IN VITRO PRODUCTION OF ANTHOCYANINS IN KARANDAFI RED SORGHUM (*SORGHUM BICOLOR* (L.) MOENCH)

By

Lateefat Bolanle HASSAN, B.Agric (ABU, Zaria) 2008

MSc/agric/1914/ 2010-2011

A THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES,

AHMADU BELLO UNIVERSITY, ZARIA

IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF A MASTER DEGREE IN PLANT PHYSIOLOGY

DEPARTMENT OF PLANT SCIENCE

FACULTY OF AGRICULTURE

AHMADU BELLO UNIVERSITY, ZARIA

DECEMBER, 2014

DECLARATION

I declare that the work in this Thesis entitled *IN VITRO* PRODUCTION OF ANTHCYANINS IN KARANDAFI RED SORGHUM (*Sorghum bicolor* (L) Moench) has been carried out by me in the Department of Plant Science under the supervision of Dr I.S. Usman, Dr M.D. Katung and Dr S.M. Bugaje. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree at this or any other institution

_____	_____	_____
Name of Student	Signature	Date

CERTIFICATION

This thesis entitled *IN VITRO* PRODUCTION OF ANTHOCYANINS IN KARANDAFI RED SORGHUM (Sorghum bicolor (L.) Moench) by Lateefat Bolanle HASSAN meets the regulations governing the award of the degree of MSc. Plant physiology of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary contribution

Dr. I.S. USMAN _____ _____

Chairman, Supervisory Committee (Signature) Date

Dr. (Mrs) M.D. Katung _____ _____

Member, Supervisory Committee (Signature) Date

Dr. S.M. Bugaje _____ _____

Member, Supervisory Committee (Signature) Date

Prof. M.F. Ishiyaku _____ _____

Head of Department (Signature) Date

Prof. A.Z. Hassan _____ _____

Dean. School of Postgraduate Studies (Signature) Date

Ahmadu Bello University, Zaria

DEDICATION

This thesis is dedicated to my parent, family and my mentor in person of late Professor Balarebe Tanimu. May Allah have mercy on him.

ACKNOWLEDGEMENT

All praise is due to Almighty Allah. I thank Him for giving me the strength and health in completing this work. My profound gratitude also goes to my major supervisor, Dr. I. S Usman for his support, constructive criticism and meaningful contributions to the success of this research work. I appreciate the contribution and support of my minor supervisors, Dr. M.D. Katung and Dr. S.M. Bugaje.

I thank my lovely husband (Engineer Nazeemdeen Ojulari) and beloved children (AbdulRahman Nazeemdeen and Fatimah Nazeemdeen) for their patience support, understanding and meaningful contributions towards the successful completion of this research work.

I appreciate the contribution of my parent (Alhaji Hassan Yusuf and Hajia Risikat Tijani) and my siblings (AbdulAkeem Hassan, Aminat Hassan, Balikis Hassan, Rasheedat Hassan, Isiaq Hassan and Maryam Hassan), thanks for your moral support and prayers towards the completion of this work

My word of appreciation also goes to Suleiman Umar, Abdul Sanni, Memuna Abdulmalik, Hadisa Gambo, Abdullahi, Jafar and Sagri of Biotechnology laboratory in the department of Plant Science, ABU, Zaria. I'm saying thanks to my colleagues and well wishers whose names are too numerous to mention.

I acknowledge the Institute of Agricultural Research, Ahmadu Bello University Zaria, for funding and supporting this research. May God reward you all.

ABSTRACT

This study was undertaken to produce and quantify anthocyanins in Karadanfi red sorghum *in vitro*. Mature embryo and immature embryo explants were used for callus induction using Murashige and Skoog basal medium supplemented with varying concentrations of 2, 4-D (0 mg/L – 4.0 mg/L) alone or in combination with 0.5 mg/L KN. Immature embryo was used for regeneration using MS media supplemented with BA (0 mg/L - 3 mg/L) alone or in combination with 2, 4-D (0.1 mg/L). The calli obtained were sub cultured on the media fortified with BA (0 mg/L – 2 mg/L) and NAA (0.2 mg/L) for anthocyanins production. Results indicated highly significant difference for percentage callus formation, callus fresh weight, total and monomeric anthocyanins. Significant differences were also observed for percentage shoot initiation. Medium fortified with 2 mg/L 2, 4-D alone or in combination with 0.5 mg/L kinetin was found to be optimum for callus induction. It was observed from this study that more 3-deoxyanthocyanindin can be obtained from *in vitro* grown sorghum at callus stage. The highest level of anthocyanin was produced in this study using MS media fortified with 1.0 mg/L BA. It is therefore concluded that *in vitro* system is suitable for the production of anthocyanin pigment in Karandafi red sorghum.

Table of Contents

page

Figure	List of Figure	Page
1	Biosynthetic pathways of 3-deoxyanthocyanindin and anthocyanindin	16

Table	List of Tables	Page

Plate	List of Plates	Page

Appendix	List of Appendices	Page
1	Means Squares for Percentage Callus Formation,Average Fresh Weight of Callus,Total and Monomeric Anthocyanins using Methanol and acidified and One way ANOVA table for Shoot Initiation using Immature Embryo and Shoot Multiplication using Meristem	76
2	Spectral characteristics of peaks corresponding to Methanol and Acidified methanol extraction of 3-deoxyanthocyanindin (Apegininidin) isolated from leaf sheath obtained *in situ,* pigmented callus, and *in vitro* plantlet using Jenway 6405 UV – Visible Spectrophotometer	77
3	Diagrammatic illustration of calendar of event undertaken	80
4	Publications	81

Abbreviations

BA	6-Benzylaminopurine
2,4-D	2,4-dichlorophenoxyl acetic acid
KN	Kinetin
PGR	Plant Growth Regulators
NAA	1-Naphthalene Acetic Acid
IAA	Indole Acetic Acid
MOH	Methanol
AMOH	Acidified Methanol
TDZ	Thiadiazuron
Apigen	Apigeninidin
Luteo	Luteolinidin
Cyn-3- glu	Cyanindin -3- glucoside
T	Taxus
MS	Murashige and Skoog,
DAP	Days After Pollination
ICRISAT	International Crop Research Institute for Semi- Arid Tropics
CoA	Co-enzyme A
IMEB	Immature Embryo
EBA	Embryonic Axes
MEB	Mature Embryo
IBA	Indole -3-Butyric Acid
CW	Coconut Water
ANOVA	Analysis of Variance
PEC	Potentially Embryogenic Calli
SAS	Statistical Analysis System
SCD	Sicke cell Disease,
D	Daucaus
A	Artemisinin

CHAPTER ONE

INTRODUCTION

Sorghum (*Sorghum bicolor* (L.) Moench) is an annual plant that belongs to the family "Poaceae" Sorghum is an important cereal crop grown in the semi-arid tropics of Africa and Asia due to its drought tolerance capability. It is well adapted to a wide range of soil types and environmental conditions (Sudhakararao, 2011). It is the fifth most important cereal crop in the world after rice, maize, wheat and barley, and is the dietary staple of more than half a billion people in over 30 countries (ICRISAT, 2010). Sorghum species is one of the main foods that is widely consumed in Nigeria. Different parts of the crop are widely used in traditional medicine.

Sorghum bicolor is a rich source of various phytochemicals including tannins, phenolic acids,anthocyanins, phytosterols and policosanols (Sudhakar *et al.,* 2008).These phytochemicals have significant impact on human health. However, recent studies suggest that this local ingredient could be the "cure all" medical scientists have been searching for (Cyrilolutayo *et al*., 2009). Anti-cancer, anti-diarrhoea, anti-microbial, anti-anaemic, immunomodulatory, neuroprotective, anti-tryosinase, anti-lipidemic activities, sedative effect as well as anti sickle cell disorder of *Sorghum bicolor* extracts were reported by Lim (2013). Anthocyanins were reported to have therapeutic benefits including anti-cancer and chemoprotective (Karaivanova *et al*., 1990), as well as anti-neoplastic properties (Kamei *et al.*, 1995).Antisickling and anti-hemolysis activities of anthocyanins extracts from *Sorghum bicolor* were reported by Mpiana *et al*. (2013).Afaq *et al.* (1991) reported that

flavonoids are well known anti-oxidants and free radical scanvengers. High potential of polyphenols to scavenge free radicals are because of their many phenolic hydroxyl groups (Sawa *et al*., 1999). For the sickle cell disease, the higher the antioxidant property of an antisickling agent, the higher its possible antisickling effect, as this enables it to reduce oxidative stress that contributes to sickle cell crisis (Ngozi, 2013).

In Nigeria, red sorghum leaves are used in combination with the *Carica papaya* pulp for the management of sickle cell anaemia. A drug called NICOSAN developed from red sorghum leaves and other medicinal plants is used for the management of sickle cell anaemia (Wambebe, *et al*., 2001). Red sorghum is used for purification of blood and stimulation of blood production (Okokoh, 1999). *Sorghum bicolor* is also reported to be antiabortive, cyanogenic, demulcent, diuretic and emollient (Cyrilolutayo *et al*., 2009). The effect of cyanate in red sorghum improves red cell survival and increases hemoglobin levels and prevents most of the minor episodes of sickle cell anemia (Cyrilolutayo *et al.*, 2009). Sorghum fractions possess high antioxidant activity *in vitro* relative to other cereals or fruits (Awika *et al*., 2004). The local name of the landrace used for this study is called Karandafi. This landrace is being used by pharmaceutical industries for development of drugs for the management of sickle cell anaemia.

1.1Justification

Sickle cell disease affects 3 to 5 million people globally. About 89% of sickle cell patients worldwide are in Africa, of which Nigeria alone constitutes 25% (Ameh *et al.*, 2009). The poor ones are more vulnerable to the disease and the cost of treatment offered by orthodox

medicine is not affordable and accessible to these poor people as a result of which they die at early stage of their lives. Two-third of the world population–mainly in developing countries relies on the traditional medicine as their primary form of healthcare. *Sorghum bicolor* has been used traditionally to cure sickle cell anaemia. Recently, anthocyanin from sorghum extract had been identified as the main bioactive compound in sorghum with antisickling property and it played important role in the management of sickle cell anaemia. It has been established that the synthesis of secondary metabolites under natural condition is stress dependent in most cases and longer time may be required to get small amount of bioactive compound. Karandafi even though identified as a major component of antisickling drugs occurs in nature as a mutant in a large population of sorghum plants. This tends to limit large scale production of this important drug. Biotechnology tools such as tissue culture technique offer an opportunity for the production of large amount of plant bioactive substances under *in vitro* condition within a short period of time. Plant tissue culture has been utilized to scale-up production of secondary metabolites from different plant species using different types of elicitors. There is paucity of information on the assessment of biosynthesis of anthocyanins (3-deoxyanthocyanindin) from red sorghum under *in vitro* condition. Studies to assess the production level of anthocyanins from *in vitro* grown sorghum could be useful for large production of the compound for the development of the drugs and this will make the drugs to be more available to the people living with the disease at an affordable price and make their state of health to be better.

1.2 Objectives of the study

In view of the above, this study was conducted with the following objectives:

i. To develop effective protocol for *in vitro* micropropagation of red sorghum

ii. To extract anthocyanins from callus and *in vitro* plantlets

iii. To determine and quantify the amount of anthocyanins present in callus and *in vitro* plantlets

CHAPTER TWO

LITERATURE REVIEW

2.1 Plant Tissue Culture

‘’Plant Tissue Culture’’ is the maintenance and propagation of plant parts, as small as a single cell, in axenic culture under controlled environmental conditions. Plant tissue culture can also be described as the technique of growing plant cells, tissues and organs in artificially prepared nutrient medium, static or liquid, under aseptic conditions. Tissue culture is proving to be useful in varieties of ways including plant propagation, raising and maintenance of high health status plants, germplasm storage and a valuable technique in plant improvement. Tissue culture has also been used in the production of secondary metabolites in plants. The production of flavours, sweeteners, natural colorants, as well as pharmaceuticals had been achieved using tissue culture technique (Pullaiah *et al.*, 2009).

2.2 Tissue Culture and Regeneration Studies *In vitro*

Explants that are derived form meristematic tissues at early stages of development are most amenable to tissue culture conditions (Puddephat*et a1.*, 1996). In cereals Immature embryos and immature inflorescences have been widely used as explants for regeneration (Indra *et al*., 2009). Sorghum flowering occurs only once in its life time and for few days only, thereby providing a small window of opportunity to initiate embryogenic cultures (Sudhakararao, 2011). Mature tissues such as seed embryos are readily accessible and are year round sources for cereal explants (Cogner *et a1.,* 1982) so as do the shoots tips and

shoot apices isolated from germinating seedlings (Zhong *et al*., 1998). *Invitro* plant regeneration can follow either of the following two different pathways (i) Organogenesis involving the development of axillary buds following inhibition of apical dominance, or *de*novo organization of shoot meristems in callus cultures, and (ii)Somatic embryogenesis in the latter case, regenerates arise from single cells either directly or follow the formation of a mass of proembryonic cells (Pullaiah *et al*., 2009)

2.3 Micropropagation through Organogenesis

The principle of micropropagation is based on the phenomenon that shoot tip when cultured on tissue culture medium, can develop large number of shoots identical to the parent plant. The technology provides many advantages over conventional propagation methods such as product development is achieved in a highly speedy manner, uniformity in product development, large population can be produced in small growing space, quick multiplication of elite and genetically engineered products, and *in vitro* storage of germplasm. The technique also helps in enhancing tissue culture products via specific product format, rooted or unrooted micro cutting, bulblets, and embryos, certified plant material free of pathogens and production can be obtained throughout the year, as most of the multiplication phase is completed under artificially controlled condition (Purohit, 2009). Protocol was developed for *Sorghum bicolor* through organogenesis by Sadia *et al.* (2010) where meristems obtained from three sorghum hybrids were cultured using MS media supplemented with BA alone or in combination with TDZ (Thiadizuron).

2.4 Somatic Embryogenesis

Somatic embryogenesis is a process by which haploid and diploid somatic cells develop into structures that resemble zygotic embryos through orderly series of characteristic embryological stages without fusions of gamete (Raemakers *et al*., 1995). However fertilization is not always essentials to stimulate the egg to undergo embryogenesis. As it is the case in parthenogenesis, the pollination stimulus alone, or simply the application of some growth regulators may induce the egg to undergo embryogenic development. Moreover, it is not the monopoly of the egg to form an embryo. Any cell of the female gametophyte (embryo sac) even that of the sporophytic tissues around the embryo sac may give rise to an embryo. Embryos formed in culture are usually referred to as somatic embryos or simply embryos. Somatic embryogenesis is the process of a single cell or a group of cells initiating the developmental pathway that leads to the reproducible regeneration of non-zygotic embryos capable of germinating to form complete plants. Embryos formed in culture have been variously designated as accessory embryos, adventive embryos, embryoid and super numerary embryos. Somatic embryos are those formed by the sporophytic cells (i.e. other than zygote) either *in vitro* or *in vivo*. (Pullaiah, *et al*., 2009).

The presence of auxin in the medium is generally essential for embryo initiation. Tissues or calli maintained continuously in an auxin free medium generally do not form embryos; therefore somatic embryogenesis is achieved in two steps. First, the callus is initiated and multiplied in a medium rich in auxin (2, 4-D 0.5mg/l), which induces the differentiation of localized of groups meristematic cells called embryogenic clumps (ECS). Second, the embryogenic clumps develop into mature embryos when transferred to medium with very

low level of auxin (0.01-0.1mg/L). Besides auxin, the form of nitrogen in the medium significantly affects *in vitro* embryogenesis. The calli initiated on medium with KNO_3 as the sole source of nitrogen failed to form embryos upon removal of auxin. However, the addition of a small amount (5mmol/L) of NH_4Cl in the presence of 55mmol/L of KNO_3 allowed embryo development (Pullaiah *et al.*, 2009).

Somatic embryogenesis is an important tool for achieving a number of objectives which involves basic physiological, biochemical and morphological studies and the development of new technologies with high practical applications (Jimnez, 2001)

Large scale propagation of elite varieties of plants through multiplication of embryogenic propagules is the most commonly attractive application of somatic embryogenesis (Merkle *et al.*, 1990). Somatic embryogenesis permits the culture of large numbers of reproductive units e.g. 60,000 to 1.35 million somatic embryos per litre of medium- with the presence of both root and shoots meristems (Jimenez, 2001). Somatic embryogenesis is frequently used in the production of plants with different levels of ploidy. For instance production of haploid plants by cultivating anthers *in vitro* (Terzi *et al.*, 1990). In recent times a reasonable success has been achieved in inducing dormancy in somatic embryos. This together with, the accomplishment of long- term storage and development of techniques for somatic embryo encapsulation have resulted in development of synthetic seed technology (Jimenez, 2001). The secondary metabolites have high economical and pharmacological importance and the industries are deeply interested in large variety of chemical substances being produced by the plants due to their lesser toxicity, the plant tissue culture technique has widened the scope and opened new vistas for the production of secondary metabolites (Purohit, 2009)

2.5 Role of Genotype on Plant Regeneration

In vitro culturing of sorghum is very much influenced by genotypes, explants, media composition and hormones (Indra *et al*., 2009). The effect of genotype on *in vitro* response in sorghum was reported by Sadia *et al*. (2010). Shoot meristems excised from different sorghum hybrids (genotypes) responded differently with respect to shoot multiplication in similar media. Similar reports of genotype-dependent variations in multiple shoot induction were recorded for barley (Sharma *et al*., 2004) and wheat (Ahmad *et al*., 2002). Genotype effect may be due to the difference in the number of morphogenetically competent cells present in the shoot meristem that have the capacity to respond to an altered signaling profile according to Sadia *et al*. (2010). Furthermore, there are many instances, both within the gramineae and in other angiosperms, where plant regeneration was obtained in almost all the genotypes tested (Bajaj, 2000).These results strongly suggest that, the physiological state and the developmental stage of the explants are critical. The same is true for *in vitro* response in sorghum in almost all the cases reported (Harsha, 2002), employment of MS basal medium supplemented with 2-4-D and low kinetin resulted in successful morphogenic response in sorghum (Sudhakar, *et al*. 2008; Sudhakar *et al*., 2009)

2.6 Callus Induction and Regeneration from Different Explants of Sorghum

Protocols had been established for *in vitro* plant regeneration of sorghum from different type of explants like immature embryos, immature inflorescences and shoot tips or apices and mature embryo (Indra *et al*., 2009). One of the most popular and most widely adopted explants for tissue culture studies, as well as genetic transformation of sorghum is the immature embryo explants. Protocols have been established for immature embryo based

regeneration in sorghum (Sudhakar *et al*., 2008). Age of the immature embryo, measured as days after pollination **(**DAP**)** is found to be critical for in *vitro* response. Immature embryos were isolated from spikelets harvested from plants 14-17 DAP for callus induction (Sudhakar *et al*., 2008). Callus was induced using the MS medium fortified with 2, 4-D alone or in combination with kinetin. He was able to regenerate plant from MS media fortified with 6-benzyaminopurine. Apart from the age of the explants, influence of the size of immature embryo used for culturing was investigated in 20 genotypes by Ma *et al.* (1987). Ma *et al. (*1987) reported from their study that the ability of the plants to differentiate was heritable and is controlled by two gene pairs acting as a dominant trait. Mackinnon *et al.* (1986) used immature and mature embryos of both grain and sweet sorghums for *in vitro* culture. They observed no major differences in response with either grain or sweet sorghum cultivars except for difference in callus pigment color (Harsha, 2002). Similar observation was made by Kresovich *et a1*. (1987), they are of the opinion that, this difference could be due to the phenotypic character of the donor plant.

Immature inflorescences also have been identified as one of the source materials capable of producing embryogenic calli, with the capacity to regenerate into whole plants. Length of the explants material has been shown to influence the *in vitro* response, and most of the reports mentioned the use of 10-40 mm length inflorescence for sorghum *in vitro* culture (Harsha, 2002). The callus induction is very much dependent on age of immature inflorescence. The effect of age of immature inflorescence on callus induction was reported by Indra *et al.* (2009). Phenolics production resulting in death of the explants is one of the most common problem associated with immature inflorescence (Addition of 150 mg L-' of asparagine to the medium, accompanied by frequent subculture has shown to mitigate this problem (Cai and Butler, 1990).

Shoot tips or shoot apices, when used as explants can overcome the seasonal limitation of the explants availability. Shoot tips are isolated from seedlings germinated in dark under aseptic conditions (Indra *et al*. 2009). For callus initiation, L_6 or MS medium supplemented with 2, 4-D and kinetin (Indra *et al*. 2009; Sudhakar, *et al*., 2008), or for direct regeneration, MS medium supplemented with BA, IAA and kinetin are used (Nahdi and De Wet, 1995). The entire process of regeneration was completed in 4-5 weeks, in direct regeneration as compared to somatic embryogenesis pathway, which took 5 weeks just for the embryos to germinate (Seetharama *et al*., 2000).

2.7 Growth Regulators

Hormones or growth regulators are not nutrients, but they influence growth and development. They are produced naturally in plants. Cultures however, usually do not manufacture sufficient quantities of growth regulators, so they must be added selectively to culture media. The four classes of growth regulators are commonly used in tissue culture media i.e, auxins, cytokinnins, gibberellins and abscisic acid. Auxins (2, 4-D, NAA, IAA or IBA), promote cell enlargement and root initiation, whereas cytokinnins promote cell division and shoot initiation. The type of growth regulators and concentration used will vary according to the cell culture purpose. (Purohit 2009). The importance of cytokinin in anthocyanin production was reported by Purohit (2009)

2.8 Secondary Metabolites

The organic compounds such as carbohydrates proteins, fats, membrane lipids, nucleic acids, chlorophylls and lemes are found throughout the plant kingdom and are central to the metabolism of plants. These compounds are known as primary metabolites. Apart from these substances, many plants particularly those of certain genera and families synthesize a number of organic compounds in them which are not in the mainstream of metabolism and appear to have no direct function in growth and development of plants. These compound are extremely numerous and chemically diverse in nature and are called as secondary metabolites or natural products and include such well known substances as alkaloids, terpenes, tannins, flavonoids etc (Jain, 2008).

