PROXIMATE COMPOSITION AND ESTABLISHMENT OF AN EFFICIENT *IN VITRO* MICROPROPAGATION PROTOCOL FOR ANCHOTE [*Coccinia abyssinica* (Lam.) Cogn.] FROM EASTERN WOLLAGA, ETHIOPIA

TOLA BAYISA GUMA

MASTER OF SCIENCE

(MOLECULAR BIOLOGY AND BIOTECHNOLOGY)

PAN AFRICAN UNIVERSITY

INSTITUTE OF BASIC SCIENCES ,TECHNOLOGY AND INNOVATION

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Proximate Composition Analysis and Establishment of an efficient *in vitro* Micropropagation Protocol for Anchote [*Coccinia abyssinca* (Lam.) Cogn]. from Eastern Wollega, Ethiopia

TOLA BAYISA GUMA

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Declaration

This thesis is my original work and has not been presented for a degree in any other University

Signature: ---------- Date: ----------

Name: Tola Bayisa Guma

Reg. No. MB 300-0012/12

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

Dr J.M. Onguso

Institute for Biotechnology Research

Jomo Kenyatta University of Agriculture and Technology (JKUAT)

Signature---------- Date ----------

Dr Peter Njenga

Botany Department

Jomo Kenyatta University of Agriculture and Technology (JKUAT)

Signature---------- Date ----------

Dr Jane W. Kahia

World Agro forestry Centre (ICRAF)

Signature……..

Date_____

DEDICATION

To all those who consciously contributed something for the betterment of humanity

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ACRONYMS

AOAC----------Association of official Analytical chemist

AVG----------Amino ethoxyvinylglcine

BAP----------Benzylaminopurine

EDTA----------Ethelenediaminetetraacetic acid

HCl---------- Hydrochloric acid

HEPA----------High efficiency particulate air

IAA----------Indole-3- acetic acid

IBA----------Indole-3-butyric acid

MS ----------Murashige and Skoog

NAA----------Naphthalene acetic acid

NaOCl----------Sodium hypochlorite

NaOH.----------Sodium hydroxide

H_2SO_4----------Sulphuric acid

TDZ----------Thidiazuron

WPM----------woody plant medium

2ip----------6-γ -γ -(dimethylallylamino)-purine

2,4-D----------2,4-dichlorophenoxyacetic acid

µM----------Micromolar

ABSTRACT

Anchote (*Cocinia abysinica* (Lam.) Cogn) is an annual trailing vine belonging to family cucurbitaceae and is a potential major root and tuber cultivated plant produced in west Oromia (East Wollega) ,Ethiopia as food, cultural ,social and economical crop. This study aimed to: i) assess the nutritional content of Anchote leaves ,stems and roots. ii) determine the optimum sterilization procedure for Anchote explants. iii) compare effects of different cytokines and auxins concentrations in callus induction and direct regeneration from Anchote explants. Standard method of Association of Official Analytical Chemist (AOAC) was used to determine the crude protein, Crude fat, total ash, crude fiber and moisture contents of the sample. The collected parameters were analyzed using INSTA GRAPPAD software (San Diego, California ,). Analyses of variance (ANOVA) was computed for each parameter and the data were summarized as mean ± SD. The mean difference were assessed by Student-Newman-Keuls Multiple Comparisons Tests at5% level of significance. For tissue culture, young nodal and leaf explants were selected from healthy plants and subjected to sterilization using 0.19% and 0.385% of NaOCl commercial bleach JIK® at 5 ,10 15 minutes intervals. As the result showed that the highest protein was observed in leaf (18.3±1) and this indicate that the leaf is good source of protein compared to the protein found in the stem and root parts .The result of ANOVA showed that protein in leaves significantly different at α=0.05. However the lowest protein was observed in roots (8.2±0.1).The highest mineral element found in the stem was Magnesium (Mg) with mean average 336±0.4 Mg/100 followed by potassium (K) which was found with mean average 323±3 Mg/100 and Calcium(Ca) was found with mean average of 313.5±1 Mg/100.Maximum clean survival explants

(84.5±0.5) were obtained with 0.19%NaOCl and 15Minutes) for nodal explants Similarly, Maximum clean survival explants (82.5±0.) were obtained with 0.19%NaOCl and 10 Minutes) for leaf explants .BAP, TDZ , Kn,2,4-D and NAA were used for callus induction. The highest percentage of callus induction (80±0.2) and (78±2) were obtained with 5µM BAP and 5µM 2,4-D) for leaf and nodal explants respectively. For regeneration of micro shoots, the nodes cultured on Murashige and Skoog's medium(MS) supplemented with 2.5 µM BAP gave the best result with (75±0.1) regeneration frequency and highest shoot number (3.2±0.5)). Similarly ,the concentration of 0.025 µM TDZ in combination with 2.5µM BAP gave highest shoot number (4.2±0.1) . The highest root initiation percentage (86 ±0.3) was shown by 0.5 µM NAA on full MS Media with maximum shoot number (4.6±4.6). Finally the best hardening (acclimatization) was obtained from Soil: Manure (1:1) mixture with mean percentage (83.33±1) of survival rate.

