ASSESSMENT OF NUTRITIONAL VARIATION AND ESTABLISHMENT OF AN EFFICIENT TISSUE CULTURE PROPAGATION PROTOCOL FOR *Vitex doniana* Sweet (LAMIACEAE) FROM BENIN (WESTERN AFRICA)

COLOMBE DADJO

MASTER OF SCIENCE
(MOLECULAR BIOLOGY AND BIOTECHNOLOGY)

PAN AFRICAN UNIVERSITY
INSTITUTE FOR BASIC SCIENCES TECHNOLOGY AND INNOVATION

2014

Assessment of Nutritional Variation and Establishment of an Efficient Tissue Culture Propagation Protocol for *Vitex doniana* Sweet (Lamiaceae) from Benin (Western Africa)

Colombe Dadjo

MB 300-014/12

A Thesis submitted to Pan African University Institute for Basic Sciences Technology and Innovation in partial fulfillment of the requirements for the degree of

Master of Science in Molecular Biology and Biotechnology

2014

DECLARATION

This thesis is my original work and has not yet been presented to any other university for examination.

Signature ..... Date.....

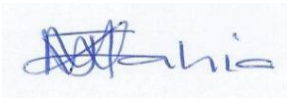
Colombe Dadjo

This thesis report has been submitted for examination with our approval as University supervisors.

Signature……Date……..

Dr Daniel Sila

Food Sciences and Technology Department, Jomo Kenyatta University of Agriculture and Technology

Signature  Date……..

Dr Jane W. Kahia

Somatic Embryogenesis Scientist, World Agroforestry Centre (ICRAF)

DEDICATION

To my beloved mother,

My late father,

My brother and sisters

ACKNOWLEDGEMENTS

I would like to express my gratitude to the African Union through the Pan African University for awarding me a scholarship and for funding my research.

My special thanks and gratitude to my supervisors Dr Daniel Sila and Dr Jane Kahia who have guided this thesis and whose useful comments and fruitful suggestions improved the manuscript considerably. I appreciate the efforts of Dr Jane Kahia who dealt with all administrative matters related to my stay in Ivory Coast; I sincerely thank her for her supportive kindness and excellent guidance.

My sincere gratitude goes to Dr. Kouassi Modeste, Scientist at Tissue Culture laboratory of "Centre National de Recherche Agronomique" Cote d'Ivoire for various suggestions which have helped to improve this work.

I am most grateful to the staff in the Somatic embryogenesis laboratory of the World Agroforestry center (ICRAF), country Program Cote d'Ivoire and the technicians of Food biochemistry laboratory of Jomo Kenyatta University of Agriculture and Technology (JKUAT) for assisting me during my laboratory work and in collecting the data.

I cannot forget the continuous support from all the teaching and administrative staff of Pan African University Institute of Basic Sciences, Technology and Innovation and Jomo Kenyatta University of Agriculture and Technology, especially Prof Naomi Maina, program coordinator Molecular Biology and Biotechnology.

Last but not least, I am grateful to my parents who have supported me in various ways throughout my studies.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

%:	Percent
°C :	Degree Celsius
2, 4-D:	Dichlorophenoxy – acetic acid
2iP:	[N6 - (2- isopentyl) adenine]
3, 4-D:	Trichlorophenoxy – acetic acid
AACC:	Approved methods of American Association of Cereal Chemists
AC:	Activated charcoal
$AgNO_{3:}$	Silver nitrate
ANOVA:	Analysis of variance
AVGSAN:	Analyse Globale de la Vulnérabilité, de la Sécurité Alimentaire et de la Nutrition
AVRDC:	Asian Vegetable Research and Development Center
BAP/BA:	Benzylamino purine
CH:	Casein hydrolysate
conc.:	Concentration
CW:	Coconut water
eq.:	Equation
FAO:	Food and Agriculture Organization
H_2O:	Water (dihydrogen monoxide)
HCl:	Hydrochloride acid

HNO_3:	Nitric acid
HPLC:	High Performance Liquid Chromatography
IBA:	Indol-3- butyric acid
ICRAF:	World Agroforestry Center
JKUAT:	Jomo Kenyatta University of Agriculture and Technology
kg/ cm^2:	Kilogram per centimeter square
Kin:	Kinetin (6-furfurylaminopurine)
KNO_3:	Potassium nitrate
MS:	Murashige and Skoog medium
N:	Normal
Na_2SO_4:	Sodium sulphate
NAA:	1-naphtylacetic acid
NaOH:	Sodium hydroxyde
NUS:	Neglected and underutilized wild Species (NUS)
OECD:	Organization for Economic Co-operation and Development
TDZ:	Thidiazuron
V. doniana:	*Vitex doniana*
v/ v:	Volume by volume
WHO:	World Health Organization

ABSTRACT

Black plum (*Vitex doniana)* tree is an indigenous wild species important for the livelihoods of rural populations in Benin. It is highly nutritious and rich in phytochemical compounds of health benefit. In Benin, the greatest economic potential of *Vitex doniana* trees probably lies in the leaves. It has emerged as a potential species for domestication. Despite some studies indicating that nutritional properties can vary according to the sample provenances, no data on nutritional variation of *V. doniana* leaves are available in Benin. On the other hand, the conventional methods of propagating the plant produce inadequate number of planting materials as seeds of this tree have a very weak germinating capacity and the macropropagation rate by stem cuttings is slow. Tissue culture is a reliable alternative method. Therefore, the objective of the current work was to evaluate the nutritional variation of leaves collected from different agro climatic zones and to establish a feasible *in vitro* propagation method for *V. doniana* using both somatic embryogenesis and micropropagation. During the current study, proximate, minerals and vitamin A and C concentrations were analyzed. In the Tissue Culture experiments, sterilization was evaluated using different concentrations of a commercial bleach, mercuric chloride and calcium hypochlorite at varying time intervals. For somatic embryogenesis, different growth regulators and additives were evaluated. In the micropropagation experiments, microshoots were regenerated using BAP and roots using IBA at concentrations 5, 10, 20, 40 µM. Results showed that *V. doniana* young leaves are highly nutritious. Protein, Calcium, vitamin A and C concentration vary significantly across agro climatic zones. The highest values of

Protein, Vitamin A and C were obtained in the Sudanian zone and the highest value of calcium was obtained in the Sudano-Guinean zone. The highest number (91%) of clean leaf explants were obtained when the leaf explants were subjected to sterilization using 2% calcium hypochlorite for 30 minutes followed by a second sterilization using 2% hypochlorite for 15 minutes (double sterilization). The highest number (40%) of clean nodal explants were obtained when the explants were subjected to sterilization using 2% calcium hypochlorite for 45 minutes followed by a second sterilization using 2% hypochlorite for 30 minutes. Inclusion of 4 µM Picloram, in the medium was observed to increase the mean number of embryos per explant. Of the four amino acids tested, only tryptophan evaluated at 150 µM had significant effect on the induction of somatic embryogenesis with 6.5 embryos per explant. On the other hand, silver nitrate and casein hydrolysate at 50 µM were found to enhance the number of embryos per explant. Liquid pulsing treatment for 24 h produced the highest (9.19) mean number of embryos compared to the control (1.2). The highest mean number of shoot/explant and the highest mean length of shoot was obtained in media supplemented with 10 µM BAP. Rooting was achieved using IBA. No optimum concentration of IBA was found for root regeneration. The protocols developed during this study would be useful for the mass propagation, and genetic transformation of selected elite lines. This work provides useful information that can be used to increase domestication of *V. doniana* in Benin. However, further investigations need to be done on the bioavailability of nutrients from the leaves of *V. doniana* and the conversion and the performance in the field of both embryo-derived plantlets and plantlets obtained from micropropagation.

CHAPTER 1 INTRODUCTION

1.1 Background

Plant genetic resources are essential for a sustainable agriculture and food security. In the past, many indigenous wild species played a crucial role in the food security, nutrition, health, income generation and food culture of the rural poor (Magbagbeola *et al.*, 2010). However, this is no longer the case. The lack of attention has meant that their potential value is under-estimated and under-exploited. It also places them in danger of continued genetic erosion and possible disappearance. Agricultural biodiversity (agro biodiversity) has steadily declined with a corresponding increase in dependence on a small number of food crops (Moore, 2010).

The Food and Agriculture Organization of the United Nations (FAO) estimates that humans have used some 10,000 species for food throughout history. However, only about 120 cultivated species provide around 90% of food requirements and 4 species (Maize, Wheat, Rice and Potatoes) provide about 60% of human dietary energy for the world's population (Muzzalupo *et al.*, 2012). Many neglected and underutilized wild species (NUS) are nutritionally rich (Ghane *et al.*, 2010; Johns and Eyzaguirre, 2006). Therefore, their erosion can have immediate consequences on the nutritional status and food security of the poor and their enhanced use can bring about better nutrition and fight hidden hunger. Even though the link between agro biodiversity and diet diversity is not automatic (Burchi *et al.*, 2011), it is agreeable that the diminution of agro biodiversity, to some extent, places considerable strain on the ease with which households are able to enjoy diversified,

balanced diets. Awareness of the importance and value of crop wild relatives and of the need to conserve them has increased. Accordingly, a number of initiatives have come forth in recognition of the importance of diversified diets notably the 2003/2004 joint Food and Agriculture Organization/World Health Organization (FAO/WHO) consultations, all of which acknowledged, explicitly or implicitly, the indispensable role of diet diversification for enhanced food security and nutrition outcomes. Food system interventions that involve carefully formulated multi-sectorial activities along the food chain- from food production to consumption and utilization (Burchi *et al.*, 2011) have been advanced as key to sustaining gains being made by short-term micronutrient control measures because they simultaneously address multiple nutrient and phytochemical needs for optimal health (Underwood, 2000). Crop diversity contribute to the stability and sustainability of farming systems and are valued for providing important attributes including *inter alia* agronomic characteristics, biotic and abiotic stresses and other factors of cultural and socio-economic importance. In addition, crop diversity contributes as direct or indirect source of several products, such as medicines, life-saving drugs, vitamins, minerals, various industrial products. Crop diversity also provide an insurance against unknown future needs/conditions as these are likely to hold still undiscovered cures for known and emerging diseases and is a fortune that can be tapped, as human needs change. FAO through the Second Global Plan of Action for the conservation and sustainable Utilization of Plant Genetic Resources for Food and agriculture supports activities improving *in situ* and *ex situ* conservation of NUS (FAO, 2011). *In situ* conservation enables to preserve evolutionary processes that generate new germplasm under conditions of natural

selection, maintain important field laboratories for crop biology and biogeography. It also serves as a continuous source of germplasm for *ex situ* conservation. *Ex- situ* conservation refers to the conservation of germplasm away from its natural habitat. This complementary approach for conservation had begun on a wide scale about three decades ago and is now practised, to some extent, in almost all countries as a means to conserve crop species diversity for posterity. This strategy is particularly important for crop gene pools, and can be achieved by propagating/ maintaining the plants in genetic resource centre, botanical gardens, tissue culture repositories or in seed gene banks (OECD, 1999).

About 187 leafy vegetables were reported in Benin by Dansi *et al.* (2008) whilst the Darwin Initiative 15/003 surveys recorded 245 plants species which include leafy, fruit and seed vegetables (Achigan-Dako *et al.*, 2010).. These species, mostly underutilized are potentially rich and important for rural populations. *Vitex doniana* (Laminaceae) is one of the high-priority NUS for domestication in Benin. It is the tallest and most frequent Pan African Vitex species, occurring from Senegal east to Somalia and south to South Africa; Comoros and Seychelles (Louppe *et al.*, 2008). It can make a great contribution to the local, regional and national economy of many West African countries (Codjia *et al.*, 2003).

1.2 Statement of Problem

V. doniana is among the Neglected and Underutilized Species (NUS) known due to their nutritional and medicinal properties (Orwa *et al.*, 2009; Dadjo *et al.*, 2012). Despite the widely known nutritional, medicinal and economic uses of *V. doniana* products, the species is still under- utilized, unimproved. Numerous studies have been conducted on the nutritional concentration of *V. doniana* fruit (Olulosa, 1992; Glew *et*

al., 1997; Solebo and Aina, 2011; Vunchi *et al.*, 2011; Osum *et al.*, 2013). However, very little information is available on the nutlritional quality of the leaves. Moreover, despite some studies indicating that nutritional properties can vary according to the sample provenances (Solebo and Aina, 2011; Assogbadjo *et al.*, 2012) no data on nutritional value of *V. doniana* leaves are available in Benin. The strong anthropic pressure affecting this species has caused its numbers to fall increasingly in its natural environment (Achigan-dako *et al.*, 2010). The planting of seedlings is negligible and the seeds of this tree have a very weak germinating capacity (Louppe *et al.*, 2008). Sanoussi *et al. (*2012) showed that macropropagation rate by stem cutting is slow. To date, there are no reports on work carried out on tissue culture of the species.

1.3 Justification

The World Food Program comprehensive food security and vulnerability analysis conducted in 2008 estimated that nearly one million people in Benin (10% of the population) are food-insecure and nutrition insecure (AGVSAN, 2009). *V. doniana* is distributed in all the climatic zones of Benin and has a great importance in the diet of rural population. More recently, the species was chosen as a model species to be domesticated in Benin (Codjia *et al.*, 2003). The fruit of *Vitex doniana* is sweet and tastes like prunes. It can be made into a jam and wine. Leaves are often used as vegetables for cooking (Achigan-Dako *et al.*, 2010; Dadjo *et al.*, 2012). The different parts of *Vitex doniana* are used in traditional medicine (Dadjo *et al.*, 2012; Orwa *et al.*, 2009). In Benin, the greatest economic potential of *Vitex doniana* trees probably lies in the leaves (Achigan-dako *et al.*, 2010). It is the most consumed vegetables during the dry season but also one of the most traded wild vegetables in Benin (Codjia *et al.*, 2003; Achigan-Dako *et al.*, 2010). Considering the importance *V.*

doniana in people's diet and for medicinal uses in Benin, it can be exploited at the commercial level. The leaves, roots, bark and fruits can be used to get plant extracts that may be helpful in overcoming nutritional related health problems among other disorders/diseases that are predominant in the many rural areas of the country. It has been demonstrated that the nutritional diversity of the plant is largely dependent on the plant part and the geographic region from which the sample is obtained from (Solebo and Aina, 2011). Therefore, elucidation of the nutritional characteristics of the leaves collected from different phytoclimatic zones in Benin would be a critical input for the selection of appropriate genotypes for cultivation and breeding purposes.

On the other hand, tissue culture is a reliable and advanced propagation method that could be more promising than the conventional propagation methods (Singh *et al.*, 2013). In addition, tissue culture ensures mass production of elite clones from hybrid or specific parental lines. Tissue culture ensures healthy seedlings with desirable characters. The tissue culture of forest trees has shown promise in obtaining regenerants and clonal multiplication for domestication of wild populations, afforestation and economically important trees that have been cultivated for generations. The application of *in vitro* culture for propagation of *V. doniana* tree will assist in sustainable availability of propagules and plant products. In that perspective, the characterization of nutritional variability for suitable material selection for tissue and the establishment of an efficient tissue culture propagation protocol for *V. doniana* presents a very important point in the biotechnology field, especially in the domestication process of the species in Benin.

1.4 Objectives

1.4.1 Main objective

To enhance the domestication of *Vitex doniana* in Benin by characterizing the nutritional variation of the species and establishing the optimum conditions for *in vitro* propagation of the species.

1.4.2 Specific objectives

The specific objectives are to:

i. Determine the nutritional variation of *Vitex doniana* by assessing the proximate composition, minerals, vitamin A and C content of its leaves collected from different climatic zones.

ii. Determine the optimum sterilization procedure for *V. doniana* explants;

iii. Evaluate effect of growth regulators on direct and indirect somatic embryogenesis in *V. doniana*;

iv. Compare effects of different cytokinin/auxin in regenerating microshoots and roots from *V. doniana* nodal explants.

1.5 Hypotheses

i. There is no significant variation in nutritional composition of *Vitex doniana* leaves according to the different climatic zones.

ii. There is no optimum sterilant concentration for sterilization of explants.

iii. There is no optimum cytokinin/auxin concentration for induction of somatic embryos.

iv. There is no optimum cytokinin/auxin concentration for optimum shooting and rooting of *Vitex doniana*.

v.

CHAPTER 2 LITERATURE REVIEW

2.1 Botanical description of *Vitex doniana*

2.1.1 Taxonomy and structure

Vitex doniana Sweet (Black plum) belongs to Vitex L. genus. The genus Vitex has been considered in Verbenaceae family by different authors but in recent works it has been transferred into Lamiaceae based on different evidences (Wagstaff *et al.*, 1998). *Vitex doniana* Sweet (syn. *Vitex cuneata* Thonn.) is the most widespread Vitex species in Africa. *V. doniana* is a diploid species, with basic chromosome number of (2n = 32). *V. doniana* is a medium-sized deciduous tree, 8-18 m high occasionally 25m, with a heavy rounded crown and a clear bole up to 5 m. Its bark is rough, pale brown or greyish-white, rather smooth with narrow vertical fissures. The bases of old trees have oblong scales.

2.1.2 Leaves

Leaves are opposite, glabrous and are 14-34 cm long. They are usually with 5 leaflets on stalks of 6-14 cm long. Leaflets distinctly stalked, ovate, obovate-elliptic or oblong, entire, are 8-22 cm long, and 2-9 cm wide.

Plate 2.1 *Vitex doniana* leaves

2.1.3 Flowers

V. doniana exhibits hermaphroditism, where both functional male and female organs are in the same flower (Orwa *et al.*, 2009). The flower petals are white except on largest lobe, which are purple, in dense opposite and axillary cymes. Flowers are numerous and small, blue or violet, 3-12 cm in diameter, only a few being open at a time. Calyx and pedicels are densely hairy.

Plate 2.2 *Vitex doniana* inflorescence

2.1.4 Fruits

The fruits are oblong, about 3 cm long and they are green when young, turning purplish-black on ripening and with a starchy black pulp. Each fruit contains 1 hard, conical seed, of 1.5-2 cm long, and 1-1.2 cm wide (Orwa *et al.*, 2009).

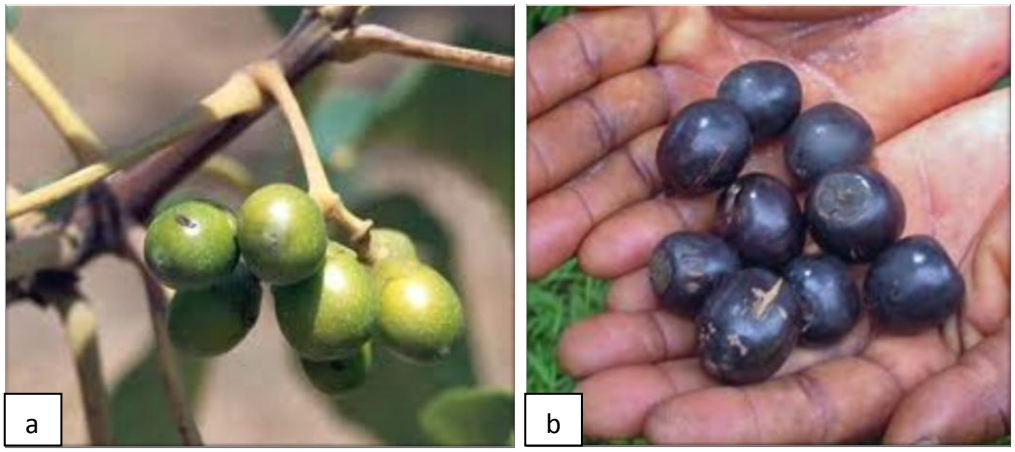


Plate 2. 3 *Vitex doniana* fruit (a) unripe (b) ripe

2.2 Ecology

The wide geographical distribution of *V. doniana* reflects its wide ecological tolerance. *V. doniana* occurs in a variety of habitats, from forest to Savannah, often in wet localities and along rivers, and on termite mounds, up to 2000 m altitude. It is a deciduous forest tree of coastal woodland, riverine and lowland forests and deciduous woodland, extending as high as upland grassland (Orwa *et al.,* 2009). It occurs in regions with a mean annual rainfall of 750–2000 mm and requires a high water table. Optimum growth is achieved when mean temperatures are between 10^{o}C and 30^{o}C. When it grows in open areas it develops a very thick bark which acts as protection against fire. In swamps it has a much smoother, thinner bark. It occurs on a variety of soils of varying origins but most commonly found on alluvial soils.