2.8.1. Secondary metabolites production through biotechnological Intervention

Generally, herbal preparations are produced from field grown plants (Murch *et al*., 2000). It was difficult to ensure the quality control as the medicinal preparations are multi-herb preparations and it is difficult to identify and quantify the active constituents**.** An efficient and most suited alternative solution to the problems faced by the phyto pharmaceutical industry is the development of *in vitro* systems for the production of medicinal plants and their extracts (sekh *et al*., 2010)

Plant tissue culture proved an important technology being used for the conservation of important plants either through organogenesis, somatic embryogenesis and genetic transformation (Mujib *et al*., 2006).

The major advantages of cell cultures for secondary metabolites production includes (i) synthesis of bioactive secondary metabolites independently from climatic and soil conditions; (ii) negative biological influences that affect secondary metabolites production in the nature are eliminated (microorganisms and insects) (iii) to select cultivars with higher production of secondary metabolites; (iv) with automatization of cell growth control and metabolic processes regulation, costprice can decrease and production increase (Sekh *et al*., 2010))

Tissue culture technique had been use for the production of different types of secondary metabolites such as taxol, alkaloids, saponins, arteminsinin, betalanin among others. Radfar *et al*. (2012) reported more production of betalanin from pigmented callus obtained from the media fortified with 1 mg/l BA+ 10% coconut water at 8 weeks of culture. Artemisinin production has been extensively studied in shoot and hairy root cultures. (Liu *et al*., 1998). An internal-loopmist bioreactor has been devised and applied to the shoot and hairy root cultures of *A. annua*, achieving an artemisinin yield of 46.9 mgl−1 in 25 days much higher than that in the shake-flasks (Liu *et al*., 1998). Various types of medium supplements have been studied aiming to enhance the taxol production. Fett- Neto *et al*. (1994) used different amino acids and phenylalanine to the culture medium of *T.cuspidata* and reported a significant increased in taxol production. Lee *et al*. (1995) reported remarkable differences in taxol content in bark and leaf tissues of *in vitro* developed taxus culture

2.9 Flavonoids

Flavonoids are common and widespread secondary plant metabolites, which have a wide range of biological and physiological activities. Flavonoids occur in plants (food products) as different glycosides, which is also preferred form for uptake in human intestine. After

uptake the glycosides are converted in to aglycon and free carbohydrates in hydrolysis reaction. Flavonoids are polyphenolic compounds, they are effective antioxidants due to their capability to scavenge free radicals of fatty acids and oxygen, their flavonol content considerably decreases atherosclerotic process, inhibits cholesterol accumulation in the blood serum and enhances resistance of vascular walls. Flavonoid decreases a risk of coronary heart diseases (Ponmonzhi, *et al*., 2011)

2.10 Anthocyanins in Sorghum

Anthocyanins are poly phenolic compounds responsible for cyanic colors ranging from salmon pink through red and violet to dark blue of most flowers, fruits, leaves and stems. They comprise the largest group of the water-soluble pigments in the plant kingdom (Ponmonzhi *et al*., 2011), and during the last few years it has been an exponential increase in the report of new anthocyanins structures (Andersen *et al*., 2006). This can be explained by the use of improved analytical techniques, but the potential use of anthocyanins as health beneficial compounds is another reason for the increased scientific interest in these pigments. They play a definite role in attracting insects in pollination and seed dispersal (Awika *et al*., 2004). The anthocyanins consist of an aglycon (anthocyanidin), sugar(s), and, in many cases, acyl group(s). The classical anthocyanin aglycon is based on a C15 skeleton (C6–C3–C6 skeleton) (Andersen *et al*., 2006). Anthocyanins are positively charged at acidic pH even though there are around 30 different anthocyanidins, approximately 90% of all anthocyanins are based on the six most common anthocyanidins pelargonidin, cyanidin, delphinidin, peonidin, petunidin and malvidin which only differ by

the hydroxylation and methoxylation pattern on their B-rings (Awika *et al*., 2004) The anthocyanins will differ with respect to glycosylation of hydroxyl groups, nature of glycosyl units, substitution pattern, and potential aliphatic and aromatic acylation (Andersen *et al*., 2006). The 3-deoxyanthocyanidins (non glycosides) is found in sorghum. In plants spagnorubins and rosacyanin B are the only anthocyanidins (aglycon) found in their non glycosidated form. Andersen *et al.* (2006) indicated the presence of cyanidin, peonidin and pelargonidin in black dried beans in glycosidated form (*Phaseolus vulgaris* L.). Pyranoanthocyanins have been discovered in small amounts in wines and grape pomace (Mateus *et al.*, 2004). More recently, glucosides of carboxypyranocyanidin have been isolated from red onion (Fossen *et al*., 2003), and carboxypyranopelargonidin 3-glucoside from strawberry (Andersen *et al.*, 2004) extracts which are all in glycosidated form.

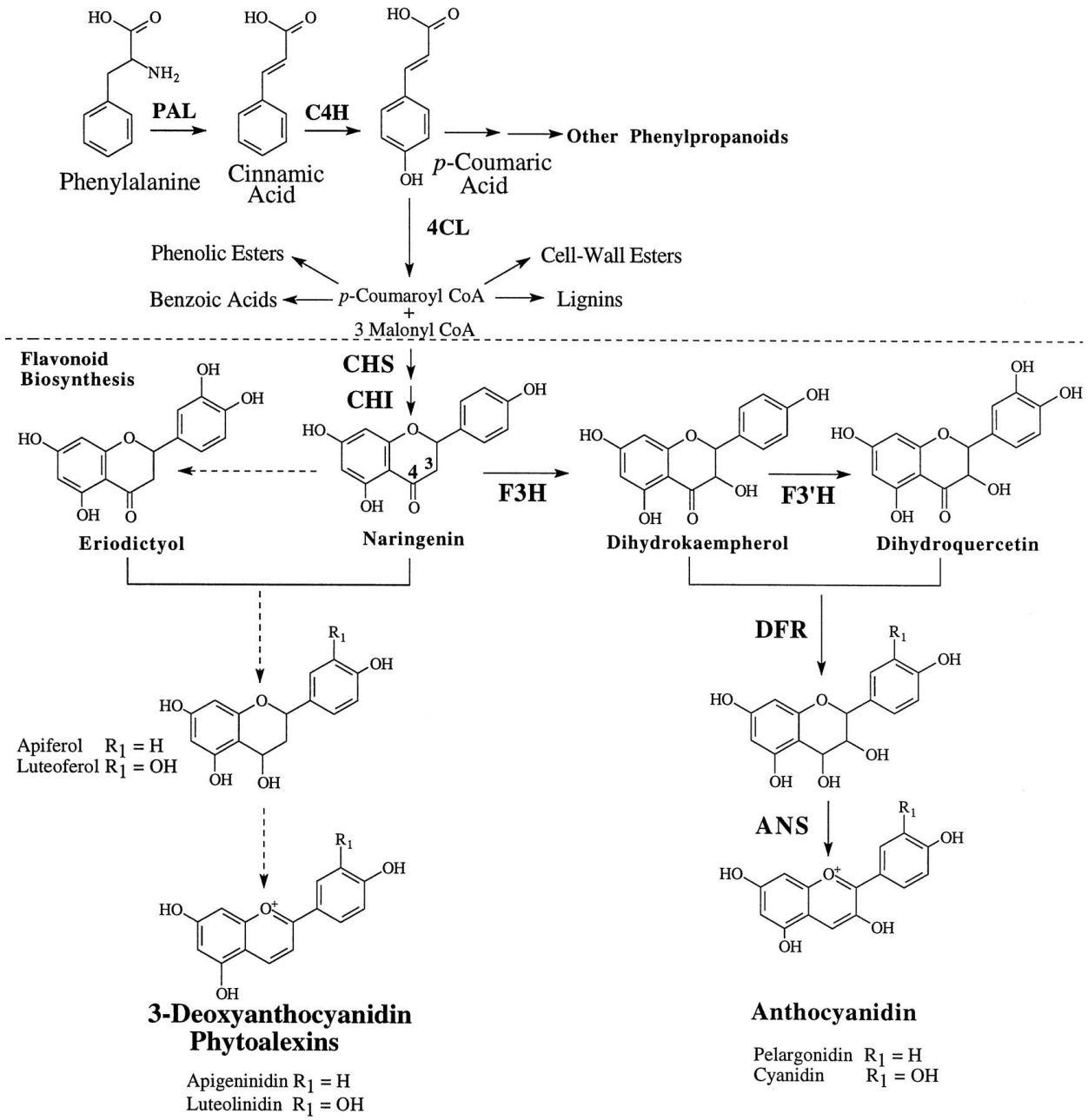


Figure1: Biosynthetic pathways for the 3-deoxyanthocyanindin and anthocyanidin

The biosynthesis of anthocyanidins and 3-deoxyanthocyanidins represents two partially overlapping, competing pathways in sorghum. Both compounds are derived from the phenylpropanoid and flavonoid pathways. Activities of the key phenylpropanoid branch-point enzymes PAL and CHS, and expression of their respective genes, are induced by light as well as by fungal infection (Orczyk *et al*., 1996). The compounds originate from the condensation of p-coumaroyl CoA and malonyl CoA to form naringenin chalcone, which is

converted to the flavanone naringenin. It is from naringenin that both the anthocyanidins and the 3-deoxyanthocyanidins are derived (Dixon *et al*., 1995; Nicholson, 1998).

2.11 Medicinal Value of Anthocyanin

Anthocyanin is becoming increasingly important not only as food colorants, but also as antioxidants. Antioxidant property of anthocyanins was reported by Ngozi (2013). Anthocyanins were also reported to have some therapeutic benefits including anti-cancer, chemoprotective, (Karaivanova *et al.*, 1990), as well as anti-neoplastic properties (Kamei *et al*., 1995). Anthocyanins are therefore, considered to contribute significantly to the beneficial effects of consuming fruits and vegetables (Wang *et al*., 1997). There is a rising demand for natural sources of food colorants with nutraceutical benefits (Boyd, 2000) and alternative sources of natural anthocyanins are becoming increasingly important. Special sorghums contain significant levels of anthocyanins and other phenols concentrated in their bran. Black sorghum was reported to have significantly more anthocyanin pigments than other sorghums. Therefore, this sorghum has a good potential for commercial exploitation. Anthocyanins have been extensively studied in fruits and vegetables. Limited data exists on the types and levels of anthocyanins in cereals, probably because they have never been regarded as a commercially significant source (Awika *et al*., 2004). They were able to isolate Apigeninidin and luteolinidin from bran of black sorghum using High Performance Liquid Chromatography (HPLC) and reported that luteolinidin and apigeninidin accounted 50% total anthocyanin in black sorghum.

2.12 Antisickling and Anti-Hemolysis Activities of Anthocyanins Extracts from *Sorghum bicolor*

According to Mpiana *et al*. (2013) drepanocytes reversed their sickle shapes to the normal biconcave when treated with anthocyanin obtained from *Sorghum bicolor* and other two medicinal plants thus comfirming the antisickling activity of anthocyanins. The properties of anthocyanins to adsorb themselves on proteins would block the polymerization of the deoxy hemoglobin S in tactoids and reduce hence the sickling process, inducing the return to the normal biconcave form of RBCs. The same results evidencing the anthocyanins antisickling activity on other plants used in Congolese traditional medicine against sickle cell disease have been already reported (Newman *et al*., 2013; Mpiana *et al*., 2009). However, the most active among the three investigated species is the anthocyanin extracts from *S. bicolor* (L.) Moench with the normalization rate evaluated to be more than 70% followed by the two others extracts from *Daucas. Colorata* C. B. Clarke and *E. hirta* L. for which the normalization rate are both greater than 50% (Mpiana *et al*., 2013). The analysis of the influence of anthocyanin extracts on the hemolysis of the drepanocytes indicates the increasing of the absorbance of 2.3% showing hence a continuous hemolysis of sickled RBCs. In contrast to this drastic situation, in the presence of anthocyanin extracts, there is a significant decreasing of absorbance especially with anthocyanins extracted from *E. hirta* L. (31.7%), followed by *S. bicolor* (L.) Moench (5.8%) and *D. colorata* C. B. Clarke (3.4%). Statistical treatment using Student T-test applied at 0.05 confidence level indicates significant difference between untreated HbSS and HbSS treated with different extracts. These results are an evidence of the anti-hemolytic activity of anthocyanins on the erythrocytes of SS blood. This fact has been already noticed for some other plants including

Trema orientalis. The anti-hemolytic activity is an important feature for an antisickling agent since it has been known so far that chronic anemia is the most frequent SCD symptom. The ability of anthocyanins to reduce the sickle erythrocytes hemolysis may be due to their capacity to act as antioxidant. Indeed, it is postulated that the sickling leads to the modification of the membrane flexibility, which makes it more sensitive and fragile towards free radicals or antioxidants. Therefore, antioxidant or free radical scavenge compounds prevent hemoglobin from oxidizing in methemoglobin and inhibit the generation of free radicals. It is thus probable that the anthocyanin extracts exert these protective capacities to prevent oxidative damages of the lipids membrane, hemoglobin and the enzymatic equipment [Hebbel *et al*., 1982].

Traditionally in Nigeria, *Sorghum bicolor* (L.) Moench is used as blood builder and used in the treatment of sickle cell crisis. A 4-5 day cold infusion of a mixture of *Sorghum bicolor* leaves and *Carica papaya* unripe fruit pulp is used by the Yorubas (Western Nigeria) to alleviate bone pains reported by (Cyrilolutayo *et al*. (2009).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Experimental site

Experiments were carried out in Biotechnology Laboratory in Department of Plant Science Institute for Agricultural Research (IAR) and Multi user Laboratory in the Department of Chemistry, Ahmadu Bello University, Samaru, Zaria. (11^0 11^1N, 07^0 $38^{1,}$ 680 m above sea level) Samaru –Zaria is situated in the northern guinea savannah ecological zone of Nigeria. It has a mean annual rainfall of about 1045mm; rainfall is well distributed over the growing season of about 130-190 days between May and October

3.2 Plant Materials

Karandafi is a sorghum landrace that has very strong anthocyanin coloration on its' leaves, stem, inflorescence, and kernels. For the purpose of this study Karandafi seeds were collected from farmers' fields in Sokoto and Katsina States. The seeds were planted in the green house in order to obtain immature seeds. The immature seeds of sorghum were harvested from main spikes of caryopsis at 17 days after anthesis, while mature ones were harvested at full maturity. Both the immature and mature seeds were washed under running tap water for 20 minutes using detergent. The immature seeds harvested for use as immature embryo were kept moist at 4^oC until embryos were excised as described by (Sudhakhar, *et al*., 2008)

Plate 1: Picture of red sorghum plant

(https// www. lancasterfarming.com (2012))

3.3 Preparation of Media for Callus Induction

The excised mature and immature embryos were used for callus induction on modified semi solid Murashige and Skoog basal medium (Murashige and Skoog, 1962) plus sucrose 30g/L. The MS basal media was supplemented with varying concentration of 2, 4-D (0.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mg/L) alone or in combination with 0.5mg/l Kinetin (KN) for callus induction. The pH of the media was adjusted to 5.8 using 0.5 M HCl or 0.5 M NaOH, agar (0.8%) was used as gelling agent and the media was heated to boiling for proper mixing. The sterilization of the media was done in an autoclave at 121^{o}C for 30 minutes and media was dispensed into petri-dishes

Table 3.1:Murashige and Skoog's (1962) basal medium

Constituents	Concentration (mg/L)
Macro-elements	
NH_4NO_3	1650
KNO_3	1900
$CaCl_2.2H_2O$	440
KH_2PO_4	170
$MgSO_4.7H_2O$	370
Micro-elements	
H_3BO_3	6.2
$NAEDTA.2H_20$	37.30
$MnSO_4.4H_2O$	22.30
$FeSO_4.4H_2O$	27.80
$ZnSO_4.7H_2O$	8.60
KI	0.83
$Na_2MO_3.2H_20$	0.25
$CuSO_4.5H_20$	0.025
$CoCl_2.6H_2O$	0.025
Vitamins	
Myoinositol	100
Nicotinic acid	0.5
Pyridoxin-HCl	0.5
Thiamine-HCl	0.5
Glycine	2.0
Carbon Source	
Sucrose	30,000

Source: Harsha (2002)

3.4 Surface Sterilization of the Explants

Immature and mature seeds were surface sterilized by dipping in 95% ethanol (v/v) for 2 minutes followed by treatment with 20% commercial bleach (containing 3.5% Sodium hypochlorite) with 2 drops of tween 20 for 20 minutes (Mackinnon *et al*, 1986). Afterwards, the seeds were rinsed three (3) times with sterile distilled water and embryos were aseptically excised using sterilized forceps and surgical blade (Sudhakhar *et al.*, 2008; Mackinnon, 1986). Mature seeds were soaked in water for 24 hours before excision.

3.5 Callus Induction

The immature embryo and the embryonic axes of both mature and immature embryo were placed on the media with their axes in contact with the media for callus induction five (5) embryos were cultivated in each petri dish (11mm diameter) and five (5) petri dishes were used per treatment for callus induction using completely randomized design. The cultures were incubated in the darkness for callus proliferation at $25^{o}C \pm 2^{o}C$. 1% activated charcoal was used to control phenolic secretion (Sudhakar *et al*., 2008).

3.6 Callus Maintenance

Part of calli obtained were sub cultured in a hormone free media and media fortified with 0.1 mg/L 2, 4-D after three weeks of age

3.7 Plant Regeneration through Somatic Embryogenesis

The whitish compact calli described as embryogenic calli were selected and transferred to culture tubes (125X25mm) containing 10ml of regeneration media consisting of MS basal

medium supplemented with 1.0 and 2.0 mg/L BA (6-benzylaminopurine) + 0.1 mg/L 2,4-D. One explant was cultivated in a test tube and this was repeated 8 times and laid out in a completely randomized design. The calli maintained in a hormone free media and the media supplemented with 0.1 mg/L 2, 4-D for somatic embryo formation under dark condition were further sub cultured after 6 weeks of age in the media supplemented with 0 mg/l BA, 1 mg/L BA, 1.5 mg/L BA + 0.2 mg/L NAA and 2.0 mg/L BA + 0.2 mg/L NAA for the production of anthocyanins. Five bottles were used per treatment.

3.8 Plant Regeneration through Organogenesis

Immature embryo obtained were used for shoot initiation using MS media supplemented with 0, 1, 2 and 3 mg/L BA. One explant was cultivated in a test tube, 5 test tubes were used per treatment and these were laid out in a completely randomized design and repeated three times. After 1 week of shoot initiation, the elongated leaves were removed and their meristem tips were cultured in the media fortified with 3 mg/L BA alone and in combination with 1 mg/L NAA (Naphthalene Acetic Acid) for shoot multiplication.

3.9 Acclimatization of Regenerated Plants

Plantlets with well-developed roots were transferred to poly-pots containing sterilized sand `under high humidity created by covering the plantlets with transparent polythene sheet. The plantlets were watered daily with half –strength MS macro and micro salts. The humidity was reduced gradually by punching hole on the polythene sheets, until they were finally removed after two weeks and were taken to screen house for further maintenance

3.10 Extraction of the Anthocyanins

Fresh weight of red calli and planlets obtained from sorghum cultured were determined. The materials were air dried and the dried weights were recorded. Dried red callus, plantlets and leaf sheath obtained from the field were extracted with methanol alone or in combination with 1% HCl in methanol in accordance with Devi *et al.* (2011). 10ml of solvent was added to 0.5 g of samples in 50 ml of centrifuge tubes and the samples were shaken for 2 hours at low speed (75rpm). Samples were stored at -20°C overnight in the dark to allow for maximum diffusion of phenolics from the cellular matrix. Samples were equilibrated to room temperature and centrifuged at 7,000rpm for 10 min Residues were rinsed with 10ml volumes of solvent two times with shaking for 5 min, then centrifuging at 7000 rpm for 10 min. Finally, the extracts were mixed very well and stored at -20°C in the dark until further biochemical analysis (Joseph *et al*., 2004)

3.11 Preparation of Buffer

pH 1.0 and pH 4.5 buffer were prepared according to Association of Official Analytical Chemists (AOAC, 2005)

(**a**) pH 1.0 buffer (potassium chloride, 0.025M)- 1.86 g of potassium chloride was weighed into a beaker and 980ml of distilled water was added. The pH was measured and adjusted to 1.0 with HCl. It was transferred to 1.0 L volumetric flask, and diluted to volume with distilled water.

(**b**) pH 4.5 buffer (sodium acetate, 0.4M). 54.43g of sodium acetate was weighed into a beaker and 960ml of distilled water added to. The pH was measured and adjusted to 4.5

with HCl. It was transferred to 1.0 L volumetric flask, and diluted to volume with distilled water (AOAC, 2005).

3.12 Stability at Variable pH.

The anthocyanin stability was tested by treating 1 ml of sample with1 ml of pH 1.0 and 4.5 solutions. The color change was observed. (Devi *et al*., 2011).