CHAPTER ONE

1.1 Background of the Study

1.1.1 Description and Uses of the Plant

Anchote [*Coccinia abyssinica* (Lam.) Cogn.] is annual trailing vine belonging to family of cucurbitacea and grown principally for its root as food. It is one of the potential major roots and tuber crop cultivated plants produced on nearly 300ha of the land on in Wollega, Ethiopia. It is produced for food, cultural, social and commercial purposes for the communities that produce it. Anchote can be propagated both vegetatively and from seeds. Vegetative propagation is carried out either by planting the whole tuber or by slicing it into two or more pieces. Low attention has been given to research and development of Anchote and no variety so far has been developed through breeding programmes (Mengesha *et al*, 2012) .

The genus *Coccinia* is made up of 30 spp. of which eight are (Anonymous,2011) reported to occur in Ethiopia. The species recorded in flora of Ethiopia since 1995 include *C. abyssinica* (Lam.) Cogn., *C. adoensis* (Hochst. Ex. A. Rich.) Cogn.), *C. grandis* (L.) Voigh (Syn. *C. indica* wight and Arn.), C. *megarhiza*, C. Jeffrey an(Abera *et al*,1973).

Anchote is cultivated in areas between 1300-2800 m above sea level where the annual rainfall ranged between 762-1016 mm as indicated by (Amare ,1973). Ethiopia has 18 agro-ecological zones which are endowed with suitable climatic and edaphically conditions for quality and quantity production of various kinds of root and tuber crops

The majority of the Ethiopian population depends mainly on cereal crops as food source. The food potential of root and tuber crops has not yet been fully exploited and utilized despite their significant contributions towards food security, income generation, provision of food energy and resource base conservation.

1.1.2 Tissue Culture Techniques

Plant tissue culture techniques are applied for crop improvement and production of higher yields. Plant cell cultures have turned into an in valuable tool for plant scientists and today *in vitro* culture techniques are standard procedures in most laboratories and now it is a well-established technology like many other technologies. It has gone through different stages of evolution; scientific curiosity, novel applications and mass exploitation. Initially, plant tissue culture was exploited as a research tool and focused on attempts to culture and study the development of small, isolated segments of plant tissues or isolated cells (Loyola-Vargas and Vaque, 2006)

In 1939 three investigators, Nobecourt & Gautheret in France, and White in the U.S.A., reported independently the indefinite culture of plant callus tissue in a synthetic medium (Wimber, 1963). The calluses increased in size by continuous cell division and were sectioned and sub cultured periodically to produce additional calluses. In a classic experiment, Skoog and Miller (1957) demonstrated that the regulation of plant organ formation (organogenesis) in tobacco callus was regulated by interactions between two plant hormones auxin and cytokinin. Callus cultures have been maintained for many years and, with the correct concentrations and ratio of growth substances, it has been

possible to induce the differentiation of shoots and roots from them, thereby producing new plants (Allan, 1994).

Experimental systems based on plant cell and tissue culture are characterized by the use of isolated parts of plants, called explants, obtained from an intact plant body and kept on, or in a suitable nutrient medium (Nair ,2008) . Such experimental systems are usually maintained under aseptic conditions. Otherwise, due to the fast growth of contaminating microorganisms, the cultured cell material would quickly be overgrown, making a rational evaluation of experimental results impossible (Neumann and Imani, 2009).

1. 2 Statement of the Problem

Nutritional value in any food item is an important aspect. In order to have a healthy population that can promote development, the relationship between food, nutrition and health should be reinforced. In developing countries, one of the ways of achieving this is through the exploitation of available local resources, in order to satisfy the needs of the increasing population. Knowledge of the nutritive value of local food ,nutritional ingredients , management and method of cultivation is necessary in order to encourage the increased cultivation and consumption of nutritive plants. The plant *C. abyssinica* locally referred to as 'Anchote' is a delicious food item in different forms and it is highly essential despite lack of proper studies to document its nutritional values in the region. Besides, as food resource the accessibility of its seed for cultivation is limited because it takes longer duration to obtain the seed, It is also eaten by birds as fruits, and the root is also eaten by wild animals. This therefore mean that there is limited chance of getting the

seed for cultivation. In addition to these factors, seed accessibility also requires physical and mechanical traditional treatment which is another burden for the farmers to get proper seeds for cultivation. These constraints are the major hindrance for the farmers to cultivate Anchote as much as they would want to scale up its production .Therefore , this study w assessed nutritional contents of Anchote and develop *in vitro* propagation .

1.3 Justification of the study

Biotechnology has been the most rapidly adopted technology in the history of agriculture and continues to expand in much of the developed and developing world. Through Cultivation of plant by micropropagation ,it is now possible to provide clean and uniform planting materials in field crops – eggplant, jojoba, pineapple, tomato; root crops – cassava, yam, sweet potato; and many ornamental plants such as orchids and anthuriums .The primary driver of adoption among farmers (both large commercial and small-scale subsistence) has been the positive impact on farm income. The adoption of biotechnology has had a very positive impact on farm income derived mainly from a combination of enhanced productivity and efficiency gains through biotechnology.