2 .3 Uses and nutritional value of *V. doniana*

2.3. 1 Food uses

The fruits and leaves are the edible part of the trees. They are either eaten raw or after processing. The blackish pulp of the fruit is edible and sweet. It is eaten raw and tastes like prunes; (Dadjo *et al.* 2012; Orwa *et al.* 2009; Louppe *et al.*2008). It is also used for juice, syrup, wine, liquor and jam production (Orwa *et al.* 2009; Louppe *et al.* 2008). Jam prepared from the fruits showed no significant difference in flavour, colour, and overall acceptability from commercial plum jam. The black plum jam is even preferred for consistency and spreadability. The syrup made from the pulp can be used instead of other syrups as a nutritive sweetener. The seed inside the fruits stone is edible (Louppe *et al.* 2008). Cooked young leaves are used as vegetable or in

sauces (Dadjo *et al.* 2012). The pounded leaves can be added to warm filtered grain beer and then drunk (Louppe *et al.*2008).

2.3.2 Medicinal uses

Vitex doniana is widely used in traditional system of medicine. The leaves, fruits, roots, barks and seed of the plant have been used as medication for liver disease, anodyne, stiffness, leprosy, backache, hemiplegia, conjunctivity, rash, measles, rachitis, febrifuge, as tonic galactagogue to aid milk production in lactating mothers, sedative, digestive regulator and treatment of eye troubles, kidney troubles. It has also been used for treatment of disease conditions such as infertility, anaemia, jaundice, dysentery, gonorrhea, headaches, diabetes, chickenpox, rash and fever (Dadjo *et al.* 2012; Orwa *et al.* 2009, Louppe *et al.* 2008; Iwueke 2006). Leaf sap is used as an eye drop to treat conjunctivitis and other eye complaints (Dadjo *et al.* 2012; Orwa *et al.* 2009; Louppe *et al.* 2008). The leaf is used against headache, stiffness, measles, rash fever chickenpox, hemiplegia, febrifuge, and to treat respiratory diseases. Paste and pounded leaves are applied to wound and burns. Leaves infusions are added to alcoholic drink to make them stronger. Dried and fresh fruits are eaten against diarrhea, dysentery, jaunice, anaemia, leprosy (Orwa *et al.* 2009). The bark is used against leprosy, bleeding after child birth, liver diseases. The powdered bark is added to water and taken to treat colic. The bark extract is used to treat kidney diseases and stomachache. A root decoction is administered orally to treat ankylostomiasis, rachitis, gastro-intestinal disorders, jaundice and as anodyne (Louppe *et al.* 2008).

2.3.3 Nutritional composition of *V. doniana* leaves

Olusola (1992) found that all the essential amino acids were present in the leave of *V. doniana* and their levels compared favourably with the FAO reference protein except for methionine and tryptophan where the level was lower. All the amino acids in the leave were found to be biologically available and were of sufficient quality to sustain normal growth (Olulosa, 1992). Adejumo *et al.,* (2013) found that the moisture content in *V. doniana* young leaves was high. *Vitex doniana* young leaf is very rich in carbohydrates. Minerals such as sodium, calcium, iron, Magnesium, Zinc, Copper were reported to be present in *V. doniana* young leaves (Adejumo *et al.,* 2013; Osum *et al.,* 2013). The result on the analysis of mineral content of the *V. doniana* young leaves revealed that Calcium (Ca) content is very high and Magnesium was moderate. Sodium was low in *V. doniana* young leaf but rich in potassium (Adejumo *et al.,* 2013).

2.3.4 Nutritional value of *V. doniana* fruits

The chemical composition of proximate, amino acids, fatty acids, vitamins, and minerals of *V. doniana* fruits has been reported (Osum *et al.,* 2013; Vunchi *et al.* 2011; Glew *et al.* 1997). Reports on the mineral content of *V. doniana* have showed that Iron, Magnesium, Manganese, Molybdenum, Phosphate, Zinc, Calcium and Sodium are present (Vunchi *et al.*, 2011; Osum *et al.*, 2013). Glew *et al.* (1997) reported that the most abundant mineral is Calcium. However, according to Vunchi *et al.* (2011), *V. doniana* fruit pulp has moderate calcium value. The fruit has also high value of vitamin C, carbohydrate and lipid. They suggested that the species could be promoted as carbohydrate and lipid supplements for cereal-based diets in rural

communities while its moderate calcium value could be used for the management of oesteomalacia. Agbede and Ibitoye (2007) studied the sugar content as well as the anti- nutritional factor in the fruit. Qualitative studies on the crude extract of the extract have detected presence of carbohydrates in moderate amount and protein in small amount (Nwachukwu and Uzueto, 2010).

2.4 Phytochemical composition and properties

The medicinal value of a plant depends on its bioactive phytochemical constituents that produce definite physiological action in the body. Several authors have evaluated the phytochemical composition of *V. doniana.* They showed the presence of flavonoids, tannins, saponins, anthraquinones, naphthoquinones, and resin in methanol, ethanol, aqueous root, stem bark and leaf extract of *V. doniana* (Iwueke, 2006). Different extracts of the leaves, stem bark and roots have been used to demonstrate the pharmacological activities of *V. doniana.* In general, the species shows good anti-oxidant property, antimicrobial, antidiarrhoeal effect, local anesthetic effect, antinociceptive activity (muscle relaxant), effect on pentobarbitone sleeping time, antidiabetic and hepatoprotective effect, anticonvulsant and antipyretic properties.

2.5 Propagation of *V. doniana*

The tree regenerates naturally by seed, coppice, wildings and root suckers. It is thought forest fires help in inducing germination because they help break the hard testa. Stones should be sown fresh after removal of the pulp and soaking in cold or warm water for 24 hours. The seeds have a very weak germinating capacity even when sown fresh. The growth rate of *V. doniana* is moderate. In Côte d'Ivoire, stones

dipped into sulphuric acid 95% for 60 minutes and subsequently in water for 72 hours germinated after 26 days, but the germination rate was only 34% (Louppe *et al.*, 2008). Seedlings were on average 70–90 cm tall after 3 years, the tallest ones reaching 170 cm. On good soils in southern Burkina Faso early growth is a bit faster (www.prota.org). Untreated fruits may take very long to germinate; fire may accelerate germination. Stones may contain several seeds and several seedlings may germinate from one stone. Seeds can be stored for up to 1 year at 3–5°C. Macropropagation by cuttings has been successful in Malawi (Louppe *et al.*, 2008). In Cameroon, Mapongmetsem *et al.*, (2012) studied the effect of growth hormones and substrate root growth using stem cuttings. They found that the rate of root growth can reach 68.31% after eleven weeks. However in Benin, Sanoussi *et al., (*2012) showed that propagation rate by stem cutting is slow while it is high (96.25%) by root cutting after three months. Survival rates in plantations are normally good, about 80–90% after 3 years (Louppe *et al.* 2008). To date there is no work reported on tissue culture of the species.

2.6 Pests and diseases

Premature leaf shedding, heart rot and die back have been reported as some of the diseases. *V. doniana* leaves were found bearing brown uredinia identified as rust the rust fungus *Olivea scitula*. The pathogen has been reported from Sierra Leone, Uganda, Nigeria and Zambia (Kapooria and Aime, 2005). According to Okia *et al.* (2008), Caterpillars and worms are the major pests that defoliate the species.

2.7 Plant tissue culture

Plant Cell and tissue culture has already contributed significantly to crop improvement and has great potential for the future (Kumar and Kumar, 1996). Research efforts in plant cell and tissue culture have increased dramatically worldwide in recent years including efforts in developing nations. The process starts with the selection of plant tissues (explant) from a healthy, vigorous mother plant (Murashige, 1974). Any part of the plant (leaf, apical meristem, bud and root) can be used as explant. Hussain *et al.*, (2012) divided the technique in 5 stages as summarized in Figure 2.1.

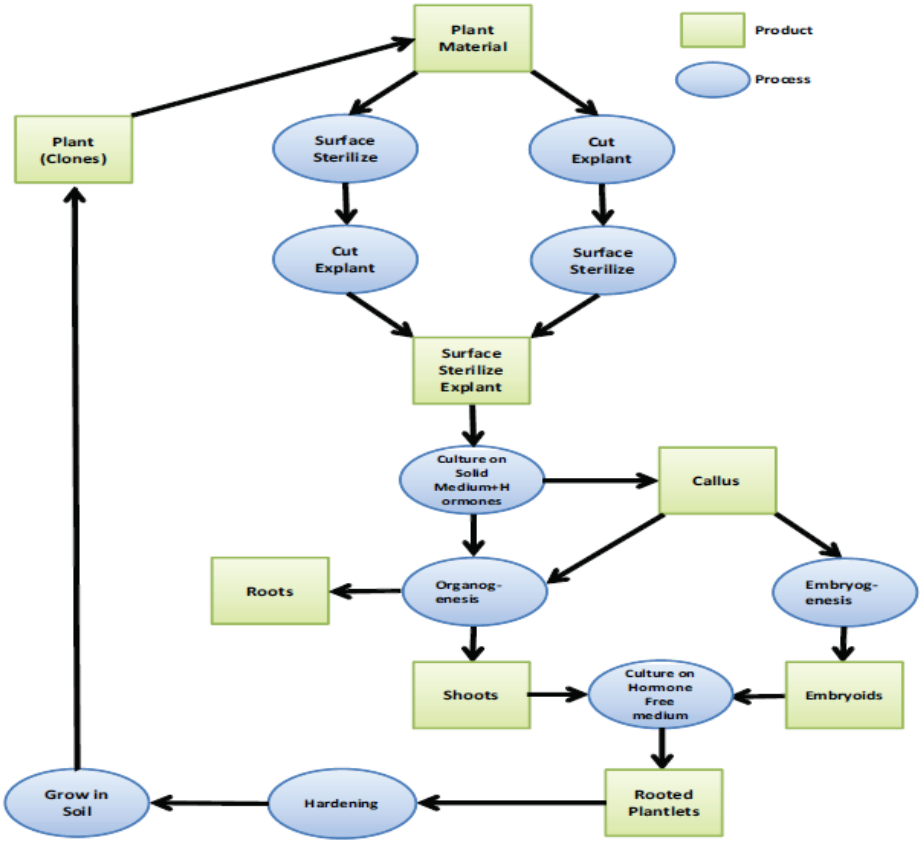


Figure 2.1 Flow chart summarizing tissue culture experiments

Source: (Hussain *et al.*, 2012).

2.7.1 Micropropagation

2.7.1.1 Stages of micropropagation

Stage 0: Preparation of donor plant: Any plant tissue can be introduced *in vitro*. To enhance the probability of success, the mother plant should be *ex vitro* cultivated under optimal conditions to minimize contamination in the *in vitro* culture.

Stage I: At the initiation stage, an explant is surface sterilized and transferred into nutrient medium. Generally, the combined application of bactericide and fungicide products is suggested. The selection of products depends on the type of explant to be introduced. The surface sterilization of explant in chemical solutions is an important step to remove contaminants with minimal damage to plant cells. The most commonly used disinfectants are sodium hypochlorite, calcium hypochlorite, ethanol and mercuric chloride ($HgCl_2$). The cultures are incubated in growth chamber either under light or dark conditions depending on the method of propagation.

Stage II: Multiplication stage aims at increasing the number of propagules. The number of propagules is multiplied by repeated subcultures until the desired (or planned) number of plants is attained.

Stage III: Rooting stage: The rooting stage may occur simultaneously in the same culture media used for multiplication of the explants. However, in some cases it is necessary to change media, including nutritional modification and growth regulator composition to induce rooting and the development of strong root growth.

Stage IV: At acclimatization Stage, the *in vitro* plants are weaned and hardened. Hardening is done gradually from high to low humidity and from low light intensity to high light intensity. The plants are then transferred to an appropriate substrate (sand, peat, compost etc.) and gradually hardened under greenhouse. In vitro growing

plantlets are under low light intensity (1,200–3,000 lux) and temperature (25 ± 2^{o}C), hence direct transfer to broad spectrum sunlight (4,000–12,000 lux) and temperature (26–36^{o}C) might cause charring of leaves and wilting of plantlets. It is therefore necessary to accustom the plant in the natural conditions by a process of hardening or acclimatization (Lavanya *et al.,* 2009). The retardation in development of cuticle, epicuticular waxes and functional stomatal apparatus during in vitro culture cause high stomatal and cuticular transpiration rates of leaves in plantlets when taken out of the culture vessels. In order to avoid this, slowly the plantlets should be transferred from high humidity to low humidity conditions. Microshoots should be kept in the shade with plugs loosened and after a week or two, they should be transferred to pots containing sterile soil and sand mixture covered with polybags. Slowly stomatal and cuticular transpiration rates gradually decreases because stomatal regulation of water loss becomes more effective and cuticle and epicuticular waxes develop (Chandra *et al.,* 2010).

2.7.1.2 Role of plant growth regulators

Auxins, abscisic acid, cytokinins, ethylene, and gibberellins are commonly recognized as the five main classes of naturally occurring plant hormones (Taiz and Zeiger 2004). Auxins, cytokinins, and auxin-cytokinin interactions are usually considered to be the most important for regulating growth and organized development in plant tissue and organ cultures, as these two classes of hormones are generally required.

- Cytokinins

The most commonly used cytokinins are the substituted purines: kinetin and BA. Cytokinin is a phytohormone that participates in events in the course of whole plant ontogeny, from fecundated ovule to senescence and death. It is present in processes such as cell division, shoot initiation and growth, senescence delay and photomorfogenic development, control of chloroplast division and growth, modulation of metabolism and morphogenesis in response to environmental stimulus (Pozo *et al.*, 2005; Hirose *et al.* 2007). Although both auxin and cytokinin are usually required for growth and morphogenesis, auxin can inhibit cytokinin accumulation, whereas cytokinins can inhibit at least some of the actions of auxin.

- Auxins

Auxins exert a strong influence over processes such as cell growth expansion, cell wall acidification, initiation of cell division, and organization of meristems giving rise to either unorganized tissue (callus) or defined organs (generally roots) and promote vascular differentiation. In organized tissue, auxins appear to be key players in maintaining apical dominance, affecting abscission, promoting root formation, and tropistic curvatures, delaying leaf senescence, and fruit ripening.

2.7.2 Somatic embryogenesis

In plants, embryo-like structures can be generated from cells other than gametes (i.e. somatic cells) by circumventing the normal fertilization process, hence the term somatic embryos (Parrott, 2000). As somatic embryos are formed without any fertilization event they are genetically identical to the parent tissue and are therefore clones. Somatic embryogenesis may be direct or indirect. Indirect somatic

embryogenesis involves dedifferentiation of organized tissue into callus prior to embryo production whereas direct somatic embryogenesis involves production of embryo from organized tissue without an intervening callus phase (Slater and Scott, 2003). A callus consists of an amorphous mass of loosely arranged thin walled parenchyma cells arising from the proliferating cells of the cultured explants. Frequently, as a result of wounding, a callus is formed at the cut end of a stem or root. Using tissue culture techniques, callus formation can be induced in numerous plant tissue and organs that do not usually develop callus in response to an injury. Many parts of a whole plant may have an ultimate potential to proliferate *in vitro*, but it is frequently found that callus cultures are more easily established from some organs than others. Young meristematic tissues are most suitable, but meristematic areas in older parts of a plant, such as the cambium, can give rise to callus. The callus formed on an original explant is called primary callus. Secondary callus cultures are initiated from pieces of tissue dissected from primary callus. Subculture can then often be continued over many years, but the longer callus is maintained, the greater is the risk that the cells thereof will suffer genetic change (George *et al.*, 2008).

2.7.2.1 Role of plant growth regulators

Plant growth regulators play a key role in process potentials and dedifferentiation and redifferention (Guo *et al*., 2011). Cytokinins are known to stimulate cells and, as such, they are also suitable candidates for induction of somatic embryogenesis and callogenesis. For example, in *P. atlantica*, callus induction was achieved by using BAP 1mg/l (Aghaei *et al.*, 2013). It has been suggested that TDZ is more effective than other cytokinins used for somatic embryogenesis (Lin *et al.*, 2004). The effects

of TDZ occur at lower concentrations than other cytokinins and it has been suggested that it either directly promotes growth due to its own biological activity or through inducing the synthesis and/or accumulation of endogenous cytokinins or auxins. Specifically, TDZ was showed to be effective in *Coffea arabica* and *Coffea canephora* somatic embryo formation (Giridhar *et al.*, 2004). These regenerative processes in cell and tissue cultures may be induced by cytokinins in collaboration with other plant growth regulators (Guo *et al.*, 2011). Many works have been reported on induction of somatic embryogenesis using a combination of cytokinins and auxins. Iin *P. atlantica*, the combination of BA 2 mg/l with NAA 1mg/1 was not effective (Aghaei *et al.*, 2013). Other growth regulators such as picloram and dicamba have been used in somatic embryogenesis. In *Phyla nodiflora,* it was found that picloram enhances formation of somatic embryos from leaf and stem explants in presence of BA (Ahmed *et al.*, 2011). Tournament of roses produced somatic embryos when cultured on medium containing dicamba (2.3µM/l). Dicamba (0.02 mg/l) was also found favourable for wheat regeneration.

2.7.2.2 Effects of carbohydrates sources and concentrations

Carbohydrates are generally considered as a carbon source needed for growth and development of tissues in *in vitro* cultures. Sucrose is the most frequently used in the culture medium compared with other sources of carbohydrates such as fructose, glucose, sorbitol and mannitol. Several reports show that carbohydrate sources affect the ability of the cells to induce somatic embryogenesis and its development as well as their concentrations (Tokuhara and Mii, 2003). In several species, sucrose is the preferred carbohydrate not only for induction, proliferation and embryos maturation

but playing a role as osmoticum (Li *et al.*, 1998). In addition, Korbes and Droste (2005) tested different maturation media by using 6% maltose, 3% sucrose or 6% sucrose. Sucrose at 6% significantly enhanced the conversion percent of soybean embryos. 0.15 M sucrose or sorbitol in induction medium while in proliferation stage was effective on date palm (*Phoenix dactylifera* L.) callus induction (Mona and Rania, 2012).

2.7.2.3 Effects of Activated charcoal and other additives

Activated charcoal has been shown to absorb 5-hydroxymethylfurfural, an inhibitor formed by the degradation of sucrose during autoclaving, as well as substantial amount of auxins and cytokinins. In sugarcane, 2% activated charcoal was found to enhance somatic embryogenesis (Manchanda and Gosal, 2012).

Amino acids (glutamine, proline and tryptophan) have been identified as enhancers of somatic embryogenesis in some species (Deo, 2010). In wheat culture, the efficiency of the amino acids was genotype-based (Duran et al., 2013). Their efficacy in embryogenesis has been attributed to their contribution to various cellular processes such as improving cell signaling processes in various signal transduction pathway (Lakshmanan and Taji, 2000), as precursor molecules for certain growth regulators.

The use of silver nitrate ($AgNO_3$), an ethylene action inhibitor has been shown to increase *in vitro* embryogenesis. $AgNO_3$ played a minor role on *in vitro* embryo induction frequency of Manchurian ash (*Fraxinus mandshurica*). However It has been shown that $AgNO_3$ enhanced synchronization and significantly inhibited abnormal somatic embryo formation suggesting that $AgNO_3$ might serve an important function in controlling the development of somatic embryos in Manchurian ash

(Kong, *et al.,* 2012). On the other hand, potassium nitrate (1.9 g/l) has been shown to enhance embryo induction rate in Cotton (*Gossypium hirsutum*) (Haq and Zafar, 2004).

The positive effect of casein hydrolysate on somatic embryogenesis has been found in species such as date palm (*Phoenix dactylifera L.*) (2g/l) (Khierallah and Hussein, 2013), maize (100 mg/l) (Dhillon and Gosal, 2012). Some researchers have reported that the inclusion of complex organic extracts, is essential for somatic embryogenesis in some species (Deo *et al.,* 2010). Coconut 10 and 20% coconut water improved the production of white-opaque somatic embryos in avocado (Per´an-Quesada *et al.,* 2004). Potato extract alone or combined with components of conventional culture media has been found to provide a useful medium for the anther culture of wheat and some other cereals.

