant by many traditional medical practioners from the wild for the treatment of diseases in the country without replacement exposes the plant to possible extinction in the near future. The only means of propagating the plant is by use of seeds which is relatively slow. Thus, an alternative mode of propagation is needed to develop planting materials for nursery and field establishment. Therefore this study aims at determining an effective sterilization regime and subsequent *in vitro* regeneration using different explants.

Intact seeds, coatless seeds, isolated embryos and nodal cutting explants were used to initiate cultures for multiplication of *C. membranaceus*. The explants were decontaminated using double sterilization. Generally, the best sterilization for seed explants was achieved by pre-treatment with 70% ethanol for 3 minutes prior to immersion in sodium hypochlorite (NaOCl). However, intact seeds were effectively decontaminated by immersion in 15% NaOCl solution for 20 minutes followed by 10% NaOCl solution for 15 minutes, whilst coatless seeds were effectively decontaminated when isolated from intact seeds immersed in 20% NaOCl solution for 20 minutes followed by 15% NaOCl solution for 15 minutes. Further, embryos isolated from intact seeds were effectively decontaminated in 20% NaOCl solution for 15 minutes followed by 15% NaOCl for 10 minutes sequentially. With these sterilization regimes, 86% intact seeds, 80% coatless seeds and 100% isolated embryos were successfully decontaminated.

Nodal cutting explants were best decontaminated by immersion in 20% NaOCl solution for 15 minutes followed by 15% NaOCl for 10 minutes sequentially without ethanol pre-treatment. With this sterilization regime, 100% of the nodal cutting explants were successfully

decontaminated. The development of shoots from explants in response to sterilization varied. Intact seeds did not develop into shoots while coatless seeds, isolated embryos and nodal cutting explants developed into shoots independent of sterilization regime. Shoot development was highest with shoot tip explants and when BAP, NAA and GA_3 were added to the medium. Shoot multiplication was best achieved on an MS basal medium amended with 5.0µM BAP, 0.5µM NAA and 5.0µM GA_3.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Importance of *Croton membranaceus*

Croton membranaceus is a plant used singly by almost all the herbal hospitals in the country for the management of benign prostatic hyperplasia (BPH) and other prostate related diseases (Obu, 2015). Several reports have shown that extracts of the plant is effective in the management of BPH (Bayor *et al*., 2007; Salatino *et al*., 2007; Nath *et al*., 2013; Obu, 2015; Asare *et al*., 2015). The root extracts have been found to exhibit general cytotoxicity and growth inhibitory activity against three cancer cell lines in humans; DLD-1(colon), MCF-7(breast) and M14(melanoma) (Bayor *et al*., 2007; Nath *et al*., 2013; Salatino *et al*., 2007; Bayor, 2008). Further report on the potency of the extract from the roots of *C. membranaceus* by (Obu 2015) shows that men with BPH had their prostate gland and prostate symptom scores reduced within two to three weeks after treatment with the extract.

Extract from the root and stem is used at Center for Plant Medicine Research (CPMR), Medi Moses Prostate Center and other herbal centers in Ghana to relieve symptoms of benign prostatic hyperplasia (Afriyie *et al*., 2014; Sarkodie *et al*., 2014). The aqueous root extract which is also used to treat measles (Schmelzer, 2008), is non-toxic yet exhibits anti-atherogenic, cardio protective and anti-ischemic potentials (Afriyie *et al*., 2013). Several studies have also shown that, both ethanolic and aqueous extracts of the roots reduce glucose levels and improve glycemic index in diabetic patients (Afriyie *et al*., 2013; Sarkodie *et al*., 2014; Asare *et al*., 2015). These findings demonstrate the potential of both ethanolic and aqueous extracts of *C. membranaceus* for the treatment of type I diabetes (i.e. diabetes mellitus). This justifies the use of extract from the leaves, in the management of diabetes by traditional healers in Ghana (

Afriyie *et al*., 2013; Sarkodie *et al*., 2014; Asare *et al*., 2015). The root and leaf extracts of *C. membranaceus* are also used to aromatize tobacco in the Bahamas whereas in Nigeria, it is used to improve digestion. The oil from the bark is essential in the treatment of cough, fever, flatulence, diarrhea and nausea (Asare *et al*., 2011; 2015).

1.2 Medicinal properties of *Croton membranaceus*

Croton membranaceus is an economically important medicinal plant. Its medicinal values are derived from the wide range of phytochemicals present in the leaves, stem and roots of the plant. The bark of the root contains scopoletin, julocrotine (a glutarimide alkaloid) and calcium oxalate crystals (Schmelzer, 2008) which make the plant potent in treating benign prostatic hyperplasia, cancer, tumors and diabetes (Aboagye, 1997; Bayor *et al*. 2007; Sarkodie *et al.* 2014). The methanolic extracts of the roots of *C. membranaceus* have been found to contain the active ingredient cajucarinolide which induces apoptosis (programmed cell death) in cancer cells (Block *et al*., 2005). Recent findings by Bayor *et al*., (2009) on the potency of the root extract revealed antihyperglycaemic properties, antimicrobial activity and cytotoxic effects against the prostate cells in human. Both ethanolic and aqueous extracts of the roots contain a compound, N[N-(2-methylbutanoyl) glutaminoyl]-2-phenylethyamine, for the reduction of glucose levels and improved glycemic index in diabetic (especially type I diabetes, i.e. diabetes mellitus) patients (Afriyie *et al*., 2013; Asare *et al*., 2015; Sarkodie *et al*., 2014). In spite of its rich phytochemicals for treatment of several diseases, the plant still grows in the wild with no or little efforts to propagate for commercial utilization.

1.3 Propagation of *Croton membranaceus* in Ghana

The propagation of croton species is by both sexual and asexual means. Asexually, techniques such as cutting, layering, division, budding and grafting are used (Ingram and Yeager, 2003; Relf and Ball, 2009; Lott and Lindgren, 2012; Devi and Shanthi, 2013). In addition to the wide range of propagation modes, few species of the genus (*Codiaeum variegatum, Croton antisyphyliticus, Croton sublyratus, Croton urucurana and Croton bonpladinum*) have been successfully cultured and multiplied *in vitro* (Nasib *et al*., 2008; Kondamudi *et al.*, 2009; Ashish and Sharma, 2011, Oliveira *et al*., 2011). However, *Croton membranaceus* is propagated only sexually by seeds which are released seasonally from the fruit by dehiscence and dispersed (Aboagye, 1997; Lott and Lindgren, 2012). Several attempts in propagating the species on the field at Center for Plant Medicine Research by asexual means specifically stem cutting and air layering yielded a comparatively low result.

1.4 Problem statement

The country report by Ghana to the Food and Agriculture Organization (FAO), (1996) on medicinal plants, indicated that *Croton membranaceus* is one of the rare species that is on the verge of becoming extinct. This is due to the high demand for the plant as a large number of stock plants is required for use in managing and treating diseases such as benign prostatic hyperplasia (BPH), measles, diabetes, cough, fever, flatulence, diarrhea and nausea (Mshana *et al*., 2000; Bayor *et al*., 2009; Asare *et al*., 2011; Devi and Shanthi, 2013;). *C. membranaceus* is treasured for its roots and bark and it is therefore prone to unsustainable harvesting (Abbiw *et al*., 2002). *C. membranaceus* is also prone to bad environmental conditions such as bush fire and

drought in the wild. Several attempts to propagate the plant on a large scale by conventional methods using seeds or stem cuttings has been unsuccessful (Aboagye, 1997). The seeds have low germination rate and it is assumed that most of the seeds are sterile (Aboagye, 1997).

1.5 Justification of the study

There is rising demand for *Croton membranaceus* in the management and treatment of diseases not only in Ghana but elsewhere in the tropics (Asare *et al*., 2011; Afriyie *et al*., 2013). *C. membranaceus* is harvested in the wild for local medicinal use and is endangered due to over exploitation and use as well as exposure to adverse environmental conditions mainly drought and bush fires. Its medicinal potency on the treatment of BPH have increased the demand for the plant (Abbiw *et al*., 2002; Afriyie *et al*., 2014; Sarkodie *et al*., 2014) while no alternative methods of propagation have been established yet. The increasing demand for this very important species is leading to over exploitation which may wipe it out of existence if measures are not taken to ensure sustainable exploitation in the wild. The limited area of confinement and the overexploitation of the species pose a great treat to its extinction hence the need to study alternative mode of propagation using *in vitro* techniques. Apart from using seeds to propagate the species, no report has yet been published on the use of any alternative mode of propagation to curb its extinction. However, since work has been done on the multiplication of some croton species by use of tissue culture techniques (Shibata *et al.,* 1996; Ashish and Sharma, 2011 Salama and Rao, 2013; Silva *et al.,* 2013), it is therefore possible that *C. membranaceus* can be propagated *in vitro* using tissue culture techniques. With efficient *in vitro* multiplication, plantlets will be made available for nursery establishment. There will also be uniform production

of the species and its conservation could be ensured. This will contribute immensely to the establishment of an alternative way of propagating the species on a large scale, prevent the possibility of genetic extinction while adding to existing scientific information on the species.

1.6 Objectives

The major objective of this current study is to use *in vitro* techniques to rapidly multiply *Croton membranaceus*. In order to achieve this, specific objectives have to be accomplished and these are: a) development of a protocol for effective sterilization of the explants, b) identification of the most appropriate totipotent explant for the initiation and proliferation of shoot and c) determination of the growth regulators for *in vitro* rapid multiplication of the species.

1.7 Hypotheses

The hypotheses to be tested are;

Null hypothesis (H_0):

1. The use of ethanol, various concentrations of sodium hypochlorite and its duration in contact with explants will not affect the decontamination and shoot development of explants.
2. Various concentrations and combinations of growth hormones in the medium will not have any significant effect on *in vitro* shoot proliferation and rapid multiplication of the species.

Alternate hypothesis (H_1):

1. The use of ethanol, various concentrations of sodium hypochlorite and its duration in contact with explants will affect the decontamination and shoot development of explants.
2. Various concentrations and combinations of growth hormones will significantly affect *in vitro* shoot proliferation and rapid multiplication of the species.

CHAPTER TWO

2.0 Literature Review

2.1 Description of *Croton membranaceus*

Croton membranaceus is an important plant of medicinal values and belongs to the family Euphorbiaceae. Its genus is referred to as *rush foil* or *croton*. Croton is derived from 'Kroton', meaning ticks in Greek due to the resemblance of the seeds to ticks (Gledhill, 2008; Nath *et al*., 2013). Croton is one of the largest genera of flowering plants described by Carolus Linnaeus in 1737 (Gledhill, 2008; Nath *et al*., 2013). While Gledhill (2008) reported that there are 313 genera in the family and over 8100 species of which 1223 species have been accepted in The World Checklist and Bibliography of Euphorbiaceae, Silva *et al*. (2013) reported that there are over 317 genera and approximately 8000 species of plants in this family. However, some other researchers have reported that there are 1797 croton species with *Croton abaitensis* being the first species and *Croton zeylanians* being the last species (Nath *et al*., 2013). But according to Bingtao *et al*. (1997), there are 322 genera and 8910 species in the family.