3.13 Flavonoid Confirmation Test

A small amount of extracted sample was treated with ferric chloride and the result was observed for the presence of flavonoids

3.14 Determination of Total and Monomeric Anthocyanins

The pH differential method as reported by Fuleki and Francis (1968) and Guisti (2003) was used for quantitative determination. Each of two 0.2 ml aliquots was diluted with 3.0 ml of pH 1.0 and pH 4.5 buffers. Total anthocyanin was determined from absorbance at different wavelengths (468nm for apigeninidin, 482nm for luteolinidin, and 520nm for cyanindin-3-glucoside) in pH1.0 buffer, while monomeric anthocyanins were determined from the differences between absorbance in pH 1.0 and 4.5 buffers. Extinction coefficients for apigeninidin (18000 L/mol/cm)) luteolinidin (13800 L/mol/cm) and cyanindin-3-glucoside (26900 L/mol/cm) were used using the formula described by Fuleki and Francis (1968), NSF international, (2004) and AOAC (2005). Jenway 6405 UV / VIS spectrophotometer

was used for the absorbance measurement. The absorbance values were read twice and their average values were recorded

Total and monomeric anthocyanins were calculated by using the following formulae:

W/w (mg/g) = $\frac{A\ X\ MWX\ DFX\ V}{\varepsilon L\ X\ Wt}$---------- Equation 1

Where,

A= (Absorbance λ vis-max- A700) pH 1.0

ε = Cyd-3-glu molar absorptivity (26,900 L/mol/cm)

ε= Apigeninidin molar absorptivity (18000 L/mol/cm)

ε= Luteolinidin molar absorptivity (13800 L/mol/cm)

MW= Cyd-3-glu molecular weight (449.2g/mol)

MW= Apigeninidin (255.24g/mol)

MW= Luteolinidin (271.24g/mol),

DF = dilution factor=15

V = final volume (ml)

Wt = sample weight (g)

L = cell path length (usually 1 cm)

Anthocyanin pigment content (mg/liter) = $\frac{A\ X\ MW\ X\ DF\ X\ 1000}{\varepsilon\ X\ 1}$ ---------- Equation 2

Where,

A= (Absorbance λ vis-max-A700) pH 1.0- (Absorbance λ vis-max-

A700) pH 4.5

3.15 Data Collected

The following set of data were taken during this study

a. Percentage callus induction

Percentage callus induction was calculated on the basis of the number of explants forming callus and the number of explants inoculated

Percentage callus induction=$\frac{Numberofexplants\quad with\ response}{Numberofexplants\quad inoculated}$ x100

b. Nature of callus

After one and three weeks of inoculation, nature of calli induced was observed and recorded

c. Weight of callus

After three weeks of inoculation, the weight of callus was measured in gram (g) with the help of a digital balance (Ohaus, AR3130, China)

d. Number of callus with shoot (percent of shoot or plant regeneration)

Number of callus with shoot was recorded and percentage of shoot regeneration

was calculated as Percentage shoot regeneration = $\frac{Number\ \ of\ \ calli\ \ with\ shoots}{Numberofinoculated\quad calli}$ X 100

e. Average number of shoots per callus

Number of shoots per callus was recorded after 2 weeks of inoculation and the mean was calculated

f. Number of shoot per immature embryo and shoot tip

Percentage shoot formation= $\frac{Numberofexplants\quad with\ shoot\ formation}{Numberofexplants\quad inoculated}$ X 100

g. Shoot length

After two weeks of shoot inoculation, the shoot length was measured in centimeter (cm) with a ruler

h. Average absorbance. This involved reading of absorbance twice using spectrophotometer (Jenway 6405, U.K) and two readings were taken per sample and the average value was recorded

i. Total and monomeric anthocyanins: The absorbances obtained at pH 1.0 buffer were used to calculate total anthocyanins while monomeric anthocyanins were determined from the differences between absorbance in pH 1.0 and 4.5 buffers.

3.16 Statistical Analysis

Means, percentages, counts were taken on the parameters. The data collected were subjected to analysis of variance (ANOVA) using the generalized linear model (glm procedure) and means were seperated using Duncan multiple range test (SAS, 2005).

CHAPTER FOUR

RESULTS

4.1 Callus Formation

Table 4.1 shows the effect of 2, 4-D alone and in combination with kinetin on percentage callus formation using mature embryo, immature embryo and embryonic axes of immature embryo. Different explants and plant growth regulators (PGR) at varying concentration levels were tested in order to determine which of the explants and phytohormones give the best callus formation. Outgrowth of immature embryo and embryonic axes were observed third day after inoculation, for mature embryo callus initiation started fifth day after induction. It was also observed that callus induction started from the radicle portion of the embryo and it grew upwardly towards the plumule. The result of this study showed highly significant difference ($p< 0.001$) for percentage callus formation among the different explants and concentrations of 2, 4-D and kinetin used. The highest callus formation was obtained from immature embryo followed by the embryonic axes; the mature embryo recorded the least percentage callus formation. Using mature embryo 4.0 mg/L 2, 4-D gave the highest callus induction frequency (60%) and this is not significantly different from what was obtained from the media fortified with 2.0 mg/L 2, 4-D combined with 0.5 mg/L KN which recorded (56%) of callus induction frequency. The least callus induction frequency was obtained from the media supplemented with 1.5 mg/L 2, 4-D (0%) (Table 4.1). No callus formation was obtained in a hormone free media and in the media supplemented with 0.5 mg/L KN alone (0%) (Table 4.1). It was observed that most of the calli obtained from mature explants were not growing very well and most of them were lost

due to phenolic secretion. Using immature embryo, 2 mg/L 2, 4-D alone and in combination with 0.5 mg/l KN and 3.5 mg/L 2, 4-D combined with 0.5 mg/L KN recorded the highest callus induction frequency (100%). This is significantly different from what was obtained from the media fortified with 1.5 mg/L 2, 4-D (80%) (Table 4.1). Also no callus formation was obtained in a hormone free media and the media supplemented with 0.5 mg/L KN instead shoot and root formation were observed. When embryonic axes were used, the media supplemented with 2.0 mg/L 2, 4-D combined with 0.5 mg/L KN recorded the highest callus induction frequency (92%) followed by the media fortified with 2.0 mg/L 2, 4-D (88%) and this is also significantly different from was obtained from the media fortified with 1.5 mg/L (60%) (Table 4.1). No callus formation was also observed in the media supplemented with no hormone and media fortified with 0.5 mg/L KN. Root and shoot formation were also observed (Plate III)

4.2 Callus Fresh Weight

Effect of 2, 4-D alone or in combination with kinetin on fresh weight of the callus obtained from immature embryo and embryonic axes three weeks after inoculation were shown in Table 4.2. For immature embryo, the highest callus fresh weight was obtained from the media supplemented with 3.5 mg/L 2,4-D and 0.5 mg/L KN which recorded 2.405g callus fresh weight followed by the media fortified with 2.5mg/L 2,4-D and 0.5 mg/L KN (2.299g) and the media fortified with 1.5 mg/L 2,4 D has the lowest fresh callus weight (0.484g) followed by the media supplemented with 2.0 mg/L 2,4-D alone (Table 4.2). Zero callus fresh weight was obtained in a hormone free media and the media supplemented with

0.5 mg/L KN. For the embryonic axes the highest callus fresh weight was obtained from the media fortified with 3.5 mg/L 2, 4-D alone which recorded 1.482g fresh callus weight followed by the media supplemented with 2.0 mg/L 2, 4-D and 0.5 mg/L KN (1.14g) and the least fresh callus weight was obtained from the media supplemented with 1.5 mg/L 2, 4-D alone (0.324g). Zero fresh callus weight was also observed from the media with no hormone and the media fortified 0.5 mg/L KN alone (Table 4.2)

4.3 The Type of Callus Induced

Tables 4.3 and 4.4 show the effect of 2, 4-D and kinetin on the nature of callus induced using immature embryo and embryonic axes as explants. Different types of calli were obtained from the media combinations tested and the explants used. When mature embryos were used, the calli obtained were initially whitish in nature and later changed to yellowish type of callus after two weeks of inoculation. Most of these calli underwent necrosis before three weeks of age. The remaining calli became reddish brown in color after three weeks of age in a hormone free media (Plate IV). Using immature embryo, the calli obtained were initially creamy compact in nature with browning at the scutellum for most of the treatments tested; (Plate II) this was observed after one week of callus formation. The browning of the scutellum affected the color of the callus obtained from immature embryo explant. After three weeks of inoculation most of the calli induced turned to globular yellowish type and these calli are friable in nature (Table 4.3 and Plate II). Most of these calli are pigmented and some do not have pigment. In the media supplemented with 3.0 mg/L and 4.0 mg/L 2, 4-D + 0.5 mg/L KN the calli later turned to whitish compact callus

after three weeks of inoculation, some of these calli were also pigmented while other did not have pigment. Most of these calli changed to reddish type of calli when they were taken to hormone free media for somatic embryo formation and cytokinin containing media (Plate II). Using embryonic axes, the calli were whitish compact in nature after one week of inoculation no browning was observed (Table 4.4 and Plate III). The calli remained whitish in color up to two weeks of inoculation. These calli changed to globular yellowish calli after three weeks of inoculation for most of the treatment tested. The media fortified with 2.0 mg/L and 2.5 mg/L 2, 4-D and 0.5 mg/L KN retained their compactness up to the three weeks after inoculation. For the media with no hormone and the media supplemented with 0.5 mg/L KN, no callus formation was observed (Plate III).

Table 4.1: Effect of 2, 4-D and Kinetin on percentage callus formation using immature embryo, embryonic axes and mature embryo explants

PGR		Callus induction for IMEB (%)	Callus induction for IMEB (T)	Callus induction for EBA (%)	Callus induction for EBA (T)	Callus induction for MEB (%)	Callus induction for MEB (T)
2,4-D (mg/L)	KINETIN (mg/L)						
0	0.0	0.00	0.00 ^{c}	0.00	0.00^{c}	0.00	0.00 ^{e}
1.5	0.0	80	1.91^{b}	60	1.77^{b}	0.00	0.00 ^{e}
2.0	0.0	100	2.00^{a}	88	1.94^{a}	44	1.65^{abc}
2.5	0.0	92	1.97^{ab}	72	1.85^{ab}	20	1.32^{d}
3.0	0.0	88	1.94^{ab}	80	1.90^{a}	40	1.59^{bc}
3.5	0.0	96	1.99^{a}	80	1.90^{a}	40	1.59^{bc}
4.0	0.0	92	1.97^{ab}	80	1.90 ^{a}	60	1.77^{a}
0	0.5	0.00	0.00^{c}	0.00	0.00^{c}	0.00	0.00^{e}
1.5	0.5	88	1.95^{ab}	84	1.92^{a}	32	1.50 ^{c}
2.0	0.5	100	2.00^{a}	92	1.97^{a}	56	1.74^{ab}
2.5	0.5	92	1.97^{ab}	84	1.92^{a}	40	1.61^{abc}
3.0	0.5	92	1.96^{ab}	84	1.92^{a}	40	1.59^{bc}
3.5	0.5	100	2.00^{a}	80	1.90^{a}	52	1.72^{ab}
4.0	0.5	96	1.99^{a}	84	1.93 ^{a}	52	1.72^{ab}
CV (%)			5.04		2.90		8.69
SE±			0.06		0.04		0.07

Means followed by the same letter(s) within a column are not significantly different at 5% level of significance using DMRT. IMEB = Immature embryo =, EBA = Embryonic axes, MEB = Mature embryo, PGR = Plant Growth Regulator, T= Transformed

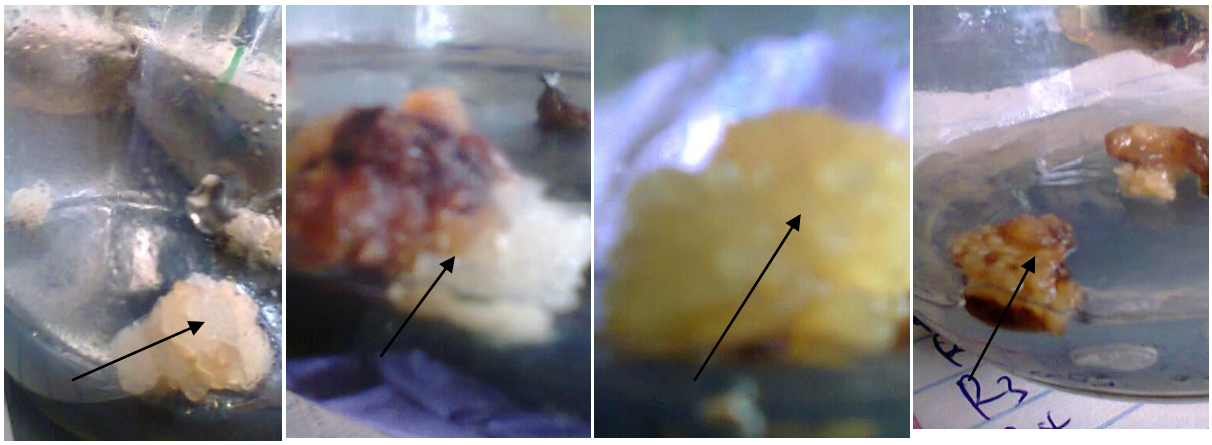
Table 4.2: Effect of 2, 4-D and kinetin on average weight of fresh callus using immature embryo and embryonic axes

PGR		Average weight of fresh callus for IMEB (g)	Average weight of fresh callus for EBA (g)
2,4-D (mg/L)	KINETIN (mg/L)		
0	0.0	0.00^{c}	0.00^{f}
1.5	0.0	0.48^{b}	0.33^{ef}
2.0	0.0	0.48^{b}	0.50^{cde}
2.5	0.0	0.52^{b}	0.47^{cde}
3.0	0.0	0.55^{b}	0.80^{c}
3.5	0.0	0.70^{b}	1.48^{a}
4.0	0.0	0.54^{b}	0.61^{cde}
0	0.5	0.00^{c}	0.00^{f}
1.5	0.5	0.51^{b}	0.35^{de}
2.0	0.5	0.52^{b}	1.14^{b}
2.5	0.5	2.30^{a}	0.76^{c}
3.0	0.5	0.65^{b}	0.71^{dc}
3.5	0.5	2.40^{a}	0.66^{cde}
CV (%)		35.82	42.12
SE±		0.17	0.56

Means followed by the same letter(s) within a column are not significantly different using DMRT (*significant at P<0.05, **highly significant at P<0.01).

Table: 4. 3: The type of callus obtained at one week and three weeks of inoculation using immature embryo

PGR		Nature of callus obtained one week after inoculation	Nature of callus obtained three weeks after inoculation
2,4D	KINETIN		
0	0	No callus formation	No callus formation
1.5	0	Creamy compact callus with browning at the scutellum	Globular yellowish callus with pigment
2.0	0	Reddish type of callus	Globular yellowish callus with pigment
2.5	0	Whitish and Creamy compact callus with browning at the scutellum	Globular yellowish callus with pigment
3.0	0	creamy compact callus with pigment	Globular yellowish callus
3.5	0	Creamy compact callus with browning at the scutellum	Globular yellowish callus with pigment
4.0	0	Whitish callus with browning at the scutellum	Globular yellowish callus with pigment
0	0.5	No callus formation	No callus formation
1.5	0.5	Creamy compact callus with browning at the scutellum	Globular yellowish callus
2.0	0.5	Creamy compact callus with browning at the scutellum	Globular yellowish callus with pigment
2.5	0.5	Creamy compact callus with pigment	Globular yellowish callus
3.0	0.5	Creamy compact callus	Whitish callus with pigment and red callus
3.5	0.5	Creamy compact callus with pigment	Globular yellowish callus with pigment
4.0	0.5	creamy compact callus with little browning at the scutellum	Whitish compact callus with and without pigment

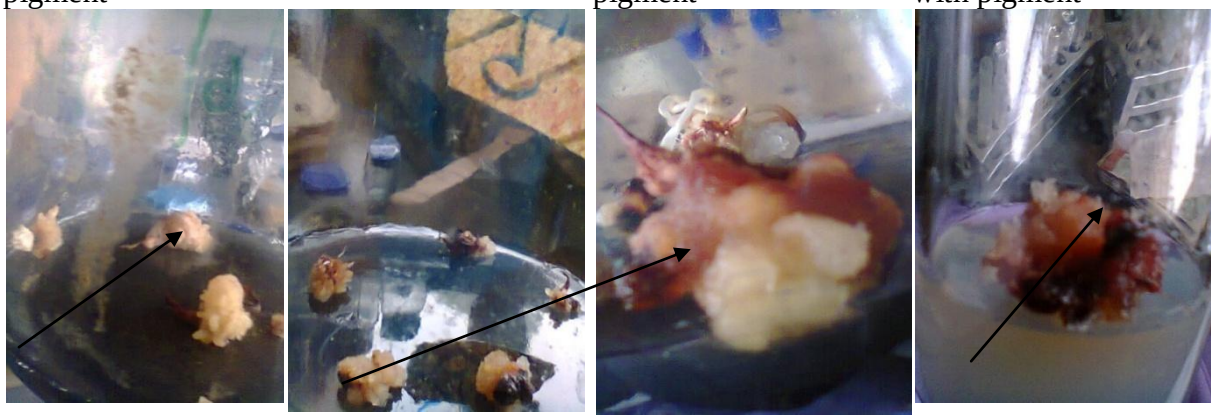


a. Whitish compact callus with no pigment

b. Whitish compact callus with pigment

c. Globular yellowish callus with no pigment

d. Globular yellowish callus with pigment



e. Creamy compact callus with no pigment

f. Creamy compact callus with pigment

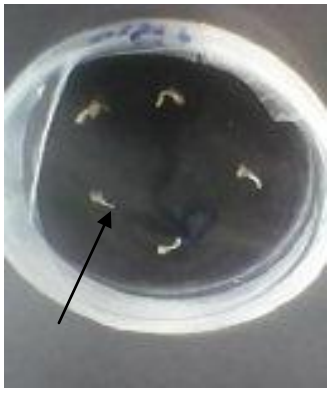
g. Creamy compact callus with pigment

h. Red callus

Plate II. Different types of calli induced using immature embryo explant

Table 4. 4: The type of callus induced at one week and three weeks of inoculation from embryonic axes cultured

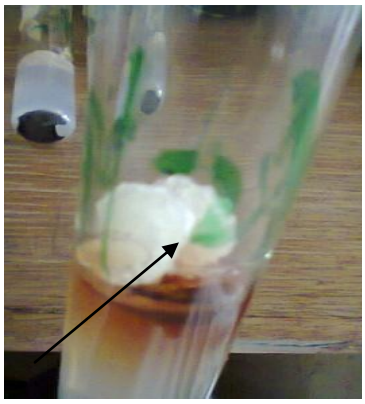
PGR		Nature of callus obtained one week after inoculation	Nature of callus obtained after two weeks of inoculation
2,4D	KINETIN		
0	0	-	No callus formation
1.5	0	Whitish compact callus	Globular yellowish callus with and without pigment
2.0	0	Whitish compact callus	Globular yellowish callus with pigment
2.5	0	Whitish compact callus	Globular yellowish callus with pigment and whitish compact callus
3.0	0	Whitish compact callus	Globular yellowish callus with and without pigment
3.5	0	Whitish compact callus	Globular yellowish callus
4.0	0	Whitish compact callus	Globular yellowish callus
0	0.5	-	No callus formation
1.5	0.5	Whitish compact callus	Globular yellowish callus
2.0	0.5	Whitish compact callus	Globular yellowish callus with pigment and whitish compact callus with and without pigment
2.5	0.5	Whitish compact callus	whitish compact callus with and without pigment and globular yellowish callus with pigment
3.0	0.5	Whitish compact callus	Globular yellowish callus with and without pigment
3.5	0.5	Whitish compact callus	Globular yellowish callus with and without pigment
4.0	0.5	Whitish compact callus	Globular yellowish callus with and without pigment



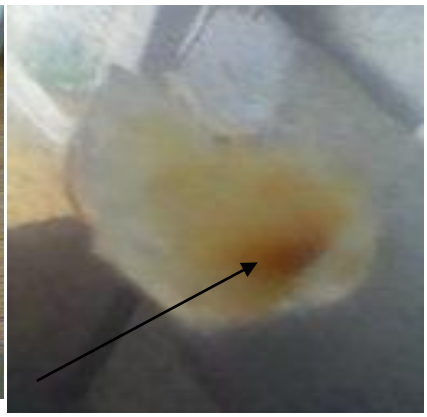
a. Outgrowth of embryonic axes 3 days after inoculation

b. Whitish type of callus 1 week after inoculation

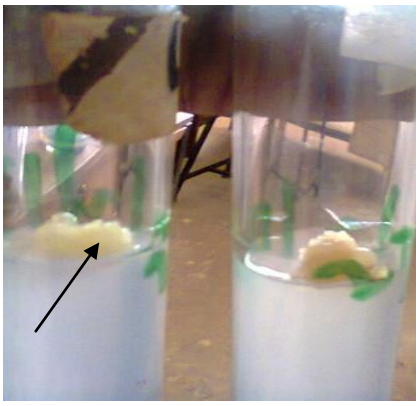
c.Whitish type of callus with no pigment after 3 weeks of inoculation



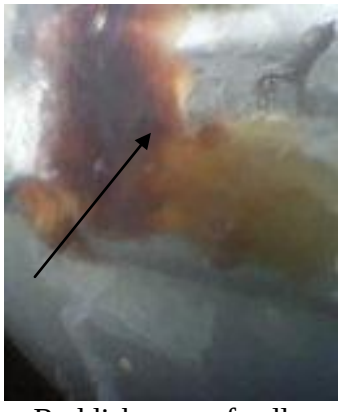
d. Whitish type of callus with pigment after 3 weeks of inoculation