Liquid culture can be a positive condition for the induction of somatic embryogenesis from various species, such as *Alstroemeria* (Akutsu and Sato 2002), *Lilium longiflorum* (Nhut *et al.,* 2006), *V. vinifera L.* (Jittayasothorn *et al.,* 2007) and *Coffea arabica* (Papanastasiou *et al.,* 2008). Furthermore, liquid pulse treatments of growth regulators could promote regeneration from plant cell culture though organogenesis or somatic embryogenesis (Madhulatha *et al.,* 2004). In addition, it has previously been reported that initial pretreatment of leaf explants of squash (*Cucurbita pepo L.*) with cytokinins for 6–48 h significantly promoted the formation of somatic embryos which developed further to the torpedo-shape stage and germinated (Kintzios *et al.,* 2002).

CHAPTER 3 MATERIALS AND METHODS

3.1 Introduction

This work was conducted in three parts. First, the plant materials were collected in Benin. Secondly, the nutritional analysis were done in the department of Food Science and Technology at Jomo Kenyatta University of Agriculture and Technology (Kenya). Lastly, the tissue culture studies were carried out in the Somatic Embryogenesis laboratory of ICRAF (Cote d'Ivoire).

3.2 Nutritional characterization

3.2.1 Sample collection Sites

Samples were collected in the Republic of Benin, situated in West Africa between latitudes $6^{o}10'$N and $12^{o}25'$N and longitudes $0^{o}45'$E and $3^{o}55'$E (Figure 3.1). It is bordered by the Republics of Togo in the west, Burkina Faso and Niger in the north, and Nigeria in the east. The mean annual rainfall varies from 900 to 1300 mm. Its lowest values are recorded in the southwest and in the far north (900-950 mm). The highest precipitation (1200-1300 mm) is confined to Southeast Benin as well as the tract Bassila-Djougou. The mean annual temperature ranges from 26 to 28°C and may exceptionally reach 35-40^{o}C in northern localities such as Kandi. The annual temperature amplitude is low in the southern part (5-10^{o}C) while it is high (11-13^{o}C) in the northern part (at least from the latitude 8^{o}N northwards). As in most West-African countries, the climate is primarily determined by the annual cycle of the "Inner Tropical Convergence Zone" (Adomou, 2005). Three climate zones can

broadly be distinguished (Akoègninou 2004). (1) The southern zone: From the coast up to the latitude 7°N, the climate is Guinean or subequatorial with two rainy seasons alternating with a longer dry season. The shorter dry season rarely exceeds two months. (2) The transition zone: Between the latitudes 7° and 9°N, the climate becomes subhumid or subsudanian with a tendency to a pattern of one rainy season and one dry season. (3) The no rthern zone is characterised by a truly Sudanian climate with a unimodal rainfall regime. The vegetation mainly consists of savannahs, grasslands, farmlands, and fallows intermingled with small islands of closed forest (semi-deciduous forest and swamp forest). Trees were randomly selected in different localities within each agro climatic zones (Figure 3.1).

3.2.2 Sampling and sample collection

Sampling of leaves was done randomly to select three individuals of *V. doniana* in each locality selected within each climatic zone of Benin. At least 1 km radius has been considered between two sample trees in a climatic zone. Fresh and newly emerging leaves (4 to 7 days) were hand harvested on each sampled tree from the top, middle and lower part of the canopy.

3.2.3 Sample handling and preparation

Samples from the same agro climatic zones were pooled together to make a composite sample, placed in polythene bag and clearly labeled. Samples were kept fresh in a cool box containing ice during transportation and stored at -20^{o}C in a deep freezer before analysis was carried out.

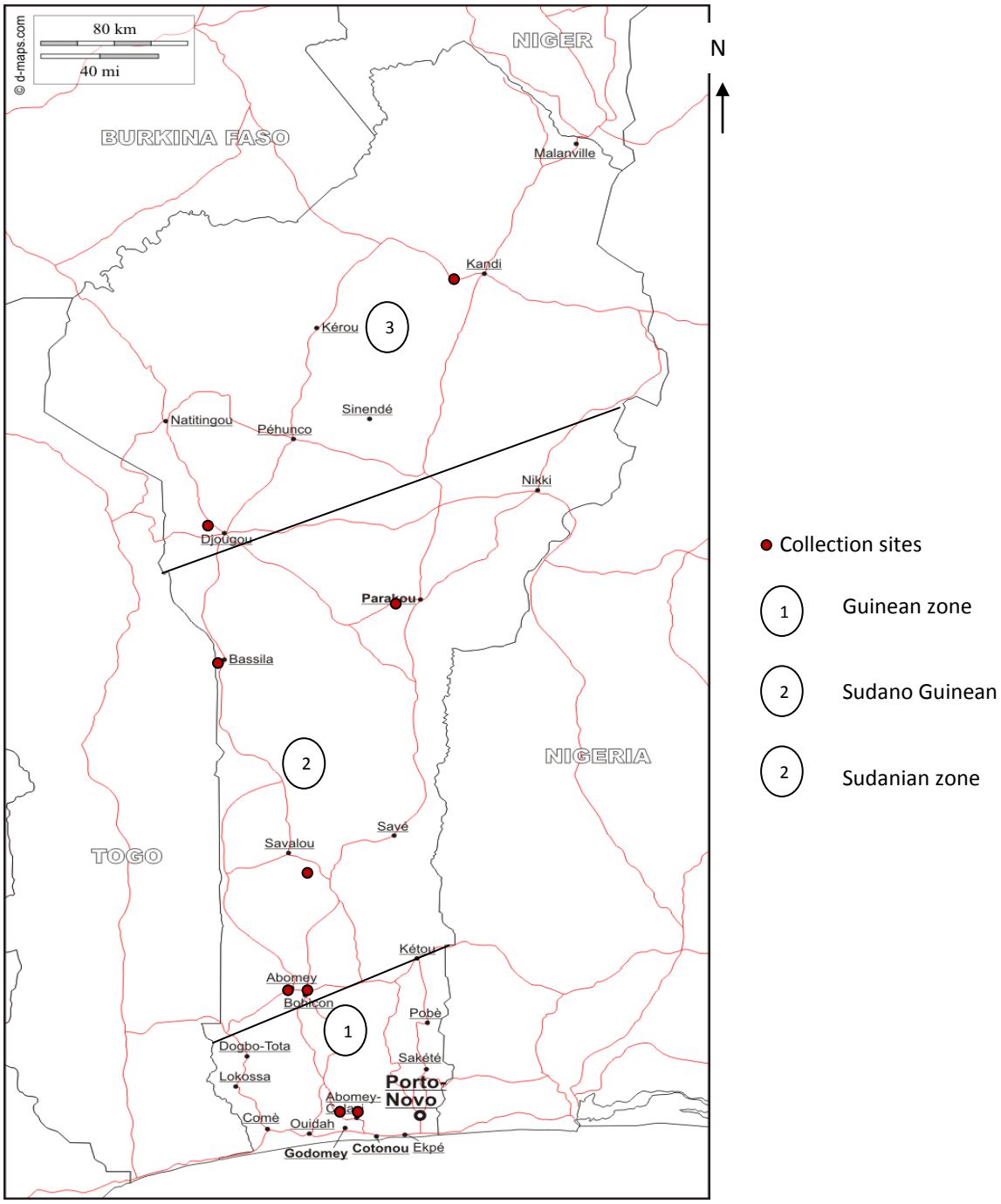


Figure 3.1 Agro-climatic zones of Benin Republic

3.2.4 Proximate analysis

3.2.4.1 Moisture Determination

Moisture content was estimated by drying 5 g of sample in an oven at 105±5°C till constant weight (AOAC, 2000). The percentage of moisture was determined as indicated in equation 1.

% Moisture = (weight of sample + dish before drying) - (weight of sample + dish after drying) x 100 / weight of sample taken) (eq. 1)

3.2.4.2 Crude Fat determination

The ether extract of leaves represents the fat and oil in the leaves. The crude fat of leaves was determined using hexane as a solvent in Soxhlet System according to the procedure give in AOAC (2000). About 150ml of an anhydrous diethyl ether (petroleum ether) of boiling point of 40-60ºC was placed in the flask. Five grams of the sample was weighed into a thimble and the thimble was plugged with cotton wool. The thimble with content was placed into the extractor; the ether in the flask was then heated. As the ether vapour reaches the condenser through the side arm of the extractor, it condenses to liquid form and drop back into the sample in the thimble, the ether soluble substances are dissolved and are carried into solution through the siphon tube back into the flask. The extraction continued for at least 4 hours. The thimble was removed and most of the solvent was distilled from the flask into the extractor. The flask was then disconnected and placed in an oven at 65ºC for 4 hours, cool in desiccator and weighed. The percentage of ether extract was determined using equation 2.

% Fat extract = weight of flask + extract- tare weight of flask x 100/ weight of sample (eq. 2)

3.2.4.3 Crude Fibre

Crude fibre of the samples was determined following the procedure in AOAC (2000). Crude fibre was estimated in fat free samples by treating with 1.25% H_2SO_4. Left

over material was subjected to further treatment with 1.25% NaOH solutions. The solution was gently boiled for about 30mins, maintaining constant volume of acid by the addition of hot water. The buckner flask funnel fitted with whatman filter was pre-heated by pouring hot water into the funnel. The boiled acid sample mixture was then vacuum filtered hot through a funnel. The residue was then washed several times with boiling water (until the residue will be neutral to litmus paper) and transferred back into the beaker. Then 200 ml of pre-heated 1.25% Na_2SO_4 was added and boiled for another 30 min; filtered under suction and washed thoroughly with hot water and twice with ethanol. The residue was dried at 65ºC for about 24hr and weighed. The residue was transferred into a crucible and placed in muffle furnace (400-600ºC) and ash for 4hours, then cooled in desiccator and weighed. The percentage of crude fibre was calculated as indicated in equation 3.

% Crude fibre = dry weight of residue before ashing - weight of residue after ashing x 100/ weight of sample (eq. 3)

3.2.4.4 Crude Protein

Crude protein was determined by kjeldahl method as described in AOAC (2000). The method involves: Digestion, Distillation and Titration.

Digestion: about 5g of the sample was weighed into kjeldahl flask and 25ml of concentrated sulphuric acid, 0.5g of copper sulphate, 5g of sodium sulphate and a speck of selenium tablet was added. Heat was applied in a fume cupboard slowly at first to prevent undue frothing, digestion continued for 45mins until the digesta became clear pale green. The digest was left until it cooled completely and 100mls of

distilled water was rapidly added. The digestion flask was rinsed 2-3 times and the rinsing added to the bulk.

Distillation: Markham distillation apparatus was used for distillation. After steaming up the distillation apparatus, about 10mls of the digest was added into the apparatus via a funnel boiled. Ten milliliters of sodium hydroxide was added from the measuring cylinder so that ammonia was not be lost. Then the distillation into 50mls of 2% boric acid containing screened methyl red indicator was done.

Titration: the alkaline ammonium borate formed was titrated directly with 0.1N HCl. The titre value which is the volume of acid used will be recorded. The volume of acid used was fitted into the formula (equation 4) which becomes:

% Nitrogen = 0.014 x Volume of acid used x 0.1 x weight of sample x 100/1000

(eq.4)

The percentage of crude protein was determined as in equation 5:

% crude protein = % Nitrogen x 6.25 (eq.5)

3.2.4.5 Ash

Ash is the inorganic residue obtained by burning off the organic matter of the leaves in muffle furnace (equation 6). Ash was estimated by direct incineration of 2 g of sample; igniting it in a muffle furnace at 550°C till grayish white residue (AOAC, 2000). The crucible was then placed in the desiccator and weighed.

% Ash = weight of dish + ash - weight of dish x 100/ weight of sample (eq.6)

3.2.4.6 Carbohydrates

The Carbohydrate content was estimated by the difference method (equation 7).

% Carbohydrate = 100- (% Protein + % fat+ % Fibre+ %Ash) (eq. 7)

3.2.5 Minerals

The mineral elements comprising iron, calcium, magnesium, and zinc were determined according to Adeyeye and Omotayo (2011) with slight modifications. The ash obtained from section 3.2.3.5 was dissolved in 5 ml of $HNO_3/HCl/H_2O$ (1:2:3) and heated gently on a hot plate until brown fumes disappeared. To the remaining material in each crucible, 5 ml of de – ionized water was added and heated until a colorless solution was obtained. The mineral solution in each crucible was transferred into a 100 ml volumetric flask by filtration through Whatman filter paper and the volume was made to the mark with de – ionized water. This solution was used for elemental analysis by flame atomic absorption spectrophotometer (Shimadzu, Kyoto, Japan).

3.2.6 Vitamin A and C determination

3.2.6.1 Vitamin C

Vitamin C was determined by HPLC method according to Vikram et al. (2005). The sample (5 g) was homogenised with 40 mls of 0.8% metaphosphoric acid (extracting solution) and was centrifuged at 10000 rpm for 10 minutes. The supernatant was filtered to obtain a clear extract and diluted with 10 mL of 0.8% metaphosphoric acid. The mixture was then filtered through a Whatman filter paper. Various concentrations of ascorbic acid standards (20 to 100 mg/l) were also made to make a calibration curve. HPLC analysis was done using Shimadzu UV-VIS detector.

3.2.6.2 Vitamin A (β-Carotene)

β-Carotene was extracted using acetone and analyzed using column chromatography and UV Spectrophotometer, (Rodriguez-Amaya and Kimura, 2004; AOAC, 2000). The sample (5 g) was added with 2g of celite and homogenised using motar and pestle. Fifty milliters of acetone was added. The filtrate was concentrated using a rotary evaporator. The resulting extract was diluted to 10 ml with petroleum-ether. Separation method involved reversed-phase liquid chromatography, with a mobile phase of petroleum ether and the absorbance was read at 450 nm in a UV-Vis spectrophotometer (Shimadzu model UV – 1601 PC, Kyoto, Japan). β-Carotene was calculated using equation 8:

β-carotene content ($\mu g/g$) = A x volume x 10000/Ap x sample weight (eq. 8)

Where A= absorbance; volume = total volume of extract (25 mL); Ap = absorption coefficient of β−carotene in Petroleum Ether (2592).

3.2.7 Statistical analysis

Samples from each climatic zones were analyzed in triplicates. Results are expressed as mean ± Standard Error (SE). The differences between the different nutritional parameters was determined by performing an one way analysis of variance (ANOVA) test on the mean values at each climatic zone and Tukey analysis was performed to assess difference between means.

3.3 Tissue culture

3.3.1 Plant materials

V. doniana seedlings, originally wildings from the forests, were collected from field in Benin and were maintained in a temporary shed in Abidjan, Cote d' Ivoire where they were watered daily. Leaves (for embryogenesis) and nodes (for microshoot and root regeneration) were harvested from the seedlings for the experiments.

Plate 3.1 *V. doniana* seedlings growing under shed in Cote d'Ivoire

3. 3.2 Media preparation

The Murashige and Skoog (1962) media was used for all the experiments (Appendix 1). Media was prepared by dissolving the organic and inorganic components in distilled water. The solution was stirred until dissolved and made up to final volume. The media pH was adjusted between 5.7 and 5.8 by using either 1N HCl or 1N NaOH before the gelling agent was added. Media was then heated on a hot plate with continuous stirring using a magnetic stirrer until agar was dissolved and media dispensed in the culture vessels. The culture vessels were capped with lids and placed in trays and autoclaved. Autoclave was set at a temperature of 121^{o}C and a pressure

of 1.1kg/cm^2 for 20 minutes. All media was autoclaved within 12 hours of preparation and when possible freshly autoclaved media was used. However, when it was not possible to use the media immediately it was stored in a refrigerator at 4^{o}C for no longer than two weeks before use.

3.3.3 Plant growth regulators

Plant growth regulators were weighed and stocks prepared using appropriate solvents (Table 3.1). The stocks were clearly labeled and stored in the refrigerator at 5^{o}C.

Table 3.1 Solvents used to dissolve plant growth substances

Plant growth substance	Solvent
BAP	0.1N NaOH and heat if required
Kinetin	0.1N NaOH
TDZ	NaOH
3,4-D	Absolute alcohol
2, 4-D	Absolute alcohol
Picloram	DMSO
Dicamba	Distilled water
IBA	Absolute alcohol

3.3.4 Aseptic techniques

The process of sterilization and dissection of plant materials was carried out under sterile conditions in lamina flow cabinet. The cabinet was switched on and swabbed down with 70% ethanol using cotton wool or sterile towel and kept running for about 15 minutes before the work in the cabinet starts. All the plant materials were dissected on the sterile petridishes. The lamina flow cabinet was frequently swabbed

down with 70% alcohol. Hands were sprayed with 70% ethanol at suitable intervals while working for protracted periods in front of the cabinets. Personal hygienic precautions were observed by wearing a clean lab coat and gloves while working in the lamina flow cabinet.

3.3.5 Dissecting tools

All tools were placed in an aluminum foil and sterilized in an autoclave. During their use in the cabinet, tools were dipped in 70% ethanol followed by heat sterilization in steribead sterilizer maintained at 250^{o}C. In between operations, the tools were frequently sterilized by dipping them in ethanol and in the steribead sterilizer for 30 seconds.

3.3.6 Washing of glassware and vessels

All glassware and vessels were washed in hot water to which few drops of liquid detergent had been added. The glassware were then rinsed in cold water three times followed by a final rinse in distilled water with a few drops of commercial bleach (super clean®). All this was carried out in a clean dust free washing room. The glassware was then dried in the oven at 60^{o}C in a clean dust free place.

3.3.7 Surface sterilization of explants

Leaf explants were harvested and transported from the greenhouse in a beaker containing tap water to the laboratory. Once in the laboratory, they were cleaned with liquid soap and cotton wool and kept under running tap water for 2 hours. They were then dipped in 0.5% fungicide (Ridomil) for 1 hour. The explants were then transferred to the lamina flow cabinet, immersed in 70% (v/v) ethanol for 30 seconds

and rinsed twice with sterile distilled water. The sterilization was carried out using two sterilizing agents: 1%, 1.5% and 2% calcium hypochlorite and 15%, 20% and 25% bleach evaluated at different time interval. They were then rinsed four times in sterile distilled water.

The Nodal explants were cleaned with cotton wool and liquid soap and soaked in 0.5% fungicide solution for 1 hour. Thereafter, they were immersed in different concentrations of mercuric chloride (0.1 and 0.5%) solution containing two drops of Tween 20 for 5 minutes. They were then, rinsed two times with sterile distilled water and dipped in 70% alcohol for 30s. Using calcium hypochlorite, the explants were immersed in 2 and 4 % calcium hypochlorite for 45 min, rinsed with distilled water and dipped in 70% alcohol for 30 s. They were sterilized again for 20 min and rinsed four times.

3.3.8 Tissue culture experiments

3.3.8.1 Somatic embryogenesis

I. Effect of plant growth regulators

a. Effect of cytokinins

Cytokinins were evaluated for their abilities to induce somatic embryogenesis in *V. doniana*. Leaf explants were cultured on half strength MS medium, 100mg/l inositol, 2% sucrose gelled with 0.3% phytagel supplemented with growth regulators. The growth regulators evaluated were BAP, Kinetin (Kin) tested at either 5, 10, 20, 40 µM and TDZ tested at either 0.1, 0.5, 1 or 2 µM. Control culture consists on MS medium without growth regulators.

b. Auxins

Four auxins were evaluated. There are 2, 4-D, 3,4-D, picloram and dicamba, at three concentrations (either 2, 4 or 8 µM). Half strength MS medium supplemented with 0.5 µM TDZ, 100 mg/l myo inositol, 2% sucrose gelled with 0.3% phytagel were supplemented with each of the auxins under evaluation in separate experiments. The media without auxin is referred to as control.