The life form categories of species under the genus Croton include herbs, shrubs, trees and occasionally lianas (woody climbers) which are widespread in tropical and subtropical secondary vegetation across the world. *Croton membranaceus* is a monoecious shrub which grows up to 2 meters tall, with slender branches, which are densely stellate and hairy (Figure 2.1). The flowers are unisexual with the inflorescence being axillary or terminal and the female flowers at the base. The fruits are ellipsoidal, slightly tri-lobed and contain a maximum of three seeds. In the wild, seeds are released through split dehiscence from the dry mature pods on the plant (Schmelzer, 2008).

Figure 2.1.*Croton membranaceus* growing on a field at Center for Plant Medicine Research (CPMR), Mampong-Akuapem in the Easter Region of Ghana. Scale bar: 2 cm.

2.2 Habitat

The species of the family Euphorbiaceae are found in the tropics, subtropics and the temperate regions of the world (Silva *et al*., 2013). Croton species are usually found in warmer regions of the world. About sixty five of the species inhabit America while a large number of a hundred and twenty are located in Madagascar (Asare *et al*., 2011). *C. membranaceus* grows in most African countries including Ghana, Cote d'Ivoire, Niger and Nigeria, mainly in the coastal belt

(Aboagye, 1997; Afriyie *et al.*, 2014; Asare *et al.*, 2015). It occurs in moist bush vegetation and savanna, at low altitude. Its distribution is restricted thus, not common (Schmelzer, 2008). In Ghana, it is found in the Krobo-Gyakiti forest reserve area of the Eastern Region (Asare *et al.*, 2015).

2.3 *In vitro* propagation of plants

The propagation of plants *in vitro* is the growing of cells, tissues or organs of plants on a nutrient medium under sterile conditions (George *et al.*, 2007) which leads to the production of true-to-type plants (Kumar and Reddy, 2011). The technique relies on the ability of totipotent cells to develop into a whole new plants and it has been applied extensively for propagation of many plant species on large scale (Varshney and Anis, 2014). *In vitro* propagation is extensively employed to rapidly multiply medicinal plants (Rout *et al*., 2000; Nalawade and Tsay, 2004; Mallick *et al*., 2012; Saini *et al*., 2012; Cheruvathur *et al*., 2015) as well as recalcitrant plant species (Engelmann, 2011). *Viola pilosa*, a plant of medicinal importance has been successfully propagated and conserved *in vitro* by slow growth and vitrification of *in vitro* shoots (Soni and Kaur, 2014). For mass multiplication of non-toxic *Jatropha curcas*, Kumar *et al.* (2011) used *in vitro* propagation through direct organogenesis from petiole explant.

The tissue culture technique can also be used to conserve germplasm (Sidhu, 2010; Patel and Nadgauda, 2014; Varshney and Anis, 2014; Karami, 2016), eliminate systemic pathogens (Chien *et al*., 2015) and produce secondary metabolites (Silva *et al*., 2013). Superior qualities of elite varieties are produced and conserved using *in vitro* propagation (Nalawade and Tsay, 2003; Kumar and Reddy, 2011). Another major advantage of the tissue culture technique is its use for

successful production of disease free plants on large scale through meristem and shoot tip culture (Russo *et al*., 2008; Sarasan *et al*., 2011; Taskin *et al*., 2013). Thus, using tissue culture techniques, Fan *et al*. (2017) produced virus-free garlic for rapid propagation.

In *vitro* propagation involves selection of donor plants, isolation of explants, effective sterilization of explants, initiation and proliferation of shoots, multiplication of *in vitro* established shoots and the formation of full plantlets in a sterile growth-promoting environment (Altman, 2000). The process is completed by acclimatization of plantlets to ambient conditions and field establishment (Kumar and Reddy, 2011).

2.4 Stages involved in *in vitro* propagation of plants

The technique employed for the multiplication of plants *in vitro*, follows several steps which include selection of donor plants, isolation and initiation of explants, multiplication of *in vitro* established cultures, plantlet formation and acclimatization (Kozai and Zobayed, 2000; Kumar and Reddy, 2011). During the first stage 0, donor or stock plants are usually grown in a green house in order to reduce the level of contamination by microorganisms (Sagare *et al*., 2000). This stage is followed by the selection of explants (stage I), i.e. the part of the donor plant used for initiation of culture. Selected explants are then thoroughly sterilized, cultured and incubated under sterile condition (Hussain *et al*., 2012). For example, in establishing an efficient micropropagation protocol for *Moringa oleifera,* seeds obtained from the plants were used as donor plants. The seeds were isolated from the coat, sterilized and cultured on MS basal medium without hormone, for regeneration of plantlets *in vitro* which served as source of leaf explants (Jun-jie *et al*., 2017). Shoot segments of *Salacia chinensis* were also used as donor plants from

which explants were prepared, sterilized and cultured on MS basal medium (Chavan *et al*., 2015). Successfully established cultures are rapidly multiplied for the production of a large number of propagules in the third stage. This is known as the multiplication stage where tissues are repeatedly subcultured under sterile conditions onto fresh media (Kumar and Reddy, 2011). Shoots are formed for subsequent propagation stages and material required for maintaining the stock (George *et al.,* 2007). This is followed by an elongation and rooting stage where full plantlets are developed, to mark the end of *in vitro* stage. The final stage of micropropagation is acclimatization. It is the gradual introduction of *in vitro* plantlets onto the field where they will grow to maturity.

2.5 Factors influencing *in vitro* propagation of plants

The regenerative potential of plants *in vitro* depends on several factors. *In vitro* propagation of plants is greatly affected by explants, type of growth medium and growth conditions for the optimization of tissue culture protocols (Tyagi *et al*., 2001; Gitonga *et al*., 2010; Alagumanian *et al*., 2004; Ali and Mirza, 2006; Kumar and Reddy, 2011; Kumar *et al*., 2011b). Seed, meristem, buds and nodal explants will affect shoot or plantlet development. This may be due to the different concentrations of endogenous growth hormones present in the explants. Explants from meristems of seedlings are more responsive than those obtained from mature plants (Feyissa *et al*., 2005). According to Feyissa *et al.*, 2005, when seedlings of *Hagenia abyssinica* were used as explants, the rate of regeneration was not better when compared to the regeneration rate for explants obtained from younger parts of mature plants. The source of explant is another important factor in the regeneration process. Hence the donor plant from which explants are

obtained must be maintained in a clean and healthy environment in order to minimize contamination of explants (Sagare *et al*., 2000). In order to reduce contamination of explants, cuttings of *Jatropha curcas* were grown in the green house (Rathore *et al*., 2015).

The source of explant may be either *in vitro* or *in vivo*. Usually *in vitro* explants have a better potential for regeneration compared to *in vivo* explant (Reddy *et al*., 2008) as this may depend on the level of endogenous hormones available in the explant. For a highly efficient and sustainable regeneration, leaves of *in vitro* established plantlets were used as explants for multiplication of *Moringa oleifera* (Jun-jie *et al*., 2017). Another factor which is equally important in the morphogenesis of *in vitro* cultures is the role played by the basal medium together with its components, pH of medium, the genotype of the species being cultured, orientation of explant on the medium, temperature, photoperiod and light intensity (Li *et al*., 2002; Kumar and Reddy, 2011).

The type of media used for the propagation of plants *in vitro* largely influences the performance of different parts of the donor plant. Specifically, the mineral composition of media affects the performance of plants *in vitro* (Kumar and Reddy 2011). Although various basal media including White, Nitcsh and Nitsch and Gamborg media (B5) have been used for the propagation of many plant species *in vitro*, Murashige and Skoog (1962) (MS) basal medium has been used most extensively, due to the fact that its optimal nutrient content is essential for the growth of plants (Prakash and Gurumurtthi, 2005; Diallo *et al*., 2008). Among three different media used, Salama and Rao (2013) found out that MS medium showed comparably best result on plantlet regeneration from embryos of *C. scabiosus* than Gamborg (B5) and Woody Plant Medium (WPM). Most plants develop shoots, leaves and roots on MS medium because it contains all the necessary nutrients which have been shown to be useful for the growth of plants (Salama and

Rao, 2013). Plantlets were obtained from coatless seeds of *M. oleifera* when cultured on MS basal medium without plant growth hormones (Jun-jie *et al*., 2017). When MS medium and WPM were used to determine the effect of basal media on the multiplication of shoot, it was observed that shoot formation was enhanced on MS medium than on WPM as the number of leaves, buds and shoot length were highest on MS medium (Nasib *et al*., 2008). Similarly, Nalawaday and Tsay, 2004, reported that the growth of embryonic callus from immature embryos of *Angelica sinensis* was faster on MS medium than it was on B5 and White media. Liang and Keng (2006) also reported that, root formation in *Phyllanthus niruri* was as high as 97-100% when MS basal medium without growth hormone was used for the regeneration of the species *in vitro*.

Regeneration and elongation of shoot are influenced by the genotype of the plant which may be due to varying concentrations of endogenous hormones in the explants, particularly cytokinin concentrations during the initiation period though the exact mechanism not yet been determined (Schween and Schwenkel, 2003). Growth hormones are used for *in vitro* propagation to enhance shoot multiplication (Kumar *et al*., 2011b). Cytokinins stimulate cell division while auxins stimulate cell elongation in plants, thus both must be present to promote growth (Dello *et al.*, 2007; Kumar and Reddy, 2011; Jana *et al.*, 2013) but gibberellins control germination through the use of nutrients stored in the endosperm (Karami, 2016) and promote shoot elongation (Sun, 2010). *In vitro* morphogenesis is controlled by the interaction and ratio between auxin and cytokinin supplemented in the culture medium and the growth hormone produced endogenously (Schween and Schwenkel, 2003; Thompson *et al*., 2008). According to Kumar and Reddy (2011), the type of *in vitro* culture determines the ratio of auxin and cytokinin. This suggests that the balance of hormones is more essential than just the concentration of hormones. Thus, for the

formation of adventitious shoots or roots, the auxin to cytokinin ratio is a necessary requirement. Maximum shoot proliferation rate has been reported for high cytokinin to auxin ratio in peach rootstock (Fotopoulos and Sotiropoulos, 2004). According to Faisal *et al*., (2017), cell division and regeneration of plants *in vitro* is synergistically affected by high levels of cytokinin and low levels of auxins. They reported that the best regeneration of shoot from R*utagra veolens* was achieved on an MS basal medium amended with higher BAP and lower NAA. Contrarily, Salama and Rao (2013) reported that low cytokinin and high auxin concentrations resulted in a considerably high regeneration rate from explants of *Croton scabiosus* but the reverse showed no response.

Gibberellins are generally used for shoot elongation and the growth of meristems or buds of higher plants *in vitro* (Kumar and Reddy, 2011). The interaction of gibberellins and auxins in micropropagated plants synergistically promotes growth in cultures. Though literature on gibberellins-auxin is scanty, Ockerse and Galston (1967) reported that the application of GA_3 and IAA to dwarf cuttings of green peas resulted in a synergistic growth when the concentration of IAA was 5µM. According to Ockerse and Galston (1967), at high concentrations of IAA there was no synergism for IAA-GA_3 interaction. When gibberellins are added to a medium to be used for rooting, the formation of adventitious roots and shoots may either be retarded or prevented though they can stimulate the formation of roots when provided in low concentrations (Kumar and Reddy, 2011).

The regeneration efficiency of explants may also be affected by its orientation in the medium. Horizontally placed explants favour high shoot proliferation than vertically placed explants since the later has little contact with the medium (Sharma and Wakhlu, 2001; Arockiasamy *et al*., 2002; Kumar and Reddy, 2010).