The result of this study will reveal nutritional status of stems, leaves and roots of Anchote seeking as alternative sources of food for farmers and consumers. Furthermore, the study will produce establishment of an efficient *in vitro* micropropagation protocol using sterile agent and different plant regulators there by enabling rapid multiplication and producing disease free propagules for Anchote .

1.4 OBJECTIVES

1.4.1 General Objective

To assess the nutritional status of the leaves, roots and stems, and develop an efficient micro propagation protocol for Anchote.

1.4. 2 Specific Objectives

1. To determine the proximate composition of *Coccinia abyssinica* leaves, roots and stems.
2. To determine optimum sterilization agent and callus induction protocol for *Coccinia abyssinica* explants
3. To determine the optimum cytokinin levels for multiple shoot production of *Coccinia abysinica*
4. To determine the optimum auxin levels for rooting of *in vitro* generated shoots.
5. To evaluate the effects of different media (soil mixture) on hardening of C*occinia abyssinica* tissue culture derived plantlets.

1.7 Research Hypothesis

1. There are no significant nutritional contents from Anchote root, stem and leaves.
2. There is no optimum sterilant concentration for sterilization and optimum growth regulators for callus induction.
3. There is no optimum cytokinins concentration for shooting.

4. There is no optimum auxin concentration for rooting
5. There is no the optimum soil mixture for hardening .

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Botanical Description

Anchote *[*Coccinia abyssinica *(Lam.) Cogn.]* is annual trailing vine belonging to family of cuccurbitacceae and grown principally for its root as food. It is one of the potential major roots and tuber cultivated plants produced on nearly 300ha of the land on in Wollega and produced for food, cultural, social and economical crop for the communities produce it(Mengesha *et al*.,2012).

The genus *Coccinia* is made up of 30 spp. of which eight are (Anonymous,2011) reported to occur in Ethiopia. The species recorded in flora of Ethiopia since 1995 include *C. abyssinica* (Lam.) Cogn., *C. adoensis* (Hochst. Ex. A. Rich.) Cogn.), *C. grandis* (L.) ,Voigh (Syn. *C. indica* wight and Arn.), C. *megarhiza*, C. Jeffrey an (Abera *et al*,1973).

2.2 Traditional Propagation

Anchote can be propagated both vegetative and from seeds. Vegetative propagation is carried out either by planting the whole tuber or by slicing it into two or more pieces, each piece having rootlets. Low attention given to the research and development of Anchote, no variety so far developed and released (Mengesha *et al*.,2012).

2.3 Agro ecological Zones

Anchote is cultivated in areas between 1300-2800 m above sea level where the annual rainfall ranged between 762-1016 mm as indicated by (Amare ,1973). Anchote is

endemic to the Western parts of Ethiopia (Getahun ,1973), mainly in the Western region of Ethiopia highlands in Eastern Wollega, Western Wollega, Kelam Wollega, and Mattu Ethiopia has 18 agro-ecological zones which are endowed with suitable climatic and edaphically conditions for quality and quantity production of various kinds of root and tuber crops

2.4 Economic Importance as Food, Income and Medicine

The majority of the Ethiopian population depends mainly on cereal crops as food source. The food potential of root and tuber crops has not yet been fully exploited and utilized despite their significant contributions towards food security, income generation, provision of food energy and resource base conservation.

Of the major tuberous vegetables such as sweet potato, Oromo potato, and others cultivated in the area, Anchote is widely recognized as cultural, social and economic crop for the local farming communities in west Oromia, Ethiopia with its annual yield of 25,000 tones (Anonymous, 2011). It is a valuable food source and according to local farmers, it helps in fast mending of broken/ fracture bones and displaced joints, as it contains high calcium, and proteins than other common and wide spread root and tuber crops (Endashaw , 2007) . Traditionally, it is also believed that, Anchote makes lactating mothers healthier and stronger (Abera , 1995). Dawit and Estifanos reported that the juice prepared from tubers of Anchote has saponin as an active substance and is used to treat Gonorrhea, Tuberculosis, and Tumor Cancer.

a  **b**.  **c**.  .

d.  **e.**  **f**. 

Plate 2.1:Different parts of Anchotes:(a) Seeds (b) Seedling from seed (c) Roots obtained from four month old seedlings (d) Boiled root (e)Mother plant (f) Unriped fruit obtained from mother plant.