II. Effect of carbon sources

c. Sucrose

Leaf explants were cultured on half strength MS media supplemented with 100 mg/l myo inositol, 0.5 µM TDZ and different concentrations (0, 60, 90, 120, 150 and 180 mM). The media without sucrose is hereby referred to as control.

d. Other carbon sources

Mannitol, sorbitol, maltose, fructose and glucose were evaluated on their effect on induction of somatic embryogenesis at concentrations of 90 mM, 120 mM and 150 mM. Explants were cultured on half strength MS media supplemented with 100mg/l myo inositol, 0.5 µM TDZ and the different sugars under evaluation. The control media had no sugar.

III. Effect of doubling potassium nitrate

The effects of doubling the amount of potassium nitrate in the MS medium on induction of *V. doniana* somatic embryos were evaluated in two separate experiments. In one of the experiments, explants were cultured in half strength MS containing normal amount of potassium nitrate (950 mg/l) and supplemented with 0.5 µM TDZ while in the other, leaf explants were cultured on half strength MS media

containing double amount of potassium nitrate (1900 mg/l) and supplemented with 0.5 µM TDZ, 2% sucrose and gelled with 0.3% phytagel.

IV. Effect of amino acids

The effects of four amino acids (leucine, tryptophan, proline and lysine) on induction and regeneration of somatic embryos were evaluated at concentration 50, 100 and 150 µM. Explants were cultured on half strength MS media supplemented with 100 mg/l myo inositol, 0.5 µM TDZ, 2 % sucrose and the different amino acids under evaluation. The media without the above four amino acids is referred to as the control.

V. Effect of silver nitrate

Leaf explants were cultured in half strength MS containing silver nitrate at either 0, 50, 100 or 150 µM and supplemented with 100 mg/l myo inositol, 0.5 µM TDZ, 2% sucrose and gelled with 0.3% phytagel.

VI. Effect of Activated Charcoal and Casein Hydrolysate

Activated charcoal (AC) and casein hydrolysate (CH) effects were evaluated on their effects on induction of somatic embryos in *V. doniana.* AC was tested at 0.1, 0.2 and 0.3% (w/v) while casein hydrolysate was evaluated at 50, 100 and 150 mg/l. The leaves were cultured on half strength MS medium supplemented with 0.5 µM TDZ and gelled with 0.3% phytagel and the additive under evaluation.

VII. Effect of organic extract

The effects of four organic extracts: corn starch, papain, and potato starch and coconut water on *V. doniana* somatic embryogenesis were evaluated. Corn starch, papain, potato starch and coconut milk were evaluated at either 50, 100, 150 mg/l while coconut water was evaluated at 10, 20 and 30% (v/v). Half strength MS medium supplemented with 100 mg/l myo inositol, 0.5 µM TDZ, 2% sucrose, gelled with 0.3% phytagel and the organic extract under evaluation was used. The media without the organic extracts is referred to as control.

VIII. Effect of liquid pulsing

Liquid pulsing has been used to improve embryogenesis of non-embryogenic callus in many plants. Leaf explants were cultured on half strength MS medium supplemented, 2% sucrose, 100 mg/l myo inositol and gelled with 0.3% phytagel. After 5 weeks on solid medium, callus tissues (non- embryogenic) were transferred to the same media devoid of phytagel and put in a rotary shaker at three different time intervals (6, 12 or 24 h). Calluses were transferred to petridishes containing half-strength MS medium solidified with 0.3% phytagel and supplemented with 2%sucrose and 0.5µM TDZ. The control cultures consisted of callus tissues cultured only on solid medium above, which were not exposed to the liquid pulses pretreatment.

3.3.8.2 Micropropagation

I. Shoot regeneration

Nodal explants of *V. doniana* were cultured on full strength MS medium supplemented with 30g/l sucrose, 100 mg/l myo-inositol, 6–Benzylaminopurine

(BAP) was evaluated at 5, 10, 20, 40 μM / l in separate experiments and gelled with 0.3% phytagel. The media without BAP was referred to as control.

II. Root regeneration

After one month on microshoot induction media. shoots were transferred to rooting medium. The induction of roots was carried out on half strength Murashige and Skoog medium supplemented with IBA at concentrations 5, 10, 20, 40 μM/l. 20g/l sucrose, and 100mg/l myo-inositol and gelled with 0.3% phytagel. The media without IBA was referred to as control.

3.3.9 Incubation conditions

For regeneration of somatic embryos, cultures were incubated in a dark room maintained at 25^{o}C. On the other hand, for regenerating microshoots and roots, the cultures were incubated in growth rooms maintained at 25^{o}C and 16 hours photoperiod.

3.3.10 Experimental design, data collection and analysis

The experiments were laid out in completely randomized design (CRD) with each treatment repeated two times. Ten (10) replicates per treatment were used at the outset of experiments. The level of replication used per treatment combination varied depending upon the availability of experimental materials. For the sterilization experiment, percent clean explants were recorded (equation 9).

% clean explant= total number of contaminated explants x 100 / total number of cultured explants (eq.9)

For the somatic embryogenesis experiment, data on the number of somatic embryos per explants and the percentage embryogenic cultures were recorded (equation 10).

% embryogenic culture = total number of explants with embryo x 100 / total number of cultured explants (eq. 10)

For the micropropagation experiment, the number of microshoots and roots per explant were recorded. The length of microshoots was also recorded. All the data were subjected to one-way analysis of variance (ANOVA) and the significant differences between treatment means were assessed by using Minitab version 14 Software. The results are expressed as percentage (%) and mean ± Standard Error.

CHAPTER 4 RESULTS

4.1 Nutritional variation of *Vitex doniana* young leaves in Benin

4.1.1 Proximate composition of *V. doniana* young leaves

The proximate composition of the samples from the varying climatic zones is indicated in Table 4.1. For the fat, carbohydrates, ash and fiber content, there were no statistical significant difference (p = 0.05) between the Sudanian, Sudano-Guinean zone and Guinean agro climatic zones. However, there were significant difference between moisture and protein content of leaves from the different climatic zones. The results show that the values of the moisture content were the highest (82.15%) in leaves collected from Guinean zone while the lowest (79.04%) was observed from those collected from the Sudanian zone. The percentage of protein in leaves collected from Sudanian zone was the highest (7.60) while the lowest (4.95) was recorded from samples collected from Guinean zone.

Table 4.1 Proximate composition of *V. doniana* young leaves

Climatic zones	% Moisture	% protein	% Fat	% Fibre	% Ash	% Carbohydrate
Guinean	82.15 ± 0.38a*	4.95 ± 0.24a	1.86 ± 0.23a	1.21± 0.4a	1.31 ± 0.17a	8.53 ± 0.35a
Sudano Guinean	80.49 ± 0.18b	5.82 ± 0.43a,b	2.69 ± 0.33a	1.95± 0.4a	1.91 ± 0.31 a	7.14 ± 0.6a
Sudanian	79.04 ± 0.28c	7.60 ± 0.64b	2.61± 0.15a	2.72 ±0 .06a	1.81 ± 0.18 a	7.54 ± 0.61a
P-value	0.001	0.018	0.102	0.063	0.216	0.067

* Values represent means ± SE. Means followed by the same letter are not significantly different at P = 0.05. n=3.

4. 1.2 Mineral composition of *Vitex doniana* young leaves

V. doniana young leaves contain minerals such as calcium, magnesium, zinc, and iron in variable proportions (Table 4.2). From the results of the analysis of variance

performed, it can be noted that there is no statistical significant difference between the climatic zones for mineral concentration such as magnesium (Mg), zinc (Zn), iron (Fe). However, statistical significant difference was noted between the climatic zones for calcium (Ca) concentration. The highest value (242.95 mg/100g) of Ca was observed in leaves collected in Sudano-Guinean zone.

Table 4.2: Mineral concentration of *V. doniana* young leaves

	Zn	Fe	Ca	Mg
Climatic zones	**Mean (mg/100g) ± SE**			
Guinean	0.22 ± 0.1a*	2.45 ± 0.52a	121.98 ± 12.3a	57.39 ± 4.62a
Sudano Guinean	0.38 ± 0.24a	2.57 ± 0.54a	242.95 ± 10.4b	57.53 ± 3.49a
Sudanian	0.49 ± 0.19a	2.42 ± 0.08a	206.64 ± 6.23b,c	69.05 ± 6.54a
P-value	0.628	0.971	P<0.001	0.251

*Means followed by the same letter are not significantly different at P = 0.05. n=3.

4.1.3 Vitamin A and C

The results of Vitamin A and C analysis showed that there was a significant difference between the vitamin A and C content of leaves collected from the different climatic zones (Table 4.3). The Vitamin A and C content of the leaves collected in the Sudanian zone was the highest at 5.5 mg /100g and 17.70 mg /100g respectively while leaves collected from the Guinean zone exhibited the lowest Vitamin A (1.37) and C (14.48 mg/100g).

Table 4.3 Vitamin A and C concentration of *V. doniana* young leaves

	Vitamin A	Vitamin C
Climatic zones	**Mean (mg/100g) ± SE**	
Guinean	1.37 ± 0.65b*	14.48 ± 0.09b
Sudano-Guinean	3.6 ± 0.9a,b	16.02 ± 0.26a,b
Sudanian	5.5 ± 0.88a	17.78 ± 0.87a
P-value	0.033	0.013

* Means followed by the same letter are not significantly different at P = 0.05. n=3.

4.2 Surface sterilization of *V. doniana* explants

4.2.1 Effects of super clean bleach and calcium hypochlorite on sterilization of *V. doniana* leaf explants

The surface sterilization of leaves was performed using the household bleach commonly called super clean®, which contains 3.85% active chlorine and calcium hypochlorite. The highest percentage (70) of clean explants was obtained when 2 % calcium hypochlorite was used for thirty (30) minutes. The results of the effect of calcium hypochlorite concentrations on per cent clean explants fourteen days after culturing are presented in Table 4.4. However, this percentage reduced further after two week and only 50% clean explants were observed. All the treatments with bleach were contaminated after fourteen days of culture. In order to achieve high rates of clean explants a double sterilization experiment was conducted. This involved sterilizing the explants using 2% calcium hypochlorite for 30 min rinsing two times with sterile distilled water followed by quick dip in 70% ethanol. During the second step, the explants were sterilized with 2% calcium hypochlorite for 15 min and finally

rinsed with sterile distilled water four times. This treatment raised the percentage of clean explants to 91% after one month and it was used for all the subsequent experiments.

Table 4.4 Effects of calcium hypochlorite on elimination of surface contamination from *V. doniana* leaf explants

Concentrations of Calcium hypochlorite (in % w/v)	Exposure time (min)	Percentage of clean explants (%)
1	25	0
1	30	0
1	35	0
1	40	0
1.5	20	0
1.5	25	0
1.5	30	0
1.5	35	20
2	15	40
2	20	70
2	25	70
2	30	70

4.2.2. Effect of calcium hypochlorite and mercuric chloride on *V. doniana* nodal explants

The surface sterilization of nodes was performed by using two sterilants: mercuric chloride and calcium hypochlorite. All the treatments with mercuric chloride were found toxic to the explants since the explants died. The highest (40%) percentage of clean explant was achieved by using 2% calcium hypochlorite in double sterilization. This consisted on sterilizing the explants using 2% calcium hypochlorite for 45 min

rinsing two times with sterile distilled water followed by quick dip in 70% ethanol. During the second step, the explants were sterilized with 2% calcium hypochlorite for 20 min and finally rinsed with sterile distilled water four times.

4.3 Evaluation of some factors affecting induction and regeneration of somatic embryogenesis

4.3.1 Effects of growth regulators on the induction of somatic embryos

4.3.1.1 Effects of cytokinins on the induction of somatic embryos

The initial aim was to regenerate direct somatic embryogenesis. However, after two weeks callus was seen to be developing form the cut edges of the explant [Plate 4.1 (a)]. The percentage of callus formation was therefore recorded as the number of explant with callus divided by total number of cultured explants.

The effects of cytokinins on callus induction are presented in Table 4.5. Callus induction was first observed after ten days in medium supplemented with 0.5 and 1 µM TDZ. Callus form in these media were brown and friable while callus formed in media containing BAP or Kin were loose and watery. The medium supplemented with TDZ at 0.5 µM produced the highest (91%) frequency of callus while the medium with 20 µM BAP produced the lowest (17%) frequency of callus. Fifty (50%) of the explants cultured on media supplemented with Kin at 5 µM produced callus. It was observed that the lower concentration of TDZ and higher concentrations of Kin did not support embryogenesis Therefore, the half strength MS media supplemented with 0.5 µM TDZ, 100mg/l inositol, 2% sucrose gelled with 0.3% phytagel was used in all subsequent experiments and considered the control.

Table 4.5 Effects of BAP, Kinetin and TDZ on callus induction

Cytokinin conc. (µM)	Callus formation (%) TDZ	BAP	Kin
Control	0	0	0
0.1	0	-	-
0.5	91	-	-
1	50	-	-
2	50	-	-
5	-	0	50
10	-	25	0
20	-	17	0
40	-	0	0

4.3.1.2 Effects of auxins on the induction of somatic embryos

The effects of Picloram on induction and regeneration of somatic embryogenesis [Plate 4.1 (b and c)] are presented in Table 4.6. Embryogenic cultures are white brownish with nodular structures. An embryogenic culture can present one or more embryos. Leaf explants cultured on medium without picloram produced the highest (91%) percentage of embryogenic culture while the lowest (37%) percentage of embryogenic culture was observed in medium supplemented with 2 µM picloram. The highest (6.1) mean number of embryos was observed in medium supplemented with 4 µM picloram and the lowest (1.2) was observed in the control.

The effects of 2,4-D on induction and regeneration of somatic embryogenesis are presented in Table 4.7. The control produced the highest (91%) percentage of embryogenic cultures. However, the lowest percentage of embryogenic cultures were observed in the medium supplemented with 2 and 4 µM 2,4-D. There is no difference

in terms of mean number of somatic embryos per explant between the different treatments.

The effects of dicamba on induction and regeneration of somatic embryogenesis are presented in Table 4.8. Leaf explants cultured on medium without dicamba produced the highest (91%) percentage of embryogenic culture. On the other hand, the lowest (11%) percentage of embryogenic cultures were observed in medium supplemented with 2 and 4 µM dicamba. There were no significant differences in terms of mean number of somatic embryos per explant among the different treatments. 3,4-D at all the concentration evaluated did not support somatic embryogenesis.

Table 4.6 Effects of Picloram on induction and regeneration of somatic embryos

Picloram conc (µM)	**Embryogenic cultures (%)**	**Mean number of embryos/explant ± SE**
0	91	1.2 ± 0.13c
2	37	3.39 ± 0.70b
4	61	6.1 ± 0.67a
8	39	2.71 ± 0.42b,c
P value		P< 0.001

*Means followed by the same letter are not significantly different at P= 0.05. n= 20

Table 4.7 Effects of 2, 4-D on induction and regeneration of somatic embryos

2,4 D conc (µM)	Embryogenic cultures (%)	Mean number of embryos/explant ± SE
0	91	1.2 ± 0.13a*
2	8	2 ± 0.0a
4	8	2 ± 0.0a
8	13	1.5 ± 0.5a
P value		0.103

*Means followed by the same letter are not significantly different at P=0.05. n=20

Table 4.8 Effects of dicamba on induction and regeneration of somatic embryos

Dicamba conc. (µM)	Embryogenic cultures (%)	Mean number of embryos/explant ± SE
0	91	1.2 ± 0.13a
2	11	2 ± 0.1a
4	11	1 ± 0a
8	0	0[x]
P value		0.193

[x]The zero responses are not included in the analyses. *Means followed by the same letter are not significantly different. n= 20

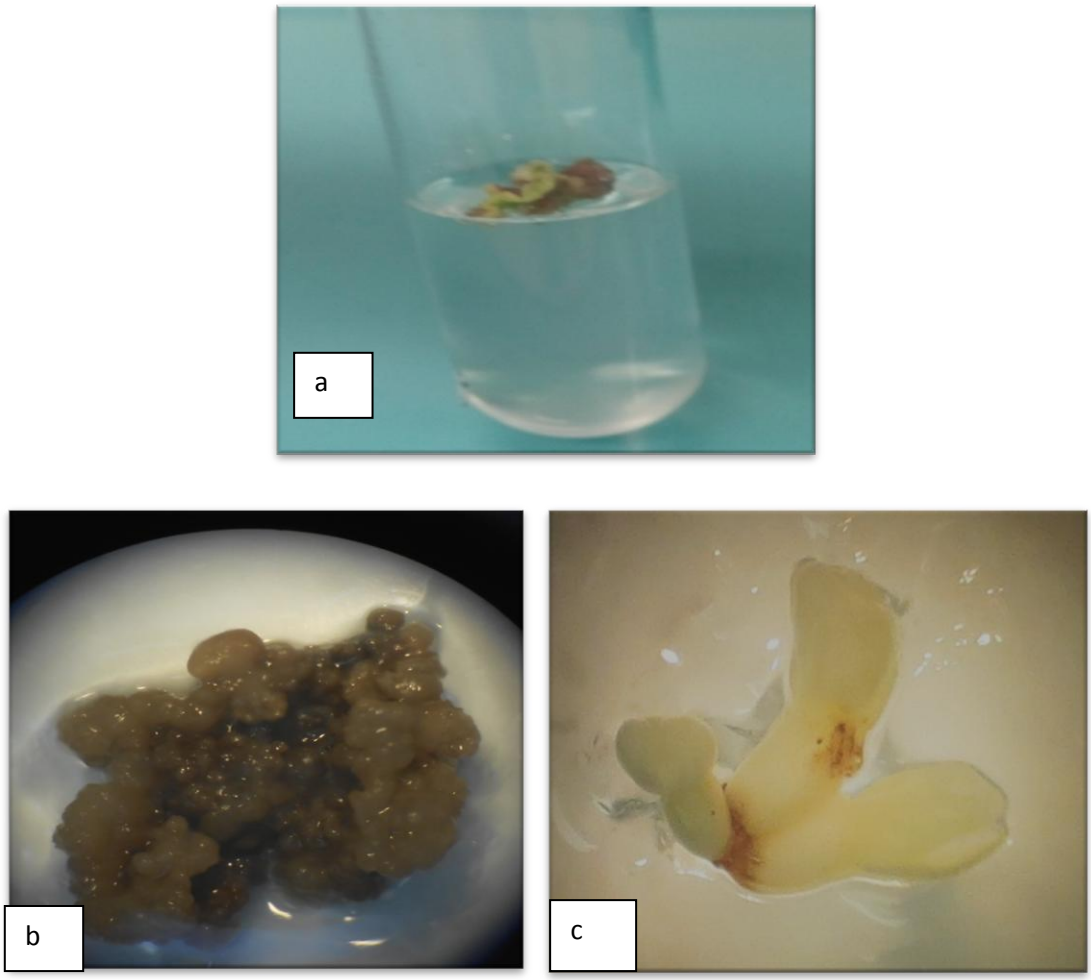


Plate 4.1 (a) Callus (b) Globular embryos (c) Fully developed embryos

4.3.2 Effect of carbon sources on induction of somatic embryogenesis

4.3.2.1 Effect of sucrose

The effects of sucrose concentrations on induction and regeneration of somatic embryos are presented in Table 4.9. The medium supplemented with 60 mM sucrose produced the highest (95%) frequency of embryogenic cultures and the lowest (14%) were produced in the medium with 180 mM. The highest (8.67) mean number of embryos were observed in the medium with 60 mM and the lowest (1.25) mean

number of embryos were produced in the medium supplemented with 180 mM sucrose. Media without sucrose did not support somatic embryogenesis.

Table 4.9 Effect of sucrose concentrations on induction of somatic embryos

Sucrose conc (mM)	Embryogenic cultures (%)	Mean number of embryos/explant ± SE
0	0	0[x]
60	95	8.67 ± 0.55a*
90	29	1.67 ± 0.33b
120	25	1.57 ± 0.3b
150	45	3.11 ± 0.589b
180	14	1.25 ± 0.25b
P value		P<0.001

[x]The zero responses are not included in the statistical analysis *Means followed by the same letter are not significantly different at P=0.05

4.3.2.2 Effect of different carbon sources on induction and regeneration of somatic embryos

The effects of different carbon sources on (a) percentage embryogenic cultures and (b) mean number of embryos are presented in Figure 4.1. The highest (40%) percentage of embryogenic cultures were observed in the medium supplemented with 90 mM maltose and the lowest (10%) percentage of embryogenic cultures were produced in the medium supplemented with 150 mM glucose. The highest (3) mean number of embryos was produced in medium supplemented with 90 mM fructose while the lowest (1) was observed in the medium supplemented with 150 mM glucose. However, no significant differences were observed in terms of mean number of somatic embryos per explant between the different sugars.