Carbon sources are functional in the synthetic pathway of many compounds, forming the building blocks of macronutrients and thus, may affect many developmental processes in the cell (Karami *et al.*, 2006). The carbon sources which are mainly carbohydrates are very important for regeneration of shoot *in vitro*, a process which requires high energy (Jain and Babbar, 2003). Carbohydrates partly influence plant morphogenesis through their nutritional value and the differences in osmotic potential, which control the rate at which cells divide or the degree of cell development (Sumaryono *et al.*, 2012). The various carbon sources used in *in vitro* culture are sucrose, glucose, maltose and galactose as well as sugar-alcohols glycerol and sorbitol, however, the most common carbon source used in micropropagation of plants is sucrose (Fowler, 2000; Ahmad *et al.*, 2007; Kumar and Reddy, 2011). Sucrose is mostly used as it is cheaper, readily available and stable to sterilization by autoclave and easily assimilated by plants. Thus, sucrose is often the choice of sugar in cell and tissue culture (Jain and Babbar, 2003; Faria *et al.*, 2004). It supplies energy for metabolism which is essential because photosynthesis is limited *in vitro*. For *in vitro* propagation of *Codiaeum variegatum*, *Phyllanthus amarus*, *Croton bonpladinum* and *Jatropha curcas*, 2.5 to 3 percent sucrose was provided in the medium as a source of carbon (Nasib *et al*., 2008; Sen *et al*., 2009; Ashish and Sharma 2011; Sharma *et al.*, 2011).

Another important factor is the pH of the media which is usually adjusted to 5.4 - 5.8. The pH affects the effectiveness of growth hormones on the development of plants (Hussain *et al*., 2012). The use of gelling agents in media also affects propagation of plants *in vitro*. Media solidified with gelling agents slows propagation rate of some species compared to those cultured in liquid medium which is more homogenous (Kumar and Reddy, 2011). Although high concentrations of gelling agents may affect rooting, high concentrations of agar, the preferred gelling agent prevent or reduce hyperhydration in plants (Debergh, 2000).

2.6 *In vitro* multiplication of croton

Investigations on the potential of multiplying croton *in vitro* have been reported by many authors (Silva *et al*., 2013; Salama and Rao, 2013). *Croton sublyratus* has been successfully propagated through shoot tip and leaf culture on MS basal medium amended with 0.2mg/l BA (Shibata *et al*., 1996). *Croton bonpladinum* was multiplied *in vitro* by Ashish and Sharma (2011) while Nasib *et al*. (2008) established an efficient, economical and reliable protocol for the propagation of *Codiaeum variegatum in vitro*. Salama and Rao (2013) propagated *Croton scabiosus* Bedd through *In vitro* callogenensis from leaves, internodes and petioles on MS basal medium amended with NAA and 2, 4-D. Leaf explants were used to induce callus from *Croton urucunara* (Lima *et al*,. 2008). Leaves were also used by Silva *et al*. (2013) for the propagation of *Croton floribundus* on MS basal medium amended with NAA and indole-3-butyric acid (IBA), singly or combined, from which secondary metabolites were produced. The success of work on these species depended on choice of explants, sterilization regimes used, type of media as well as the growth hormones and their concentrations.

Explants commonly used for *in vitro* multiplication of croton species are seeds, embryos, leaves, shoot tips and nodal cuttings. In order to develop a standard protocol for the multiplication of *Croton scabiosus*, an endemic medicinal plant on a large scale, Salama and Rao (2013), used embryos as explants. Shoot tips were used as explants for the propagation of *Codiaeum variegatum in vitro* (Nasib *et al*., 2008) and micropropagation of *Croton sublyratus* (Shibata *et al*.,1996) while nodal stem, intermodal segments and leaves were used for micropropagation of *Croton bonpladinum* (Ashish and Sharma, 2011).

2.7 Decontamination of explants for culture

Successful *in vitro* plant multiplication depends largely on effective decontamination of explants which in turn depends on the concentration of sterilants and duration of which explants are exposed to the sterilizing agents (Sen *et al*., 2013; Evtushenko *et al*., 2016). Some disinfecting agents used for sterilizing explants are sodium hypochlorite (NaOCl), calcium hypochlorite ($Ca(OCl)_2$), ethyl alcohol, hydrogen peroxide (H_2O_2), chlorine gas (Cl_2), sodium dichloroisocyanurate (NaDCC), isothiazolone biocide (PPM) and antibiotics such as gentamicin and ampicillin (Miyazaki *et al*., 2010, 2011). Mercuric chloride ($HgCl_2$) and sodium hypochlorite (NaOCl) are widely used by researchers to prevent microbial growth during *in vitro* multiplication of some species of croton (Nasib *et al*., 2008; Ashish and Sharma, 2011; Salamma, and Rao, 2013). Working on *Croton scabiosus*, Salama and Rao (2013) reported that 30% of NaOCl was the best for surface sterilization of seeds, after washing seeds in running water containing 2% Labolien as surfactant followed by immersion in 70% alcohol for 60 seconds. Shoot tips of *Codiaeum variegatum* were surface sterilized with 0.5% NaOCl containing few drops of Tween-20 as surfactant, for 20 minutes (Nasib et al., 2008). Nodal stem explants, inter nodal segments and leaves from *Croton bonpladinum* were effectively sterilized using 0.1% $HgCl_2$ for 3-5 minutes (Ashish and Sharma, 2011).

2.8 Culture media and growth hormones

Many plant media are available for *in vitro* multiplication of plants. The commonly used media for multiplying croton species *in vitro* include Murashige and Skoog (MS), Gamborg (B5), White's and Woody Plant Medium (WPM). Among these media, MS medium has received wide

usage. For example, MS medium has been used as basal medium to investigate the effect of the removal of leaf and shoot tips as well as the orientation of explant on axillary shoot regeneration of *Codiaeum variegatum* (Orlikowska *et al*., 2000). MS medium was used for dedifferentiation and establishment of callus tissues from nodal, intermodal and leaf segments of *Croton bonpladinum* in culture (Ashish and Sharma 2011). MS medium was successfully used by Salamma and Rao (2013) for embryo culture of *Croton scabiosus*. The most commonly used growth hormones for *in* vitro multiplication of croton are the auxins and cytokinins. MS medium fortified with BAP enhanced multiple shoot proliferation of nodal stem segment of *Croton bonpladinum* (Ashish and Sharma, 2011). IAA and BAP were used by Salamma and Rao (2013) for regeneration of embryos from *Croton scabiosus.* Gibberellins have been used in few cases to enhance internode elongation in some cultured croton species. When GA_3 was combined with BAP used for the regeneration of axillary shoots from *Codiaeum variegatum*, the number of shoots produced doubled (Orlikowska *et al*., 2000). Even though no research findings have yet been reported on *in vitro* multiplication of *Croton membranaceus*, the reports on the success of the propagation of other species of croton *in vitro*, are indications that the species for the current study can be successfully multiplied *in vitro*.

CHAPTER THREE

3.0 Materials and Methods

3.1 Research site

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

3.2 Plant material

Seeds and seedlings of *Croton membranaceus* were obtained from 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 for the development of shoots to be used as explants. Explants used for the research were dry mature seeds with seed coat (intact seeds), seeds without seed coat (coatless seeds), embryos isolated from seeds (isolated embryos) and cuttings (nodal cuttings) from young shoots of *C. membranaceus*.

3.3 Culture medium

Murashige and Skoog (MS) basal medium (1962) (Appendix, Table A.1) was used for the experiment. The ingredients used for the preparation of the medium were produced by Sigma-Aldrich Company, Spain. Stock solutions of macronutrients, micronutrients, vitamins and growth

hormones were prepared based on the composition and quantity of nutrient required. All stock solutions were kept in a refrigerator and stored at 4°C. Media were prepared from stock solutions (Appendix, Table A.2) 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 in to test tubes after which they were sterilized in an autoclave at a temperature of 121°C and a pressure 102.97KPa for 15 minutes. The medium was allowed to cool after autoclaving before used.

3.4 Sterilization of Lab ware

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

3.5 Experiment 1: Decontamination of explants

A decontamination experiment was done using seeds and nodal cuttings to determine the optimal sterilization regime for Croton explants. The effects of ethanol and various concentrations of sodium hypochlorite were determined. Seeds of 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 minutes, followed by 10% NaOCl for 15 minutes, without ethanol pretreatment (A_0) or with ethanol pretreatment (A_1), 20% NaOCl for 15 minutes followed by 15% for 10 minutes, without ethanol pretreatment (B_0) or with ethanol pretreatment (B_1) and 20% NaOCl for 20 minutes followed by 15% NaOCl for 15 minutes, without ethanol

pretreatment (C_0) or with ethanol pretreatment (C_1). Nodal cuttings were first washed under running tap water containing few drops of house hold liquid detergent (Klene lene) containing sodium laureth sulfate for 5 minutes and rinsed with sterile distilled water. The nodal cuttings were further trimmed to a length of 1.5cm with two nodes and then sterilized as described for intact seeds.

Seeds and nodal cuttings were placed in separate bottles, completely immersed in NaOCl solution and carefully agitated by hand. Four drops of household liquid detergent (Klene lene) was added to every 100ml of NaOCl solution used. Seeds and nodal cuttings were thoroughly washed with sterile distilled water in order 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 1cm. Sterilized explants were inoculated on MS basal medium supplemented with 5.0µM BAP and 5.0µM NAA. One explant was inoculated in a test tube containing 10ml of medium. The cultures were incubated in 16 hours light and 8 hours darkness, at a temperature of 26±1^{o}C. Each explant was replicated five times and the experiment repeated three times. The number of decontaminated explants as well as the number of explants that developed shoots or plantlets were recorded.

3.6 Experiment 2: Initiation of cultures

3.6.1 Initiation of cultures from seeds.

Intact seeds were sterilized using the best sterilization regime from decontamination experiment and cultured on MS basal medium supplemented with 100mg/l *myo*-inositol, 0.1mg/l thiamine HCl and varying concentrations of BAP, NAA and GA_3 (Table 3. 1). Coatless seeds and

embryos were obtained from sterilized intact seeds and cultured on MS medium as described for intact seeds.

Table 3.1. Concentrations of BAP, NAA and GA_3 in MS basal medium used for the initiation experiments.

Growth hormones (µM)		
BAP	NAA	GA_3
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

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

3.6.2 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 100mg/l *myo*-inositol, 0.1mg/l thiamine HCl, 5.0µM BAP only, 5.0µM BAP and 0.5µM NAA, 5.0µM BAP and 5.0µM NAA, 5.0µM BAP and 5.0µM GA_3, 5.0µM BAP, 0.5µM NAA and 5.0µM GA_3 or 5.0µM BAP, 5.0µM NAA and 5.0µM GA_3. 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 this 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. The height of shoots was measured with a meter rule. All data were recorded four weeks after culture.