2.5 Nutrition

There is an increasing prevalence of nutrition related illnesses especially in Africa due to poverty and insufficient knowledge on the nutritional and economic importance of locally available and easily accessible foods and foodstuffs. Studies on Cucurbitacceae seeds have shown that they contain high protein levels with high levels of most essential amino acids except lysine (Sharma, 1988). Research on nutritional, genetic and medicinal aspects was carried out in different *cucurbitaceous* members. The fruits, leaves, flowers and roots of the cucurbitaceae members are consumed as food. They have medicinal

importance as diuretics, alexiterics, laxatives, hepato protectives, antivenumous, antihypertension, anti-diabetic, astringent, antihelminthic, aphrodisiac, antiseptic characteristics and capable of curing pimples or acne (Chunduri,2013)

Food is essential to human survival. It provides the proteins, carbohydrates, fat, fiber, vitamins, and minerals needed to stay alive, grow, and stay healthy. Ash content is also essential to a food's nutrition and longevity. Some foods are energy-rich because they contain little or no water, fiber or other material which does not yield energy; examples are metabolizable sugars, fats and oils. Foods with much water and dietary fiber are usually energy-poor. For example, 100 g table sugar (sucrose) will provide 400 kcal, whereas 100 g of items such as lettuce, tomatoes or cucumber, which contain about 95 per cent water plus fiber, will provide only about 20 kcal. Eating most salad items instead of sugar, fats and oils greatly reduces the energy intake (Wise M.,2004).

2.5.1 Carbohydrates

All the energy needed for growth and repair of the body, for muscular activity of all kinds and for all the work done by cells comes from the metabolism of carbohydrate, fat, protein and alcohol. The numerous other items of the diet, even though essential for other reasons, do not provide energy, although many are directly involved in the chemical reactions which yield energy. If the diet is adequate and properly balanced the energy normally comes chiefly from carbohydrate and fat, while most of the protein is used for cell growth and repair. When there is not enough carbohydrate and fat, the protein is used for energy and is then not available for other purposes (Newton, 2007).

2.5.2 Moisture Content

Moisture content is defined as the mass of water per unit mass of dry mater .Understanding the water content of material is a common interest and concern to many diverse industries. Moisture content is important for the processing and handling of cosmetics, pharmaceuticals, food, personal care products, pulp and paper products and specialty chemicals, to name just a few. The amount of available water will also dictate the shelf life and stability of many systems. For example, the presence of water in food greatly impacts its susceptibility to chemical, enzymatic and microbial activity. Moisture content has been established as an important indicator of shelf life for foods. Moisture can determine the aesthetics of food, giving estimates to product shelf life regardless of sample properties in a wet or dry state (Wise M.,2004).

2.5.3 Protein

Proteins play a central role in biological systems. Although the information for evolution and biological organization of cells is contained in DNA, the chemical and biochemical processes that sustain the life of a cell/organism are performed exclusively by enzymes.. The myriad of biological functions performed by proteins might not be possible but for the complexity in their composition, which gives rise to a multitude of three dimensional structural forms with different biological functions. All biologically produced proteins can be used as food proteins. However, for practical purposes, food proteins may be defined as those that are easily digestible, nontoxic, nutritionally adequate, functionally useable in food products, and available in abundance. Traditionally, milk, meats

(including fish and poultry), eggs, cereals, legumes, and oilseeds have been the major sources of food proteins. However, because of the burgeoning world population, nontraditional sources of proteins for human nutrition need to be developed to meet the future demand (Fenna,1996).

2.5.4 Dietary Fiber

Dietary fiber means carbohydrate polymers with a degree of polymerization (DP) not lower than 3 which are neither digested nor absorbed in the small intestine. A degree of polymerization not lower than 3 is intended to exclude mono- and disaccharides. It is not intended to reflect the average DP of a mixture. Dietary fiber consists of one or more of: Edible carbohydrate polymers naturally occurring in the food as consumed, carbohydrate polymers, which have been obtained from food raw material by physical, enzymatic or chemical means, synthetic carbohydrate polymers." The Association of Official Analytical Chemist (AOAC)method (995.43) requires enzymic digestion of protein and non-resistant starch, followed by precipitation of soluble fiber with 95% alcohol and weighing (IFST, 2007).

2.6 Plant Tissue Culture

Unique in biology, plant cells are totipotent; whole plants can be regenerated from single nonsexual cells (Lack and Evan, 2005) .As a necessary precursor to most plant transformation systems, there must be methods established to manipulate plant tissues and cells in sterile media. From tissues taken from plants, media components and hormones can be manipulated to recover organs or induce somatic embryos.

Tissue culture techniques are used extensively to grow many different plants for commercial and research purposes (Hussain *et al*, 2012). New plants are grown from small pieces of plant tissue in a nutrient medium under sterile conditions. When conditions are suitable, plants can be induced to rapidly produce new shoots, which can be subdivided to produce more plants. The addition of suitable hormones can then induce root growth, and the plants can then be placed in soil and grown in the normal manner (Rand, 2001*).* Plant Cell and tissue culture has already contributed significantly to crop improvement and has great potential for the future . Research efforts in plant cell and tissue culture have increased dramatically worldwide in recent years including efforts in developing nations (Sidorov ,2013).

Micropropagation starts with the selection of plant tissues (explant) from a healthy, vigorous mother plant (Murashige and Skoog, 1974). According to (Hussain *et al.,* 2012) there are five stages in micro propagation:

2.6.1 Stage 0: Preparation of Donor Plant

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

2.6.2 Stage I: Initiation Stage

In this stage an explant is surface sterilized and transferred into nutrient medium. Generally, the combined application of bactericide and fungicide products is suggested.