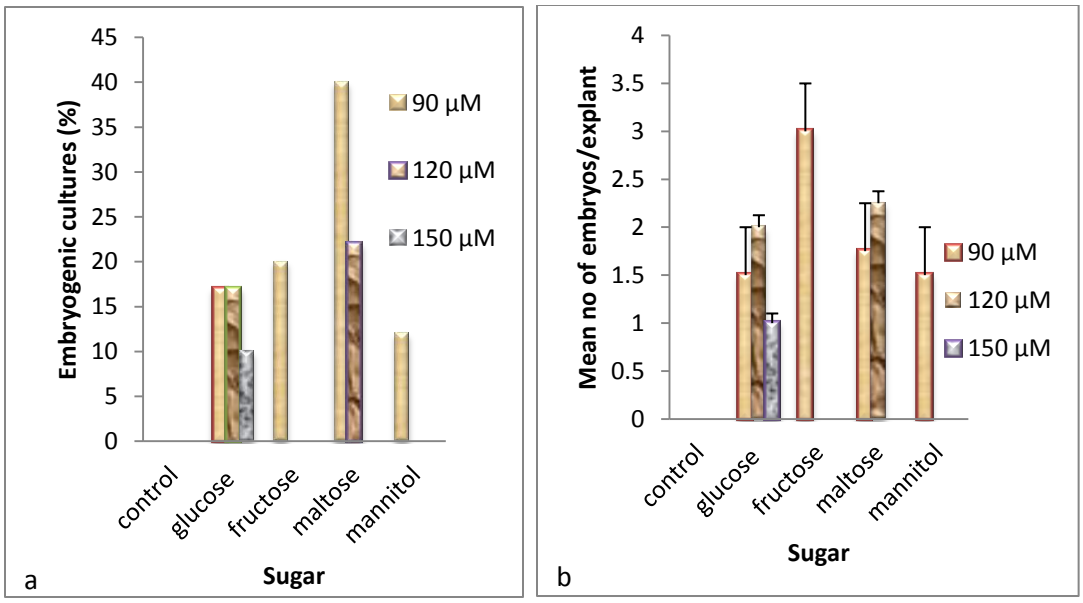


Figure 4.1 Effects of different sugars sources on (a) percentage embryogenic cultures and (b) mean number of embryos

4.3.3 Effect of doubling potassium nitrate on the induction of somatic embryos

Leaf explants cultured on medium supplemented with normal amount of potassium nitrate produced the highest (91%) percentage of embryogenic cultures (Table 4.10). Medium with double amount of potassium nitrate produced the lowest (33%) frequency of embryogenic cultures. There were no differences in terms of mean number of embryos per explant in the two concentration of potassium nitrate evaluated.

Table 4.10 Effects of potassium nitrate on induction of somatic embryos

KNO_3 concentration (mg/l)	**Embryogenic cultures (%)**	**Mean number of embryos/explant ± SE**
normal (950)	91	1.20 ± 0.13a*
Double (1900)	33	1.25 ± 0.25a
P Value		0.852

*Means followed by the same letter are not significantly different at P=0.05. n=20

4.3.4 Effects of some amino acids on the induction of somatic embryos

The effects of the amino acids on (a) percentage embryogenic cultures and (b) mean number of embryos are presented in Figure 4.2**.** The highest (91%) frequency of embryogenic cultures were observed in the control and the lowest (6%) percentage of embryogenic cultures were produced in medium supplemented with 150 µM proline. Leaf explants cultured on medium supplemented with 150 µM tryptophan produced the highest (6.5) mean number of somatic embryos and the lowest (1.2) mean number of somatic embryos were produced in the control. The medium supplementing with 150 µM leucine did not support embryogenesis.

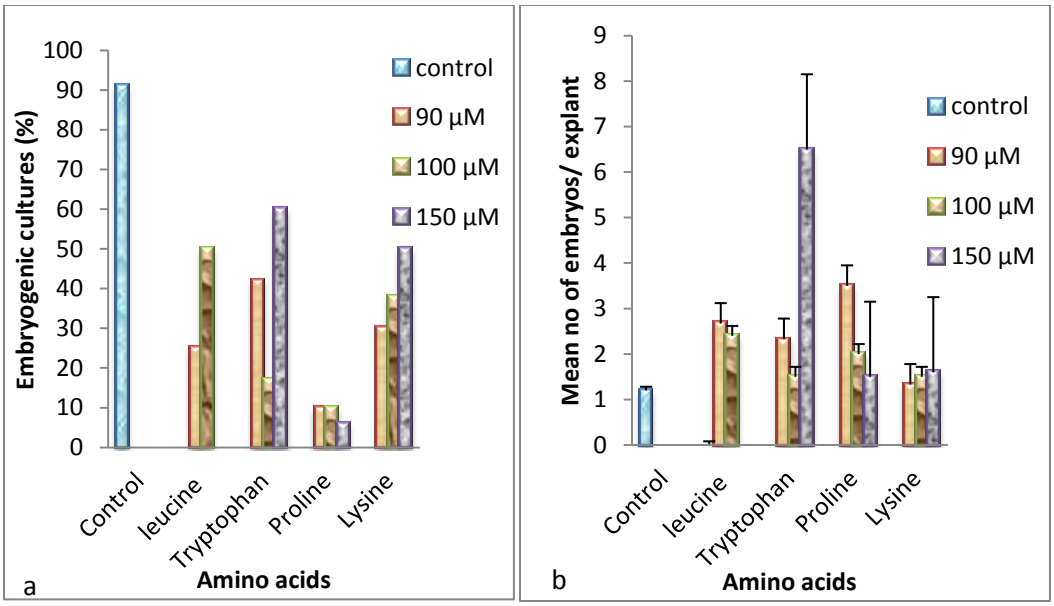


Figure 4.2 Effects of some amino acids on (a) percentage embryogenic cultures and (b) mean number of embryos

4.3.5 Effects of silver nitrate on the induction of somatic embryos

The highest (91%) frequency of embryogenic cultures were observed in the control and the lowest (25%) percentage of embryogenic cultures were produced in medium with 150 µM silver nitrate (Table 4.11). Leaf explants cultured on medium supplemented with 50 µM silver nitrate produced the highest (6.5) mean number of somatic embryos. On the other hand, the lowest (1.2) mean number of somatic embryos was produced in the control.

Table 4.11 Effects of silver nitrate on induction of somatic embryos

$AgNO_3$ conc (µM)	Embryogenic cultures (%)	Mean number of embryos/explant ± SE
0	91	1.2 ± 0.13b*
50	90	6.49 ± 0.38a
100	67	1.5 ± 0.27b
150	25	1.75 ± 0.25b
P-value		P<0.001

*Means followed by the same letter are not significantly different at P=0.05. n= 20

4.3.6 Effects of activated charcoal and casein hydrolysate on the induction of somatic embryos

The effects of CH are presented in Table 4.12. The highest (91%) percentage of embryogenic cultures were observed in the control while the lowest (30%) were observed in the medium supplemented with 100 mg/l CH. The highest mean number of embryos were produced in the medium supplemented with 50 mg/l CH. On the other hand, the lowest (1.2) mean number of embryos were produced in the control. AC at all the concentrations evaluated did not support somatic embryogenesis.

Table 4.12 Effects of casein hydrolysate on induction of somatic embryos

CH conc. (mg/l)	Embryogenic cultures (%)	Mean number of embryos/explant ± SE
0	91	1.2 ± 0.13b*
50	58	4.15 ± 0.5a
100	50	3.38 ± 0.42a
150	30	2.67 ± 0.67a
P-value		P<0.01

*Means followed by the same letter are not significantly different at P= 0.05

4.3.7 Effect of some organic extracts on induction of somatic embryogenesis

The results of the effects of potato starch, corn starch and papain on percentage embryogenic cultures and mean number of embryos are presented in Figure 4.3. The highest (91%) percentage of embryogenic cultures were observed in the control and the lowest (4%) were observed in the medium with 150 mg/l papain. The highest (1.7) mean number of somatic embryos were produced in the medium supplemented with corn starch and the lowest (1) in the medium with papain 100 and 150 mg/l. Potato starch at concentrations 50 and 100 mg/l did not support embryogenesis. However, there was an insignificant difference between the mean number of in potato, corn starch and papain and the control ($P \geq 0.05$).

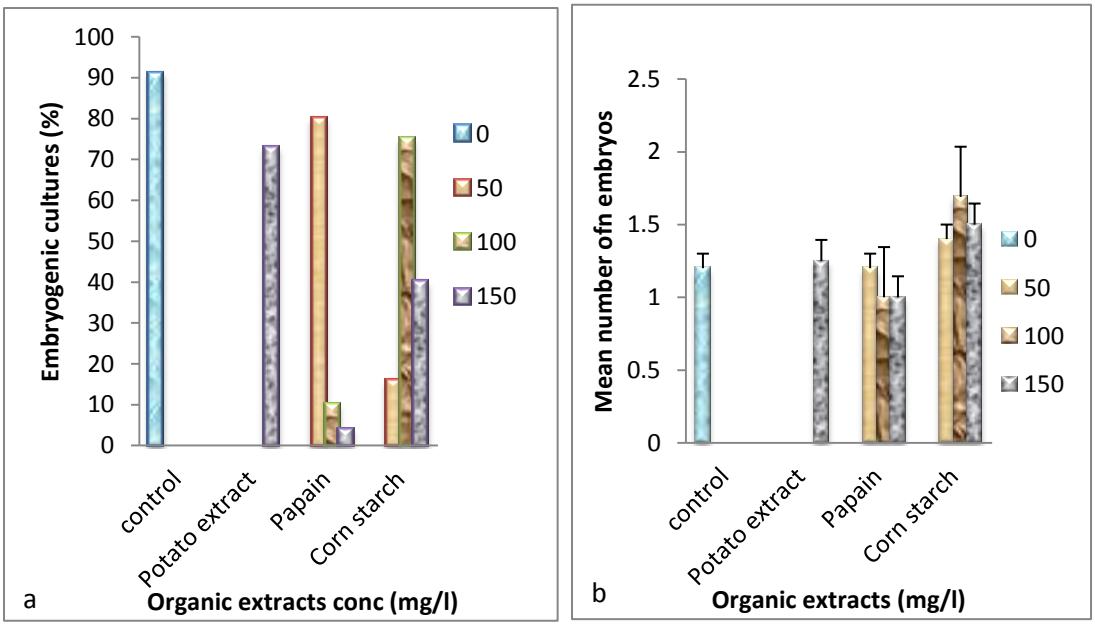


Figure 4.3 Effects of some organic extracts on (a) percentage embryogenic cultures and (b) mean number of embryos

The results of the effects of coconut milk on induction and regeneration of somatic embryos are presented in Table 4.13. The highest (91%) percentage of embryogenic cultures were observed in the control and the lowest (30%) were observed in the medium with 30% coconut milk. The highest (3.18) mean number of somatic embryos were produced in the medium supplemented with 20% coconut milk and the lowest (1.2) in the control.

Table 4.13 Effects of coconut water on induction and regeneration of embryos

Coconut water conc.(%)	**Embryogenic cultures (%)**	**Mean number of embryos/explant ± SE**
0	91	1.2 ±0.13 b*
10	50	1.6± 0.4 b
20	85	3.18± 0.3a
30	30	2.33±0.33a, b
P value		P<0.001

*Means followed by the same letter are not significantly different at P= 0.05

4.3.8 Effect of liquid pulsing on the induction of somatic embryos

The effects of liquid pulsing on percentage embryogenic cultures and mean number of embryos are presented in Figure 4.4. Callus pretreatment in liquid medium for different periods had a very significant effect on somatic embryo induction and proliferation. Callus tissues pretreated in liquid medium for 24 hours produced the highest (94%) frequency of embryogenic cultures while the lowest frequency embryogenic cultures was observed in calluses pretreated in liquid medium for 6

hours. The highest (9.19) mean number of somatic embryos were observed on callus tissues pretreated in liquid medium for 24 hours and the lowest mean number (1.2) of embryo somatic embryos was produced in the control.

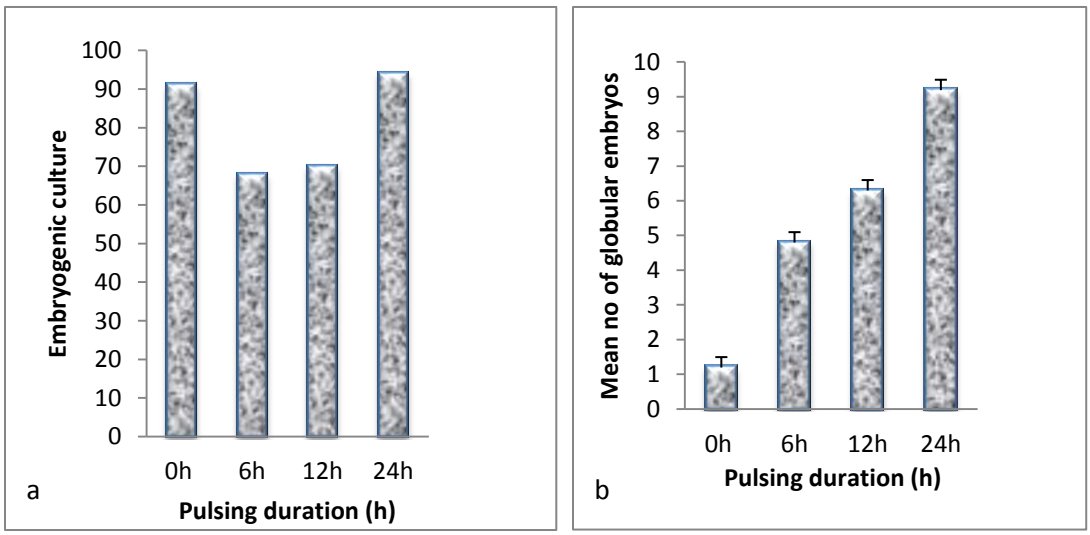


Figure 4.4 Effect of liquid pulsing on (a) percentage embryogenic cultures and (b) mean number of embryos

4.4 Effect of growth regulators on shoot and root regeneration from nodal explants

4.4.1 Effect of BAP on microshoot regeneration

Shoots were first observed two weeks after culture. The effect of BAP on microshoot regeneration is presented in Table 4.14 and Plate 4.2 (a). BAP at 10µM/l gave the highest mean number (2.25) of shoots per explant and the highest mean length (0.9). Increasing the concentration of BAP from 10 to 40 µM/l decreased the number of microshoots/node and the mean shoot length.

Table 4.14 Effect of BAP on microshoot regeneration

Concentration (μM/l)	Mean no. of microshoot /explants (±SE)	Mean length of microshoot (cm) (±SE)
0	0[x]	0
5	1.4 ± 0.25b*	0.32 ± 0.049b
10	2.25 ± 0.16a	0.9± 0.05a
20	2.0 ± 0b	0.38 ± 0.138b
40	1.67 ± 0.33b	0.33 ± 0.03b
P value	0.023	P<0.001

[x] The zero responses are not included in the statistical analysis. *Means followed by the same letter are not significantly different. n= 20

4.5.2 Effect of IBA on root regeneration

After one month on microshoot induction media, shoots were transferred to rooting medium. The effect of IBA on root regeneration is presented in Table 4.15 and plate 4.2 (b). IBA at 10 μM/l gave the highest mean number (2.2) of roots per explant. However, there were no significant differences between the different treatments.

Table 4. 15 Effect of IBA on root regeneration

Concentration (μM/l)	Mean no. of root /explants (±SE)
0	0[x]
5	2 ± 0.37a*
10	2.2 ± 0.37a
20	1.6 ± 0.25a
40	1 ± 0.00 a
P value	0.168

[x] The zero responses are not included in the statistical analysis. *Means followed by the same letter are not significantly different. n= 20.

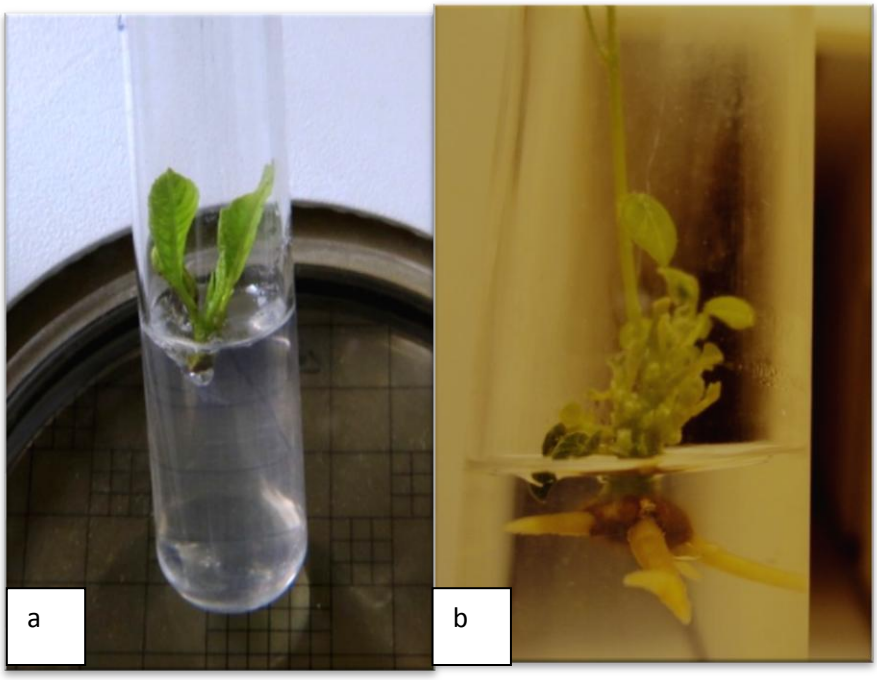


Plate 4.2 (a) Regenerated microshoot after four weeks on shooting media (b) Rooting of *in vitro* shoots after four weeks of culture

CHAPTER 5 DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

5.1.1 Nutritional variation of *V. doniana* young leaves

Green leafy vegetables such as *V. doniana* young leaves form an indispensable constituent of diet in Africa and in Benin in particular. In the present study, the nutritional variation of *V. doniana* young leaves obtained from different climatic zones in Benin were analyzed. Previous studies conducted on *V. doniana* young leaves from different provenances in Nigeria have shown that the protein concentration varies from 5.85% to 13.85 % in raw samples (Otitoju *et al.*, 2014; Osum *et al.* 2013; Adejumo *et al.*, 2013 while the moisture content is in the range of 21.14 to 78.76% (Otitoju *et al.*, 2014; Osum *et al.* 2013; Adejumo *et al.*, 2013). Fat, fiber, carbohydrate, ash content range from 1.10 and 2.92%, 1.85 and 2.75% , 7.57 and 12.92%, 1.63 and 6.21% respectively (Otitoju *et al.*, 2014; Osum *et al.* 2013; Adejumo *et al.*, 2013). Most of the reported values are similar to the results obtained in this study except for the moisture content. The Fe concentration found in the present study is within range of the values reported by other authors (Otitoju *et al.*, 2014; Osum *et al.*, 2013). However, the Ca concentration is twice to four times higher than the maximum reported in the literature (Otitoju *et al.*, 2014; Osum *et al.*, 2013).

Vitamin C in *V. doniana* leaves is reported in the literature to vary from 20.80 to 91.28 mg/100g while Vitamin A varies from 0.53 to 2.15 mg/100g (Otitoju *et al.*, 2014; Osum *et al.*, 2013; Adejumo *et al.*, 2013). The reported values for Vitamin C concentration in previous studies are higher than the results obtained in the current

study. On the other hand, the Vitamin A concentration in the Guinean zone was found to be similar to the reported values but the concentration in the Sudanian zone was found to be double the previously reported values. Among the nutritional parameters evaluated, only the moisture, protein, calcium (Ca) and the vitamins A and C concentrations showed significant differences due to the agro-climatic zones. Other nutritive concentration of *V. doniana* leaves do not vary significantly ($p > 0.05$), across agro-climatic zones. They could not therefore linked to the agro climatic zones of provenance of the trees.