3.6.3 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. 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 minutes and rinsed thoroughly with sterile distilled water. This was followed by double sterilizing with 15% sodium hypochlorite (NaOCl) solution for 20 minutes and 10% NaOCl solution for 15 minutes sequentially. The nodal explants were rinsed thoroughly with sterile distilled water to remove any remaining sterilant on the explants. The sterilized nodal and shoot tip explants were aseptically trimmed to obtain cuttings of 1cm long with two buds each. The sterilized explants

were cultured on solid MS basal medium amended with 100mg/l *myo*-inositol, 0.1mg/l thiamine HCl, 30g/L sucrose without growth hormone, 5.0µM BAP and 5.0µMNAA. One nodal explant was cultured in one test tube which contained 10ml of MS medium and replicated ten times. The cultures were incubated in the growth room in light for 16 hours and in 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 shoot was measured with a meter rule.

3.7 Multiplication of *Croton membranaceus*

Two experiments were conducted to determine an optimum medium for multiplication of *Croton membranaceus*. In experiment one, *in vitro* shoots were subcultured on MS basal medium containing 100mg/l *myo*-inositol, 0.1mg/L thiamine HCl, 30g/l sucrose without growth hormone, with 5.0µM BAP only or in combination with 0.5µM or 5.0µM NAA , 5.0µM GA_3, 0.5µM NAA and 5.0µM GA_3 or 5.0µM NAA and 5.0µM GA_3. One nodal explant was cultured in a test tube and this was replicated ten times. All cultures were incubated under growth room condition of 25 ± 1^0C and light intensity of 3000 Lux for four weeks. After four weeks, the number of cultures that developed shoots was recorded. Also, the number of shoot as well as leaves per culture was counted. The height of shoot was measured with a meter rule.

In experiment two, the response of *in vitro* nodal cuttings of *C. membranaceus* on MS basal medium amended with BAP, NAA and varying concentrations of GA_3 was investigated. *In vitro* shoots were subcultured on MS basal medium amended with 5.0µM BAP and 0.5µM NAA (control) and in combination with 5.0µM GA_3 or 50µM GA_3. One nodal cutting was cultured in a test tube and replicated ten times. All cultures were incubated as described above. The number of

shoot per explant, number of leaves per shoot and the height of shoots were recorded after four weeks.

3.8 Design and data analysis

The experiments were laid out in a Completely Randomized Block Design. Data collected were quantified and analyzed statistically by One-way Analysis of Variance (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.

CHAPTER FOUR

4.0 Results

4.1 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 4.1.

When intact seeds were double sterilized with 15% NaOCl solution for 20 minutes and 10% NaOCl solution for 15 minutes sequentially, the least explant decontamination was 33% and was obtained without pretreatment with ethanol (A_0) while the highest explant decontamination was 86.7% and was obtained when intact seeds were immersed in 70% ethanol for 3 minutes prior to immersion in NaOCl (A_1). 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 solution for 15 minutes and 15% NaOCl solution for 10 minutes sequentially without pretreatment with ethanol (B_0) while the highest explant decontamination was 80% and was obtained when intact seeds were immersed in 70% ethanol for 3 minutes followed by double sterilization with 20% NaOCl solution for 15 minutes and 15% NaOCl solution for 10 minutes sequentially (B_1). With treatment C, the least explant decontamination was 46.7% and obtained when intact seeds were double sterilized with 20% NaOCl solution for 15 minutes and 15% NaOCl solution for 10 minutes sequentially without pretreatment with ethanol (C_0) while the highest explant decontamination was 80% and was obtained when intact seeds were immersed in 70% ethanol for 3 minutes followed by double sterilization with 20% NaOCl solution for 20 minutes and 15% NaOCl solution for 15 minutes sequentially (C_1). None of the intact seeds developed shoots or roots when sterilized with all the sterilization regimes used (Table 4.1).

Table 4.1. Effect of ethanol and various concentrations of sodium hypochlorite (NaOCl) solutions on decontamination of explants. Explants were cultured on MS basal medium amended with 5µM BAP and 5µM NAA.

Treatment	No. of cultures	Intact seeds		Coatless seeds		Isolated embryos		Nodal explants	
		Decontaminated (%)	Plantlets (%)	Decontaminated (%)	Plantlets (%)	Decontaminated (%)	Plantlets (%)	Decontaminated (%)	Plantlets (%)
A_0	15	33.33	0.00	13.33	6.67	73.33	33.33	80.00	93.33
A_1	15	86.67	0.00	66.67	0.00	93.33	40.00	93.33	80.00
B_0	15	60.00	0.00	66.67	13.33	93.33	40.00	100.00	66.67
B_1	15	80.00	0.00	80.00	46.70	100.00	80.00	93.33	66.67
C_0	15	46.67	0.00	73.33	46.70	80.00	60.00	93.33	73.33
C_1	15	80.00	0.00	80.00	26.67	100.00	60.00	100.00	66.67

Note: *A_0 – 15% NaOCl for 20min, 10%NaOCl for 15min*

A_1 – 70% ethanol for 3min, 15% NaOCl for 20min, 10% NaOCl for 15min

B_0 – 20% NaOCl for 15min, 15% NaOCl for 10min

B_1 – 70% ethanol for 3min, 20% NaOCl for 15min, 15% NaOCl for 10min

C_0 – 20% NaOCl for 20min, 15% NaOCl for 15min

C_1 – 70% ethanol for 3min, 20% NaOCl for 20min, 15% NaOCl for 15min

Coatless seeds were least decontaminated (13.7%) when sterilized with treatment A_0 (15% NaOCl for 20min, 10% NaOCl for 15min) but the rate of decontamination increased to 66.67% when coatless seeds were treated with A_1 (70% ethanol for 3min, 15% NaOCl for 20min, 10% NaOCl for 15min) (Table 4.1). With treatment B, 66.67% coatless seeds were decontaminated when sterilized B_0 (20% NaOCl for 15min, 15% NaOCl for 10min) while 80% of the coatless seeds were decontaminated with B_1 (70% ethanol for 3min, 20% NaOCl for 15min, 15% NaOCl for 10min). With treatment C_0 (20% NaOCl for 20min, 15% NaOCl for 15min), 73.33% coatless seeds were decontaminated whereas with C_1 (70% ethanol for 3min, 20% NaOCl for 20min, 15% NaOCl for 15min), 80% decontamination was recorded. There was no development of shoot or root when coatless seeds were treated with A_1, however, when coatless seeds were treated with A_0, 6.7% developed shoots and roots. Shoot development increased (13.33%) with treatment B_0 though this was lower than 26.67% shoot development when coatless seeds were decontaminated with treatment C_1. The optimum number of 46.7% coatless seeds developed shoots and roots when treated with B_1 and C_0.

Embryos isolated from intact seeds sterilized with treatment A_0 (15% NaOCl for 20min, 10% NaOCl for 15min) were least decontaminated 73.3% while with Treatment A_1 (70% ethanol for 3min, 15% NaOCl for 20min, 10% NaOCl for 15min), 93.33% isolated embryos were decontaminated (Table 4.1). When the percentage of NaOCl solution was increased as in B_0 (20% NaOCl for 15min, 15% NaOCl for 10min), the number of decontaminated embryos observed was 93.33% but 100% decontamination was observed when explants were pretreated with ethanol prior to immersion in the same concentration of NaOCl solution for the same time, B_1 (70% ethanol for 3min, 20% NaOCl for 15min, 15% NaOCl for 10min). With treatment C, 80% of isolated embryos were decontaminated when ethanol was not used C_0 (20% NaOCl for

20min, 15% NaOCl for 15min), while an optimum of 100% isolated embryos were decontaminated when sterilized treatment C_1 (70% ethanol for 3min, 20% NaOCl for 20min, 15% NaOCl for 15min). The Embryos decontaminated with the various sterilization regimes developed shoots and roots (Table 4.1). The least number of embryos which developed shoots with roots was 33.3% and this was observed in embryos decontaminated with treatment A_0. The number increased to 40% for embryos treated with A_1 and B_0. A further increase of 60% shoot development was observed for embryos treated with C_0 and C_1 while an optimum of 80% embryos developed into shoots with roots when decontaminated with Treatment B_1 as described earlier.

Nodal cutting explants were least decontaminated (80%) when sterilized with treatment A_0 (15% NaOCl for 20min, 10% NaOCl for 15min) (Table 4.1). Decontamination rate increased (93.33%) when nodal cutting explants were sterilized with treatment A_1 (70% ethanol for 3min, 15% NaOCl for 20min, 10% NaOCl for 15min), B_1 (70% ethanol for 3min, 20% NaOCl for 15min, 15% NaOCl for 10min) and C_0 (20% NaOCl for 20min, 15% NaOCl for 15min). An optimum rate of 100% decontamination was observed when nodal cutting explants were sterilized with treatments B_0 (20% NaOCl for 15min, 15% NaOCl for 10min) and C_1 (70% ethanol for 3min, 20% NaOCl for 20min, 15% NaOCl for 15min). Nodal cutting explants developed shoots with roots when decontaminated using all the sterilization regimes (Table 4.1). However, nodal cutting explants decontaminated with treatments B_0, B_1 and C_1 had the least rate of plantlet regeneration. With these treatments, 66.7% 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 C_0 and A_1 respectively. An optimum number of 93.3% nodal explants developed shoots and roots when nodal explants were sterilized with treatment A_0.

4.2 Response of explants to BAP, NAA and GA_3 in the culture medium

The effect of BAP, NAA and GA_3 in MS basal medium on shoot development is shown in Tables 4.2 to 4.4.

Intact seeds failed to develop into shoots when cultured on MS basal medium amended with BAP alone, BAP with NAA or BAP, NAA and GA_3. However, only one intact seed developed radicle on MS basal medium amended with 5.0µM BAP and 5.0µM NAA after twelve weeks of culture (Figure 4.1).

Figure 4.1. Intact seed explant developing root on MS basal medium amended with 5.0µM BAP and 5.0µM NAA after twelve weeks of culture.

Coatless seed explants developed into shoots nine days after culture on MS basal medium with or without hormone (control). However, the number of shoots developed depended on the combination of growth hormones present in the culture medium (Table 4.2).

Table 4.2. Response of coatless seeds to MS basal medium amended with BAP and NAA. Data was 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 GA_3	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 GA_3	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 GA_3	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 from each other significantly by LSD ($\alpha = 0.05$).

All explants cultured on MS basal medium with 5.0µM 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 GA_3 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 (control) and MS basal medium amended with BAP and NAA only or BAP with GA_3 only (Table 4.2). The mean number of shoot per explant was 0.10 independent of the hormone manipulation and combination in the culture medium. However, the number of leaf and root per explant varied as well as the height of shoot. The mean number of leaves was 0.2 on MS medium without hormone and MS basal medium fortified with 5.0µM BAP and 5.0µM NAA or GA_3. The mean number of leaves increased (0.5) when an MS basal medium amended with

5.0µM BAP and 0.5µM NAA. Statistically, the differences observed were not significant (P≤0.712) (Appendix, Table 6). The mean number of root per explant on MS basal medium without hormone treatment was 0.4. This was higher than the mean number (0.1) observed on MS basal medium amended with 5.0µM BAP and 5.0µM NAA. The mean number of root increased to 0.5 per explant when an MS basal medium was amended with 5.0µM BAP and 5.0µM GA_3 with an optimum of 0.7 root per shoot on MS basal medium amended with 5.0µM BAP and 0.5µM NAA. A statistical analysis showed that the differences observed were not significant (P≤0.700) (Appendix, Table 7). The mean height of shoot was 0.12cm on MS basal medium without hormone and this was higher than 0.10cm and 0.11cm as observed on MS basal medium amended with 5.0µM BAP and 5.0µM NAA or 5.0µM GA_3 respectively. The highest shoot (0.38cm) was obtained on an MS basal medium amended with 5.0µM BAP and 0.5µM NAA (Figure 4.2). However, the differences observed in shoot height was not statistically significant (P≤0.649) (Appendix, Table 8).