The selection of products depends on the type of explants to be introduced. The surface sterilization of explants in chemical solutions is an important step to remove contaminants with minimal damage to plant cells. The most commonly used disinfectants are sodium hypochlorite, calcium hypochlorite, ethanol and mercuric chloride ($HgCl_2$). The cultures are incubated in growth chamber either under light or dark conditions according to the method of propagation.

2.6.3 Stage II: Multiplication Stage

The aim of this phase is to increase the number of propagules. The number of propagules is multiplied by repeated subcultures until the desired (or planned) number of plants is attained.

2.6.4 Stage III: Rooting Stage

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

2.6.5 Stage IV: Acclimatization Stage

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

It is important to recognize and understand the differences between an in vitro and a greenhouse environment. Roots formed in culture can be beneficial for enhancing early growth following transfer from culture. The optimum growth rate of deflasked plantlets frequently does not occur until new leaves and roots develop in the greenhouse environment. However, as species differ greatly in their requirements there is no universal acclimatizing protocol (Seelye *et al.*,2003).

Tissue culture is not only a necessary enabling technology for transgenic plant production but is also used for *in vitro* propagation of valuable plants (Persley and MacIntyre, 2002). Plant tissue culture is the *in vitro* (literally "under glass") manipulation of plant cells and tissues, which is a keystone in the foundation of plant biotechnology. It is useful for plant propagation and the study of plant hormones, and is generally required to manipulate and regenerate transgenic plants. Whole plants can be regenerated *in vitro* using tissues, or a single cell to form whole plants by culturing them on a nutrient medium in a sterile environment. Elite varieties can be clonally propagated, endangered plants can be conserved, virus-free plants can be produced by meristem culture, germplasm can be conserved, and secondary metabolites can be produced by cell culture (Rai, 2007).

Tissue culture serves as an indispensable tool for transgenic plant production (Gupta and Ibaraki, 2006) and for nearly any transformation system, an efficient regeneration protocol is imperative. This can be attributed to totipotency of plant cells and manipulation other growth medium and hormones. Plant cells are unique in the sense that

every cell has the potency to form whole new plantlike stem cells (stem cell production in mammals is located in time and space, and most mammalian cells cannot be converted to stem cells). However, having an understanding of each plant species and explants (donor tissue that is placed in culture) is essential to the development of an efficient regeneration system. The physiological stage of the explants plays a very important role in its response to tissue culture. For example, young explants generally respond better than do older one (Loyola-Vargas and Vaque-Mota, 2006)

The major breakthrough in the field of plant tissue culture has enabled nearly all. MS medium seems to be ideal for many cultures since it has all the nutrients that plants require for growth and contains them in the proper relative ratios (Bejwani and Razdan, 1996). The medium has high macronutrients, sufficient micronutrients, and iron in the slowly available chelated form. The success of tobacco culture using MS medium laid the foundation for future tissue culture work, and this has now become the medium of choice for most tissue culture work. MS medium has been improved on in the past 45 years, but the article by (Murashige and Skoog, 1962) remains one of the most highly cited papers in plant biology.

2.7 Media and Culture Conditions for Plant Tissue Cultures

2.7.1 Basal Media

The success of tissue culture lies in the composition of the growth medium, hormones, and culture conditions such as temperature, pH, light, and humidity (Ziv and Naor , 2006). The growth medium is a composition of essential minerals and vitamins that are

necessary for a plant's growth and development; everything, including sugar, which the plant needs to thrive all, must be in sterile or axenic conditions. The minerals consist of macronutrients such as nitrogen, potassium, phosphorus, calcium, magnesium, and sulfur, and micronutrients such as iron, manganese, zinc, boron, copper, molybdenum, and cobalt (Bradley and White, 2005). Iron is seldom added directly to the medium; it is chelated with EDTA (ethylenediaminetetraacetic acid) so that it is more stable in culture and can be absorbed by plants over a wide pH range. Note that EDTA is used in many foods as a preservative. If iron is not chelated with EDTA, it forms a precipitate, especially in alkaline pH. Vitamins are necessary for the healthy growth of plant cultures. The vitamins used are thiamine (vitamin B1), pyridoxine (B6), nicotinic acid (niacin), and thiamine. Other vitamins such as biotin, folic acid, ascorbic acid (vitamin C), and vitamin E (tocopherol) are sometimes added to media formulations. Myoinositol, a sugar alcohol, is added to most plant culture media to improve the growth of cultures.

In addition, plants require an external carbon source—sugar—since cultures grown *in vitro* rarely photosynthesizes sufficiently to support the tissues' carbon needs. Sometimes cultures are grown in the dark and do not photosynthesize at all. The most commonly used carbon source is sucrose. Other sources used are glucose, maltose, and sorbitol. The pH of the medium is important since it influences the uptake of various components of the medium as well as regulating a wide range of biochemical reactions occurring in plant tissue culture(Murashige and Skoog ,1962).