5.1.2 Surface sterilization of *V. doniana* explants

The study was conducted with an aim of optimizing the sterilization of *Vitex doniana* explants. The use of field grown plants as a direct source of explant material for obtaining 'clean' explant, presents a major challenge. Previous attempts to initiate clean explants from field grown coffee, especially those from canopy close to the ground, resulted in 100% contamination (Kahia, 1999). The disinfectant widely used for surface sterilization is sodium hypochlorite (Miche and Balandreau, 2001). It is usually purchased as household laundry bleach. A balance between concentration and time must be determined empirically for each type of explant because of phytotoxicity. Calcium hypochlorite being a mild sterilant, is rarely used for decontaminating explants. It may be less injurious to plant tissues than sodium hypochlorite (CSS 451, 2009). Bleach (1.4% NaOCl solution for 1 minute) has the capability to be an effective sterilization agent for leaves as observed in *A. crasna* and *A. sinensis* from greenhouse source (Okudera and Ito, 2009). Hassan *et al.* (2011) achieved effective sterilization when using 50% bleach for 20 minutes on shoot tips

of *A. hirta*, from greenhouse while leaf explants of *Allanblackia stuhlmannii* were best surface sterilized using 8% sodium hypochlorite (Neondo *et al.,* 2011). During the present study, sodium hypochlorite was found to be less effective than calcium hypochlorite in sterilizing *Vitex doniana* leaf explants. Explants treated with sodium hypochlorite turned brown and died. This is probably due to the fact that leaves used were too young and the sterilant caused phytotoxicity. Mercuric chloride (0.1 and 0.5 %) was found toxic in sterilizing node explants. This result is contrary to those of Vadawale *et al.* (2006) who achieved surface sterilization of *Vitex negundo* nodes using 0.05% mercuric chloride. The toxicity observed in this study can explained by the relatively high concentrations used. Many authors succeeded in surface sterilization using calcium hypochlorite. Mihaljević *et al.,* (2013) while working on shoots of sour cherry recorded 80% clean explants when 3% $Ca(OCl)_2$ for 15 minutes was used. In order to increase the percent clean explant and reduce as well the toxicity observed both with sodium hypochlorite and mercuric chlorite, a two- step sterilization procedure was evaluated. This procedureallows the explants to be sterilized twice and at the same reduces the number of dead explants due to toxicity. Indeed, between the two steps explants are allowed to breathe while they are rinsed. Similar results were reported by Nieves and Aspuria, (2011) who recorded 90 % clean explants from cotyledon of *Moringa oleifera* using two steps comprising of 5% $Ca(OCl)_2$ for 15 minutes.

5.1.3 Factors affecting somatic embryogenesis in *V. doniana*

In vitro somatic embryogenesis may follow two routes: Direct embryogenesis in which the embryos arise directly from the explants tissue in the absence of callus

proliferation. This occurs through pre-embryonic determined cells (PEDC). The other route is indirect embryogenesis where there is a callus phase prior to embryo formation. The cells from which embryos arise are referred to as induced embryogenic determined cells (IEDC). In the majority of cases embryogenesis is through the indirect method. In this type, specific growth regulator concentrations are required for somatic embryos to arise. Media supplemented with cytokinins have been known to induce direct somatic embryos (Kahia, 1999). Most of the reports have indicated the requirement of an auxin alone or in combination with a cytokinin for callus formation (Sheeba *et al.,* 2013. Danya *et al*., 2012). In this study different types and concentrations of cytokinins namely BAP, Kin and TDZ were tested for induction of direct embryogenesis. Ten days after initiation callus induction was observed. Similar report was made by Manisha and Rajesh (2013) who obtained moderate to good callus growth in *Tecomella undulata* with different concentrations of BAP and Kin alone. The formation of callus in media supplemented with cytokinin alone may be explained by the presence of endogenous auxin and the sensitivity of the species to the hormones. In woody trees, auxin is mainly produced in young leaves (Aloni, 2007). During the current study very young leaves and this could be the reason why wt callus was obtained instead of embryos. In the work being reported, the best callus induction was obtained in the medium supplemented with TDZ at concentration 0.5 µM compared to Kin and BAP. Similarly, Lin *et al.* (2004) have suggested that TDZ is more effective than other cytokinins used for somatic embryogenesis in sweet potato and bamboo respectively. It has been suggested that TDZ either directly promotes growth due to its own biological activity or through

inducing the synthesis and/or accumulation of endogenous cytokinins or auxins (Panaia *et al.,* 2004). The latter could explain the effectiveness of TDZ as it may be mediating levels of endogenous auxin levels within the cultured tissue.

The most commonly used protocol for induction of embryogenesis involves the induction of callus in an auxin-supplemented medium (Deo *et al.,* 2010). Contrary to our findings, 2, 4-D has been found to be effective in many plant species. Wang *et al.* (2006) reported that 2, 4-D (0.2 mg/L) induced the embryogenic callus in *Chorispora bungeana*; similar results were observed in *Phoenix dactylifera* (Lin *et al.,* 2004). It was also observed that 3,4-D and dicamba were not effective. This probably is because those growth regulators share similarities in structure and activity to 2, 4-D (Balieldin *et al.,* 2000). However, the results showed that picloram at concentration 4 µM significantly enhanced the mean number of embryos. Similarly, Surdarmonowati and Henshaw (1996) observed that picloram (6 mg/l) improved embryogenesis in cassava. It can therefore be concluded that the initiation of embryos cannot be ascribed to any one plant growth regulator (Deo *et al.,* 2010). It also appears that different species have different levels of sensitivity towards various plant growth regulators; hence, their response to embryogenesis is variable.

Carbohydrates such as sucrose, fructose and glucose, maltose, mannitol, sorbitol were tested for their potential to induce and regenerate embryos. At low sucrose concentration (60 mM), the highest percentage of embryogenic culture were observed as well as the highest mean number of embryos. Increasing sucrose concentration did not improve the mean number of embryos. These results contrary to those of Gerdakaneh *et al.,* (2009) who found that increasing sucrose concentration from 2 to

6 % in the medium improved the development of somatic embryos in strawberry. However, they found that increasing the sucrose concentration from 6 to 12% decrease the number of embryos per explant. Similarly, Pande and Khetmalas, (2012) showed that culture medium supplied with 25 g/l sucrose did not influence somatic embryogenesis in *S. rebaudiana.* Increased sucrose concentration to 45g/l in culture medium resulted in less proliferation of callus and generation of somatic embryos. This demonstrated that optimum concentration of sucrose is important for embryogenesis. During the current study, mannitol, fructose, glucose, sorbitol did not improve somatic embryogenesis. This showed that the best carbon source for embryo culture in *V. doniana* is sucrose. Similar conclusion was made by Gerdakaneh *et al.*, (2009) while comparing sucrose, fructose and glucose in strawberry. Our study demonstrated that the type and concentration of sugar have important effects on the embryo development in *V. doniana*.

The recalcitrance of some species can be overcome by manipulating media components. During the current study the amount of KNO_3 in MS media was doubled. This resulted in no effect on the frequency of embryogenic cultures and the mean number of embryos. Similar report was made by Haq and Zafar (2004) in cotton where doubling the amount of KNO_3 in half MS (1/2 MS + 1.9 g/l KNO_3) decreased the number of embryos from 100 i to 88.5.

Requirement of exogenous supply of amino acids for *in vitro* somatic embryogenesis has been reported in a number of plant species (Gill *et al.,* 2004; Coste *et al.,* 2011). For instance, glutamine has been found beneficial for embryo development in date palm (El-Shiaty *et al.,* 2004). MS medium supplemented with 100 mg /l glutamine

gave the highest number of embryos (3.33) followed by MS medium with 50 mg/l glutamine which gave (3.00) with no significant difference between them. Sarker et al., (2007) found that significant response was found using L-asparagine at 150 mg/l concentration in four popular wheat Bangladeshi cultivars viz Kanchan, Shourav, Gourav and Satabdi. In the work being reported, four amino acids namely proline, lysine, leucine and tryptophan were evaluated. It was found that exogenous application of a specific amino acid, especially tryptophan, promoted the process of somatic embryogenesis and regeneration. A significant response was found using tryptophan at 150 µM concentration giving the highest (6.5) mean number of embryos. Similarly, when tryptophan (100 to 300 µM) was added to callus induction medium of rice, it was observed a great enhancement of the frequency of embryogenic cultures and regeneration (Shahsavari, 2011). Gill et al. (2004) reported that proline at 560 mg/l was able to increase somatic embryogenesisin surgarcane. In *Peucedanum oreoselinum*, embryo formation and maturation was enhanced by addition of proline (5 mM) in MS medium (Coste et al., 2011). Although there was no significant difference between the mean number of embryos in the media supplemented with proline, it was observed that the lowest concentration of proline increased almost 3 folds the mean number of embryos compared to the control. It was also found that an increase in proline concentration has resulted in decrease in the frequency of embryogenic cultures and the number of embryos. This results were contrary to those found in rice by Chowdhri et al., (1993) who showed that proline enhanced embryogenesis in rice. These authors reported that an increase in proline concentration from 3 M to 12 mM resulted in a proportional increase of the

percentage of culture showing regeneration (the maximum being 63% as compared to 23% without proline). The results obtained in the work being reported with leucine are contrary to those of Gill et al.(2004) and Coste et al. (2011) as. leucine at the highest concentration (150 µM) was found to inhibit embryogenesis. On the other hand, an increase in the frequency of embryogenic cultures and in the mean number of embryos was observed when the concentration of lysine increased from 50 to 150 µM even if there were no significant differences between the different concentrations and the control. Usefulness of tryptophan can be considered to provide organic nitrogen supply associated with *in vitro* culture and somatic embryogenesis. On the contrary, lysine, leucine and proline are not effective in providing nitrogen. Differential responses of different amino acids indicate the requirement of specific amino acids for somatic embryo regeneration.

The exact mechanism by which $AgNO_3$ affects somatic embryogenesis is not completely understood (Kong *et al.,* 2012). In the present study, $AgNO_3$ has been found to enhance somatic embryogenesis in *Vitex doniana*. Media supplemented with $AgNO_3$ has also been shown to improve somatic embryogenesis in species such as *Coffea canephora* (Rubiaceae) (Fuentes *et al.,* 2000), *Spinacia oleracea (*Ishizaki *et al.,* 2000), *Carthamus tinctorius* (Asteraceae) (Mandal *et al.,* 2001), *Paspalum scrobiculatum* (Poaceae) (Vikrant and Rashid, 2002), *Bactris gasipaes* (Arecaceae) (Steinmacher *et al.*, 2007), *Paspalum scrobiculatum* (Poaceae) and *Eleusine coracana* (Poaceae) (Kothari-Chajer *et al.,* 2008), *Hedychiurn bousigonianum* (Gingeberacea) (Sakhanokho *et al.*, 2009), *Gossypium nelsonii* (Malvaceae) and *G. australe* (Yan *et al.*, 2010), and *Pinus taeda* (Pinaceae) (Pullman *et al.*, 2003). Kong *et al.,* (2012)

demonstrated that $AgNO_3$ stimulates embryo formation in Manchurian ash. Ishizaki *et al.,* (2000) observed that in *Spinacia oleracea* addition of 10 mM $AgNO_3$ to the medium resulted in formation of about three times more embryos as compared with controls. In the present study, 50 µM of $AgNO_3$ was able to increase the number of embryos These results are contrary to those of Hatanaka *et al.* (1995) who reported that addition of $AgNO_3$ (50 µM) inhibited somatic embryo formation in leaf explants of *Coffea canephora*. Fuentes *et al.* (2000) found that the addition of $AgNO_3$ caused only small modifications in the ionic equilibrium of the medium and concluded the effects of the compound on somatic embryogenesis were not attributable to any substantial changes in available nutrients. In addition, the effects of $AgNO_3$ on somatic embryogenesis varied with the compound concentration. The lowest number (one) of embryos per explant was induced in Manchurian ash at 2.5 mg L^{-1} $AgNO_3$ while when 10 mg/L $AgNO_3$ was included in the medium, the total number of somatic embryos was the highest, with an average of 3.86 embryos per explant (Kong *et al*., 2012). Kong *et al.,* (2012) reported that the number of somatic embryos per explant in Manchurian ash increased with increasing $AgNO_3$ concentration. During the current study, the higher concentrations of $AgNO_3$ reduced the numbers of visible embryos.

In the work being reported, addition of casein hydrolysate to the medium increased the mean number of embryos. The highest mean number of embryos was observed in media supplemented with 50 mg/l casein hydrolysate. Khierallah and Hussein, (2013) observed the same result in date palm when 2 g/l casein hydrolysate was added to the medium. Angeel and Elmeer (2011) found that the best result of somatic embryos per

treatment was achieved with 3 g/ l casein hydrolysate in date palm. The Increase in the number of embryos by adding casein hydrolysate may be attributed to the fact that casein as a simple protein decomposes into galactosamine and glucosamine or mannosamine as well as simple sugars linked with protein with covalent bonds or glycoside bond with hydroxyl group of the amino acids serine and threonine (Al-Dalaly and Al-Rekaby, 1995). Activated charcoal has been found to have a positive effect on many species such as *Vitis vinifera* (Lopez-Perez *et al.,* 2005), pepper (Ellialtıoğlu *et al.*, 2001) and *Aesculus hippocastanum* (Calic-Dragosavac *et al.*, 2010). Lopez-Perez *et al.*, (2005) found that addition of AC (0.25 %) to the medium used for callus culture was an essential prerequisite to obtain somatic embryos in the case of *Vitis vinifera var*. Crimson Seedless. In Italia and Don Mariano; it greatly increased the frequency of embryogenic calli in *var.* Sugraone from 5.8 % in medium without AC to 99.5 % with AC. Activated charcoal is commonly used in tissue culture media to darken the immediate media surroundings and to absorb inhibitory or toxic substances (Moshkov *et al.,* 2008). Moreover, activated charcoal also has been shown to absorb 5-hydroxymethylfurfural, an inhibitor formed by the degradation of sucrose during autoclaving, as well as substantial amount of auxins and cytokinins. Consequently, apart from removing inhibitors that would prevent growth, it may also adsorb and reduce the levels of growth regulators. (Deo *et al.,* 2010). In this study, supplementation of the media with activated charcoal inhibited embryogenesis at all the concentrations evaluated. This observation might be due to the fact that AC degraded the growth regulator present in the medium and this affected embryogenesis.

Some researchers have underscored the need to include complex organic extracts, such as coconut water (CW), potato extract (PE), corn extract (CE) and papaya extract (PAE) for inducing somatic embryogenesis in some species (Ichihashi and Islam, 1999; Islam *et al.*, 2003; Rahman *et al.*, 2004). The current study investigated the effects of coconut water, potato and corn starch, papain on somatic embryogenesis in *V. doniana* and the results indicated that with potato starch, corn starch and papain did not improve embryogenesis. On the contrary, the best result was obtained when the modified VW medium supplemented with 20% potato extract produced the best results on proliferation of Vanda Kasem's Delight (VKD) orchid's protocorm-like bodies (Gnasekaran *et al*., 2012). Potato is a carbohydrate-rich food which contains about 80% water and 20% dry matter, with 60 to 80% of the latter composed of starch (Prokop and Albert 2008). There are antioxdants present in potatoes and beside them, notable amounts of proteins and vitamin B6 of high biological value could also promote somatic embryogenesis. Substantial amounts of alkaline salts in the form of potassium may thwart acid overdose (Demigne et al., 2004) and prevent medium fermentation, which in turn supports the production somatic embryos. In the current study, Potato starch was used and the negative response observed might be due to the concentrations evaluated which might be too low. Low concentrations of the potato starch could not provide sufficient amounts of some of the biological compounds present at trace levels in the potato starch. Similarly, concentrations of papain and corn starch did not improve embryogenesis in *V. doniana*. Complex organic extracts used in the present experiment contain carbohydrates, protein, fat, several vitamins, phenolic compounds, a lower level of some amino acids, and organic acids. However,

there could be other unknown substances, alone or in combination which might affect embryogenesis.

During the current study, supplementation of the media with coconut water improved somatic embryogenesis in *V. doniana*, with 20 % coconut water giving the highest response. Similarly, Khieralla and Hussein (2013) found that media supplemented with 20 % coconut water gave the highest number of embryos (65 embryos) in date palm. In contradictory to our results, Al-Khayri (2010) observed in date palm that 20 % coconut water decreased the number of embryos. The addition of coconut water to the culture media resulted in the plants with a greater nutritional and carbohydrates content as coconut itself contains 21.8 mg/L in total (George, 1993). The enhancement of the number of mean number of embryos compared to the control might be due to this high carbohydrate content. Mauney *et al.* (1952) purified a growth factor from the aqueous extract of coconut meat which was found to be very potent in promoting growth of tissue cultured plants. Coconut water was shown to have cytokinin like activity by Kuraishi and Okumura (1961) and recognised natural cytokinin substances have since been isolated [9-β-D-ribofuranosyl zeatin, and several unidentified compounds but the levels of these compounds in various samples of coconut water have not been reported. It has been observed that coconut contains natural cytokinins, adding it to medium culture often has the same effect as adding recognised cytokinin. Higher or lower concentrations (10 and 30%) of coconut water did not significantly increase the number of embryos in the current study probably due to reduction of the balance of nutrients or their availability.

It has been previously demonstrated that liquid pulse treatments with growth regulators could promote regeneration from plant cell culture through organogenesis or somatic embryogenesis (Madhulatha *et al.*, 2004). In addition, it has previously been reported that initial pretreatment of leaf explants of squash (*Cucurbita pepo L.*) with cytokinins for 6–48 h significantly promoted the formation of somatic embryos which developed further to the torpedo-shape stage and germinated (Kintzios *et al.*, 2002). Therefore, during the current study, it was found necessary to evaluate the effect of the pretreatment of *V. doniana* callus in a liquid nutrient medium on the induction and development of somatic embryos. In the present study, pretreatment in liquid nutrient medium for regeneration of *Vitex doniana* plants have been established for the first time. The induction and enhancement of somatic embryogenesis in leaf derived callus tissues within the experimental period of the present study was absolutely dependent on the physical state of the culture medium, since it only occurred when cultures were liquid-pulsed for 6, 12 or 24 hrs. The stimulating effect of liquid culture on *Vitex doniana* somatic embryogenesis could be associated with increased uptake of TDZ by the cultured cells. All the treatments increased the mean number of embryos compared to the control (leaf explants continuously cultured on solid medium). The highest number of embryos was observed on callus tissues pretreated in liquid medium for 24 h. This number was 8 fold higher than the mean number recorded in the control. This results is similar to those of Papanastasiou *et al.* (2008) who found that the highest number of embryos was observed on *Coffea arabica* callus tissues pretreated in 50 µM BA for 24 h and subcultured on BA-supplemented medium. However, they found that control and callus pretreated for 6 h

did not show any embryogenic response during the 5-weeks incubation period. On the contrary, we observed a mean number of embryos four fold higher than the control when callus were pretreated for 6 h.

5.1.4 Micropropagation in *V. doniana*

Cytokinin formulations were earlier shown to be critical for shoot elongation of many plant species, including medicinal plants (Rout *et al.* 2000, Rout, 2004). A range of cytokinins (Kinetin, BA, 2-ip and zeatin) has been used in micropropagation work (Bhojwani and Razdan, 1992). BAP has been shown to be the most reliable and useful cytokinin. A number of plants such as *Vitex negundo* (Usha *et al.*, 2007), *Vitex trifolia* (Sujin and De Britto, 2013) were successfully multiplied on medium containing BAP. In the present study, the concentration of BAP influenced the average number of shoots produced per explants as well as the mean length of the shoots. BAP evaluated at 10 µM was the most effective concentration. These results are similar to those of Usha *et al.* (2007) while regenerating shoot from *Vitex negundo*. They reported the highest (4.2) mean number of shoots per explant and the highest (1.8 cm) length of shoot in media supplemented with 8.87 µM BAP. BAP at 2 mgl/1, proved to be optimal, producing an average mean number of shoot of 4 and an average shoot length of 5.73 cm per explants in *Vitex trifolia* (Sujin and De Britto, 2013). During the present study it was observed that lower (5 µM) level of BAP is not suitable for shoot regeneration as well as higher concentrations (20 and 40 µM).. Similarly, in *Vitex trifolia*, 2 mg /1 was found to be the optimal because at higher concentration of BAP, the number of shoot per explant decreased (Sujin and De Britto, 2013).