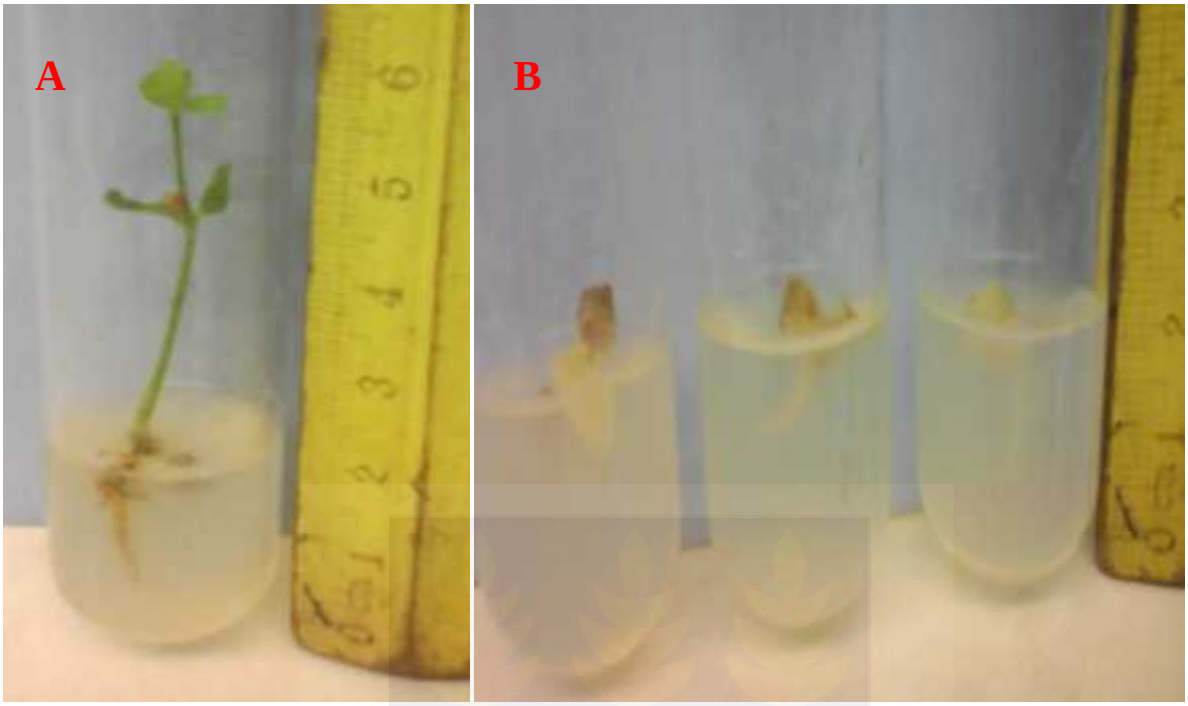

Figure 4.2. Coatless seed explants developing shoots on MS basal medium amended with A) 5.0µM BAP and 0.5µM NAA and B) 5.0µM BAP and 5.0µM NAA four weeks after culture.

Isolated embryos developed into shoots five days after culture. However, the survival rate and development of the shoots depended on combination of growth hormones in the MS medium (Table 4.3).

Table 4.3 Response of isolated embryos to MS basal medium amended with BAP, NAA and GA_3. Data was 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 GA_3	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 GA_3	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 GA_3	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 from each other significantly by LSD ($\alpha = 0.05$).

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 5.0µM BAP only or with 0.5µM NAA did not develop shoots. Shoot development occurred when the concentration of NAA in the culture medium was increased or when GA_3 was added to the medium. Isolated embryos cultured on MS basal medium amended with 5.0µM BAP and 5.0µM GA_3, 5.0µM BAP, 0.5µM NAA and 5.0µM GA_3 or 5.0µM BAP, 5.0µM NAA and 5.0µM GA_3 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 5.0µM BAP and 5.0µM NAA ($P \leq 0.546$) (Appendix, Table 8). Similarly, shoot development was lower with a mean of 0.1 shoot per explant on an MS basal medium amended with 5µM BAP and 5µM GA_3 or together

with either 0.5 or 5.0 µM NAA while an optimum of 0.2 shoot per explant was observed on an MS basal medium amended with 5.0µM BAP and 0.5µM NAA but not statistically different (P≤0.546) (Appendix, Table 9).

Leaf development was least (0.1) when an MS basal medium was amended with 5.0µM BAP, 5.0µM GA_3 and 5.0µM NAA but the number increased to 0.2 when NAA was reduced to 0.5µM. An optimum mean (0.7) was observed when an MS basal medium amended with 5.0µM BAP and 5.0µM NAA or 5.0µM GA_3. Statistically, the differences observed for leaf development were not significant (P≤0.587) (Appendix, Table 10).

Root development was not evident for shoots on MS basal medium amended with 5.0µM BAP and 0.5µM NAA and 5µM GA_3. When an MS basal medium was amended with 5.0µM BAP and 5.0µM GA_3, a mean of 0.2 root per explant was observed. This increased to 0.4 root per explant when an MS basal medium was amended with 5.0µM BAP and 5.0µM NAA and 5.0µM GA_3 while the highest mean of 0.8 root per shoot was observed on an MS basal medium amended with 5.0µM BAP and 5.0µM NAA (Table 4.3). However, the differences observed were not statistically significant (P≤0.590) (Appendix, Table 11).

The height of shoot was least (0.08cm) on MS basal medium amended with 5.0µM BAP, 5.0µM GA_3 and 0.5µM NAA but increased to 0.1cm when NAA was increased to 5.0µM. The height of shoot increased from 0.25cm when an MS medium was amended with 5.0µM BAP and 5.0µM GA_3 to 0.28cm when an MS medium was amended with 5.0µM BAP and 5.0µM NAA. However, the differences observed in the height of shoot were not statistically significant (P≤ 0.757) (Appendix, Table 12).

Thus, only a medium amended with 5.0µM BAP in combination with 5.0µM NAA, 5.0µM GA_3 and 5.0µM BAP in combination with both NAA and GA_3 irrespective of NAA concentration, developed into shoots (Figure 4.3).

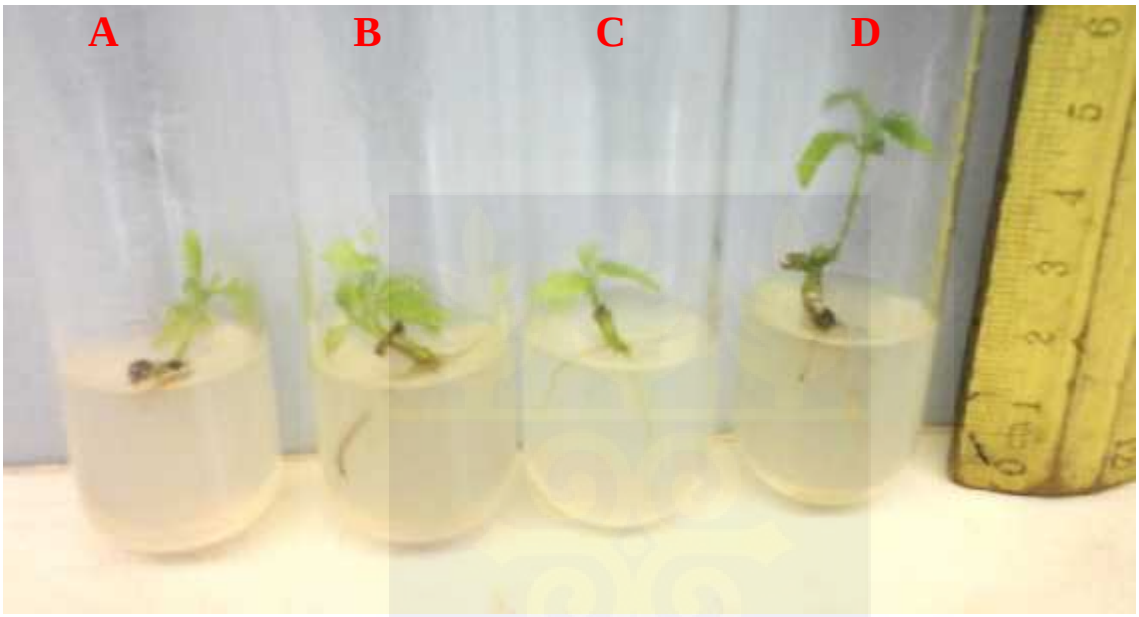

Figure 4.3. Response of isolated embryos to BAP, NAA and GA_3 treatments; A) 5.0µM BAP, 0.5µM NAA and 5.0µM GA_3, B) 5.0µM BAP and 5.0µM GA_3, C) 5.0µM BAP, 5.0µM NAA and 5.0µM GA_3 and D) 5.0µM BAP and 5.0µM NAA. Photos were taken four weeks after culture.

4.3. 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 4.4).

Table 4.4. Effect of MS basal medium amended with BAP, NAA and GA_3 on nodal cutting and shoot tip explants. Data was 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 GA_3	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 GA_3	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 GA_3	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 from each other significantly by LSD ($\alpha = 0.05$).

Explants cultured on MS basal medium without hormone (control) had a surviving rate of 0.3 explant (Table 4.4). This was higher than 0.10 as observed for explants cultured on an MS basal medium amended with 5.0µM BAP and 5.0µM NAA or 5.0µM BAP, 0.5µM NAA and 5.0µM GA_3. When an MS basal medium was amended with 5.0µM BAP and 0.5µM NAA, the survival rate increased to 0.2 explant. A further increase (0.4) was observed when an MS basal medium was amended with 5.0µM BAP only or combined with 5.0µM GA_3. The survival rate was optimum (0.6) when an MS basal medium was amended with 5.0µM BAP, 5.0µM NAA and 5.0µM GA_3. Statistically, the differences observed in the survival of explants were not significant ($P \leq 0.147$) (Appendix, Table A. 13).

The number of shoot 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 5.0µM BAP and 5.0µM NAA or 5.0µM BAP, 0.5µM NAA and 5.0µM GA_3, the number of shoot per explant reduced to 0.1. The number of shoot per explant observed (0.2) when an MS basal medium was amended with 5.0µM BAP and 0.5µM NAA, was still lower than that of the control medium. An increase of 0.6 shoots per explant was observed when explants were cultured on an MS basal medium amended with 5.0µM BAP and 5.0µM GA_3. When an MS basal medium was amended with 5.0µM BAP, 5.0µM NAA and 5.0µM GA_3, 0.7 shoots per explants were observed. The number of shoot was optimum (0.8) when nodal cutting and shoot tip explants were cultured on MS basal medium amended with 5.0µM BAP only. Statistically, the differences observed in shoot development from nodal cutting and shoot tip explants were not significant ($P \leq 0.165$) (Appendix, Table A. 14).