Optimal growth and morphogenesis of tissues may vary for different plants according to their nutritional requirements.(Murashige and Skoog ,1962) has indicated that moreover, tissues from different parts of plants may also have different requirements for satisfactory growth . Tissue culture media were first developed from nutrient solutions used for culturing whole plants e.g. root culture medium of White and callus culture medium of Gautheret. White's medium was based on Uspenski and Uspenska's medium for algae, Gautheret's medium was based on Knop's salt solution . Basic medium that is frequently used is Murashige and Skoog (MS) medium .

Most media are adjusted to a pH of 5.2–5.8. The acidic pH does not seem to negatively affect plant tissues but delays the growth of many potential contaminants. However, a higher pH is required for certain cultures. Nutrient media occupy a central significance for the success of a cell culture system. Although almost all intact higher plants are able to grow autotrophically in light under normal air conditions and sufficient supply of water and mineral nutrients, this is not the case for all plant organs and tissue.For example, roots or the developing seeds require the import of assimilates from shoot tissue, or phytohormones produced in other, remote tissue to stay alive, function, and grow (Bejwani and Razdan, 1996).

In micropropagation, *in vitro* environmental conditions (i.e., environmental conditions surrounding plantlets within culture vessels such as light conditions, temperature, and gaseous composition), have an important role in plantlet growth. Normally, *in vitro* environmental conditions cannot be controlled directly; instead, they are largely

determined by regulated culture conditions outside the vessel. Therefore, culture conditions should be optimized for plantlet growth. It is necessary for optimization of culture conditions to understand relationships between culture conditions and *in vitro* plant growth, physiological state, or both (Bejwani and Razdan, 1996).*In vitro* environmental conditions may change with plantlet growth during culture because the plantlet itself affects them. Therefore, non-destructive evaluation of the growth of micro propagated plantlets and their physiological state without disturbing the *in vitro* environmental conditions is desirable for investigating these relationships and considering their dynamics (Gupta and Ibaraki, 2006).

Cultures can be grown in either liquid or solid medium. The medium is most often solidified as it provides a support system for the explants and is easier to handle. Explant is the term denoting the starting plant parts used in tissue culture. Solidification is done using agar derived from seaweed or agar substitutes such as Gelrite™ or Phytagel™ commercially available as a variety of gellan gums. These are much clearer than agar. Other than this membrane rafts or filters paper also used for support in liquid medium.

A plethora of media formulations are available for plant tissue culture other than MS (Murashige and Skoog, 1962).They are also used Gamborg (1968); Schenk and Nitsch and Nitsch (1969).Knudson's medium Knudson (1946) was developed for orchid tissue culture and is also used for fern tissue culture. With so many choices in media formulations, one might wonder about how to choose a medium to culture the species of interest. The choice of medium is typically determined empirically for optimal response

of the plant species; explants used for culture and plant taxonomy are good starting points. For example, nearly all tissue cultures of plants in the Solanaceae (the nightshade family) use MS media. The composition of nutrients varies from medium to medium. for example, ms medium has higher macronutrients than does wood Plant Medium (WPM), which is suitable for most plant species, but woody plants respond better in WPM than MS medium. It is important to select the right medium for culture according to how the plant empirically responds in tissue culture. Hildebrandt, (1972); White (1963); Linsmaier and Skoog(1965); MCCown and Lloyd (1981). has been widely used for tree tissue culture.

2.7 .2 Growth Regulators

Plant hormones (phytohormones) are small organic molecules that affect diverse developmental processes. Alterations in hormone responses have been responsible for several important agricultural advances, such as the breeding of semi-dwarf varieties and increased grain production (Ashikari et al., 2005; Silverstone and Sun, 2000).Virtually every aspect of plant development from embryogenesis to senescence is under hormonal control. In general, this developmental control is exerted by controlling cell division, expansion, differentiation and cell death. In this manner, diverse developmental processes can be controlled, including formation of the apical-basal and radial pattern, seed germination and shedding of leaves.

The basal medium (e.g., Murashige and Skoog) is designed to keep plant tissues alive and thriving. Plant growth regulators or hormones are needed to manipulate the

developmental program of tissues—say, to make callus tissue proliferate, or produce roots from shoots (Ali *et al.*,2007). Growth regulators are the items most often manipulated as experimental factors to enhance tissue culture conditions.

The most important growth regulators for tissue culture are auxins, cytokinins, and gibberellins. Both natural and synthetic auxins and cytokinins are used in tissue culture. Auxins promote cell growth and root growth (Stewart, 2008; Verma *et al.*,2011) ;Auxins are an important factor involved in rooting because they promote adventitious root formation in the vast majority of species (De Klerk, 2002). They also acts to promote cell division and growth, they do so mainly by increasing the expression of genes that encode required proteins for these processes. Thus, researchers have sought to understand the steps between auxin perception and the final gene expression regulation. The most commonly used auxins are IAA (indoleacetic acid), IBA (indole butyric acid), NAA (naphthalene acetic acid), and 2, 4-D (2, 4 dichlorophenoxy acetic acid). Cytokinins promote cell division and shoot growth (Silva , 2010). Similarly , (Sunitibala and Kishor , 2009) also reported that BAP showed best response for multiple shoot induction of *Dendrobium transpare L.gv*. (Baque *et al.*,2010) reported as IBA and NAA showed differential effects on adventitious root induction from leaf explants of M. *citrifolia*. **(**Louerguioui *et al*.,2011).Both the concentration and type of auxins used markedly influenced the percentage of root formation.