Shoots derived from nodal explants exhibited good response for root induction in a medium supplemented with IBA. These results are similar to those of Rathore and Shekhawat (2011) who reported *in vitro* rooting of micropropagated shoots of *Vitex negundo* using IBA and maximum number of roots (6-7) of with a length of 4-5 cm with 3 mg/l.

5.2 Conclusion

Results of this study showed that *V. doniana* young leaves are highly nutritious. The protein, Calcium, vitamin A and C concentration vary significantly across agro climatic zones. The highest content of protein (7.6 %), Vitamin A (0.55 mg/100g) and C (17.78 mg/100g) were obtained in the Sudanian zone while the highest concentration of calcium (242.95 mg/100g) was obtained in the Sudano-Guinean zone.

It can be concluded that a double sterilization is more effective for sterilization of field-grown derived leaf and nodal explants of *Vitex doniana*. *V. doniana* is ameniable to tissue culture. Among all the treatments evaluated the liquid state of the medium gave the best result on embryogenesis given a mean number of embryos eight fold higher (9.19) than the control (1.2).

BAP was used for shoot regeneration. The best treatment was BAP 10µM which gave the highest mean shoot number and mean shoot length. IBA at all evaluated concentrations was able to regenerate roots but no optimum concentration was found.

This works provides useful information that can be used to catalyse increased domestication of *V. doniana* in Benin based on the nutritional properties of the

species and the *in vitro propagation* protocols developed. However, more work needs to be done to strengthen the current outputs as described in section 5.3.

5.3 Recommendations

V. doniana has a high content of crude protein, oils, minerals, and vitamins. We recommend this species as a complex food supply. Further to the findings of our study, we recommend:

- more investigations on the bioavailability of nutrients from the leaves of *V. doniana* as well as its anti-nutritional composition.
- more investigations on the nutritional variation of *V. doniana* by analysis the link between the soil types and the samples' provenances

Mature somatic embryos were obtained in this study after a long period. For more efficient and complete tissue culture protocol, we recommend:

- Optimization of maturation process in future studies which will provide enough material for germination studies
- Evaluation of other growth regulators on micropropagation for optimum plantlets' regeneration
- Studying the somaclonal variation
- Conversion and the performance in the field of both embryo-derived plantlets and plantlets obtained from micropropagation.

REFERENCES

Achigan-Dako EG, Pasquini MW, Assogba-Komlan F, N'danikou S, Yédomonhan H, Dansi A Ambrose-Oji B (2010). Traditional vegetables in Benin: diversity, distribution, ecology, agronomy, and utilisation. Institut National des RecherchesAgricoles du Bénin, Benin. 252 pp.

Adejumo AA, Alaye SA, Ajagbe RO, Abi EA, Adedokun FT (2013). Nutritional And Anti – Nutritional Composition Of Black – Plum (*Vitex doniana) Journal of Natural Sciences Research* 3 (12): 144-148

Adeyeye IA, Omotayo FO (2011). *Agriculture and Biology Journal of North America*, p 502

Adomou AC (2005): Vegetation patterns and environmental gradients in Benin: Implications for biogeography and conservation. PhD Thesis Wageningen University, Wageningen

Agbedje JO, Ibitoye AA (2007). Chemical composition of black plum (*Vitex doniana*): an underutilised fruit. *Journal of Food Agriculture and Environment* 5: 95-96.

Angeel S, Elmeer K (2011). Effects of Casein Hydrolysates and Glutamine on Callus and Somatic Embryogenesis of Date Palm (*Phoenix dactylifera* L.). *New York Science Journal* 4(7): 121-125

Aghaei P, Bahramnejad B, Mozafari AA (2013). Effect of different plant growth regulators on callus induction of stem explants in *Pistacia atlantica subsp. kurdica Plant Knowledge Journal* 2(3): 108-112

Ahmed ABA, Suryanarayana R A, Venkateswara MR, Taha RM (2011). Effect of picloram, additives and plant growth regulators on somatic embryogenesis of *Phyla nodiflora* (L.) Greene. *Brazilian archives of biology and technology* 54 (1): 7-13

Akoègninou A (2004). Recherches botaniques et écologiques sur les forêts actuelles du Bénin. Thèse d'Etat. Université de Cocody-Abidjan (Côte d'Ivoire), 326 pp.

Akutsu M, Sato H (2002). Induction of proembryos in liquid culture increases the efficiency of plant regeneration from *Alstroemeria* calli. *Plant Sci*ence 163:475–479

Al-Dalaly BK, Al-Rekaby K (1995). Food Chemistry, (in Arabic) Al-Kendy Publisher, Baghdad, Iraq. pp. 234.

Al- Khayri JM (2010). Somatic embryogenesis of Date Palm (*Phoenix dactylifera* L.) improved by coconut water. *Biotechnology*, 2010: 1-8

Aloni R (2007). Phytohormonal mechanisms may control wood quantity formation in young and mature trees. In K Entwiistle, P Harris, J Walker (eds). The compromised wood workshop. The Wood Technology Research Center, University of Canterbury, Christchurch, New Zealand

Analyse Globale de la Vulnérabilité, de la Sécurité Alimentaire et de la Nutrition, AGVSAN (2009). République du Bénin, Program Alimentaire Mondial (PAM), UNICEF, FAO. PAM, Rome – Italy.

AOAC. (2000). Official Methods of Analysis 14th ed. Association of Official Analytical Chemists Washington, D.C.

Assogbadjo AE, Chadare FJ, Glele Kakaï R, Fandohan B, Baidu-Forson JJ (2012). Variation in biochemical composition of baobab (*Adansonia digitata*) pulp, leaves,

and seeds in relation to soil types and tree provenances. *Agriculture, Ecosystems and Environment* 157: 94– 99

Bahieldin A, Dyer WE, QU R (2000). Concentration effects of dicamba on shoot regeneration in wheat. *Plant Breeding* 119, 437-439

Barna KS, Wakhlu AK (1994) Whole plant regeneration of *Cicer arietium* from callus culture via organogenesis. *Plant Cell Reports* 13:510-513.

Bhojwani SS, Razdan MK (1992). Plant tissue culture: Theory and practice, Elsevier, Amsterdam, London, New York, Tokyo. *Trifolium repens* genotypes. *Physiologia Plantarum* 52:187-190.

Burchi F, Fanzo J, Frison E (2011). The Role of Food and Nutrition System Approaches in Tackling Hidden Hunger. *International Journal of Environmental Research and Public Health* 8, 358-373.

Calic-Dragosavac D, Zdravkovi-Korac S, Bohanec B, Radojevic L, Vinterhalter B, Stevovic S, Cingel A, Savic J (2010). Effect of activated charcoal, abscisic acid and polyethylene glycol on maturation, germination and conversion of *Aesculus hippocastanum* androgenic embryos. *African Journal of Biotechnology* 9 (25): 3786-3793

Chandra S, Bandopadhyay Rajib, Kumar V, Chandra R (2010). Acclimatization of tissue cultured plantlets: from laboratory to land. Biotechnology Letter 32, 1199–1205

Chowdhry CN, Tyagi AK, Maheshwari N, Maheshwari SC (1993). Effect of L-proline and L-tryptophan on somatic embryogenesis and plantlet regeneration of rice (Oryza sativa L. cv. Pusa 169). Plant Cell Tissue and Organ. Culture. 32: 357-361.

Codjia JTC, Assogbadjo AE, Ekue MRM (2003). Diversite et valorization au niveau local des resource vegetales forestieres alimentaires du Benin. *Cahiers Agricultures* 12: 321-331.

Coste A, Oltean B, Halmagyi A, Deliu C (2011). Direct somatic embryogenesis and plant regeneration in Peucedanum oreoselinum (L.) Moench**.** *Romanian Biotechnological Letter* 16(4):6451-6459

CSS 451 (2009) Biotechnology Applications for Plant Breeding and Genetic. January 13, Course and laboratory orientation; sterile techniques and tissue culture https://www.msu.edu/course/css/451/ accesed on 24th May 2014

Dadjo C, Assogbadjo AE, Fandohan B, Glèlè Kakaï R, Chakeredza S, Houehanou TD, Van Damme P, Sinsin B (2012). Uses and management of black plum (*Vitex doniana* Sweet) in Southern Benin. *Fruits* 67(4) 239-248.

Dansi A, Adjatin A, Adoukonou-Sagbadja H, Faladé V, Yedomonhan H, Odou D, Dossou B (2008). Traditional leafy vegetables and their use in the Benin Republic. *Genetic Resources and Crop Evolution* 55, 1239-1256.

Danya U, MR Udhayasankar, K Arumugasamy, D Punitha (2012). An efficient protocol devised for rapid callus induction from leaf explants of Stephania wightii (Arn.) Dunn. - an endemic medicinal plant. *Research in Plant Biology* 2(5): 18-22

Demigne C, Sabboh H, Puel C, Remsey C, Coxam V (2004) Organic anions and potassium salts in nutrition and metabolism, *Nutrition Research Reviews*, Cambridge journals, Cambridge University Press 17: 249-258.

Deo PC, Tyagi AP, Taylor M, Harding RM, Becker DK (2010). Factors affecting somatic embryogenesis and transformation in modern plant breeding. *The South Pacific Journal of Natural and Applied Sciences* , 28(1): 27-40.

Dhillon NK, Gosal SS (2012). Effect of growth adjuvents on somatic embryogenesis in maize (*Zea mays* L.) *Journal of Cell & Tissue Research*, 12(2), p3205

Duran RE, Coskun Y, Demırcı T (2013). Comparıson of amıno acıds for theır efficıency on regeneratıon ın wheat embryo culture. *Asian Journal of Plant Science and Research* 3(1):115-119

Ellialtıoğlu Ş, Kaplan F, Abak K (2001). The effect of carrot extract and activated charcoal on the androgenesis of pepper. XIth EUCARPIA Meeting on Genetics and Breeding of Capsicum & Eggplant, 2001, Antalya-Turkey

El-Shiaty OH, El-Sharabasy SF, El- Kareim AHA (2004). Effect of some amino acids and biotin on callus and proliferation of date palm (Phoenix dactylifera L.) Sewy cultivar. *Arabian Journal of Biotechnology* 7: 265-272.

FAO (2011). Second Global Plan of Action for the conservation and sustainable Utilization of Plant Genetic Ressources for Food and agriculture ISBN 978-92-5-107163-2 Rome, Italy.(**http://www.fao.org/docrep/015/i2624e/i2624e00.htm**) retrieved on 27, April, 2013.

FAO/WHO (2003). Diet, nutrition and the prevention of chronic diseases. Report of a Joint FAO/WHO Expert Consultation. WHO Technical Report Series No. 916. Geneva.

FAO/WHO (2004). Promoting fruit and vegetable consumption around the world. Fruit and vegetables for health. Report of a Joint FAO/WHO Workshop, 1–3 September, 2004, Kobe, Japan.

Fuentes SRL, Calheiros MBP, Manetti-Filho J, Vieira LGE (2000). The effects of silver nitrate and different carbohydrate sources on somatic embryogenesis in *Coffea canephora*. *Plant Cell Tissue and Organ Culture.* 60: 5-13.

George EF, Hall MA, De Klerk G-J (2008). Plant Propagation by Tissue Culture: Volume 1. The Background - Originally published by Exegetics, Basingstoke, UK 3rd ed.Springer. pp:81

Georges EF (1993). Plant propagation by tissue culture. *The technology* 2nd edition London Exegetics

Gerdakaneh M, Mozafari AA, Khalighi A, Sioseh-mardah A (2009). The Effects of Carbohydrate Source and Concentration on Somatic Embryogenesis of Strawberry (Fragaria x ananassa Duch.). *American-Eurasian Journal Agriculture. & Environmental. Sciences.* 6 (1): 76-80

Ghane SG, Lokhande V H., Ahire M L, Nikam TD (2010). *Indigofera glandulosa* Wendl. (Barbada) a potential source of nutritious food: underutilized and neglected legume in India. *Genetic Resources and Crop Evolution* 57(1): 147-153

Gill NK, Gill R, Gosal SS (2004). Factors enhancing somatic embryogenesis and plant regeneration in surgarcane (*Saccharum officinarum* L.)**.** *Indian Journal of Biotechnology*, 3: 119-123

Giridhar P, Indu EP, Ravishankar GA, Chandrasekar A (2004). Influence of TRIA on somatic embryogenesis in *Coffea arabica* L. and *Coffea canephora* P. EX FR. *in vitro cellular and Development Biology Plant* 40:200-203.

Glew RH, Dorothy JV, Cassius L, Louis E, Garrett CS, Andrzej P, Mark M (1997). Amino acid, fatty acid, and mineral composition of indigenous plants of Burkina Faso. *Journal of Food Composition and Analysis* 10: 205-217.

Guo B, Abbasi BH, Zeb A, Xu LL, Wei YH (2011). Thidiazuron: A multi-dimensional plant growth regulator. *African Journal of Biotechnology*, 10(45): 8984-9000

Haq IU, Zafar Y, (2004). Effect of nitrates on embryo induction efficiency in cotton (Gossypium hirsutum L.) *African Journal of Biotechnology* 3(6): 319-323

Hassan NH, Ali NAM, Zainudin F, Ismail H (2011). Effect of 6-benzylaminopurine (BAP) in different basal media on shoot multiplication of *Aquilaria hirta* and detection of essential oils in the *in vitro* shoots**.** *African Journal of Biotechnology*, 10(51):10500-10503

Hatanaka T, Sawabe E, Azuma T, Uchida N, Yasuda T (1995). The role of ethylene in somatic embryogenesis from leaf discs of Coffea canephora**.** *Plant Sciences.* 107:199-204.

Hierallah HSM., Hussein NH (2013). The role of coconut water and casein hydrolysate in somatic embryogenesis of date palm and genetic stability detection using RAPD markers. *Research in Biotechnology*, 4(3): 20-28

Hirose N, Takei K, Kuroha T, Kamada-Nobusada T, Hayashi H, Sakakibara H (2007). Regulation of cytokinin biosynthesis, compartmentalization, and translocation. *J Exp Bot* 14: 1-9.

Hussain A, Qarshi IA, Nazir H, Ullah I (2012). available from http://www.intechopen.com/download/pdf/40180. retrieved on 25th august 2013

Ichihashi S, Islam M O (1999). Effects of complex organic additives on callus growth in three orchid genera, Phalaenopsis, Doritaenopsis and Neofinetia. *Journal of Japanese Society for Horticultural Science*, 68: 269-274

Ishizaki T, Komai F, Msuda K (2000). Exogenous Ethylene Enhances Formation of Embryogenic Callus and Inhibits Embryogenesis in Cultures of Explants of Spinach Roots. *ournal of the American Society for Horticultural Science.* 125(1):21–24.

Islam M O, Rahman ARMM, Matsui S, Prodhan AKMA (2003). Effects of complex organic extracts on callus growth and PLB regeneration through embryogenesis in the Doritaenopsis orchid. *Japan Agricultural Research Quarterly* 37: 229-235.

Iwueke AV, Nwodo OFC, Okoli CO (2006). Evaluation of the anti-inflammatory and analgesic activities of *Vitex doniana* leaves. *African Journal of Biotechnology* 5 (20): 1929-1935.

Jittayasothorn Y, LU J, Xu X, Thipyagong P, Boonkerd N (2007). A simple and highly efficient protocol for somatic embryogenesis and plant regeneration from proembryonic mass suspension culture in 'Autumn Royal Seedless' *Vitis* **46** (1): 45–46

Johns T, Eyzaguirre PB (2006). Symposium on Wild-gathered plants: basic nutrition, health and survival‟ Linking biodiversity, diet and health in policy and practice. *Proceedings of the Nutrition Society* 65: 182–189

Jonsson U (2009). Paradigms in Applied Nutrition Paper .19th International Congress of Nutrition (ICN) Bangkok, 4-9 October 2009

Kahia WJ (1999). *In vitro* propagation of the new *Coffea arabica* cultivar- Ruiru 11. PhD thesis. University of London . United Kingdom

Kapooria RG, Aime MC (2005). First report of the rust fungus *Olivea scitula* on *Vitex doniana* in Zambia. *Plant Disease* 89:431.

Kintzios S, Seretti E, Blouhos P, Drossopoulos JB, Kitsaki CK, Liopa-Tsakalidis A (2002). Growth regulator pre-treatment improves somatic embryogenesis from leaves of squash and melon. *Plant Cell Reports* 21:1–8

Kong D, Shen H, Li **N** (2012). Influence of AgNO3 on somatic embryo induction and development in Manchurian ash (*Fraxinus mandshurica* Rupr.). *African Journal of Biotechnology* 11(1): 120-125

Körbes AP, Droste A (2005). Carbon sources and polyethylene glycol on soybean somatic embryo conversion. *Pesquisa Agropecuária Brasileira,* 40 (3): 211-216,

Kothari-Chajer A, Sharma M, Kachhwaha S, Kothari SL (2008). Micronutrient optimization results into highly improved *in vitro* plant regeneration in kodo (*Paspalum scrobiculatum* L.) and finger millets (*Eleusine coracana (*L.) Gaertn.). *Plant Cell Tissue Organ Culture* 94: 105-112.

Kumar A, Kumar VA (1996). Plant Biotechnology and Tissue Culture Principles and Perpectives, International Book Distributing Co, Lucknow.

Kuraishi S, Okumura FS (1961). A new green-leaf growth stimulating factor, phyllococosine, from coconut water. *Nature* 189:148-149

Lakshmanan P, Taji A (2000). Somatic embryogenesis in leguminous plants. *Plant Biology* 2: 136-148.

Lavanya M, Venkateshwarlu B, Devi BP (2009). Acclimatization of neem microshoots adaptable to semi-sterile conditions. Indian Journal of Biotechnology 8:218–222

Li XY, Huang FH, Murphy JB,. Gbur Jr E.E (1998). Polyethylene glycol and maltose enhance somatic embryo maturation in loblolly pine (*Pinus taeda* L.). *In vitro Cellular and Development Biology Plant* 34:2–26.

Lin CS, Lin CC, Chang WC (2004). Effect of thidiazuron on vegetative tissue–derived somatic embryogenesis and flowering of bamboo *Bambusa edulis. Plant Cell, Tissue and Organ Culture,* 76, 75-82.

Lopez-Perez AJ, Carreno J, Martinez-Cutillas A, Dabauza M (2005) High embryogenic ability and plant regeneration of table grapevine cultivars (Vitis vinifera L.) induced by activated charcoal. *Vitis* 44 (2): 79–85

Louppe D, Oteng-Amoako AA, Brink M(2008). Timbers 1. Plant Resources of Tropical Africa. Wageningen, the Netherlands. PROTA Foundation. Backhuys Publishers. CTA. 7 (1) 704pp.

Madhulatha P, Anbalagan M, Jayachandran S, Sakthivel N (2004). Influence of liquid pulse treatment with growth regulators on *in vitro* propagation of banana (Musa spp. AAA)**.** *Plant Cell Tissue and Organ Culture* 76:189–191

Magbagbeola JAO, Adetoso JA, Owolabi OA (2010). Neglected and underutilized species (NUS): A panacea for community focused development to poverty alleviation/poverty reduction in Nigeria. *Journal of Economics and International Finance* 2(10): 208-211.

Manchanda P, Gosal S (2012). Effect of activated charcoal, carbon sources and gelling agents on direct somatic embryogenesis and regeneration in sugarcane via leaf roll segments. *Sugar Technology.*14:168-173.

Mandal AKA, Gupta DS, Chatterji AK (2001). Factors affecting somatic embryogenesis from cotyledonary explants of safflower. *Biologia Plantarum* 44: 503-507.