Leaf development was 2.2 leaves per shoot as observed when an MS medium was not amended with hormones (control) (Table 4.4). The number of leaves per shoot reduced to 0.2 when an MS basal medium was amended with 5.0µM BAP, 0.5µM NAA and 5.0µM GA_3. An increase of 0.4 leaves per explant was observed when an MS basal medium was amended with 5.0µM BAP and 5.0µM NAA. This was followed by 0.8 leaves per explant when an MS basal medium was amended with 5.0µM BAP and 0.5µM NAA. Leaf development increased to 2.3 leaves per shoot when an MS basal medium was amended with 5.0µM BAP and 5.0µM GA_3. 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 5.0µM BAP only or 5.0µM BAP, 5.0µM NAA and 5.0µM GA_3. However, the differences observed in leaf development were not statistically significant ($P \leq 0.183$) (Appendix, Table A. 15).

The height of shoot observed on MS basal medium without hormone was 0.61cm (Table 4.4). This was higher than the height of shoot observed on four treatments with growth hormones. When an MS basal medium was amended with 5.0µM BAP, 0.5µM NAA and 5.0µM GA_3, 5.0µM BAP and 0.5µM NAA, 5.0µM BAP and 5.0µM NAA and 5.0µM 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 5.0µM BAP and 5.0µM GA_3 (Figure 4.4c). On this medium, nodal cutting and shoot tip explants grew to the height of 0.64cm. The optimum shoot height (0.80cm) was observed when an MS basal medium was amended with 5.0µM BAP, 5.0µM NAA and 5.0µM GA_3 (Figure 4.4e). Statistically, the differences observed were not significant ($P \leq 0.270$) (Appendix, Table A. 16). However, the overall response of nodal cutting explants to the development of shoots, leaves roots and the height of shoots was optimum on MS basal medium amended with 5.0µM BAP, 5.0µM NAA and 5.0µM GA_3 (Figure 4.4).

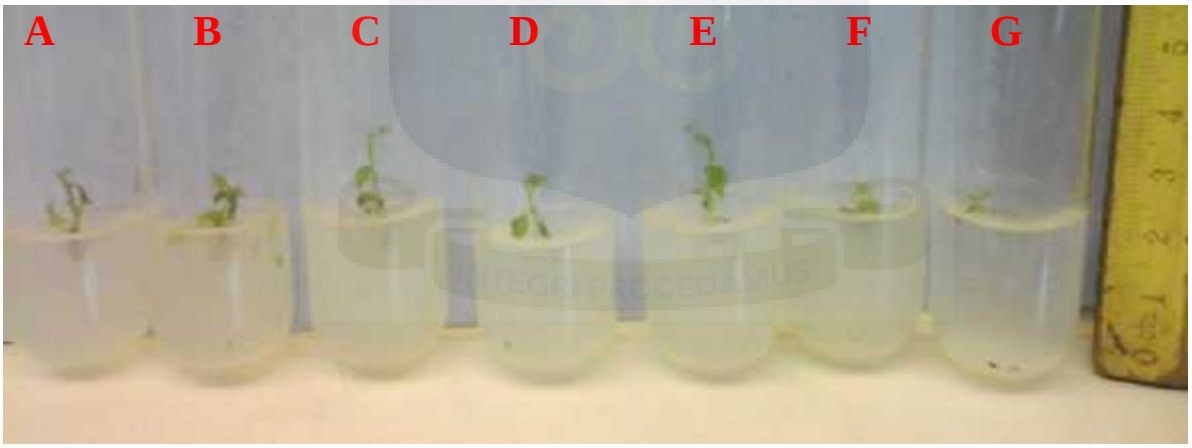

Figure 4.4: Response of nodal explants to BAP, NAA and GA_3 treatments; A) Control, B) 5.0µM BAP only, C) 5.0µM BAP and 5.0µM GA_3, D) 5.0µM BAP and 5.0µM NAA, E) 5.0µM BAP, 5.0µM NAA and 5.0µM GA_3, F) 5.0µM BAP and 0.5µM NAA and G) 5.0µM BAP, 0.5µM NAA and 5.0µM GA_3. Photos were taken four weeks after culture.

4.4 Comparison of nodal cutting and shoot tip explants

The finding that an MS basal medium amended with a combination of the growth hormones 5.0µM BAP, 5.0µM NAA and 5.0µM GA_3, was effective in the response of nodal cutting and shoot tip explants (Table 4.4), 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 5.0µM BAP, 5.0µM NAA and 5.0µM GA_3, 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 4.5).

Table 4.5 Comparison of nodal cutting and shoot tip explants on shoot regeneration of *Croton membranaceus*. Explants were cultured on MS basal medium amended with 5.0µM BAP, 5.0µM NAA and 5.0µM GA_3. Data was 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 from each other significantly by LSD ($\alpha = 0.05$).

The number of surviving explants from nodal cutting explants was not significantly lower (0.50) (P≤ 0.177) (Appendix, Table A.17) than shoot tip explants (0.80).

Similarly, shoot development in nodal cutting explants was lower (0.50) shoots per explant. The number of shoot tip explants which developed into shoots was more than twice (1.20) that of nodal cutting explants. Statistically, there is evidence of significant difference (P≤ 0.031) (Appendix, Table A.17), 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 explants were observed for shoot tip explants. Although the difference observed was more than twice, there was no statistical evidence of significant difference in leaf development for nodal cutting and shoot tip explants (P≤ 0.092) Appendix, Table A.17).

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 significant difference (P≤0.003) (Appendix, Table A.17) in the height of shoot 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 regeneration of shoots better (Figure 4.5).

Figure 4.5. Shoot development from A) shoot tip and B) nodal cutting explants at four weeks of culture on MS basal medium amended with 5.0µM BAP, 5.0µM NAA and 5.0µM GA_3.

4.5 Effect of BAP, NAA and GA_3 on *in vitro* nodal cutting explants.

This experiment was conducted to investigate the effect of BAP, NAA and GA_3 on shoot development using *in vitro* nodal cuttings as explants. Shoot development was observed on MS basal medium without growth hormone as well as MS basal medium amended with BAP, NAA and GA_3 irrespective of the concentration or combination (Table 4.6).

The mean number of explants which survived on MS basal medium without hormonal modification (control) (0.5), was less than the number of surviving explants on MS basal medium amended with growth hormones. When an MS basal medium was amended with 5.0µM BAP only or in combination with 5.0µM GA_3, 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 5.0µM BAP, 5.0µM GA_3 combined with either 0.5µM or 5.0µM NAA. The highest mean of surviving explant was 1.0 and this was observed on an MS basal medium amended with either 5.0µM BAP and 0.5µM NAA or 5.0µM BAP and 5.0µM NAA. However, there was no statistical evidence of significant difference ($P \leq 0.187$) (Appendix, Table A.18) in the number of surviving explants.

The mean number of shoot per explant observed on an MS basal medium without hormone (0.6), was the least. This increased to 0.9 when MS medium was amended with 5.0µM BAP, 5.0µM NAA and 5.0µM GA_3. A further increase (1.0) was observed when an MS medium was amended with 5.0µM BAP and 5.0µM GA_3 or (1.2) when an MS medium was amended with 5.0µM BAP, 0.5µM NAA and 5.0µM GA_3. The highest mean number of shoot per explant (1.4) was observed on an MS medium amended with 5.0µM BAP only and 5.0µM BAP combined with either 0.5µM or 5µM NAA. Statistically, the differences observed were not significant ($P \leq 0.121$) (Appendix, Table A.19)

Leaf development was least (1.4) on MS medium without hormone. When 5.0µM BAP and 5.0µM GA_3 were added to an MS medium, leaf development increased to 2.3 leaves per shoot. When an MS basal medium was amended with 5.0µM BAP, 5.0µM NAA and 5.0µM GA_3, or 5.0µM BAP and 5.0µM 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 5.0µM BAP only was added to an MS medium, 3.8 when 5.0µM BAP and 0.5µM NAA were added to an MS basal medium and the highest mean number of leaves per explant (3.9) were observed when an MS basal medium was amended with 5.0µM BAP, 0.5µM NAA and 5.0µM GA_3. However, there was no

statistical evidence of significant difference (P≤ 0.193) (Appendix, Table A.20) in the number of leaves per explant on each MS medium.

The height of 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 5.0µM BAP and 5.0µM GA_3. The height of shoot increased to 0.70cm on an MS basal medium amended with 5.0µM BAP, 5.0µM NAA and 5.0µM GA_3. A further increase in shoot height (0.80cm) was observed when an MS basal medium was amended with 5.0µM BAP and 0.5µM NAA while a mean height of 0.84cm was observed when an MS basal medium was amended with 5.0µM BAP only. A mean shoot height of 0.92cm was observed on an MS basal medium amended with 5.0µM BAP, 0.5µM NAA and 5.0µMGA_3 with an optimum mean height of 1.03cm observed when an MS basal medium was amended with 5.0µM BAP and 5.0µM NAA. Statistically, the differences observed were not significant (P≤0.156) (Appendix, Table A.21).

Table 4.6. Response of *in vitro* nodal cutting explants to BAP, NAA and GA_3. Data was 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	0.50 ± 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 GA_3	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 GA_3	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 GA_3	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 from each other significantly by LSD ($\alpha = 0.05$).

4.6: Effect of different GA_3 concentrations on shoot multiplication of *in vitro* nodal cuttings

Modification of an MS basal medium amended with 5.0µM BAP and 0.5µM NAA with either 5.0µM GA_3 or 50µM GA_3 had different effects on shoot development of *in vitro* nodal cutting explants (Table 4.7).

Table 4.7. Response of *in vitro* nodal cutting explants to different GA_3 concentrations on an MS basal medium supplemented with BAP and NAA. Data was collected four weeks after culture.

GA_3 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 different from each other significantly by LSD (α = 0.05).

The mean number of surviving explants on MS basal medium amended with 5.0µM BAP and 0.5µM NAA without GA_3 (control) was 0.8 and the same was observed when an MS basal medium was amended with 5.0µM BAP, 0.5µM NAA and 50.0µM GA_3 (Table 4.7). Explant survival was enhanced when an MS basal medium was amended with 5.0µM BAP, 0.5µM NAA and 5.0µM GA_3. The number of surviving explants increased when the concentration of GA_3 was increased from 0 to 5.0µM and then finally decreased as the hormone concentration was increased to 50.0µM suggesting that GA_3 was phytotoxic at this concentration. However, there was no statistical evidence of significant difference ($P \leq 0.354$) (Appendix, Table A.22) in the survival rate.

The number of shoot per explant observed when an MS basal medium was amended with 5.0µM BAP and 0.5µM NAA without GA_3 (control), was 1.8. This increased to 2.0 shoots per explant when GA_3 was increased to 50.0µM. An optimum number of shoot per explant (2.7) was observed when GA_3 was reduced to 5.0µM. However, there was no evidence of statistical significance ($P \leq 0.169$) (Appendix, Table A.23) in the differences observed.

Leaf development on MS basal medium amended with 5.0µM BAP and 0.5µM NAA without GA_3 was 7.0 leaves per explant. This was higher than 6.8 leaves per explants observed when an MS basal medium was amended with 5.0µM BAP, 0.5µM NAA and 50.0µM GA_3. An increase to 9.3 leaves per explant was observed when an MS basal medium was amended with 5.0µM BAP, 0.5µM NAA and 5.0µM GA_3. Statistically, the differences observed for leaf development were not significant (P≤0.281) (Appendix, Table A.24).