An auxin like compound TDZ (thidiazuron) has increased success rate of plant regeneration in many species (Lack and Evan, 2005) (Mok *et al*., 1987). Huettman and

Preece ,1993) described TDZ However, according to (Mok *et al*. 1987) the reason for the high activity of low concentrations of TDZ in woody plant . In general, TDZ increases shoot formation of many woody plant species more efficiently than purine adenine derivatives, but it is ineffective for the proliferation in some species (Huntsman and Preece,1993). TDZ at used concentrations has not induced expected rise of multiplication level in cv. Lapin On the other hand, TDZ may inhibit shoot elongation (Huetteman, Preece 1993) andit induces formation of shortened internodes in apple cv. (van Nieuwkerk *et al*., 1986). In the present work, TDZ has also an effect on reduction of multipliedshoot length of cv. Lapins, especially with the concentrations of 5–15μM. The results obtained with *L. corniculatus* shoots seedling showed that even in the lowest concentrations (0.08 and 0.22μM), TDZ retarded elongation (Nikolić *et al*. 2006).

The most commonly used cytokinins are BAP (benzylaminopurine), zeatin, and kinetin. In addition to auxins and cytokines, other hormones such as abscisic acid (Decoteau, 2005) and Stewart, 2008) and jasmonic acid have also been used in plant cell culture. Critical aspect in plant tissue cultures is the management of the gaseous hormone ethylene. When plants are grown *in vitro* in closed culture vessels, there is a buildup of ethylene, which is typically detrimental to the cultures. The addition of ethylene biosynthetic inhibitors such as silver nitrate (Giridhar *et al*.,2001),AVG (aminoethoxyvinylglycine), and silver thiosulphate (Reis *et al*,.2003) have been shown to increase the formation of shoots. Tissues are transferred to fresh media periodically—every week to monthly, depending on the species and experiment. Without sub culturing,

tissues will deplete the media and often crowd each other, competing for decreasing resources (Cutler and Bonita, 2009).

When a small portion of a plant is isolated, it is no longer able to receive nutrients or hormones from the plant, and these must be provided to allow growth *in vitro (*Loriz and Nagata, 2010). In addition, the culture must be provided with the ability to excrete the waste products of cell metabolism. This is accomplished by culturing on or in a defined culture medium. The medium must be periodically replenished (Saad and Elshahed, 2012). A controlled environment is needed for the growth of cells *in vitro*. Tissue cultures, sustained by the nutritive medium and confined in a protective vessel, require a stable and suitable climate. Thus, light and temperature must be more carefully regulated than in the case of the whole plant (Faeroe, 2009).

2.8 Sterile Techniques

2.8 .1 Clean Equipment

Successful tissue culture requires the maintenance of a sterile environment. All tissue culture work is done in a laminar flow hood. The laminar flow hood filters air with a dust filter and a high-efficiency particulate air (HEPA) filter (Gupta and Ibaraki, 2006). It is important to keep the hood clean, which can be done by wiping it with 70% alcohol. The instruments used should also be dipped in 70% ethanol and sterilized using flame or glass beads. Hands should be disinfected with ethanol before handling cultures in order to avoid contamination. It is imperative to maintain axenic conditions throughout the life of cultures: from explants to the production of whole plants. Entire experiments have been

lost because of an episode of fungal or bacterial contamination at any stage of culture (Cutler and Bonita, 2009). Especially problematic are fungal contaminants that are propagated by spores that might blow into a hood from an environmental source. Therefore, it is important to work away from the unsterile edge of a laminar flow hood. Culture rooms or chambers must be maintained as clean as possible to control any airborne contaminants (Saad and Elshahed, 2012.)

2.8.2 Surface Sterilization of Explants

Sterilization is the process of making explants contamination free before establishment of cultures. Various sterilization agents are used to decontaminate the tissues. These sterilants are also toxic to the plant tissues, hence proper concentration of sterilants, duration of exposing the explant to the various sterilants, the sequences of using these sterilants has to be standardized to minimize explant injury and achieve better survival (CPRI, 1992).

Plant tissues inherently have various bacteria and fungi on their surfaces. It is important that the explants be devoid of any surface contaminants prior to tissue culture since contaminants can grow in the culture medium, rendering the culture non sterile. In addition, they compete with the plant tissue for nutrition, thus depriving the plant tissue of nutrients. Bacteria and especially fungi can rapidly overtake plant . Some researchers (Badoni and Chauh ,2010)showed that amongst the two sterilants i.e. NaOCl and HgCl2, NaOCl was found better for controlling the infection and it had not any adverse effect on explants even in long duration .