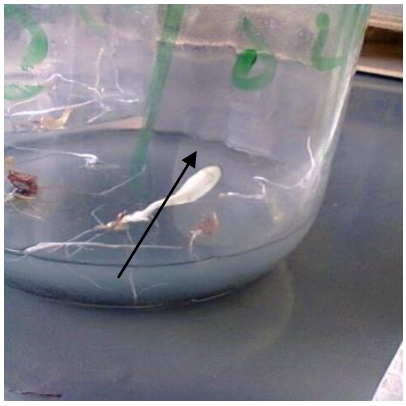
e. Yellowish type of callus



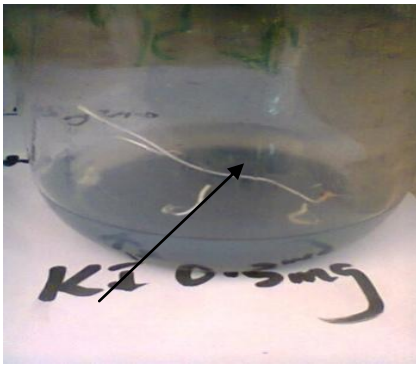
f. Globular yellowish callus after 3 weeks of inoculation



g. Reddish type of callus



h. Control A (Hormone free media)



i. Control B (0.5KN)

Plate III. Different types of calli induced using embryonic axes

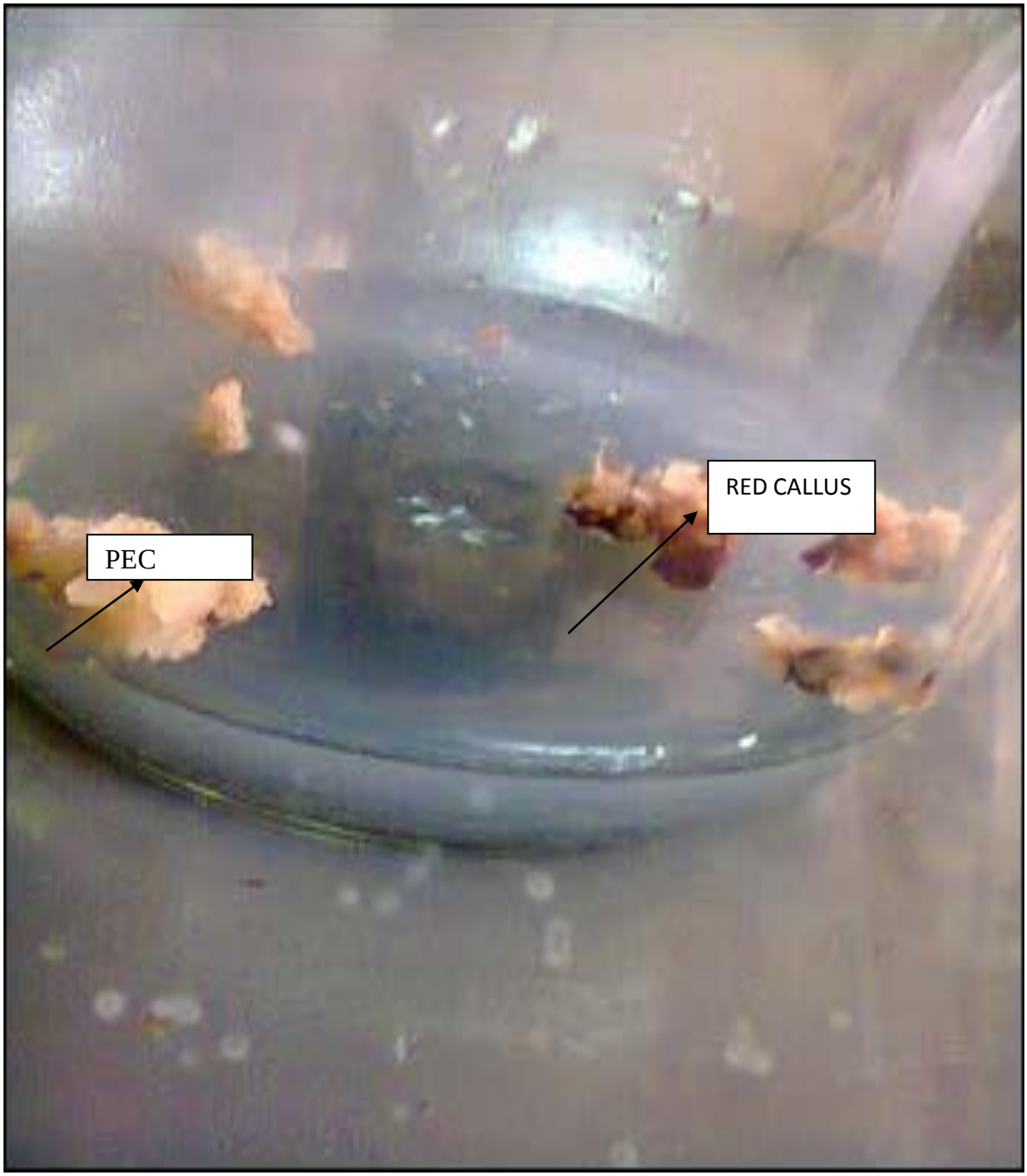


Plate IV: The calli obtained from mature embryo

PEC – Potentially Embryogenic callus

4.4 Plant Regeneration through Somatic Embryogenesis

Whitish compact calli taken to regeneration media turned greenish after few days of culture on regeneration media and plantlets development was observed after two weeks of culture. More percentage shoot formation was obtained from the media supplemented with 1mg/L BA (75%) compared to 50% obtained from the media supplemented 2 mg/L BA + 0.1 mg/L, 2, 4–D. The average number of shoot per callus was higher in the media fortified with 1 mg/L BA (2.25) but this is not significantly differently at 5% level of significance with what was obtained from the media supplemented with 2 mg /L BA+0.1 mg/L 2,4-D which recorded 1.88 (Table 4.5). The average shoot length was 2.26 cm in the media fortified with 1 mg/l BA which was not significantly different from average shoot length recorded by the media supplemented with 2 mg/L BA+0.1 mg/L 2,4-D (Table 4.5). No response was observed in the media fortified with 0.1 mg/L 2, 4-D but in a hormone free media the greening of some calli was observed but these calli couldn't differentiate further to form shoot under light condition (Plate V).

4.5 Regeneration through Organogenesis

Shoot formation was observed in all the treatments tested. Media supplemented with 2 mg/L BA recorded the highest shoot formation (86.67%) followed by the media fortified with 1 mg/L BA (73.30%). Both root and shoot formations were observed in a hormone free media and it recorded the least percentage shoot initiation (46.67%) (Table 4.6). The primary leaves were removed from the shoot apices and the meristems were cultured for multiple shoots formation. 1-4 shoots per explant were obtained from the apical meristem cultured. The mean shoot number per explant was higher in the media supplemented 3.0 mg/L BA combined with 1 mg/L NAA (2.4) and this not significantly different from what

was obtained from the media supplemented with 3 mg/L BA alone (2.0).The plant height in the media fortified with 3.0 mg/L+1 mg/L NAA was 7.2cm and this is not significantly different from what was the media supplemented with 3.0 mg/l BA (6.2cm) (Table 4.7). The plantlets obtained from hormone free media were acclimatized after one week of regeneration (Plate VI).

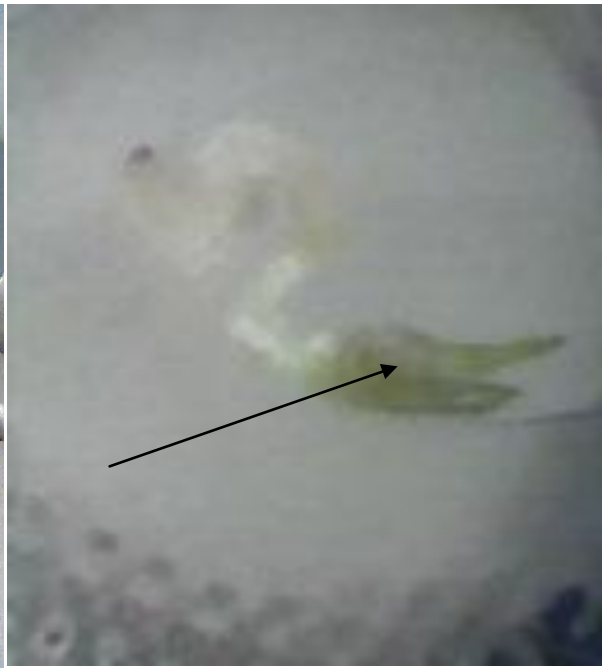
Table 4.5. Effect of BA alone and in combination with 2, 4-D on plant regeneration

PGR USED		No of callus inoculated	No of callus with shoot formation	Percentage shoots formation (%)	Total number of shoots /treatment	Average number of shoot/callus	Average shoot length(cm)
BA (mg/L)	2,4-D (mg/L)						
0	0.0	8	0	-	-	-	-
0	0.1	8	0	-	-	-	-
I	0.0	8	6	75	18	2.25[a]	2.26[a]
2	0.1	8	4	50	15	1.88[a]	1.48[a]

a. Shoot initiation stage

b.Shoot multiplication stage

c. Shoot elongation stage



d. Greening of callus in a hormone free media

Plate V. Plant regeneration through somatic embryogenesis.

Table 4.6: Effect of BA on shoot initiation using immature embryo

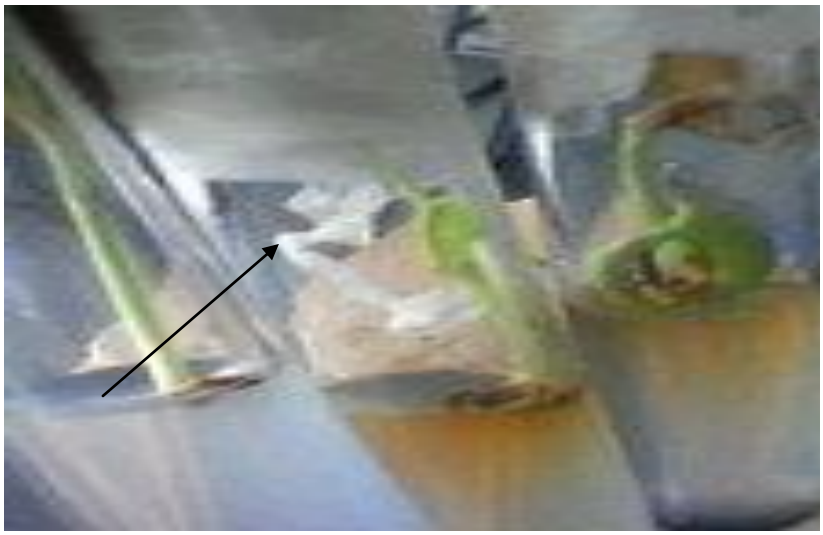
PGR USED	No of explants inoculated	No of explants that formed shoot	Shoot formation (%)	Shoot formation (T)
BA (mg/L)				
0.0	15	7	46.67	1.67[b]
1.0	15	11	73.3	1.85[ab]
2.0	15	13	86.67	1.94[a]
3.0	15	8	53.33	1.73[b]
CV				5.40
SE±				0.03

Means followed by the same letter(s) within a column are not significantly different using DMRT (*significant at P<0.05, **highly significant at P<0.01). T= Transformed

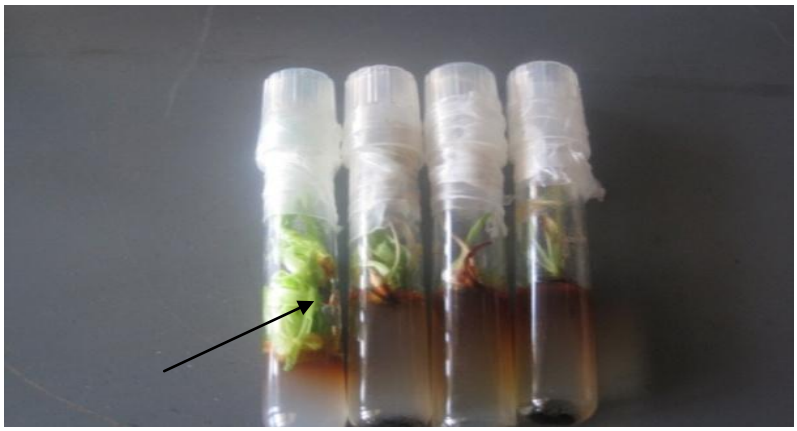
Table 4.7 :Effect BA and NAA on shoot multiplication

PGR		No of explants inoculated	No of shoot /treatment	No of shoot/explants	Average plant height (cm)
BA(mg/L)	NAA(mg/L)				
3.0	0.0	10	20	2.0a	6.2a
3.0	1.0	10	24	2.4a	7.2a
CV				50.71	25.21
SE±				0.49	0.75

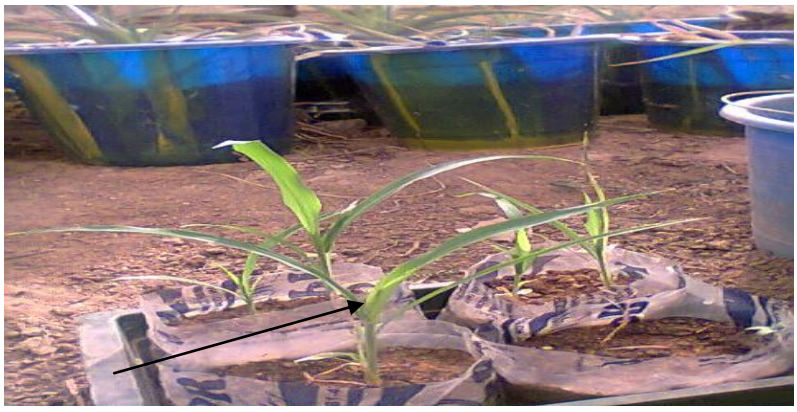
Means followed by the same letter(s) within a column are not significantly different using DMRT (*significant at P <0.05, **highly significant at P<0.01).



a. Shoot initiated from immature embryo culture



b. Shoot multiplication stage.



c. Graduated plantlets in the green house

Plate VI: Plant regeneration through organogenesis.

4.6 Extraction of Anthocyanins and pH Stability Test

The fresh weights and dried weights of the samples used were shown in Table 4.8. Methanol and 1% HCl in methanol were used for the extraction. The color of the extracts was red in most of the samples used except the extract obtained from plantlets that was greenish in color (Table 4.9). The stability of the extracts was tested at different pH. The extracts were yellow in pH 1.0 buffer but when the pH increased to 4.5, the extract became light orange. The same results were observed in methanol extracts and acidified methanol (Table 4.9 and Plate VII).

4.7 Flavonoids Confirmation Test

Flavonoid is known to be an important antioxidant; its presence in the extracts was confirmed by using 0.1ml of ferric chloride. When 0.1ml of ferric chloride was added to 1ml of each extract, the color change was observed. It was observed that the reddish color of most of the extracts changed to deep brown coloration while light brown coloration was observed in one week old plantlets obtained *in vitro* (Table 4.10 and Plate VIII).

4.8 Average Absorbance using Methanol and Acidified Methanol.

Average absorbance values obtained from the extracts at different wavelengths using methanol alone and acidified methanol are shown in Table 4.11 and 4.12 respectively. Using methanol alone the highest absorbance was obtained from the leaf sheath obtained *in situ* at 16weeks of age for all the wavelengths followed by the callus in 1 mg/l BA (Table 4.11) while the callus in 1 mg/L BA recorded the highest absorbance at 8 weeks of age using acidified methanol followed by the callus in 1.5 mg/L BA+0.2 mg/L NAA (Table 4.12).

Table 4.8: The fresh and dried weight of the samples used

Plant samples	Fresh weight (g)	Dried weight(g)
Callus in 1 mg/L BA	10.09	1.110
callus in 1.5 mg/L BA +0.2mg/L NAA	9.85	1.188
Callus in 2 mg/L BA+0.2 mg/L NAA	6.902	0.843
Callus in a hormone free media	9.05	1.280
Leaf sheath obtained *in situ*	-	1.01
Plantlet obtained *in vitro*	6.736	0.708

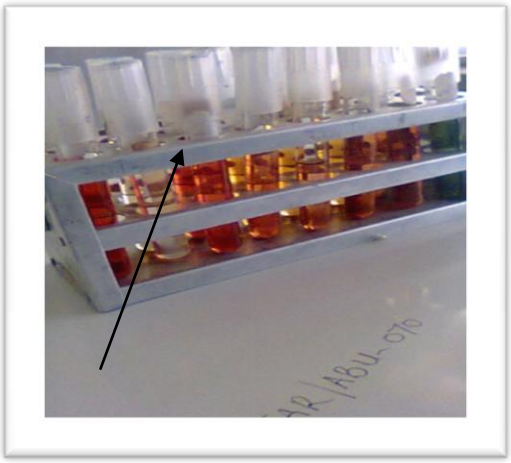
Table 4. 9: Color and age of the plant samples used for the extraction

Plant sample	Color of the plant sample	Age of the plant at sampling	Color of the extracts in pH 1.0 buffer	Color of the extracts in pH 4.5 buffer
Callus in a hormone free media	Red	8weeks	Yellow	Light orange
Callus in 1 mg/L BA	Red	8weeks	Yellow	Light orange
callus in 1.5 mg/L BA +0.2mg/L NAA	Red	8weeks	Yellow	Light orange
Callus in 2 mg/L BA+0.2 mg/L NAA	Red	8weeks	Yellow	Light orange
Leaf sheath obtained *in situ*	Red	16weeks	Yellow	Light orange
Plantlet obtained *in vitro*	Green	1week	Light green	Colorless

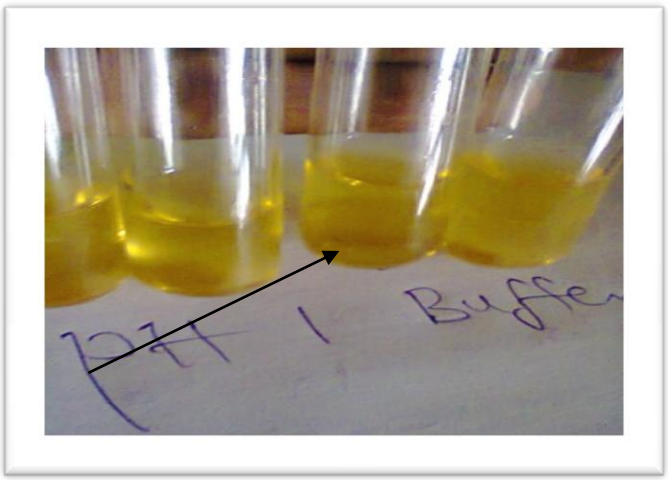
Table 4. 10: Flavonoids confirmation in red sorghum using ferric chloride

Plant sample	Color of the extracts in methanol	Color of the extract after adding the ferric chloride	Color of the extracts in acidified methanol	Color of the extracts after adding the ferric chloride
Callus in a hormone free media	Red	Deep brown	Red	Deep brown
Callus in 1 mg/L BA	Red	Deep brown	Red	Deep brown
Callus in 1.5 mg/L BA +0.2 mg/L NAA	Red	Deep brown	Red	Deep brown
Callus in 2 mg/L BA+0.2 mg/L NAA	Red	Deep brown	Red	Deep brown
Leaf sheath obtained *in situ*	Red	Deep brown	Red	Deep brown
Plantlet obtained i*n vitro*	Green	Light brown	Green	Light brown

Air-dried sample



Extracts obtained from the sample using methanol and acidified methanol

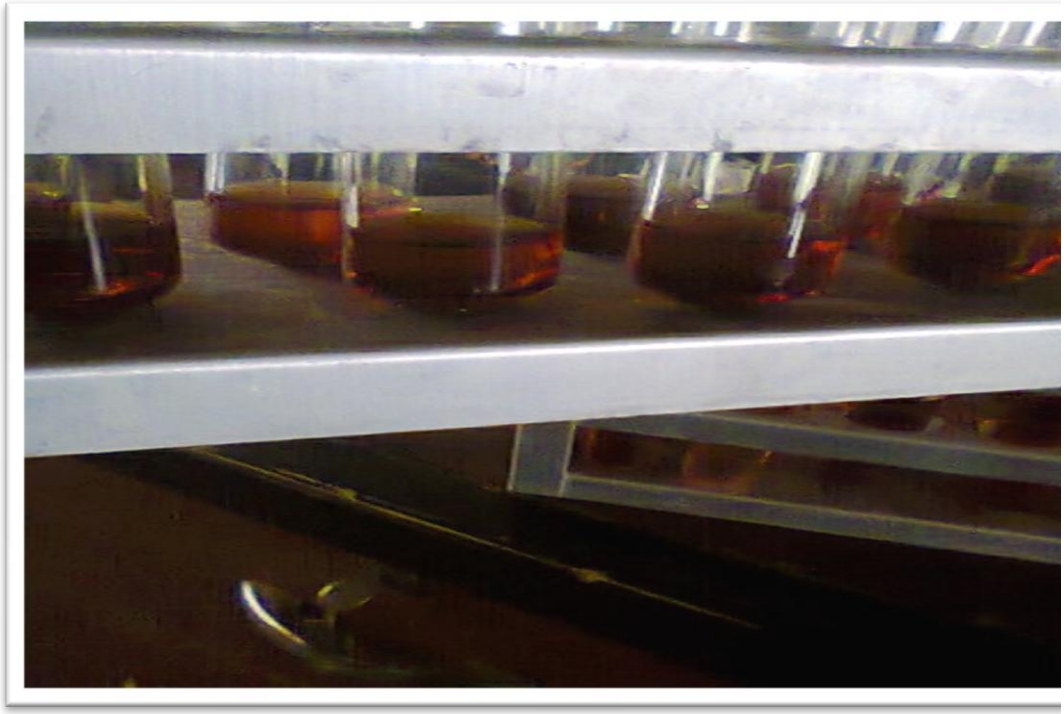


Color of the extracts after adding pH 1.0 buffer

Color of the extracts after adding pH 4.5 buffer

Plate VII. Air-dried samples and the extracts obtained after adding the methanol, acidified methanol and buffers