The height of shoot followed a similar trend as leaf development. Shoot height was 1.3cm on an MS basal medium was amended with 5.0µM BAP and 0.5µM NAA without GA_3. Shoot height increased to 1.4cm on an MS basal medium amended with 5.0µM BAP, 0.5µM NAA and 5.0µM GA_3 while it reduced to 1.2cm when an MS basal medium amended with 5.0µM BAP, 0.5µM NAA and 50.0µM GA_3. Although, the differences observed in the height of shoot were not statistically significant (P≤ 0.643) (Appendix, Table A.25), shoot development was vigorous on MS basal medium amended with 5.0µM BAP, 0.5µM NAA and 5.0µM GA_3, (Figure 4.6).

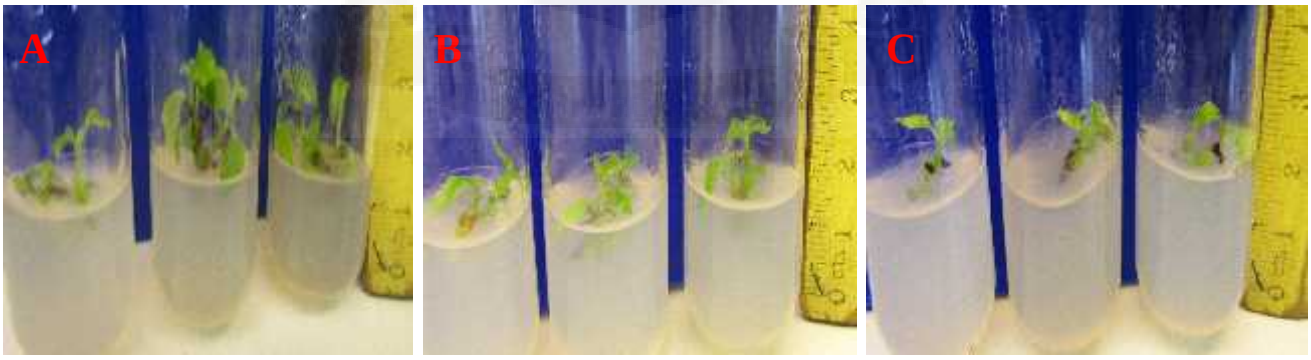

Figure 4.6: Effect of different GA_3 concentrations on shoot multiplication of *in vitro* nodal cuttings; A) 5.0µM BAP, 0.5µM NAA and 5.0µM GA_3, B) 5.0µM BAP and 0.5µM NAA and C) 5.0µM BAP, 0.5µM NAA and 50µM GA_3.

CHAPTER FIVE

5.0 Discussions

5.1 Decontamination of explants

Reports on *in vitro* propagation of *Croton membranaceus* are scanty, with few propagation methods using seeds as explants with low germination rate due to the sterility of the seeds (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* (Orlikowska *et al*., 2000; Shibata *et al*., 1996; Nasib *et al*., 2008; Ashish and Sharma, 2011; Salamma and Rao, 2013). The present study was therefore conducted to establish an effective sterilization regime and determine an appropriate totipotent tissue for rapid multiplication of *C. membranaceus* plant.

The selection of appropriate sterilization scheme may have a great effect on the efficiency of subsequent regeneration of explants (Sen *et al*., 2013; Evtushenko *et al*., 2016). In addition, the duration of exposure of explants to the sterilants may equally be vital in producing viable explants for *in vitro* propagation (Badoni and Chauhan, 2010). These treatments tend to remove pathogens which may be present in explant such as seeds, nodal cuttings and shoot tips (Donnaruma *et al*., 2011; Srivastava *et al*., 2010; Garg *et al*., 2011). Further, the contamination of explants depends on such factors as plant species, the age of the donor plant, source of explant and the existing weather conditions (Srivastava *et al*., 2010). Depending on the source of explants, single or double sterilization regimes may be used for decontamination. In this study, double sterilization with NaOCl was used to effectively sterilize explants including seeds, prior to culture. This was necessary as explants were obtained from the field under ambient conditions and may be highly susceptible to contaminants. Even though NaOCl was used in this study, double sterilization of field grown *Aloe vera* has been effectively decontaminated prior to culture

using 0.1% mercuric chloride and 0.04% streptomycin in a time duration of 5-6 minutes (Singh and Sood, 2009). Coatless seeds and embryos were not immersed in the sterilant after isolation since they are well protected in the intact seeds which were double sterilized. With double sterilization of explants, 86.7%, 80% and 100% of intact seeds, coatless seeds and isolated embryos were respectively decontaminated. The frequency of decontamination was high when seed explants were immersed in ethanol prior to sterilizing in NaOCl solution. While a lower percentage of NaOCl solution and more time was optimum for decontaminating intact seeds, a higher percentage of NaOCl solution irrespective of time was optimum for decontaminating seeds prior to the removal of the seed coat and isolation of embryos. Thus, the increase in concentration of sterilant and timing did not improve decontamination of intact seeds but necessary when the seed coat ought to be removed. The use of 15% NaOCl solution for 20 minutes followed by 10% NaOCl solution gave the optimal decontamination frequency and did not improve when the concentration and time were increased. Pretreatment with ethanol and double sterilization may have accounted for the high decontamination rates at a lower concentration of the sterilant. For each treatment A, B and C, the use of ethanol in the sterilization regime (A_1, B_1 and C_1) resulted in higher numbers of decontaminated intact seeds than when ethanol was not used prior to sterilization (A_0, B_0 and C_0). Although the frequency of decontaminated coatless seeds and embryos was higher when isolated from seeds immersed in ethanol prior to sterilizing in NaOCl solution, the frequency improved with increased NaOCl solution (20%) while the time was either maintained (20 minutes) or reduced (15 minutes). A detailed review of work on other croton species revealed that only single sterilization was employed. Salama 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 minutes. Again, effective double sterilization of *Croton variegatum* (croton) leaf explants, using ethanol pretreatment and followed by 25% NaOCl and $HgCl_2$ has been reported by Radice (2010). This 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 regeneration of explants. None of the decontaminated intact seeds developed shoots. The failure of the intact seeds to develop shoots could be due to factors other than the effect of the sterilant. One factor may be the presence of the hard seed coat limiting the uptake of water from the medium needed to initiate the germination process. It could also be due to the size of the embryo. According to Koornnef *et al*. (2002), seed germination is influenced by the potential of the embryo to grow and the limitation caused by tissues surrounding it. The size of the embryo which may be under developed but differentiated as well as the presence of seed coat are reported as limiting to the germination of most seeds (Finch-Savage and Leubner-Metzger, 2006).

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

Nodal cutting explants were effectively decontaminated with double sterilization irrespective of the use of ethanol as pretreatment or not. There was 100% rate of decontamination with or without ethanol pretreatment. This established the effectiveness of double sterilization of the

nodal cutting explants. With double sterilization, 20% of NaOCl solution for 15 or 20 minutes followed by 15% NaOCl solution for 10 or 15 minutes was optimum for decontaminating nodal explants. The ineffectiveness of single sterilization with NaOCl solution had been reported by Salama and Rao (2013) who found out that single application of 5-20% NaOCl solution was not effective for surface sterilization of nodal explant of *Croton scabiosus* obtained from the wild. Comparison of the three sterilization regimes used showed that the higher percentage of 20% NaOCl solution (treatments B and C) resulted in optimum number of decontaminated nodal explants (Table 4.1). However, shoot development from nodal cutting explants was higher (73.3-93.3%) when explants were not pretreated with ethanol than (67-80%) when explants were pretreated with ethanol. The low rate of shoot development when explants were pretreated with ethanol and sterilized with high concentrations of NaOCl could be due to the fact that meristematic tissues are delicate and may have been scorched by ethanol and higher concentrations of NaOCl (Sen *et al*., 2013).

5.2 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*., 2011b), the growth regulators (Kumar *et al*., 2011b) 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 that varying responses of different explants in culture media with only nodal segments and cotyledons of *Macadamia* spp form shoots whilst leaf explants dried up within the first week of culture. In this thesis, four explants were used 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 GA_3 and compared with MS basal medium without growth hormone (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 5.0µM BAP and 5.0µM NAA after twelve weeks of culture. Rather than growth hormones, the hard seed coat may be the limiting factor to shoot development from intact seeds as inadequate amount of water is absorbed and the comparatively small embryos were unable to break through the seed coat. Further, the intact seeds did not develop shoots in this study suggesting that the media formulated for *C. membranaceus* in this study may require further additives such as peptone to aid shoot development from seeds. Even though the use of peptone on seed germination was not investigated in this study, peptone has been reported to support *in vitro* germination of *Dendrobium lasianthera,* an endangered medicinal plant (Utami *et al*., 2017) and *Phalaenopsis* hybrid (Shekrriz *et al*., 2014).

Development of coatless seeds into plantlets was observed on MS basal medium (control) and MS basal medium amended with BAP combined with NAA or GA_3. The number of plantlets depended on hormonal treatment. A high BAP to low NAA concentration ratio gave the highest plantlet formation (Table 4.3). A similar result on using 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 have been reported in inducing shoots from different sources of explants such as embryos in *Croton scabiosus* (Salamma and Rao, 2013) and callus induction and shoot regeneration of *Lantara camara,* a medicinal plant (Veraplakron, 2016). A combination of BAP, NAA and GA_3 did not

support plantlet formation from coatless seeds. Similar results were obtained by Masekesa *et al.* (2016) who reported a near similar observation that 0.5mg/l (2.7 µM) NAA + 1 mg/l (4.4 µM) BAP and 10 mg/l (28.9 µM) GA_3 did not support the induction of shoot from cultured meristems of sweet potato. It seems, therefore, that the presence of GA_3 may be having an inhibitory effect on NAA for the production of shoots from coatless seeds in this study.

Unlike coatless seeds, plantlet development from isolated embryos was highest (Table 4.5) when cultured on an MS basal medium amended with 5.0µM BAP and 5.0µM NAA. Contrary to the present findings, Karami (2016) has reported that addition of 3mg/l (17.1µM) IAA+ 0.5 mg/l (2.2µM) BAP, a high auxin to cytokinin ratio enhanced the development of embryos in *Croton scabiosus*.

The high regeneration rate of isolated embryos in comparison with coatless seed and intact seed explants in this research 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 GA_3 (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 regeneration of plantlets from recalcitrant plant species have been reported by Moura *et al*., (2009) whilst working with macaw palm (*Arocomia aculeata*).

5.3 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 in regards to the number of shoots, leaves per explant and the height of shoots. These observations from this study were similar to findings in

Croton bonpladinum 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 5.0µM BAP alone. This was followed by 0.7 shoots per explant when an MS basal medium was amended with 5.0µM BAP, 5.0µM NAA and 5.0µM GA_3. Multiple shoot induction from nodal cuttings of *Croton bonplandinum* were obtained on MS basal medium amended with 0.5mg/l (2.22µM) BAP alone (Ashish and Sharma, 2011). 5.0µM BAP alone or in combination with 5.0µM NAA and 5.0µM GA_3 equally promoted leaf development but shoot height was optimum with all three hormones at the same concentration. The addition of GA_3 was more effective in the formation of shoot from nodal explants of *C. membranaceus* than BAP alone or BAP combined with NAA as GA_3 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 GA_3 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 reported by Nasib *et al*. (2008), who observed that the formation of shoot from nodal cuttings of *Codiaeum variegatum* produced axillary buds on MS basal medium amended with 0.5mg/l (2.22µM) BAP alone. This 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 in the medium in which the plant is being cultured (Thompson *et al*., 2008). Apart from growth hormones, shoot regeneration depended on the part of stem segment used. Shoot tip explants showed faster bud break and rapid shoot growth over nodal cuttings for the same period of time. There were 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 suggests that the response of nodal cuttings and shoot tips could be species specific.