Manisha BP, Rajesh SP (2013). Effects of Plant Growth Regulators (PGRs) on callus induction from leaf segment explant of *Tecomella undulata* (Sm.) Seem - An Important Medicinal plant. *International Journal of Scientific and Research Publications* 3(12): 1-4

Mapongmetsem PM, Djoumessi MC, Yemele MT, Fawa G, Doumara DG, Noubissie JBT, Tientcheu A, Bellefontaine MLR (2012). Domestication de *Vitex doniana* Sweet. (Verbenaceae): influence du type de substrat, de la stimulation hormonale, de la surface foliaire et de la position du noeud sur l'enracinement des boutures uninodales *Journal of Agriculture and Environment for International Development* 106 (1): 23 - 45

Mauney JK, Hillman WS, Miller CO, Skoog F, Clayton RA, Strong FM (1952). Bioassay, purification and properties of Growth Factor from coconut. *Physiologia Plantarum* 5(4):485-497

Miche L, Balandreau J (2001). Effects of rice seeds sterilization with Hypochlorite on inoculated Burkholderia vietnamiensis. *Applied and Environment Microbiology* 67(7): 3046-3052

Mihaljević I, Dugalić K, Tomaš V, Viljevac M, Pranjić A, Čmelik Z, Puškar B, Jurković Z (2013). *In vitro* sterilization procedures for micropropagation of 'OBLAČINSKA' sour cherry. *Journal of Agricultural Sciences* 58 (20): 117-126

Mona HM, Rania TA (2012). Callogenesis, Somatic Embryogenesis and Regeneration of Date Palm *Phoenix dactylifera* L. Cultivars Affected by Carbohydrate Sources. *International Journal of Agricultural Research* 7(5): p231

Moore G (2010). Multilateral and national regulatory regimes for agrobiodiversity. In Lockie, S., and Carpenter, D. (Eds.). Agriculture, biodiversity and markets: Livelihoods and agroecology in comparative perspective. Washington DC, Earthscan.

Moshkov IE, Novikova GV, Hall MA, George EF (2008). Plant growth regulators III: gibberellins, ethylene, abscisic acid, their analogues and inhibitors; miscellaneous compounds. In: George EF, Hall MA, De Klerk GJ (eds). Plant propagation by tissue culture, 3rd edn, The background. Springer, Dordrecht, The Netherlands, 1: 227-282.

Murashige T and Skoog F (1962). A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Plant Physiology*, 15 : 473-497

Murashige T. (1974) Plant propagation through tissue culture. *Ann. Rev. Plant Physiol.* 25: 135.

Neondo J, Machua J, Muigai A, Nyende AB, Munjuga M, Jamnadass R, Muchugi A (2011). Micropropagation of *Allanblackia stuhlmanii*: Amenability to tissue culture

technique. *International Journal for Biotechnology and Molecular Biology search* 2(11):185-194

Nhut DT, Hanh NTM, Tuan PQ, Nquyet LTM, Tram NTH, Chinh NC, Nguyen NH, Vinh DN (2006). Liquid culture as a positive condition to induce and enhance quality and quantity of somatic embryogenesis of *Lilium longiflorum***.** *Scientia Horticulturae* Amsterdam 110:93–97

Nieves MC, Aspuria ET (2011). Callus Induction in Cotyledons of *Moringa oleifera* Lam. *Phillip Agric scientist* 94 (3): 239-247

Nwachukwu E, Uzueto HO (2010). Antimicrobial activity of leaf of *Vitex doniana* and *Cajanus cajan* on some bacteria. *Researcher* 2(3), 11p

OECD (1999). Handbook of Incentives Measures for biodiversity – Design and Implementation.

Okia CA, Opolot VI, Sekatuba J, Katumba BM, Esegu JF (2008). Use and Conservation of Indigenous Fruit Tree Diversity for Improved Livelihoods in Eastern Africa Uganda Country Report National Forestry Resources Research Institute (NaFORRI)

Olusola L (1992). An evaluation of the nutritive and medicinal values of *Vitex doniana* available from http://dspace.unijos.edu.ng/handle/10485/1484 accessed on 4 june 2013.

Orwa C, Mutua A, Kindt R, Jamnadass R, Anthony S (2009). Agroforestree Database: a tree reference and selection guide version 4.0 http://www.worldagroforestry.org/sites/treedbs/treedatabases.asp) retrieved on 23 April 2013.

Osum FI, Okonkwo TM, Okafor GI (2013). Effect of processing methods on the chemical composition of *Vitex doniana* leaf and leaf products. *Food Science and Nutrition* 3: 241-245.

Otitoju GTO, Nwamarah JU, Otitoju O, Iyeghe LU (2014). Nutrient composition of some lesser known green leafy vegetables in Nsukka Lga of Enugu State. *Journal of Biodiversity and Environmental Sciences* 4(4): 233-239

Pande S, Khetmalas M (2012). Effect of Concentration of Sucrose On Callus Induction and Somatic Embryogenesis of Anti-Diabetic Plant: *Stevia rebaudiana. Asian Journal of Biochemical and Pharmaceutical Research* 2 (1): 27-31

Papanastasiou I, Soukouli K, Moschopoulou G, Kahia J, Kintzios S. (2008). Effect of liquid pulses with 6-benzyladenine on the induction of somatic embryogenesis from coffee (*Coffea arabica* L.) callus cultures. *Plant Cell Tissue and Organ Culture* 92:215–225

Parrott (2000). Somatic embryogenesis of soybean [Electronic Version] from http://www.cropsoil.uga.edu/homesoybean/somprot.htm.

Perán-Quesada R, Sánchez-Romero C, Barceló-Muñoz A, Pliego-Alfaro F (2004). Factors affecting maturation of avocado somatic embryos. *Scientia Horticulturae* 102: 61–73

Pozo JC, Lopez-Matas MA, Ramirez-Parra E, Gutierrez C (2005) Hormonal control of the plant cell cycle. *Physiol Plant* 123: 173-183.

Prokop S and Albert J, Potatoes, nutrition and diet, International year of the potato (2008). http://www.potato2008.org/en/index.html [28th October 2014].

Protabase. *Vitex doniana* Sweet. www. prota.org retrieved on 14th April 2013

Pullman GS, Namjoshi K, Zhang Y (2003). Somatic embryogenesis in loblolly pine (Pinus taeda L.): Improving culture initiation with abscisic acid, silver nitrate, and cytokinin adjustments**.** *Plant Cell Reports.* 22: 85-95.

Rahman ARMM., Islam MO, Prodhan AKMA, Ichihashi S (2004). Effects of complex organic extracts on plantlet regeneration from PLBs and plantlet growth in the Doritaenopsis orchid. *Japan Agricultural Research Quarterly*, 37: 229-235.

Rathore MS, Shekhawat NS (2011). Micropropagation of *Vitex negundo* through *in vitro* bud proliferation Agricultural Science Research Journals 1(8) : 191-198

Rodriguez-Amaya DB, and Kimura M (2004). HarvestPlus Handbook for Carotenoid Analysis. Harvest Plus Technical Monograph 2.

Rout GR, Samantaray S, Dass P (2000). *In vitro* manipulation and propagation of medicinal plants. *Biotechnology Advances*.18: 91–120.

Rout GR (2004). Effect of cytokinins and auxins on micropropagation of *Clitoria ternatea* L. *Biology letter*, 41(1): 21-26

Sakhanokho HF, Rajasekaran K, Kelley RY (2009) Somatic embryogenesis in *Hedychium bousigonianum. Hort. Sci.* 44: 1487-1490.

Sanoussi A, Ahoton LE. and Odjo, Th. (2012). Propagation of Black Plum (Sweet) Using *Vitex donania* Stem and Root Cuttings in the Ecological Conditions of South Benin. *Tropicultura (*2), 107-112.

Sarker KK, Kabir AH, Sharmin SA, Nasrin Z, Alam MF (2007). Improved somatic embryogenesis using L-Asparagine in wheat (Triticum aestivum L.)**.** *Sjemenarstvo* 24(3):187-196

Sebastin J, Barna KS (2003). Plant regeneration through callus culture of *Iresine lindenii*. *In vitro Cellular and Developmental Biology,* Communicated.

Shahsavari E (2011). Contribution of sorbitol on regeneration of embryogenic calli in upland rice. *Int. J. Agric. Biol*. 13: 838–840

Sharma SK, Bryan GJ, Millam S (2007). Auxin pulse treatment holds the potential to enhance efficiency and practicability of somatic embryogenesis in potato. *Plant Cell Reports*, 26: 945–950.

Sheeba E, Palanivel S, Parvathi S (2013) Effect of plant growth regulators on callus induction in *Physalis minima* Linn. *International Journal of Innovative Research in Science,Engineering and Technology* 2(9): 4847-4851

Singh SR, Singh R, Kalia S, Dalal S, Dhawan AK, Kalia RK (2013). Limitations, progress and prospects of application of biotechnological tools in improvement of bamboo—a plant with extraordinary qualities Physiology and Molecular Biology of Plants, 19 (1):21–41

Slater A, Scott NW (2003). Plant Biotechnology, The genetic Manipulation of Plant. Oxford Univeristy Press.

Solebo SO, Aina JO (2011). Physico-Chemical Properties And Sensory Evaluation Of Jam Made From Black-Plum Fruit (*Vitex doniana*). *African Journal of Food, Agriculture, Nutrition and Development* 11(3): 4772-4784

Spory S, Jacobsen E, Wendel G (1978). Production of monohaploids embryoids and plantlets in cultured anthers of *Solanum tuberosis*. *Plant Sciences12*, 47-54

Steinmacher DA, Cangahuala-Inocente GC, Clement CR, Guerra MP (2007). Somatic embryogenesis from peach palm zygotic embryos. *In vitro Cellular Development Biology- Plant* 43: 124-132.

Taiz L, Zeiger E (2004). Fisiologia Vegetal. 3ª ed. Artmed, Porto Alegre. 719 p.

Tokuhara K, Mii M (2003). Highly efficient somatic embryogenesis from cell suspension cultures of Phalaenopsis orchids by adjusting carbohydrate sources. *In vitro Cellular and Developmental Biology Plant*, 39: 635-639..

Underwood BA (2000). Overcoming micronutrient deficiencies in developing countries: Is there a role for agriculture? *Food and Nutrition Bulletin* 21(4). The United Nations University.

Usha PK, Sailas B, Mohanan BV, Rhaghu AV (2007). An efficient micropropagation system for *Vitex negundo* L., an important woody aromatic medicinal plant through shoot tip culture. *Research journal of botany* 2(2): 102-107.

Vadawale AV, Barve DM, Dave AM (2006). In vitro flowering and rapid propagation of *Vitex negundo* L.- A medicinal plant. *Indian Journal of Biotechnology* 5: 112-116

Vikram VB, Ramesh MN, Prapulla SG (2005). Thermal degradation kinetics of nutrients in orange juice heated by electromagnetic and conventional methods. *Journal of Food Engeneering*, 69: 31-40.

Vikrant RA (2002). Somatic embryogenesis from immature and mature embryos of a minor millet *Paspalum scrobiculatum* L**.** Plant *Cell Tissue and Organ Culture* 69: 71-77.

Vunchi MA, Umar AN, King M, Liman AA, Jeremiah G, Aigbe CO (2011). Proximate, Vitamins and Mineral Composition of *Vitex doniana* (black plum) Fruit Pulp. *Nigerian Journal of Basic and Applied Sciences* 19(1): 97-101.

Wagstaff SJ, Hickerson L, Spangelr R, Reeves PA, Olmstead G (1998). Phylogeny in Labiatae S.L., inferred from cpDNA Sequences. *Plant Systematic and Evolution*, 265-274.

Wang J, AN L, Wang R, Yang D, Si J, Fu X, Chang J, Xu S (2006). Plant regeneration of *Chorispora bungeana* via somatic embryogenesis. *In vitro Cellular and Developmental Biology - Plant* 42(2):148-151.

Yan SF, Zhang Q, Wang JE, Sun YQ, Daud MK, Zhu SJ (2010). Somatic embryogenesis and plant regeneration in two wild cotton species belong to G genome**.** *In vitro Cellular and Developmental Biology - Plant* 46: 298-305.

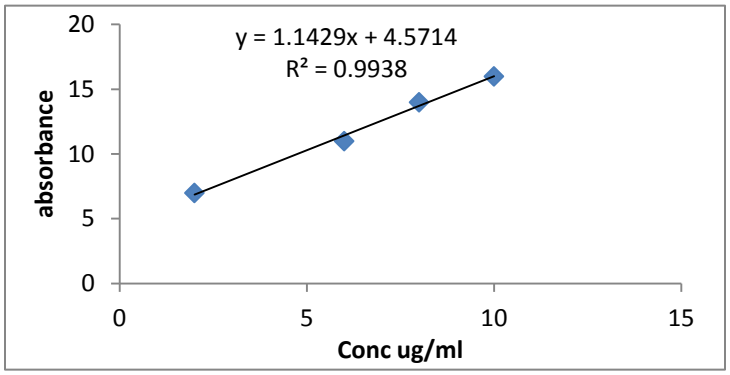
APPENDICES

Appendix 1 Composition of Murashige and Skoog's Medium

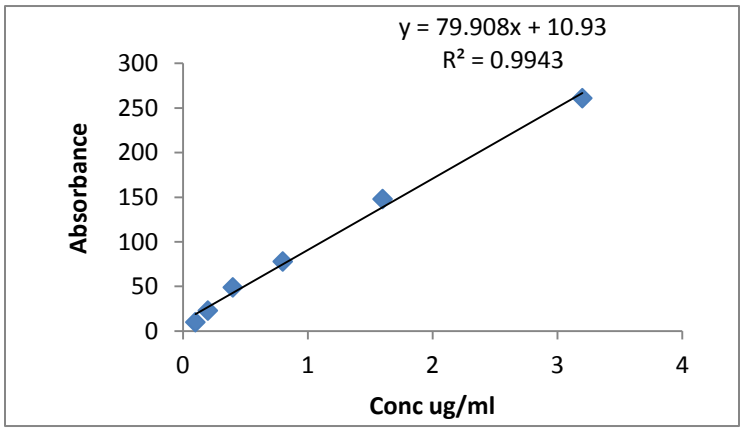
Stock solution	**Constituents**	**Concentration in stock solution g/l**	**Volume of stock solution in final medium ml/l**	**Final concentration in medium mg/l**
A	NH_4NO_3	82.5	20	1650
B	KNO_3	95.0	20	1900
C	H_3BO_3	1.24	5	6.2
	KH_2PO_4	34		170
	KI	0.166		0.83
	$Na_2MoO_4.2H_2O$	0.05		0.25
	$COcl_2.6H_2O$	0.005		0.025
D	$Cacl_2.2H_2O$	88.0	5	440.0
E	$MgSO_4.7\ H_2O$	74.0	5	370.0
	$MnSO_4.4\ H_2O$	4.46		22.3
	$ZnSO_{4.}7\ H_2O$	1.72		8.6
	$CuSO_{4.}5\ H_2O$	0.005		0.025
F	Na_2.EDTA	7.45	5	37.35
	$FeSO_4.7\ H_2O$	5.57		27.85
G	Thiamine HCL	0.02	5	0.1
	Nicotinic Acid	0.1		0.5
	Pyridoxine HCL	0.1		0.5
	Glycine	0.4		2.0

Appendix 2 Standard curve used in determination of nutritional parameters

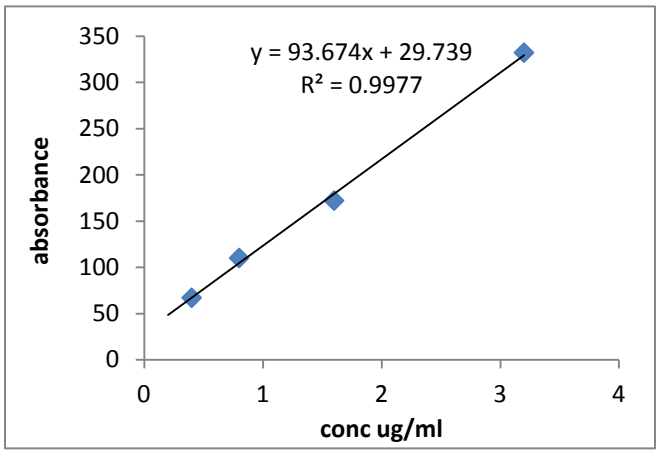
- Standard curve for Ca determination



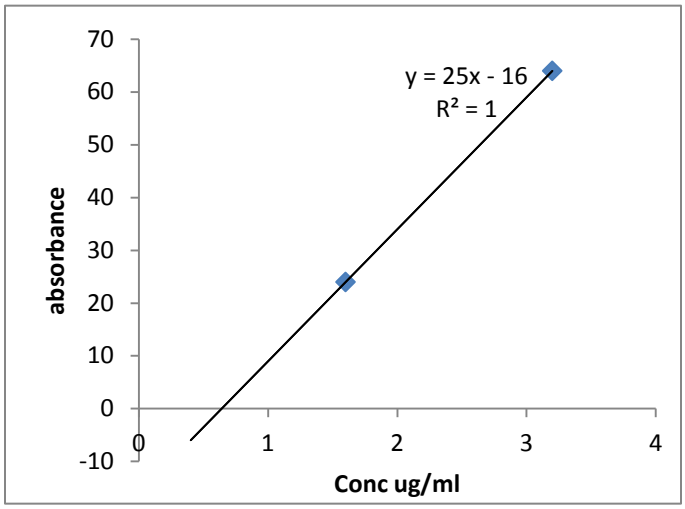
- Standard curve for Mg determination



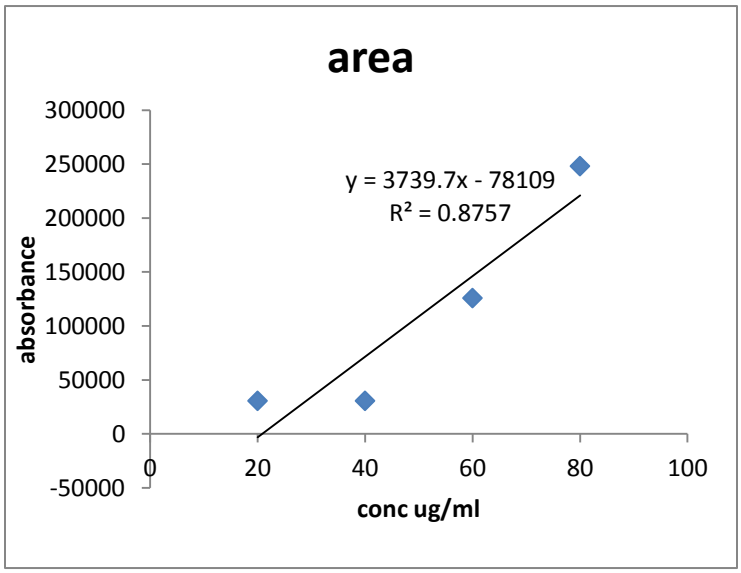
- Standard curve for Zn determination



- Standard curve for Fe determination



- Standard curve for Vitamin C determination



Appendix 3 Statistical analysis outcomes

One-way ANOVA: Protein versus Climatic Zones

Source	DF	SS	MS	F	P
Climatic Zones	2	10.977	5.488	8.38	0.018
Error	6	3.932	0.655		
Total	8	14.909			

S = 0.8095 R-Sq = 73.63% R-Sq(adj) = 64.84%

One-way ANOVA: Moisture versus Climatic Zones

Source	DF	SS	MS	F	P
Climatic Zones	2	14.496	7.248	27.95	0.001
Error	6	1.556	0.259		
Total	8	16.052			

One-way ANOVA: Vitamin C versus Climatic Zones

Source	DF	SS	MS	F	P
Climatic Zones	2	16.296	8.148	9.79	0.013
Error	6	4.996	0.833		
Total	8	21.292			

S = 0.9125 R-Sq = 76.54% R-Sq(adj) = 68.71%

One-way ANOVA: Vitamin A versus Climatic Zones

Source	DF	SS	MS	F	P
Climatic Zones	2	25.53	12.76	6.37	0.033
Error	6	12.02	2.00		
Total	8	37.54			

S = 1.415 R-Sq = 67.99% R-Sq(adj) = 57.33%

One-way ANOVA: Ca versus Climatic Zones

Source	DF	SS	MS	F	P
Climatic Zones	2	23121	11560	38.68	0.000
Error	6	1793	299		
Total	8	24914			

S = 17.29 R-Sq = 92.80% R-Sq(adj) = 90.40%