Color of the extracts before adding the ferric chloride



Color of the extracts after adding the ferric chloride

Plate VIII. Flavonoid Confirmation Test

Table 4.11: Average absorbance obtained from the extracts at different wavelengths using methanol alone

Treatments	Apigen (468nm)		Luteo(482nm)		Cyanindin-3-glucoside (520nm)		700nm	
Treatments	pH 1.0	pH 4.5	pH1.0	pH 4.5	pH 1.0	pH 4.5	pH 1.0	pH 4.5
Callus in a hormone free media	0.557	0.435	0.486	0.487	0.203	0.327	0.066	0.092
Callus in 1 mg/L BA	1.266	0.702	1.037	0.691	0.274	0.471	0.072	0.121
callus in 1.5 mg/L BA +0.2mg/L NAA	1.074	0.646	0.968	0.646	0.310	0.435	0.093	0.115
Callus in 2 mg/L BA+0.2 mg/L NAA	1.106	0.599	0.996	0.589	0.404	0.413	0.178	0.127
Leaf sheath obtained *in situ*	1.645	0.962	1.548	0.964	0.561	0.669	0.181	0.177
Plantlet obtained i*n vitro*	0.636	0.912	0.549	0.823	0.504	0.718	0.341	0.467

Table 4.12: Average absorbance obtained from the extracts at different wavelengths using acidified methanol

Treatments	Apigen (468nm)		Luteo (482nm)		Cyn-3-glu(520nm)		700nm	
	pH 1.0	pH 4.5	pH1.0	pH 4.5	pH 1.0	pH 4.5	pH 1.0	pH 4.5
Callus in a hormone free media	0.429	0.451	0.335	0.366	0.198	0.291	0.063	0.115
Callus in 1mg/L BA	1.853	0.987	1.686	0.885	0.452	0.607	0.094	0.113
Callus in 1.5mg/L BA+0.2 mg/L NAA	1.704	1.229	1.580	1.169	0.504	0.791	0.088	0.144
Callus in 2 mg/L BA+0.2 mg/L NAA	1.112	0.520	0.960	0.438	0.338	0.309	0.087	0.089
Leaf sheath obtained *in situ*	1.529	0.940	1.416	0.854	0.498	0.577	0.144	0.144
Plantlets obtained *in vitro*	1.136	0.866	1.036	0.880	0.880	0.707	0.665	0.502

4.9 Quantification of Total anthocyanins and Monomeric anthocyanins using Methanol and Acidified methanol

The result of this study showed highly significant difference ($p<0.001$) for total and monomeric anthocyanins content among the different samples used for the extraction. When methanol was used, the red leaf sheath obtained *in situ* at sixteen weeks of age recorded the highest total anthocyanin content when expressed as apigeninidin (18 mg/g), luteolinidin (24 mg/g) and cyanindin-3-glucoside (Table 4.13). This is significantly different from what was obtained from green plantlet obtained *in vitro* at one week of age which recorded the least for apigeninidin and luteolinidin respectively. Using acidified methanol, among the different concentrations of hormone used, the pigmented callus in the media supplemented with 1 mg/L BA at eight weeks of age recorded the highest total anthocyanin content when expressed as apigeninidin (22.44 mg/L), and luteolinidin (28.16 mg/g) followed by the callus in the media fortified with 1.5 mg/L BA and 0.2 mg/L NAA which recorded 20.62 mg/g and 26.43 mg/g for apigeninidin and luteolinidin respectively. This is significantly different from what was obtained from the callus in a hormone free media (0 BA) which recorded the least for apigeninidin and luteolinidin. The callus in the media fortified with I.0 mg/L BA and callus in the media supplemented with 1.5 mg/L BA+0.2 mg/L NAA recorded the highest for total monomeric anthocyanin which is 415.075 mg/L and 415.084 mg/L respectively (Table 4.14). The lowest total monomeric anthocyanin was obtained from callus in a hormone free media when methanol was used (Table 4.13) while plantlet obtained *in vitro* recorded the least when acidified methanol was used (Table 14). The absence of monomeric anthocyanin was observed from green plantlet obtained *in vitro* using methanol

Table 4.13: Total anthocyanin and monomeric anthocyanin concentration obtained from red sorghum using methanol alone for the extraction and jenway, 6405 UV/VIS spectrophotometer for absorbance determination

Treatment	Total apigen (mg/g)	Total luteol (mg/g)	Total cyn-3-glu (mg/g)	Monomeric apigen(mg/L)	Monomeric luteol (mg/L)	Total monomeric (mg/L)
Callus in a hormone free media	6.26[e]	7.42[e]	2.05[f]	43.07[d]	42.90[d]	85.97[d]
Callus in 1 mg/L BA	15.24[b]	17.08[b]	3.04[d]	109.12[b]	116.60[b]	255.72[b]
Callus in 1.5 mg/L BA+0.2 mg/L NAA	12.53[c]	15.49[c]	3.26[c]	95.93[c]	101.72[c]	197.65[c]
Callus in 2 mg/L BA+0.2 mg/L NAA	11.84[d]	14.47[d]	3.39[ab]	96.99[c]	104.81[c]	201.80[c]
Leaf sheath obtained in *situ*	18.68[a]	24.18[a]	5.76[a]	144.32[a]	170.99[a]	315.31[a]
Plantlets obtained *in vitro*	3.76[f]	3.67[f]	2.44[e]	-32.01[e]	-43.78[e]	-75.79[e]
SE (±)	0.06	0.08	0.05	1.36	2.97	3.52
CV(%)	0.55	0.56	1.45	1.78	3.62	2.22

Means followed by the same letter(s) within a column are not significantly different at 5% level of significance using DMRT

Table4.14: Total anthocyanin and monomeric anthocyanin concentration obtained from red sorghum using acidified methanol for the extraction and jenway6405 UV/VIS spectrophotometer for absorbance determination

Treatment	Total apigen (mg/g)	Total luteol (mg/g)	Total cyn-3-glu (mg/g)	Monomeric apigen (mg/L)	Monomeric luteol (mg/L)	Total monomeric anthocyanins (mg/L)
Callus in a hormone free media	4.66^{f}	4.79^{f}	2.02^{e}	6.17^{e}	74.15^{e}	80.32^{d}
Callus in 1 mg/L BA	22.44^{a}	28.16^{a}	5.75^{ab}	189.09^{a}	225.98^{b}	415.07^{a}
callus in 1.5 mg/L BA +0.2 mg/L NAA	20.62^{b}	26.43^{b}	6.25^{a}	113.05^{c}	302.03^{a}	415.08^{a}
Callus in 2 mg/L BA+0.2 mg/L NAA	13.06^{d}	15.43^{d}	3.75^{c}	126.45^{b}	102.75^{d}	228.78^{c}
Leaf sheath obtained *in situ*	17.68^{c}	22.49^{c}	5.31^{b}	126.03^{b}	209.47^{c}	335.92^{b}
Plantlets obtained *in vitro*	6.01^{e}	6.56^{e}	3.23^{d}	20.08^{d}	-2.06^{f}	20.08^{e}
SE(±)	0.03	0.10	0.19	1.23	0.75	1.24
CV (%)	0.19	0.55	4.44	1.20	0.45	0.49

Means followed by the same letter(s) within a column are not significantly different at 5% level of significance using DMRT

CHAPTER FIVE

5.0 DISCUSSION

5.1 Callus Induction

Callus induction involves the use of different types of explants such as immature embryo, mature embryo, shoot tip, leaf etc. Auxin (2, 4-D) causes cell division, cell elongation and swelling of tissues. The auxins are mostly used in combination with cytokinins for callus induction. Cytokinins are adenine derivatives and they also promote cell division and somatic embryogenesis (Pullaiah *et al*., 2009). Both mature and immature embryos were used for callus induction in this study. Different concentrations of 2, 4-D alone or in combination with 0.5 mg/L KN were used for callus induction. Outgrowth of explants was observed after 3 days of inoculation except for mature embryo that took up to five days before it began to show response. Indra *et al.* (2009) also reported that fully mature healthy seeds showed callus induction after 8-10 days of culturing on MS medium supplemented with 2, 4-D.

Analysis of variance showed highly significant difference for percentage callus formation among the varying concentrations of the phytohormone used. Using mature embryo, the media fortified with 4.0 mg/L 2, 4-D recorded the highest percentage callus formation followed by the media supplemented with 2.0 mg/L 2, 4-D combined with 0.5 mg/L KN. When immature embryo was used, the media supplemented with 2.0 mg/L 2, 4-D alone or in combination with 0.5 mg/L KN and the media supplemented with 3.5 mg/L 2, 4-D combined with 0.5 mg/L KN recorded the highest. Using embryonic axes alone, the media fortified with 2.0 mg/L 2,4-D+0.5 mg/L KN recorded the highest percentage callus formation The result of this study revealed that different level of phytohormones (2, 4-D)

and the type of explants used have significant impact on percentage callus formation and callus fresh weight. This result agreed with the findings of Indra *et al*. (2009) who reported the effect of different phytohormones level on percentage callus formation where highest percentage callus induction was observed from the media fortified with 2 mg/L 2, 4-D+0.5 mg/L KN. The result of this study also showed that 2.0 mg/L 2, 4-D alone or in combination with 0.5 mg/L KN are optimum concentrations of 2, 4-D and kinetin that can be used to induce callus in sorghum. This is similar to what was obtained when immature embryo was cultured by Sudhakar *et al.* (2008). Bi *et al*. (2008), also reported that cereals in general require 2, 4- D to initiate callus and higher concentration have been found to be less effective in the formation of embryogenic callus (Lu *et al*., 1983).

Low level of 2, 4-D has been the most commonly used callus inducing hormone in the cereals according to Hagio (1994). The similar trend was previously reported by Maheswari *et al*. (2006) and Pola *et al*. (2006). The formation of callus from immature inflorescence cultured in MS medium supplemented with 2.0 mg/L 2, 4-D and 0.2 mg/L KN was reported by Manjula *et al.* (2000). Sudhakar *et al. (*2009) also reported the long-term maintenance of callus cultured from immature embryo of sorghum with highest callus formation obtained from medium supplemented with 2.0 mg/L 2, 4-D and 0.5 mg/L kinetin. The highest callus weight was obtained from the media supplemented with 3.5 mg/L 2, 4-D combined with 0.5 mg/L KN followed by the media fortified with 2.5 mg/L 2, 4-D for immature embryo while for embryonic axes the media fortified with 3.5 mg/L 2, 4-D alone recorded highest followed by the media supplemented with 2mg/L 2, 4-D. The lowest average weight of fresh callus recorded by media fortified with 2 mg/L 2, 4-D using immature embryo may be due to addition of activated charcoal that lowered the effectiveness of auxin or due to

secretion of phenolic compound at early stage which hindered the callus growth. Exudation of phenolic compounds resulted in the browning and necrosis of callus were reported by Indra *et al*. (2009) and Sudhakar *et al.* (2008). The calli obtained from all the explants, usually appeared in 3 types based on their color, friability or compactness. For mature embryo, the calli were initially whitish in color and this later changed to yellowish type of calli and most of these calli went in to necrosis before three weeks of age and the survived ones became reddish in color after three weeks of age. The calli obtained from immature embryo was initially creamy compact in nature; the browning of the scutellum affected the color of the calli obtained at early stage for most of the treatments tested. The scutellum fell off from some of the calli obtained before 3 weeks of age which enabled these calli to become whitish compact in nature after three weeks of age, most of the calli turned to yellowish friable calli after three weeks of age some produced pigment while other did not. Using embryonic axes, most of the calli obtained were whitish compact in nature at 1 week up to 2 weeks of age. They later changed to yellowish type. The similar result was obtained by Sudhakar *et al*. (2008) when callus was induced using immature embryo. These types of calli were also similar to the embryogenic calli described by Cai and Butler (1990). Mackinnon *et al.* (1986) also reported reddish type of calli in sweet sorghum. Formation of whitish embryogenic callus was also reported by Sudhakar *et al.* (2009) using leaf explant. A distinct feature of callusing in cereals and grasses is the formation of two different types viz. embryogenic and non embryogenic calli which differed markedly in their regenerative potential (Sudhakhar *et al.*, 2008). Nature of the calli induced is highly dependent on genotype, explant type, age, size and medium composition according to (indra*et al.*, 2009). Induction frequency of primary calli and embryogenic calli were markedly influenced by different treatments reported by (liming *et al*. 2010)

5.2 Regeneration

It was observed from this study that 1 mg/L BA was sufficient for shoot initiation for regeneration through somatic embryogenesis, this agreed with the findings of Indra *et al.* (2009) who obtained the highest percentage shoot formation from the media fortified 1mg/L kinetin. Sufficiency of 2mg/L BA was observed for shoot initiation through organogenesis. The proficiency of shoot regeneration at a concentration of 2 mg/L BA was reported by Sudhakar *et al*. (2008). Prathibha *et al*. (2001) reported that 2 mg/L BA+ 0.5 mg/L 2, 4-D was found to be suitable to obtain maximum percentage of regeneration in sorghum. Pola *et al.* (2009) reported multiple shoot formation using 2 mg/L BAP + TDZ (Thidiazuron). Sadia *et al*. (2010) observed maximum number of shoot formation in sorghum using 3mg/L BA+1mg/L TDZ. The effect of different concentrations and combinations of phytohormones and cultivars on shoot induction frequency was reported by Liming *et al*. (2010). He obtained highest induction frequency on MS media supplemented with 1 mg/L 1AA and 3 mg/L BA for a variety and obtained highest shoot induction frequency on the same media supplemented with 2 mg/L BA + 0.5 mg/L KN for another variety. The greening of callus was obtained when hormone free media was used but no further differentiation was observed. The use of hormone free media and media fortified with 0.1 mg/L 2, 4-D under dark condition for somatic embryo formation was reported by purohit, (2009). The effect of phenolics on regeneration potential of this variety was also observed. Maheswari *et al*. (2006) noted that major hurdles in the micropropagation of sorghum to be the species'genotype dependant response, its very low rate of regeneration, the production of phenolics, and its problems in acclimatization

5.3 Extraction of Anthocyanins and pH Stability at Variable pH

It was observed from this study the color of the extracts was red in all the sample tested except plantlets obtained *in vitro* that was greenish in nature, 3-deoxyanthocyanidin tended to be more stable at pH 1.0 buffer because the reddish color changed to yellowish color in both methanol and acidified methanol after adding pH 1.0 buffer and this color is peculiar to apigeninidin. This agrees with findings of Devi *et al.* (2011) that reported the stability of anthocyanin at low pH.

5.4 Flavonoids Confirmation Test

Flavonoids are naturally occurring phenolic compounds in plants. In fact, the majority of antioxidants, both natural and synthetic, are phenolic compounds (Brown *et al.*, 2006). These are all phenols and several thousand different structural variants are found in nature. It has been estimated that the total intake of these compounds in the typical diet is close to 1000 mg per day (Brown *et al.*, 2006). Due to their remarkable importance, antioxidants have been the focus of considerable research and the antioxidant properties of several plants *in vivo* and *in vitro* have been shown to be of great advantage to nutrition today (Ngozi, 2013). For sickle cell disease (SCD), the study of antioxidants especially in various antisickling agents is of great importance because different antisickling agents have different degrees of effect (Ngozi, 2013). Antioxidants (scavengers of free radicals) are believed to be major components of these antisickling agents that add to their potential (Tatum, 1996). Thus, it is believed that the higher the antioxidant property of an antisickling agent, the higher its possible antisickling effect, as this enables it to reduce

oxidative stress that contributes to sickle cell crisis (Ngozi, 2013). The color changed after adding ferric chloride showed the presence flavonoids in all the samples tested. The extracted anthocyanin from the red sorghum bran using acidified methanol and methanol solvents showed brown coloration. The formation of brown color had indicated the presence of flavonoids according to Devi *et al*. (2011). In the presence of $FeCl_3$, methanol and acidified methanol extracts showed brown coloration, this shows the presence of flavonoids reported by (Ponmonzhi *et al*., 2011)

5.5 Quantification of total and monomeric anthocyanin using methanol and acidified methanol

The result of this study showed that acidified methanol resulted in significant higher values for the total and mono anthocyanin than using methanol alone. The total anthocyanin extracted by acidified methanol were 32% and 39.35% higher than methanol alone for callus in 1 mg/L BA when expressed as apigeninidin and luteolinidin respectively, it was 47% for cyanindin-3- glucoside, and 45.62% higher for monomeric anthocyanin. For callus in 1.5 mg/L BA combined with 0.2mg/L NAA, the total anthocyanin extracted by acidified methanol were 39.23% and 42.26% higher than methanol when expressed as apigeninidin and luteolinidin respectively, 47.84% when expressed as cyanidin -3-glucoside while it was 52.38% higher for monomeric anthocyanin. For the leaf sheath obtained *in situ* the total anthocyanin extracted by acidified methanol were 5.65% and 7.51% lower than using methanol alone when expressed as apigeninidin and luteolinidin respectively, while for monomeic anthocyanin it was 6.14% higher. In this study it was identified that the acidified methanol with low pH is the most suitable solvent for extraction, quantification and analysis of anthocyanins in red sorghum. Devi *et al.* (2011) reported that total

anthocyanin content in acidified methanol was more than what was obtained from using methanol alone. The reason is that the acidified methanol preserves the extracted anthocyanin in their original form for long duration (Joseph, *et al*., 2004). At low level of pH (1.0) the stability of anthocyanin was more (Guisti, 2003). The total anthocyanins extracted by acidified methanol were as an average of 57% higher than the methanol extracts (Devi, *et al.*, 2011). However, since acidified methanol preserves the extracted anthocyanins in their original form better, it should be the solvent of choice for quantification and analysis of anthocyanins (Devi *et al*., 2011). Similar result was reported by Awika *et al*. (2004). The total monomeric anthocyanin obtained from pigmented callus in this study also was more (415.08mg/L) than what was obtained from sorghum bran (242.7mg/L) by Devi *et al*. (2011)

It was also observed from this study that light had effect on the synthesis of 3-deoxyanthocyanidin because the synthesis tended to be more when the calli were being exposed to light. Reduction of total anthocyanin when expressed as cyanindin -3- glucoside might due to effect of light on accumulation of 3-deoxyanthocyanindin. Similar trend was reported by (Nicholson, 1998) when certain sorghum plant (cv DK18) produced appreciably higher levels of 3-deoxyanthocyanindin in response to light and accumulated less amount of anthocyanins According to him, in the absence of anthocyanin accumulation, it is likely that more resources, including substrate and energy were available to cv DK18 plants for phytoalexin accumulation. Plant growth regulators play important role in the synthesis of secondary metabolites. More 3-deoxyanthocyanindin was obtained from cytokinin containing media compared to what was obtained from hormone free media. Cytokinins have been demonstrated to stimulate production of anthocyanins according to Purohit (2009). Accumulation of anthocyanins is induced by various environmental stimuli,

including UV and red irradiation (Reddy *et al*., 1994), low temperature (Christie *et al*., 1994), pathogen attack (Hipskind *et al*., 1996), and several plant-growth regulators, such as cytokinins (Deikman and Hammer, 1995).The luxuriant pigmented callus achieved on MS medium supplemented with BAP (1.0 mg L-1) + CW (20%) as well as IBA (0.5 mg L-1) + KN (0.5 mg L-1) + CW (15%) after 8 weeks of subculture was also reported by (Radfar *et al*., 2012)

CHAPTER SIX

SUMMARY, CONCLUSION AND RECOMMENDATIONS

This study was undertaken to produce and quantify anthocyanins in Karadanfi red sorghum *in vitro*. Experiments were carried out in Biotechnology laboratory in Institute for Agricultural Research (IAR) and Multi user laboratory in the Department of Chemistry, Ahmadu Bello University, Zaria. Mature and immature embryos were used as explants for callus induction while only immature embryo was used for shoot initiation. Murashige and Skoog basal medium supplemented with different concentrations of 2, 4-D alone or in combination with 0.5 mg/l KN were used for callus induction while BA alone or in combination with 2, 4-D and NAA were used for regeneration and anthocyanins production. Ferric chloride test was used for flavonoids confirmation while methanol and acidified methanol methods were used for the extraction of anthocyanins. Analysis of variance showed highly significant difference for percentage callus formation; callus fresh weight, total and monomeric anthocyanins. Significant difference was also observed for percentage shoot initiation. There was no significant difference observed between hormone treatments for shoot multiplication.

In conclusion, Optimum protocol for shoot formation was developed for Karandafi cultured in *vitro* and it was observed that 2, 4-D is an important hormone necessary for callus formation in sorghum, 2 mg/L 2, 4-D alone or in combination with 0.5 mg/L KN are the optimum concentrations of hormones for callus induction in this study. 1 mg/L and 2 mg/L BA are sufficient for shoot initiation through somatic embryogenesis and organogenesis, respectively. The brown coloration observed after treating the extracts with ferric chloride confirmed the presence of flavonoids. The result of this findings showed that *in vitro*

system is a suitable method for the production of anthocyanins. This metabolite had been successfully produced and quantified at callus stage and based on the result of this study, MS media fortified with 1.0 mg/L BA is sufficient for the *in vitro* production of this important secondary metabolite.

1. The use of *in vitro* system for the production of 3-deaxyanthocyanindin is recommended for the pharmaceutical industries because more total anthocyanins content was obtained from pigmented callus at 8 weeks of age when acidified methanol was used compared to what was obtained from red leaf sheath obtained *in situ* which took up to 16 weeks of age before anthocyanins was produced. The *in vitro* production of this important metabolite using bioreactor could serve as a more prosperous alternative for the production of anthocyanins by the pharmaceutics.