5.4 Shoot multiplication from *in vitro* nodal cuttings

The investigation of the effect of various combinations and concentrations of BAP, NAA and GA_3 on 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 5.0µM BAP alone or in combination with 0.5µM NAA or 5.0µM NAA resulted in the highest number of shoots per explant. This is comparable to optimum multiple shoot formation on MS medium amended with 5.0µM BAP and 0.5µM 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 the addition of 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, addition of 5.0µM GA_3 to 5.0µM BAP and 0.5µM NAA resulted in the highest number of leaves per explant. Further experimentation with two different concentrations (5.0µM and 50µM) of GA_3 combined with 5.0µM BAP and 0.5µM NAA to investigate their effect on the multiplication of *C. membranaceus in vitro*, showed the highest shoot regeneration

when 5.0µM GA_3 was added and the least when 50 µM GA_3 was added (Table 4.13). Generally in culture, GA_3 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). From the current study, it was observed that the concentration of GA_3 in combination with BAP and NAA, had a profound effect on shoot development, a situation which conforms to the fact that an interaction and ratio between growth hormones in the medium can have an effect on the growth and development of plants *in vitro* (Kumar and Reddy, 2011).

The addition of 5.0µM GA_3 to 5.0µM BAP and 0.5µM NAA greatly enhanced the number of shoots and leaves per explant as well as the height of shoots. In this study, it was shown that a ten-fold increase in the concentration of GA_3 from 5.0µM to 50µM was inhibitory to shoot development in *C. membranaceus.* Even though in all reports on the multiplication of other croton species, GA_3 was not used, similar findings were observed and reported by Fotopoulos and Sotiropoulos, (2004) that the addition of 0.028 to 0.28µM GA_3 enhanced vigorous shoot formation whilst a ten-fold increase from 0.28 to 2.8µM GA_3 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 GA_3 may be inhibitory to shoot development.

5.5 Conclusions

The current research has established a protocol for effective decontamination of explants for initiation and multiplication of culture of *Croton membranaceus in vitro*. Decontamination was achieved using 70% ethanol for 3 minutes 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 source 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 5.0µM BAP, 0.5µM NAA and 5.0µM GA_3. The various hormone combinations and concentrations used did not promote root development of *C. membranaceus*.

5.6 Recommendations

Although this study has established protocol for sterilization and initiation of *Croton membranaceus*, further investigations are needed to;

(i) Increase the number of shoots to enhance rapid multiplication

(ii) Identify the effect of other growth regulators on shoot development.

(iii) Stimulate root development in the developed shoots

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APPENDIX

Table A.1. Composition of MS basal medium used for culture initiation and multiplication.

Constituents	Concentration (mg/l)
Macronutrients	
NH_4NO_3	1,650
KNO_3	1,900
$CaCl_2.2H_2O$	450
$MgSO_4.7H_2O$	370
KH_2PO_4	170
$FeSO_4.7H_2O$	29.0
Na_2EDTA	37.3
Micronutrients	
H_3BO_3	6.2
$MnSO_4.H_2O$	22.3
$ZnSO_4.7H_2O$	8.6
$Na_2MoO_4.2H_2O$	0.25
$CuSO_2.5H_2O$	0.25
$CoCl_2.6H_2O$	0.25
KI	0.83
Vitamins	
Myo-inositol	100
Thiamine HCl	0.1
Carbohydrate source	
Sucrose	30,000
Growth hormones	
BAP	
NAA	
GA_3	
Agar	8500

Table A.2. Required volumes of stock solutions used for preparing MS basal medium.

Stock	Reagent	Amount in stock (g/l)	Volume of stock (ml)	Final volume in medium (ml/l)
1	NH_4NO_3 KNO_3 $MgSO_4.7H2O$ KH_2PO_4	82.5 85.0 16.5 8.5	100	20
2	H_3BO_3 $MnSO_4.H_2O$ $ZnSO_4.7H_2O$ $NaMoO_4.2H_2O$ $CuSO_4.5H_2O$ $CoCl_2.6H_2O$	0.62 2.176 0.86 0.025 0.0025 0.0025	100	1
3	KI	0.075	100	1
4	$CaCl_2.2H_2O$	15.0	100	3
5	Na_2EDTA $FeSO_4.7H_2O$	0.001492 0.001114	200	5
6	Thiamine-HCl	0.01	200	5
7	*Myo*-inositol	0.8	200	6.75

Table A.3. One – way Analysis of Variance for the survival of coatless seeds on MS basal medium amended with BAP, NAA and GA_3.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Accession	6	0.1714	0.02857	0.50	0.806
Error	63	3.6000	0.5714		
Total	69	3.7714			

Table A.4. One – way Analysis of Variance for the development of shoot in coatless seeds on MS basal medium amended with BAP, NAA and GA_3.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Accession	6	0.1714	0.02857	0.50	0.806
Error	63	3.6000	0.5714		
Total	69	3.7714			

Table A.5. One – way Analysis of Variance for the development of leaves in coatless seeds on MS basal medium amended with BAP, NAA and GA_3.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Accession	6	1.971	0.3286	0.62	0.712
Error	63	33.300	0.5286		
Total	69	35.271			

Table A.6. One – way Analysis of Variance for the development of roots in coatless seeds on MS basal medium amended with BAP, NAA and GA_3.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Accession	6	4.971	0.8286	0.64	0.700
Error	63	81.900	1.3000		
Total	69	86.871			

Table A.7. One – way Analysis of Variance for the height of shoot in coatless seeds on MS basal medium amended with BAP, NAA and GA_3.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Accession	6	1.089	0.1815	0.70	0.649
Error	63	16.281	0.2584		
Total	69	17.370			

Table A.8. One – way Analysis of Variance for survival of isolated embryos on MS basal medium amended with BAP, NAA and GA_3.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Treatment	6	0.3429	4.05714	0.84	0.546
Error	63	4.3000	0.06825		
Total	69	4.6429			

Table A.9. One – way Analysis of Variance for the development shoot in isolated embryos on MS basal medium amended with BAP, NAA and GA_3.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Treatment	6	0.3429	4.05714	0.84	0.546
Error	63	4.3000	0.06825		
Total	69	4.6429			

Table A.10. One – way Analysis of Variance for the development of leaves in isolated embryos on MS basal medium amended with BAP, NAA and GA_3.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Treatment	6	3.743	0.6238	0.78	0.587
Error	63	50.200	0.7968		
Total	69	53.943			

Table A.11. One – way Analysis of Variance for the development of roots in isolated embryos on MS basal medium amended with BAP, NAA and GA_3.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Treatment	6	5.600	0.9333	0.78	0.590
Error	63	75.600	1.2000		
Total	69	81.200			

Table A.12. One – way Analysis of Variance for the height of shoot in isolated embryos on MS basal medium amended with BAP, NAA and GA_3.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Treatment	6	1.110	0.1850	0.56	0.757
Error	63	20.637	0.3276		
Total	69	21.747			

Table A.13. One – way Analysis of Variance for the survival of nodal cuttings on MS basal medium amended with BAP, NAA and GA_3.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Treatment	6	2.000	0.3333	1.65	0.147
Error	63	12.700	0.2016		
Total	69	14.700			

Table A.14. One – way Analysis of Variance for the development of shoot in nodal cuttings on MS basal medium amended with BAP, NAA and GA_3.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Treatment	6	5.143	0.8571	1.59	0.165
Error	63	34.000	0.5397		
Total	69	39.143			

Table A.15. One – way Analysis of Variance for the development of leaves in nodal cuttings on MS basal medium supplemented with BAP, NAA and GA_3.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Treatment	6	93.29	15.55	1.53	0.183
Error	63	640.50	10.17		
Total	69	733.79			

Table A.16. One – way Analysis of Variance for the height of shoot in nodal cutting explants on MS basal medium amended with BAP, NAA and GA_3.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Treatment	6	4.847	0.8079	1.30	0.270
Error	63	39.124	0.6210		
Total	69	43.971			

Table A.17. Two-sample T-Test for the performance of nodal cutting and shoot tip explants on MS basal medium amended with BAP, NAA and GA_3 for regeneration shoot in *Croton membranaceus*.

Source	DF	T-Test of difference	T-value	P-Value
Surviving cultures	18	0	-1.41	0.177
Shoot per explant	18	0	-2.33	0.031
Leaf per explant	18	0	-1.78	0.092
Shoot height	18	0	-3.40	0.003

Table A.18. One – way Analysis of Variance for the survival of *in vitro* nodal cutting explants on MS basal medium amended with BAP, NAA and GA_3.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Treatment	6	1.171	0.1952	1.52	0.187
Error	63	8.100	0.1286		
Total	69	9.271			

Table A.19. One – way Analysis of Variance for the development of shoot from *in vitro* nodal cutting explants on MS basal medium amended with BAP, NAA and GA_3.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Treatment	6	5.800	0.9667	1.77	0.121
Error	63	34.500	0.5476		
Total	69	40.300			

Table A.20. One – way Analysis of Variance for the development of leaves from *in vitro* nodal cutting explants on MS basal medium amended with BAP, NAA and GA_3.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Treatment	6	49.94	8.324	1.50	0.193
Error	63	350.00	5.556		
Total	69	399.94			

Table A.21. One – way Analysis of Variance for the height of shoot of *in vitro* nodal cutting explants on MS basal medium amended with BAP, NAA and GA_3.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Treatment	6	1.878	0.3130	1.62	0.156
Error	63	12.171	0.1932		
Total	69	14.049			

Table A.22. One – way Analysis of Variance for the survival of *in vitro* nodal cutting explants on MS basal medium amended with BAP, NAA and different concentrations of GA_3.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Treatment	2	0.2000	0.10000	1.08	0.354
Error	27	2.5000	0.09259		
Total	29	2.7000			

Table A.23. One – way Analysis of Variance for the development of shoot from *in vitro* nodal cutting explants on MS basal medium amended with BAP, NAA and different concentrations of GA_3.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Treatment	2	4.467	2.233	1.90	0.169
Error	27	31.700	1.174		
Total	29	36.167			

Table A.24. One – way Analysis of Variance for the development of leaves from *in vitro* nodal cutting explants on MS basal medium amended with BAP, NAA and different concentrations of GA_3.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Treatment	2	38.60	19.30	1.33	0.281
Error	27	391.70	14.51		
Total	29	430.30			

Table A.25. One – way Analysis of Variance for the height of shoot of *in vitro* nodal cutting explants on MS basal medium amended with BAP, NAA and different concentrations of GA_3.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Treatment	2	0.3500	0.1750	0.45	0.643
Error	27	10.5170	38.95		
Total	29	10.8670			

Table A.9. Growth hormones added to the MS basal medium.

Growth hormones	Molecular weight	µM for 1 mg/l
BAP	225.3	4.44
GA_3	346.4	2.89
NAA	186.2	5.37

Source: Sigma Aldrich, A part of Merck. Growth Regulators-Plant Tissue Culture Protocol; www sigmaaldrich.com