2. Challenges due to phenolic secretion affected regeneration potential and rooting of the plantlets so further research is needed to improve the regeneration potential and rooting of the plantlets. Also there is need to subject some of IAR sorghum varieties to tissue culture technique to see whether the response is genotype dependent.

REFERENCES

Afaq, H.S., Shamee, l.M., Usmanghani, K. and Ahmad, M. (1991). Phytochemical Studies on *Dermonema abbottiae* (Nemaliales – Rhodophyta). *Botanical Marin*e, 34(3): 215–220.

Ahmad, A., Zhong, H., Wengling, W. and Sticklen, M.B. (2002). Shoot apical meristem: *In vitro* regeneration and morphogenesis in wheat (*Triticum aestivum* L.). *In Vitro Cellular and Developmental Biology – Plant*, 38: 163-167.

Ameh, S.J., Obodozie, O.O., Afolabi, E.K., Oyedele, E.O., Ache, T.A., Onanuga, C.E., Ibe, M.C. and Inyang, U.S. (2009).Some basic requirement for preparing antisickling herbal medicine-NIPRSAN.*African Journal of Pharmacy and Pharmacology*, 3(5):259-264

Andersen, Q.M. and Jordheim, M. (2006).The Anthocyanins. In Flavonoids and Chemistry, *Biochemistry and Applications*, CRC Press: Boca Raton, pp., 471–553.

AOAC. (2005). Total Monomeric Anthocyanin Pigment Content of Fruit Juices, Beverages, Natural Colorants, and Wines. *Journal of AOAC Int*ernational, 88:1269

Awika, J.M. and Rooney, L.W. (2004).Sorghum phytochemicals and their potential impact on human health.*Phytochemical*, 65:1199-1221

Bajaj, P.S. (2000).*Biotechnology in agriculture and forestry Transgenic crops* In (Eds) Springer-Verlag Berlin Heidelberg, pp.3-34

Bi, R., Kou, M.M., Chen, L.G., Mao, S.R., Wang, H.G. (2008). Plant regeneration through callus initiation from mature embryo of *Triticumaestivum*.*Plant Breeding.*126: 9-12.

Boyd, W. (2000).Natural colors as functional ingredients in healthy foods. *Cereal Foods World*, 45(5), 221–222

Brown, M. J. David, H. E. and Hunt, C. (2006). "Comparison of the antioxidant properties of supercritical fluid extracts of herbs and the confirmation of Pinocembrin as a principal antioxidant of Mexican oregano (*Lippa gravolens*),"*Electronic Journal of Environmental, Agricultural and Food Chemistry*, 3:102–107.

Cai, T. and Butler, L. (1990). Plant regeneration from embryogenic callus initiated from immature inflorescences of several high tannin sorghums. *Plant Cell Tissue Organ Culture*, 20: 101-110

Christie, P.J., Alfenito, M.R. and Walbot, V. (1994). Impact of low-temperature stress on general phenylpropanoid and anthocyanin pathways: enhancement of transcript abundance and anthocyanin pigmentation in maize seedlings. *Planta*, 194:541–549.

Conger, B.V., Hilenski, L.L, Lowe, K.W. and Carabia, J.V. (1982). Influence of different auxins at varyng concentrations on callus induction and growth from embryo and leaf-tip explants in graminaceae. *Environment of Experimental Botany*, 22:39 – 48

Cyrilolutayo, C.M., Elujoba A.A and Durosimi.M.A. (2009). Antisickling Properties of the Fermented Mixture of *Carica papaya* Linn and *Sorghum bicolor* (L.) Moench" *African Journal of Pharmacy and Pharmacology*, 3 (4):140-143

Deikman, J. and Hammer, P.E. (1995).Induction of anthocyanin accumulation by cytokinins in *Arabidopsis thaliana*. *Plant Physiolology*, 108:47–57.

Devi, P.S., Saravanaka, M. and Mohandas, S. (2011). Identification of 3-deoxyanthocyanin from red sorghum (*Sorghum bicolor*) bran and its biological properties.*African Journal of Pure and Applied Chemistry*.7:181-193

Dixon, R.A. and Paiva N.L. (1995).Stress-induced phenylpropanoid metabolism.*Plant Cell Report*, 7:1085–1097.

Fett-Neto, A.G., J.M. Stewart, S.A. Nicholson, J.J. Pennington and DiCosmo, F. (1994) Improved taxol yield by aromatic carboxylic acid and amino acid feeding to cell cultures of *T. cuspidata*. *Biotechnology and Bioengineering,* 44:967-971.

Fossen, T. and Andersen, Ø.M. (2003).Anthocyanins from red onion, *Allium cepa*, with novel aglycone.*Phytochemistry*, 62**:**1217–1220.

Fuleki, T., and Francis, F.J. (1968).Quantitative methods for anthocyanins extraction and determination of total anthocyanin in cranberries.*Journal of Food Science*, 33:72–77

Giusti, M.M., and Wrolstad, R.E. (2003).Acylated anthocyanins from edible sources and their applications in food systems.*Biochemistry Engineering Journal*, 14(3): 217–225.

Hagio, T. (1994).Varietal difference of plant regeneration from callus of sorghum mature seed.*Breeding Science, 44*: 121-126.

Harsha, D.V. (2002). Establishment of Genetic transformation protocol for the production of transgenic plants in sorghum, (A PhD. Thesis). Centre for plant molecular biology Osmania University, AP, India. Pp 25-30

Hebbel, R.P., Eaton, J.W., Balasingam, M. and Steinberg, M.H. (1982)."Spontaneous Oxygen radical generation by sickle cell erythrocytes,"*Journal of Clinical Investigation*, (6):1253-1259.

Hipskind, J., DeNeergaard, E., Goldsbrough P. and Nicholson, R.L. (1996).Stimulation of phenylalanine ammonia-lyase in sorghum in response to inoculation with *Bipolaris maydis.Physiology of Molecular Plant Pathology*. 48:55–64.

ICRISAT, (2010). http//test/icrisat.org/sorghum/sorghum.htm, retrieved on 30th March, 2010.

Indra, A.P. and Krishnaveni S. (2009).Effect of hormones, explants and genotypes on *in vitro* culturing of sorghum.*Journal of Biochemistry Technology*, 4: 96-103

Jain, V.K. (2008). *Fundamentals of Plant Physiology*.S.chand and Company Ltd Ram Nagar, New Delhi. pp. 523-535.

Jimenez, V.M., Guevara, E., Herrera, J. and Bangerth, F. (2001). Endogenous hormone levels in habituated nucellar citrus callus during the initial stages of regeneration. *Plant Cell Reports*, 20:92-100

Joseph M.A., Lloyd W.R., and Ralph D.W. (2004). Anthocaynins from black sorghum and their antioxidant properties.*Food Chemistry*, 90: 293 –301.

Kamei, H., Kojima, T., Hasegawa, M., Koide, T., Umeda, T. and Terabe, K. (1995). Suppression of tumor cell growth by anthocyanins.*In vitro Cancer Investigations*, 13(6):590–594.

Karaivanova, M., Drenska, D. and Ovcharov, R. (1990). A modification of the toxic effects of platinum complexes with anthocyanins. *Eksperimetnalna Meditsna IMorfologiia*, 29(2):19–24.

Kresovich, S., McGee, R.E., Panelia, L., Realley, A.A. and Miller, T.R. (1987).Application of cell and tissue culture techniques for the genetic improvement of sorghum, *Sorghum bicolor* (L) Moench progress and potential.*Advances in Agronomy*, 41: 147 – 170

Lee, C.Y., Lin, F.L., Yang, C.T., Wang, L.H., Wei, H.L. and Tsay, H. S. (1995). Taxol production by cell cultures of *Taxus mairei*. *Proceeding of Symposium on development and utilization of resources of medicinal plants in Taiwan*, Taiwan Agricultural Research Institute, Taiwan, April 21, TARI Special Publication 48:137-148.

Lim, T.K. (2013). *Edible Medicinal and Non Medicinal Plants*.Springer Dordrecht Heidelberg, New York, London. pp 375-381

Liming, Z., Shujun L. and Songquan, S. (2010). Optimization of callus induction and plant regeneration from germinating seeds of sweet sorghum (*Sorghum bicolor* (L) Moench). *African Journal of Biotechnology*, 9 (16):2367-2374.

Liu, C.Z., Y.C. Wang, Y.C.C. Guo, C.F. Ouyang, F.H.C. Ye, H.C. and G.F. Li. G.F. (1998). Production of artemisinin by shoot cultures of *Artemisia annua* L. in a modified inner-loopmist bioreactor.*Plant Science.*135:211–217.

Lu, C., Vasil, V. and Vasil, I.K. (1983). Improved efficiency of somatic embryogenesis and plant regeneration in tissue culture of maize (*Zea mays*), *Theoretical Application of Genet*ics, 66: 285-289

Ma, H., Gu, M. and Liang, G.H. (1987). Plant regeneration from cultured immature embryos of *Sorghum bicolor* (L.)Moench.*Theoretical Application of Genetics,* 73:389 – 394

MacKinnon,C. Gunderson. G. and Nabors.M.W. (1986). Plant regeneration by somatic embryogenesis from callus cultures of sweet sorghum. *Plant Cell Report,* 349-351

Maheswari, M., Jyothi, N., Lakshmi, S.K. Yadav, Y. Varalaxmi, A., VijayaLakshmi, M.V. and Venkateshwarlu, B. (2006). Efficient plant regeneration from shoot apices of sorghum.*Biologia Plantarum*, 50 (4): 741-744.

Manjula, S.M., Kuruvinasetti, M.S. and Harti, C.C.(2000). Regeneration establishment and evaluation of somaclones in *Sorghum bicolor* (L.)Moench.*Euphytical*, 115:173-180

Mateus, N., Oliveira, J., Haettich-Motta, M., and de Freitas, V. (2004).New family of bluish Pyranoanthocyanins.*Journal of Biomedical and Biotechnology***,** *5*:299–305.

Merkle, S.A., Parrot,W.A.& Williams, E.G. (1990). Applications of somatic embryogenesis and embryo cloning.In; Bhojwani, S.S., (Ed.). *Plant Tissue Culture: Applications and Limitations*. Amsterdam.*Elsevier*, pp. 67-101.

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

Mujib, A. and Samaj, J. (2006).Somatic embryogenesis. Springer-Verlag, Berlin, Heidelberg, New York; pp 157-176

Murch, S.J., Raj, S.K. and Saxena, P.K. (2000). Phytopharmaceuticals mass production, standard, and conservation. *Science Review of Alternative Medicin*e4:39–43.

Mpiana,P.T., Lombe,B.K, Ombeni, A.M., Ngbolua, K., Tshibangu, D.S.T., Wimba, L..K., Tshilanda, D.D., Mushagalusa, Muyisa, F.K.(2013). I*n vitro* effects of anti-sickle erythrocytes hemolysis of *Diclipteracolorata*, C.B. Clarke, *Euphorbia hirta* L. and *Sorghum bicolor* (L.) Moench.*Open Journal of Blood Diseases*, 3:43-48

Mpiana, P.T., Mudogo, V., Tshibangu, D.S.T. Ngbolua, K.N., Atibu, E.K., Kitwa, E.K.A., Kanangila, B. and Makelele, L.K. (2009). Activité antifalcémianteet Thermo

degradationuneFractionD'anthocyanesextraitsde*Zizyphusmucronata*,"*AnnalesAfrica inesde Medecine*, 2: 91-97.

Nahdi, S. and Dewet.J.J. (1995).*In vitro* regeneration of *Sorghum bicolor* lines from shoots apices. *International Sorghum and Millet News letter*, 36: 89 -90

Newman, D. J. and Cragg, G.M. (2012). Natural Products as Sources of New Drugs over the 30 Years from 1981 to 2010," *Journal of Natural Products*, 3: 311-335

Nicholson, R.L. (1998). Reduction of light induced anthocyanin accumulation in inoculated sorghum mesocotyl. *Plant physiology*, 3: 979-989

Ngozi, A.I. (2013). Phytomedicine and nutraceutical.Alternative therapeutics for sickle cell disease.*The Scientific World Journals*, 1-13

NSF International (2004).*Anthocyanin content in bilberry by pH differential spectrophotometryINAmethod*. Retrieved from http:/www.nsf.org/business/ina bilberry.asp

Okokoh , L. (1999). Quick guide to Natural Health Care. Capstone Herbal Health Centre, Lagos. pp. 29-36

Orczyk, W., Hipskind, J., DeNeergaard, E., Goldsbrough, P. and Nicholson, R.L. (1996) Stimulation of phenylalanine ammonia-lyase in sorghum in response to inoculation with *Bipolaris maydis.Physiological and Molecular Plant Pathology*, 48:55–64.

Pola, S.R. and Sarada M.N. (2006).Somatic embryogenesis and plant regeneration in *Sorghum bicolor* (L.)Moench from leaf segment. *Cell Molecular Biology*, 5:99-107.

Pola S, Mani N.S and Ramana, T. (2009). Long term maintenance of callus cultures from immature embryo of *Sorghum bicolor. World Journal of Agricultural Sciences*, 5:415-421.

Ponmozhi, P., Greetha, M., Kumar, M.S. and Devi, P.S (2011). Extraction of anthocyanin and analyzing its antioxidant properties from *Pithecello biumdulce* fruit preicarp. *Asian Journal of Pharmaceutical and Clinical Research*, 4:41-45

Prathibha, B. and Sticklen, M.B. (2001). Culturing shoot tip clumps of *Sorghum bicolor* and optimal micro projectile bombardment parameters for transient expression. *Journal of Cytological Genetics* 2: 89-96

Puddephat, I.J., T.J. and Fenning, T.M. (1996). Transformation of *Brassica oleracea* L. A *Critical review of Molecular Breeding*, 2:185 – 210

Pullalah, T. and SubbaRao, M.V. (2009).*Somatic Embryogenesis in Plant Tissue Culture Theory and Practical*, pp 60-67.

Purohit, S.S. (2009). *Micropropagation through organogenesis in plant Tissue Culture*, pp 49-73

Raemakers, C.J.J.M, Jacobsen, E. and Visser, R.G.F. (1995).Secondary somatic embryogenesis and application in plant breeding.*Euphytica*, 81: 93-107

Radfar, M., Sudarshana, M.S. and Niranjan, M.H.(2012). Betalains from stem of callus cultures of *Zaleya decandra* L.N. Burn. f.-A medicinal plants. *Journal of Medicinal Plants Research*, 6, 12: 2443-2447.

Red sorghum[Image]. (2012). Retrieved from https// www.lancasterfarming.com/ scientists tap sorghum's potential for cold tolerance

Reddy, V.S, Goud, K.V., Sharma, R., and Reddy, A.R. (1994). Ultraviolet-B-responsive anthocyanin production in rice is associated with a specific phase of phenylalanine ammonialyase biosynthesis. *Plant Physiology*, 105:1059–1066.

Sadia, B., Josekutty, R.C., Potlakayya, S.D., Patel, P., Goldman, S. and Rudrabhatla, S.V. (2010). An efficient protocol for culturing meristems of sorghum hybrids. *International Journal of Experimental Botany*, 79: 177-181

SAS Institute Inc. (2005). SAS Online DocVersion 8.[Internet]. SAS Institute Inc. Available from: http://www.sas.com/

Seetharama, N. Sairam, R.V. and Ran, T.S. (2000). Regeneration of sorghum shoots tip cultures and field performance of progeny. *Plant Cell Tissue and Organ Culture*, 61: 69 -173

Sekh A. N, Junaid A., Rashmi K. and Saeed A.K. (2010). Secondary metabolites production through biotechnological intervention: A Review. *Emir Journal of Food Agriculture*, 22 (3): 147-161

Sharma, V.K., Hansch, R., Mendel, R.R. and Schulze, J. (2004). A highly efficient plant regeneration system through multiple shoot differentiation from commercial cultivars of barley (*Hordeum vulgare* L.) using meristematic shoot segments excised from germinated mature embryos. *Plant Cell Reports*, 23: 9-16.

Sudhakhar, P., Sarada Mani, N., Ramana, T. (2008). Plant tissue culture studies in *Sorghum bicolor:* immature embryo explants as the source material. *International Journal of Production.*2:1-14.

Sudhakhar, P., Sarada Mani, N. and Ramana, T. (2009). Long- term maintenance of callus cultured from immature embryo of *Sorghum bicolor. World Journal of Agricultural Sciences*, 5(4): 415-421.

Sudhakararao, P. (2011). Leaf discs as a source material for plant tissue culture studies of *sorghum bicolor* (L.)Moench.*Nortulae Scientia Biologicae,* 3(1):70-78

Tatum, V.L. and Chow, C.K. (1996).Antioxidant status and susceptibility of sickle erythrocytes to oxidative and osmotic stress.*Free Radical Research*, 25(2):133–139,

Terzi, M. and Lo Schiavo, F. (1990). Somatic embryogenesis in : Bhajwanii, S.S. (Ed.). *Plant Tissue Culture: Application and Limitation*. Amsterdam. *Elsevier*. pp. 54-56

Wambebe, C., Khamofu, H. and Momoh, J.A. (2001).Double-blind, placebo-controlled, randomized cross-over clinical trial of NIPRISAN in patients with sickle cell disorder.*Phytomedicine,* 8(4):252-61

Wang, H., Cao, G., and Prior, R.L. (1997).Oxygen radical absorbing capacity of anthocyanins.*Journal of Agricultural and Food Chemistry,* 45:304–309.

Zhong, H., Wang, W. and Sticklen, M.B. (1998).*In vitro* morphogenesis of *Sorghum bicolor* (L.) Moench: Efficient plant regeneration from shoot apices. *Journal of Plant Physiology*,153:719- 726

APPENDICES

Appendix 1

Means Squares for Percentage Callus Formation, Average Fresh Weight (AVFW) of Callus, Total and Monomeric Anthocyanins using Methanol and acidified and One way ANOVA table for Shoot Initiation using Immature Embryo and ShootMultiplication using Meristem

Source of Variation	DF	T%CF for IMEB	T%CF for EBA	T%CF for MEB	AVFW for IMEB	AVFW for EBA
Treatments	13	2.393**	2.562**	2.432**	2.564**	0.7814**
Error	56	0.006	0.002	0.012	0.071	0.063

Source of Variation	Total A	Total L	Total C	Mono A	Mono L	Total M
Treatment	101.17**	182.42**	5.96**	8774.87**	17503.38**	55190.05**
Error	0.0009	0.0114	0.0421	1.8051	0.6676	1.5377

Source of Variation	Total A	Total L	Total C	Mono A	Mono L	Total M
Treatment	61.61**	106.03**	3.36**	7740.83**	10950.90**	37068.62**
Error	0.0040	0.0059	0.0023	1.8513	8.8371	12.3792

Source of Variation	DF	SS	MS	F-VALUE	Pr
Treatments	3	3033.33	1011.11	4.33	0.042
Error	8	1866.67	233.33		
Corrected Total	11	4900.00			

Source of Variation	DF	SS	MS for No of shoot/explants	MS forAverage plant height (cm)	Pr
Treatments	1	0.80	0.80	5.30	0.433
Error	18	22.40	1.24	2.85	0.893
Corrected Total	19				

Appendix II

Spectral characteristics of peaks corresponding to Methanol and Acidified methanol extraction of 3-deoxyanthocyanindin (Apegininidin) isolated from leaf sheath obtained *in situ,* pigmented callus, and *in vitro* plantlet using Jenway 6405 UV – Visible Spectrophotometer

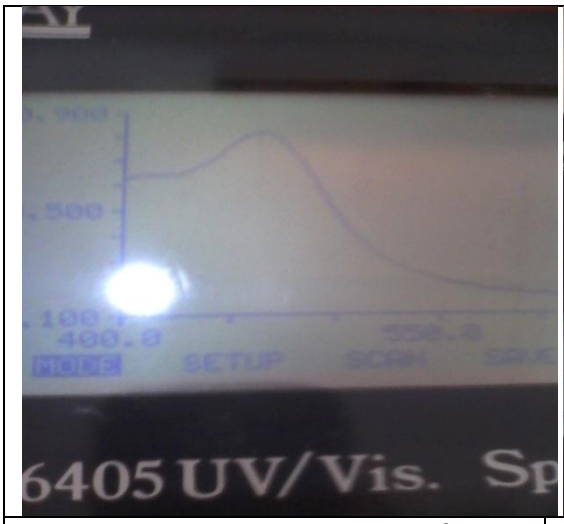
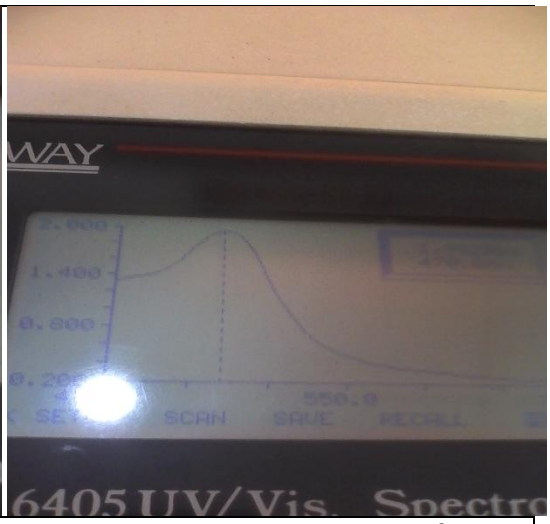
	

Plate I: Spectral characteristics of peak of 3-deoxyanthocyaninidin (apegininidin) isolated from leaf sheath using methanol for the extraction	Plate II: Spectral characteristics of peak of 3-deoxyanthocyaninidin (apegininidin) isolated from leaf sheath using acidified methanol for the extraction

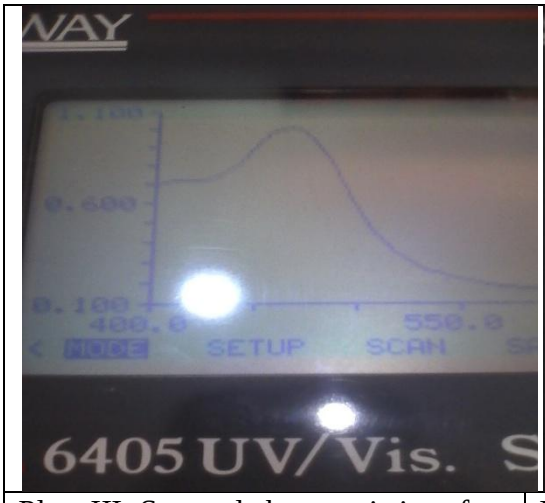
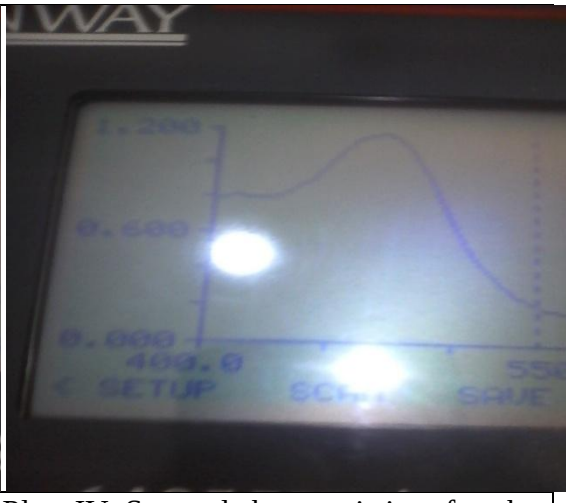

	

Plate III: Spectral characteristics of peak of 3-deoxyanthocyaninidin (apegininidin) isolated from callus in 2.0 mg/L BA + 0.2 mg/L NAA using methanol for the extraction	Plate IV: Spectral characteristics of peak of 3-deoxyanthocyaninidin (apegininidin) isolated from callus in 2.0 mg/L BA + 0.2 mg/L NAA using acidified methanol for the extraction

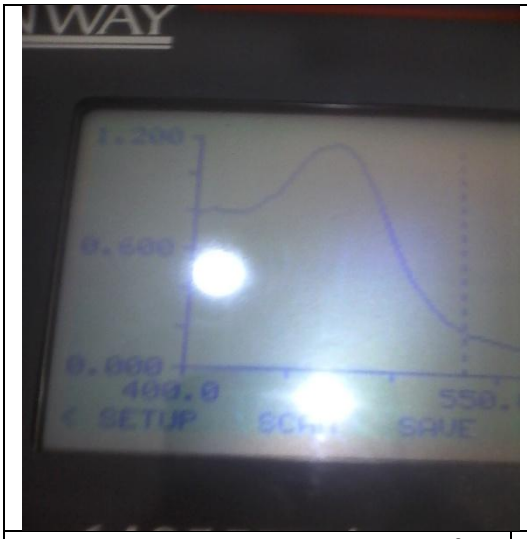
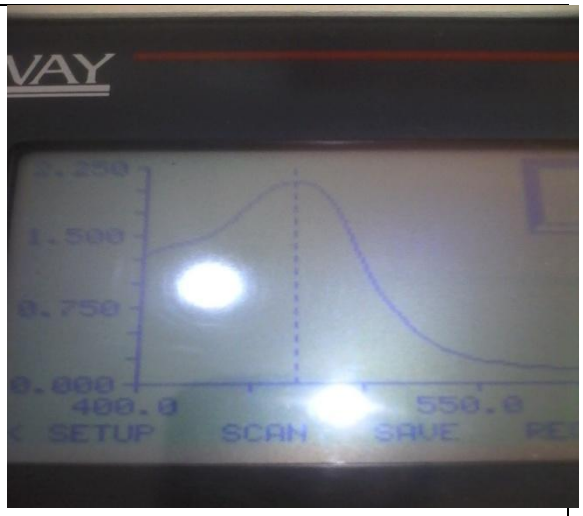
	

Plate V: Spectral characteristics of peak of 3-deoxyanthocyaninidin (apegininidin) isolated from callus in 1.0 mg/L BA using methanol for the extraction	Plate VI: Spectral characteristics of peak of 3-deoxyanthocyaninidin (apegininidin) isolated from callus in 1.0 mg/L BA using acidified methanol for the extraction

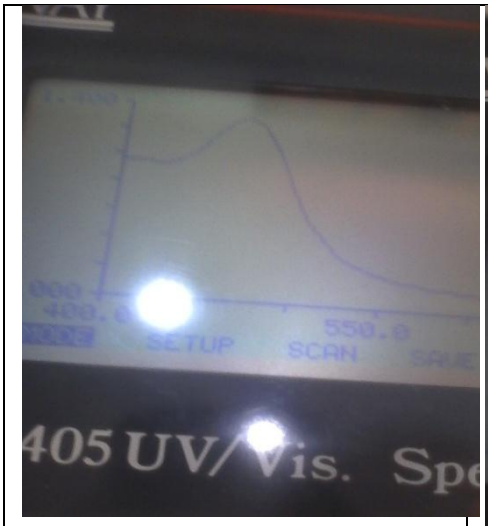
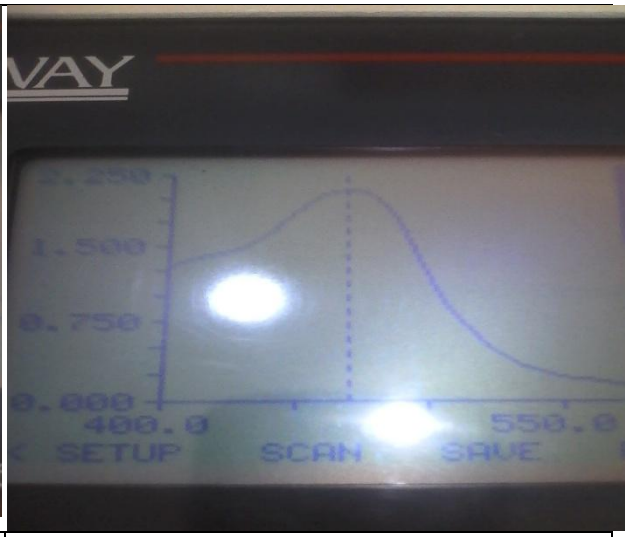
	

Plate VII: Spectral characteristics of peak of 3-deoxyanthocyaninidin (apegininidin) isolated from callus in 1.5 mg/L BA+ 0.2 mg/L NAA using methanol for the extraction	Plate VIII: Spectral characteristics of peak of 3-deoxyanthocyaninidin (apegininidin) isolated from callus in 1.5 mg/L BA + 0.2 mg/L using acidified methanol for the extraction

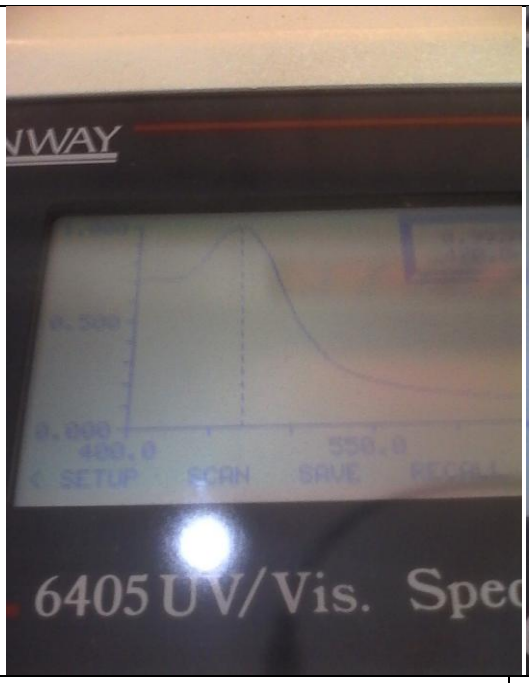


Plate IX: Spectral characteristics of peak of 3-deoxyanthocyaninidin (apegininidin) isolated from callus in a hormone free media using methanol for the extraction

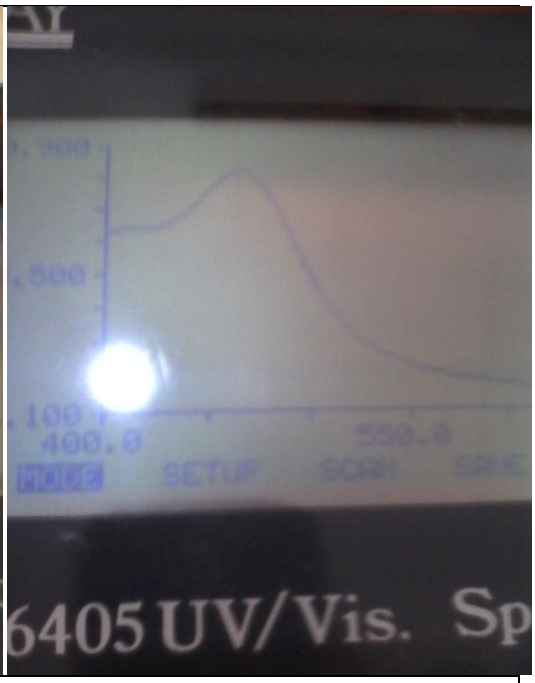


Plate X: Spectral characteristics of peak of 3-deoxyanthocyaninidin (apegininidin) isolated from callus in a hormone free media using acidified methanol for the extraction

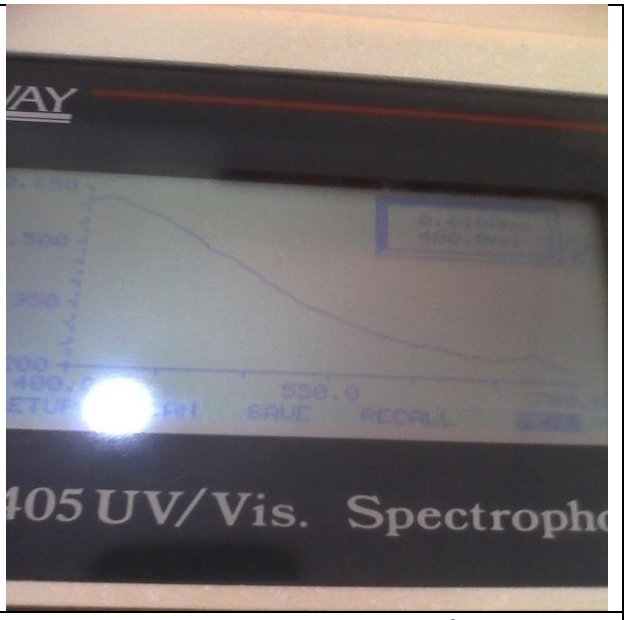
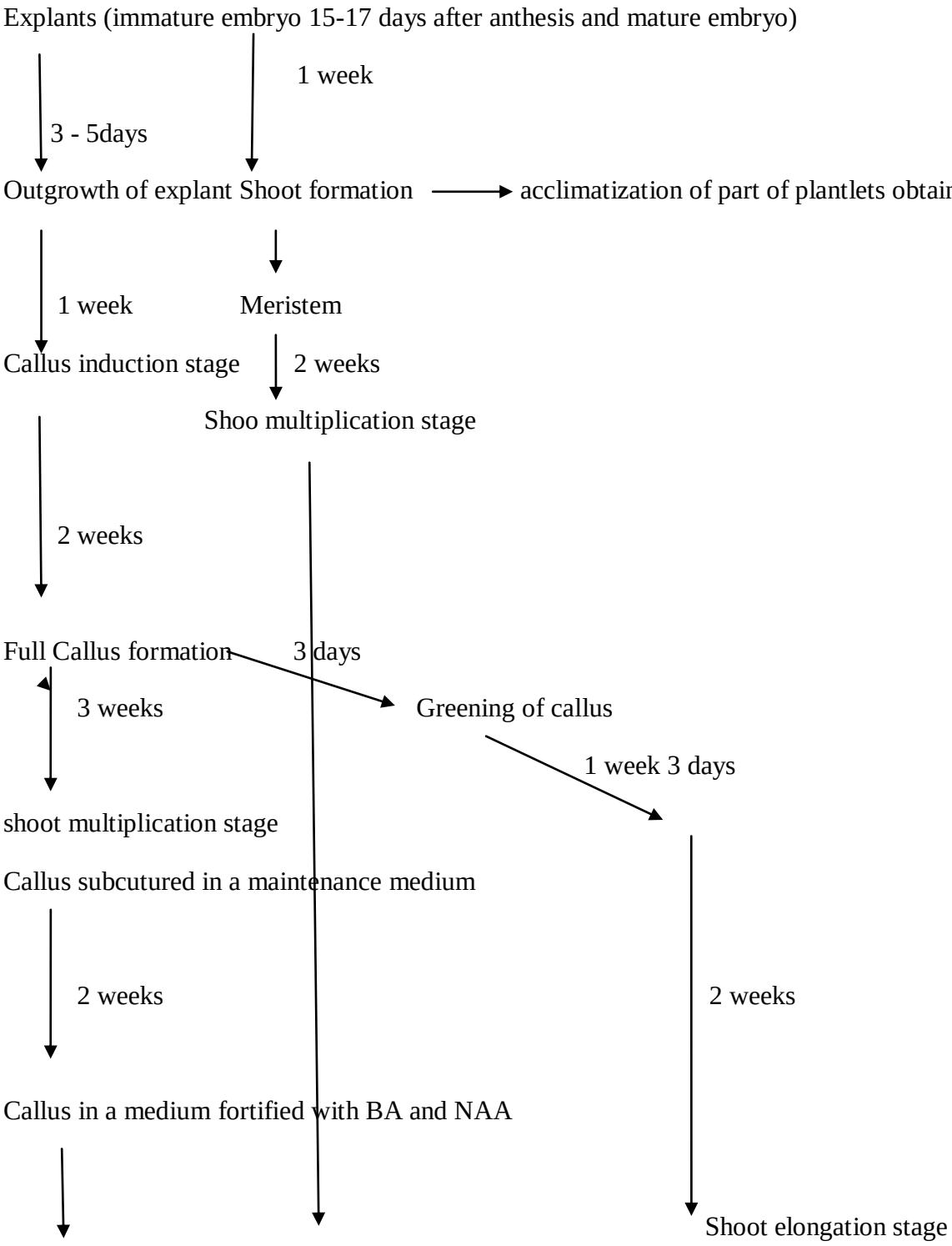


Plate XI: Spectral characteristics of peak obtained from plantlets obtained *in vitro* using methanol for the extraction

Appendix III



Diagrammatic illustration of calendar of event undertaken

Appendix IV

Publications

International Journal of Applied and Natural Sciences (IJANS)
ISSN (P): 2319-4014; ISSN (E): 2319-4022
Vol. 3, Issue 1, Jan 2014, 85-88

OPTIMUM PROTOCOL FOR SHOOT FORMATION IN SORGHUM (Sorghum bicolor (L)Moench) THROUGH ORGANOGENESIS

***Hassan L.B., I.S.Usman, M.D. Katung and S.M . Bugaje**

Department of Plant Science, P.M.B 1044 Ahmadu Bello University Zaria, Nigeria.

***Corresponding author haslat2000@yahoo.com**

ABSTRACT

A protocol for shoot formation in Sorghum bicolor (L) Moench was developed using immature embryo. Murashige and Skoog (MS) basal medium supplemented with varying concentration of 6- benzyl aminopurine (0, 1, 2 and 3mg/l) was used for shoot initiation. After one week of inoculation the primary leaves of the shoot obtained were removed and meristems were cultured for shoot multiplication using MS medium fortified with 3mg/l BAP (6-benzylaminopurine) alone or in combination with 1mg/l NAA (Naphthalene Acetic Acid). For shoot initiation the media fortified with 2mg/l BAP recorded the highest percentage shoot formation (86.67%), the least was obtained from hormone free media (46.67%). The average shoot number per plant was more in the media fortified with 3mg/l BAP + I mg/l NAA (2.4) but this is not significantly different from what was obtained from the media fortified with 3mg/l BAP alone

INTRODUCTION.

Sorghum bicolor belongs to the family poacea and it is an important cereal crop worldwide. It is the fifth most important cereal crop after rice, maize, wheat and barley ((ICRISAT, 2010). Sorghum bicolor is used as food for human consumption and as feeds for animal. Sorghum bicolor is rich in different phytochemicals and these important bioactive compounds are used by pharmaceutical industries for the development of various drugs for the treatment of different kinds of ailments such as cancer, sickle cell anaemia, tubercular swellings, and measles among others. Tissue culture technique has been used as an alternative method of genetic improvement in sorghum because the use of conventional breeding method may take many years to be realized and it has also been used by pharmaceutical industries for the production of different types of secondary metabolites. According to Sadia *et al.* (2010) genetic engineering allows for the generation of novel varieties of a certain crop by introducing genes from sources outside that crop. To achieve

efficient genetic transformation in sorghum, the development of an *in vitro* regeneration system specific to this crop is required. The aim of this work is to develop efficient protocol for shoot formation in sorghum bicolor through organogenesis

MATERIALS AND METHODS

Karandafi seeds collected from farmers' fields in Sokoto and Katsina States were used for this study. The seeds were planted in the green house in order to obtain immature seeds. The immature seeds of sorghum containing immature embryo were harvested from main spikes of caryopsis of 15-17 days after anthesis according to Sudhakar *et al*. (2008), the immature seeds were washed under running tap water for 20 minutes using detergent and surface sterilized in 95%(v/v) ethanol for 2 minutes and further sterilized in 20% (v/v) commercial bleach for 20minutes. The seeds were rinsed three times using double sterilized distilled water. The immature embryos were aseptically excised from immature seeds. Immature embryos obtained were used for shoot initiation using MS media supplemented with varying concentration of BAP (0, 1, 2 and 3mg/l), the pH of the media was adjusted to 5.8 using 0.5M NaOH and 0.5M HCl and gelled with 8g/l agar. Phenolic secretion was controlled using activated charcoal. Fifteen explants were used per treatment and percentage shoot formations were recorded after 1 week of inoculation. Afterwards the primary leaves were removed from the shoot apices and the meristems were cultured for multiple shoots formation in the media fortified with 3mg/l BAP alone or in combination with 1mg/l NAA for shoot multiplication using ten test tubes per treatment. The number of shoots per treatment, number of shoots per explant and average plant height were recorded. The data collected were subjected to analysis of variance and their means were separated using Duncan multiple range test

RESULTS AND DISCUSSION

Shoot formation was observed third day after inoculation in all the treatments tested. Percentage shoot formation was recorded after one week of culture, media supplemented with 2mg/l BAP recorded the highest shoot formation (86.67%) followed by the media fortified with 1mg/l BAP (73.30%). Both root and shoot formations were observed on hormone free media and it recorded the lowest percentage shoot initiation (46.67%) (Table 1 and Fig.1.). The primary leaves were removed from the shoot apices and the meristems were cultured for multiple shoots formation. 1-4 shoots per explant were obtained from the apical meristem cultured after 2 weeks of inoculation. The mean shoot number per explant was more in the media supplemented 3.0mg/l BAP combined with 1mg/l NAA (2.4) and this not significantly different from what was obtained from the media supplemented with 3mg/l BAP alone (2.0) (Table 2). The plant height in the media fortified with 3.0mg/l+1NAA was 7.2cm and this is not significantly different from the media supplemented with 3BAP (6.2) (Table 2). The plantlets obtained from hormone free media were acclimatized after one week of regeneration (Fig. 1).

a.	b.	c.

Fig.`1. plant regeneration through organogenesis. A. Shoots initiated from immature embryo B. Shoot multiplication stage. C. Graduated plantlets in the green house

Table 1: Effect of BAP on shoot initiation using immature embryo

PGR USED	No of explants inoculated	No of explants with response	Frequency of shoot formation (%)
BAP (mg/l)			
0.0	15	7	46.67b
1.0	15	11	73.3ab
2.0	15	13	86.67a
3.0	15	8	53.33b
CV(%)			23.50
SE±			12.4

Means followed by the same letter(s) within a column are not significantly different using DMRT (*significant atP<0.05, **highly significant at P<0.01).

Table 2.**Effect BAP and NAA on Shoot Multiplication**

PGR USED		No of explants inoculated	No of shoots/ treatment	No of shoot/explant	Average plant height (cm)
BAP (mg/l)	NAA (mg/l)				
3.0	0.0	10	20	2.0a	6.2a
3.0	0.1	10	24	2.4a	7.2a
CV (%)				50.71	25.21
SE±				0.49	0.75

Means followed by the same letter(s) within a column are not significantly different using DMRT (*significant atP<0.05, **highly significant at P<0.01).

From the result of this study, it was observed that 2mg/l BAP is sufficient for shoot initiation in sorghum using immature embryo while 3mg/l combined with 1mg/l NAA

recorded more number of shoot per meristem cultured. Anju verma and Anandkumar (2005) reported multiple shoot induction by using 2 mg/L of BAP in the culture medium. Sudhakar *et al*. (2008) reported that shoot regeneration was proficient at a concentration of 2mg/l BAP. Prathibha *et al*. (2001) reported that 2mg/l BAP+ 0.5mg/l 2,4-D were found to be suitable to obtain maximum percentage of regeneration in sorghum. Pola *et al.* (2009) reported multiple shoot formation using 2mg/l BAP + TDZ (Thidiazuron). Sadia *et al.* (2010) reported multiple shoot formation using 3mg/l BAP combined with 1mg/l TDZ. Formation of roots and shoots was also observed from this study when hormone free media was used which shows that it is possible to obtain both organs at a time using hormone free media but the limitation is that only one plantlet can be obtained from an explant but these plantlets are pathogen free. It was observed from this study that this variety contains high amount of phenolic compounds and its usage for the management of sickle cell anaemia may be attributed to high amount of phenols it contains because of the high antioxidant properties of phenolic compounds such as anthocyanin and flavonoids

CONCLUSION

From the result of this study it was observed that the use of BAP in shoot initiation gave better result because more percentage shoot initiation was obtained from the media fortified with BAP while the least was obtained from the media with zero hormones. In conclusion, the protocol described here is optimum for shoot formation in sorghum but more varieties need to be subjected to tissue culture technique to see whether the response is genotype dependent.

REFERENCES

Anju Verma and Anand Kumar, P. 2005. *In vitro* plant regeneration from different explants of *Sorghum bicolor* (L.)Moench.*Plant Cell Biotechnology and MolecularBiology* 6 (3&4): 101-108.

ICRISAT, (2010). http//test/icrisat.org/sorghum/sorghum.htm. retrieved on 30th March, 2010.

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

Pola S, Mani N.S and Ramana, T. (2009). Long term maintenance of callus cultures from immature embryo of *Sorghum bicolor. World Journal of Agricultural Sciences*, 5:415-421.

Prathibha, B. and Sticklen, M.B. (2001). Culturing shoot tip clumps of *Sorghum bicolor* and optimal microprojectile bombardment parameters for transient expression. *J. Cytol. Genet*. 2: 89-96

Sadia, B., Josekutty, R.C., Potlakayya, S.D., Patel, P., Goldman, S. and Rudrabhatla, S.V. (2010). An efficient protocol for culturing meristems of sorghum hybrids. *International Journal of Experimental Botany*, 79: 177-181

Sudhakhar, P., Sarada Mani, N., Ramana, T. (2008). Plant tissue culture studies in *Sorghum bicolor:* immature embryo explants as the source material. *International Journal of Production.*2:1-14

American Journal of Plant Sciences, 2014, 5, 671-675

Published Online March 2014 in SciRes. http://www.scirp.org/journal/ajps
http://dx.doi.org/10.4236/ajps.2014.55081
How to cite this paper: Hassan, L.B., *et al*. (2014) Optimum Protocol for Shoot Formation in *karandafi* Red Sorghum (*Sorghum bicolor* (L.) Moench) through Somatic Embryogenesis Using Mature Embryo.*American Journal of Plant Sciences*, **5**,671-675.
http://dx.doi.org/10.4236/ajps.2014.55081

Optimum Protocol for Shoot Formation in *karandafi* Red Sorghum (*Sorghum bicolor* (L.) Moench) through Somatic EmbryogenesisUsing Mature Embryo

Lateefat Bolanle Hassan*, Inuwa S. Usman, Mary D. Katung, Sanusi M. Bugaje
Department of Plant Science, Ahmadu Bello University, Zaria, Nigeria
Email: *haslat2000@yahoo.com

Received 18 December 2013; revised 14 February 2014; accepted 4 March 2014

Abstract.

In this study an optimum protocol for shoot formation through somatic embryogenesis using mature embryo explants was developed. Calli were initiated on Murashige and Skoog (MS) media supplemented with varying concentration of 2,4-Dichlorophenoxyl acetic acid (2,4-D) ranging from 1.5 mg/l-4.0 mg/l alone or in combination with 0.5mg/l Kinetin (KN). Significance difference ($p<0.05$) was observed among the different concentrations of hormone used for callus induction. The highest percentage callus formation was obtained from the media fortified with 4mg/l 2, 4-D for mature embryo obtained from imbibed seed while for the preconditioned mature embryo, the media supplemented with 2m/l 2,4-D + 0.5mg/l kinetin (KN) recorded more percentage callus formation compared to what was obtained from the media supplemented with 2.5mg/l 2,4-D + 0.5mg/l. More percentage shoot formation was obtained from the media supplemented with 1mg/l 6-benzylaminopurine (BA). Average number of shoot per callus was also more in the media fortified with 1mg/l BA (2.25) but this is not significantly different from what was obtained from the media fortified with 2mg/l BA+ 0.1mg/l 2,4-D at 5% level of significance

Keywords.*Sorghum bicolor*, Callus induction, *in-vitro* regeneration

Introduction

Sorghum bicolor is an important cereal crop grown in the tropics, sub-tropic and temperate regions as well as in poor quality soils and semi-arid regions [2]. It is the fifth most important cereal crop in the world, and is the dietary staple of more than half a billion people in over 30 countries [3]. *Sorghum bicolor* is used as food for human consumption and feed for animals. Its use as a medicinal plant is getting prominent due to the various

phyto-chemicals it contains that are used for the treatment of different ailments. Being an important crop with different uses

there is need to breed better crops that are disease resistant and high yielding varieties. With the use of conventional breeding many years may be required to develop an improved variety. Genetic transformation through biotechnology is an alternative method of genetic improvement which can be achieved within a short period of time. An essential step of this technique is the development of efficient protocol for plant regeneration.
Explants such as immature inflorescences, immature embryos and shoot tip had been reported by [4]. To obtain immature embryo throughout the year is not easy because sorghum flowering occurs once in its life time according to [11]. Zygotic embryos from mature seed were therefore used as explants for callus induction [6]. These explants, however, present some difficulties in regeneration [5]. The objective of the study is to optimize protocol for somatic embryogenesis and plant regeneration from mature zygotic embryos of sorghum

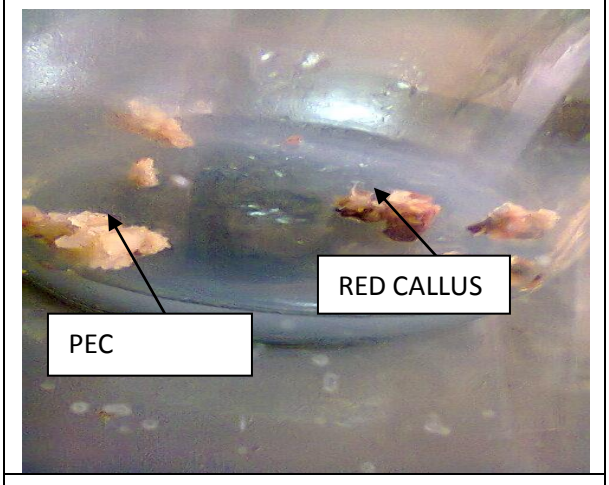
Materials and Methods

For the purpose of this study seed of Karandafi (a mutant redsorghum) were collected from farmers' fields in Sokoto and Katsina States. The seeds were washed with mild detergent under running tapwater and surface sterilized by dipping in 95% ethanol (v/v) for 2 minutes followed by treatment with 20% commercial bleach (containing 3.5% Sodium hypochlorite) with few drops of tween 20 for 20 minutes. Afterwards, the seeds were rinsed three (3) times with sterile distilled water and parts were soaked in distilled water and some were preconditioned on hormone free MS (Murashige and Skoog) media for 24 hours. Embryos were aseptically excised from imbibed seeds using sterilized forceps and surgical blade.The excised embryos were used for callus induction on amodified semi solidMS medium [8] supplemented with sucrose 30g/L, different concentration of 2,4-D (1.5-4.0mg/L, 2,4-D) with or without 0.5mg/L Kinetin (KN). In another experiment, to evaluate the effect of preconditioning of the embryo by culture on hormone free MS media for 24hrs, preconditioned embryos were excised and culture on MS media supplemented with 2.0mg/l +0.5mg/l KN or 2.5mg/l 2,4-D)+0.5 mg/l KN. The pH of the media were adjusted to 5.8 using 0.5M HCl and 0.5M NaOH, agar (0.8%) was used as gelling agent and the media were heated to boiling for proper mixing. The sterilization of the media was done in an autoclave at 121^{o}C for 15 minutes and media were dispensed into petri dishes. Five Embryos were cultivated in each Petri dish (11mm diameter) and five (5) petri dishes were used per treatment and two (2) petri dishes were used for preconditioned mature embryo per treatment. The cultures were incubated in the darkness for callus proliferation at 25^{o}C±2^{o}C. Activated charcoal was used to control phenolic secretion [10]. The number of explants that formed calli and percentage callus formation were recorded. For the regeneration of the explants, the calli obtained from preconditioned mature embryo were taken to regeneration media after 2 weeks of callus induction using MS medium supplemented with 1-2mg/l BA + 0.1 mg/l 2,4-D. Eight test tubes were used per treatment. Percentage shoot formation, Average number of shoots per calli for each treatment and average shoot length were recorded. The data collected were subjected to analysis of variance and the mean were separated using Duncan Multiple Range Test (DMRT)

Results and Discussion

Callus induction

Sticking out of embryo was observed after fifth day of inoculation. The calli were initially whitish in nature and this later changed to yellowish type of calli. They became reddish type on hormone free media (**Figure 1.**) There was significant difference in percentage callus formation among the different concentrations of hormone used at 5% level of significance. Treating the explant with 4.0mg/l 2,4-D gave the highest callus induction frequency (60%) followed by the media fortified with 2.0mg/l 2,4-D combined with 0.5mg/l KN which recorded (56%) of callus induction frequency. The least callus induction frequency was obtained from the media supplemented with 1.5mg/l 2,4-D (0%) (**Figure 2)**. No callus formation was obtained on hormone free media and in the media supplemented with 0.5mg/l KN alone (0%) (**Figure 2)**. Across all the treatments, calli with significant phenolic excretion demonstrated slow growth and most of them were lost after 3 weeks of culture. Complete (100%) callus induction was obtained from the media fortified with 2mg/l 2,4-D+0.5KN when this mature embryo was preconditioned on hormone free media before inoculation (**Table 1**). It was observed from this study that 2mg/l 2,4-D+ 0.5KN is an optimum concentration of hormone that can be used for callus induction. This is similar to what was obtained when immature embryo was cultured by Sudhakar *et al.* (2008). [5] reported that the highest percentage callus formation was obtained from germinating seeds using 4mg/l 2,4-D and the callus induction frequency was said to reduce when the concentration of 2,4-D was increased to 6mg/l. [7] reported highest callus induction frequency from immature inflorescence using 2.0mg/l 2,4-D + 0.2KN



Callus obtained from mature embryo cultured –

Figure1. The calli obtained from mature embryo

PEC- Potentially Embryogenic Calli

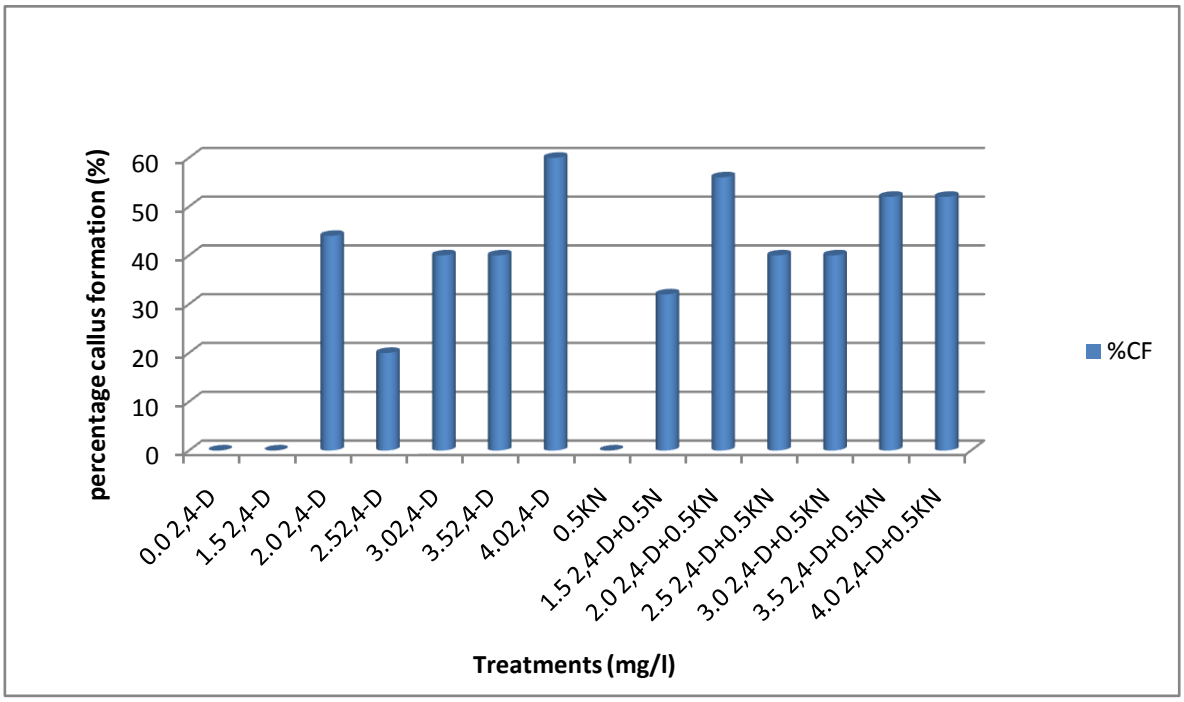
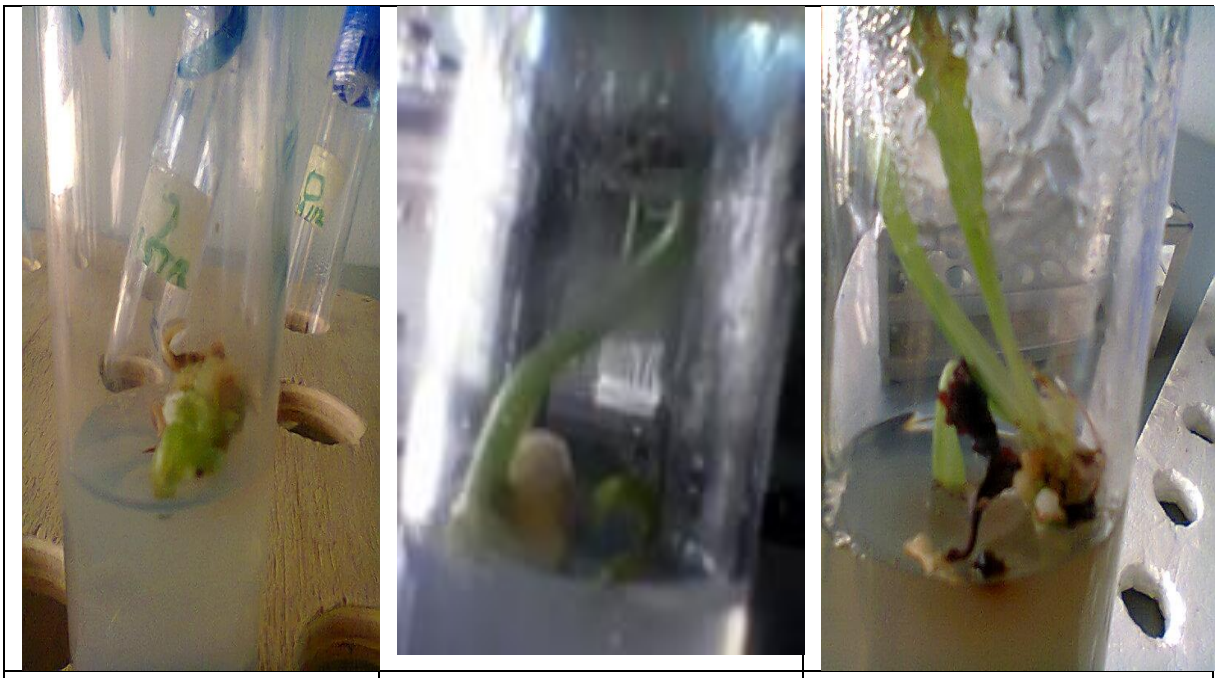


Figure2. Effect of 2, 4-D and kinetin on callus induction using mature embryo

Regeneration

To induce somatic embryogenesis, calli were cultured on hormone free media and media fortified with 0.1mg/l 2,4-D. Somatic embryo formations were observed in most of the calli transferred after three week of culture on both hormone free and media supplemented with low concentration of 2,4-D.To regenerate plantlets, developing somatic embryos were taking to regeneration media. Low level of regeneration and death of the developing embryos was observed after 3-6 weeks of culture on regeneration media. To ensure improvement in the frequency of regeneration, whitish and compact calli obtained from preconditioned embryo were selected and cultured directly on regeneration media fortified with different concentrations of BA. The calli turned greenish after few days of culture on regeneration media and plantlets development was observed after two weeks of culture (**Figure 3**). More percentage shoot formation was obtained from the media supplemented with 1mg/l BA (75%) compared to 50% obtained from the media supplemented 2mg/l BA+ 0.1, 2, 4–D. The average number of shoot per callus was higher in the media fortified with 1mg/l BA (2.25) but this not significantly differently at 5% level of significance with what was obtained from the media supplemented with 2mg/l BA+0.1mg/l 2,4-D which recorded 1.88 (Table 2). The average shoot length was 2.26cm in the media fortified with 1mg/l BA which is not significantly different from average shoot length recorded by the media supplemented with 2mg/l BA + 0.1mg/l 2,4-D (Table 2). Proficiency of shoot regeneration at a concentration of 2mg/l BA was reported by [10]



A	B	C

Figure 3.plant regeneration through somatic embryogenesis. A. shoots initiation stage. B. shoots multiplication stage. C. shoots elongation stage

Table 1: Effect of 2, 4-D and Kinetin on callus induction using mature embryo preconditioned on hormone free media for 24 hours

PGR USED		No of explants inoculated	No of explants that showed response	Frequency of callus formation (%)
2,4D	KINETIN			
2.0	0.5	10	10	100
2.5	0.5	10	4	40

Table 2: Effect of BA alone or in combination with 2, 4-D on plant regeneration

PGRUSED		No of callus inoculated	No of explants with response	Percentage shoots formation (%)	Total number of shoot /treatment	Average number of shoot/callus	Average shoot length(cm)
BA (mg/l)	2,4-D (mg/l)						
I	0	8	6	75	18	2.25a	2.26a
2	0.1	8	4	50	15	1.88a	1.48a

Better shoot regeneration using 2.5mg/l TDZ plus 1.0mg/l BA and 0.5mg/l IAA was reported by [11]. [9] reported multiple shoot formation using 2mg/l BA + TDZ (Thidiazuron). [1] reported maximum number of shoot formation in sorghum using 13.3uM BA and 2.3uM 2,4-D. Research had shown that the major problems of using tissue culture technique for genetic improvement in sorghum is due to production of phenolic compounds, low regeneration frequency of mature seeds and longer time required to obtain immature embryo. With efficient method of regeneration, improved crop variety can be made available to farmers using tissue culture technique. So in this study it was observed that better response was obtained when the mature seeds were preconditioned on hormone free media before it was taken to callus induction media. In conclusion, a protocol for shoot formation had been optimized using mature embryo. This shows that there is possibility of improving regeneration from mature embryo by preconditioning the mature seed on hormone free media before inoculation.

Acknowledgements
My word of appreciation goes to Dr. I.S. Usman, the Head of Plant Science Department, ABU, Zaria, the technologists in the biotechnology laboratory and Institute for Agricultural Research (IAR) for their financial support throughout the course of this research work

REFERENCES

[1] Baskaran, B.R. Rajeswari, and N. Jayabalan. Development of an in vitro regeneration system in sorghum (*Sorghum bicolor* (L.) Moench using root transverse thin cell layers (tTLCs). *Turk. J. Bot*., Vol. 30, 2006. pp1-9

[2] G. Grassi. Sweet sorghum lament network. Munich. 2001. Pp 10-25

[3] ICRISAT,(2010). Http//test1.icrisat.org/sorghum/sorghum.htm

[4] A.P. Indra and S. Krishnaveni . Effect of hormones, explants and genotypes on *in vitro* culturing of sorghum. *Journal of Biochemistry Technology*, Vol. 1, No. 4, 2009, pp. 96-103

[5] Z. Liming, L. Shujun and S. Songquan.Optimization of callus induction and plant regeneration from germinating seeds of sweet sorghum (*Sorghum bicolor* (L) Moench). *African Journal of Biotechnology*, Vol. 9, No. 16, 2010, pp. 2367-2374.

[6] C. MacKinnon, G.Gunderson and M.W.Nabors. Plant regeneration by somatic embryogenesis from callus cultures of sweet sorghum. *Plant Cell Rep*. Vol. 5, 1986, pp. 349-351. http://dx.doi.org/10.1007/BF0026859

[7] S.M. Manjula, M.S. Kuruvinasetti and C.C. Harti . Regeneration establishment and evaluation of somaclones in Sorghum bicolor (L.) Moench. *Euphytical*, Vol.115, 2000, pp. 173-180. http://dx.doi.org/10.1023/A:1004010315991

[8] T. Murashige and F. Skoog. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiology of Plantarum*, Vol 15, No.43, 1962, pp.473-497 http://dx.doi.org/10.1111/j.1399-3054.1962.tb08052.x

[9] S. Pola, N.S. Mani and T. Ramana. Long term maintenance of callus cultures from immature embryo of *Sorghum bicolor. World Journal of Agricultural Sciences,* Vol. 5, No. 4, 2009, pp. 415-421.

[10] P. Sudhakhar, M.N. Sarada, T. Ramana. Plant tissue culture studies in *Sorghum bicolor:* immature embryo explants as the source material. *International Journal of plant Production,* Vol. 2, No.1, 2008, pp. 1-14.

[11] P. Sudhakararao. Leaf discs as a source material for plant tissue culture studies of *sorghum bicolor* (L.) Moench. *Nortulae Scientia Biologicae,* Vol. 3, No. 1, 2011, pp. 70-78