The surface sterility chosen for an experiment typically depend on the type of explants and also plant species **(**Rezadost *et a.l,*2013).Explants are commonly surface-sterilized using sodium hypochlorite (household bleach), ethanol, and fungicides when using field-grown tissues. Some researchers (Badoni and Chauh ,2010) showed that amongst the two sterilants i.e. NaOCl and HgCl2, NaOCl was found better for controlling the infection and it had not any adverse effect on explants even in long duration,

The time of sterilization is dependent on the type of tissue; for example, leaf tissue will require a shorter sterilization time than will seeds with a tough seed coat (Funguomali *et al.*,2013, Sharma and Nautiya, 2009). Wetting agents such as Tween added to the sterilant can improve surface contact with the tissue. Although surface contamination can be eliminated by sterilization, it is very difficult to remove contaminants that are present inside the explants that may show up at a later stage in culture (Olew *et al.*,2014). This internal contamination can be controlled to a certain extent by frequent transfer to fresh medium or by the use of a low concentration of antibiotics in the medium. Overexposing tissues to decontaminating chemicals can also kill tissues, so there is a balancing act between sterilizing explants and killing the explants themselves (Qin *et a.l,* 2012 and Olew *et al.*,2014).

2.9 Culture Conditions and Vessels

Cultures are grown in walk-in growth rooms or growth chambers. Humidity, light, and temperature have to be controlled for proper growth of cultures. A light intensity of 25–50 mmol m22 s21 is typical for tissue cultures and is supplied by cool white fluorescent

lamps. A relative humidity of 50–60% is maintained in the growth chambers. Some cultures are also incubated in the dark. Cultures can be grown in various kinds of vessels such as Petri plates, test tubes, "Magenta boxes," bottles, and flask (Gupta and Ibaraki, 2006).

2.9.1 Culture types and their uses

2.9.1.1 Callus Culture

Callus is an unorganized mass of cells that develops when cells are wounded and is very useful for many *in vitro* cultures. Callus is developed when the explant is cultured on media conducive to undifferentiated cell production—usually the absence of organogenesis (organ production) can lead to callus proliferation (Mungole *et al.*, 2011) .Stated another way, callus production often leads to organogenesis, but once callus begins to form organs, callus production is halted. Auxins and cytokinins both aid in the formation of most callus cells (Ali *et al.*, 2007).Callus can be continuously proliferated using plant growth hormones or then directed to form organs or somatic embryos . Callus cultures can be transferred to a new medium for organogenesis or embryogenesis or sadder as callus in culture.

2.9.1.2 Plant Regeneration from Different Organs

Differentiated plant organs can usually be grown in culture without loss of integrity. They can be of two types: Determinate organs which are destined to have only a defined size and shape (e.g. leaves, flowers and fruits) (Hopkins, 2007); indeterminate organs, where growth is potentially unlimited (apical meristems of roots and non flowering

shoots). In the past, it has been thought that the meristematic cells within root or shoot apices were not committed to a particular kind of development. It is now accepted that, like the primordial of determinate organs such as leaves, apical meristems also become inherently programmed (or determined) into either root or shoot pathways (Kayser and Quax, 2007) .The eventual pattern of development of both indeterminate and determinate organs is often established at a very early stage. For example, the meristematic protrusions in a shoot apex become programmed to develop as either lateral buds or leaves after only a few cell divisions have taken place (Nain, 2008).

2.9.1.3 Explants Culture

Explants culture is actually the tissue culture. Culturing of any excised tissue or plant parts such as leaf tissue, stem parts, cotyledon, hypocotyls, root parts, etc., is called explants culture. The primary purpose of explants culturing is to induce callus cultures or to regenerate whole plantlets directly from it without the formation of callus. Shoot apical meristemetic culture is an example, and its important uses are the following; Production of virus-free germplasm or plantlets, mass production of desirable genotypes, facilitation of exchange between locations (production of clean material), and cryopreservation (cold storage) or *in vitro* conservation of germplasm, etc., are the main purposes of meristem or shoot apex culture.

CHAPTER THREE

3.0 Materials and Methods

3.1 Plant Materials

The Anchote root samples (12kg) of roots were bought from the market in Sibu Sire, West Ethiopia and transported to Kenya where they were grown in a green house, at Jomo Kenyatta University of Agriculture and Technology, Juja. The nutritional analysis were conducted at the Food biochemistry while the tissue culture studies were carried out at the Institute of Biotechnology Research (IBR) laboratory of Jomo Kenyatta university of agriculture and technology (Kenya).

Plate 3.1:Anchote (*Coccinia abyssinica*) grown in net greenhouse

3.2 Study Site and Research Design

Anchote (*Coccinia abyssinica*) roots were collected from West Ethiopia transported to Kenya where they were grown in the greenhouse in Juja. Leaves (for callus induction) and nodes (for microshoot and root regeneration) were harvested from the seedlings and used for all the experiments. The research design summarized as the following:

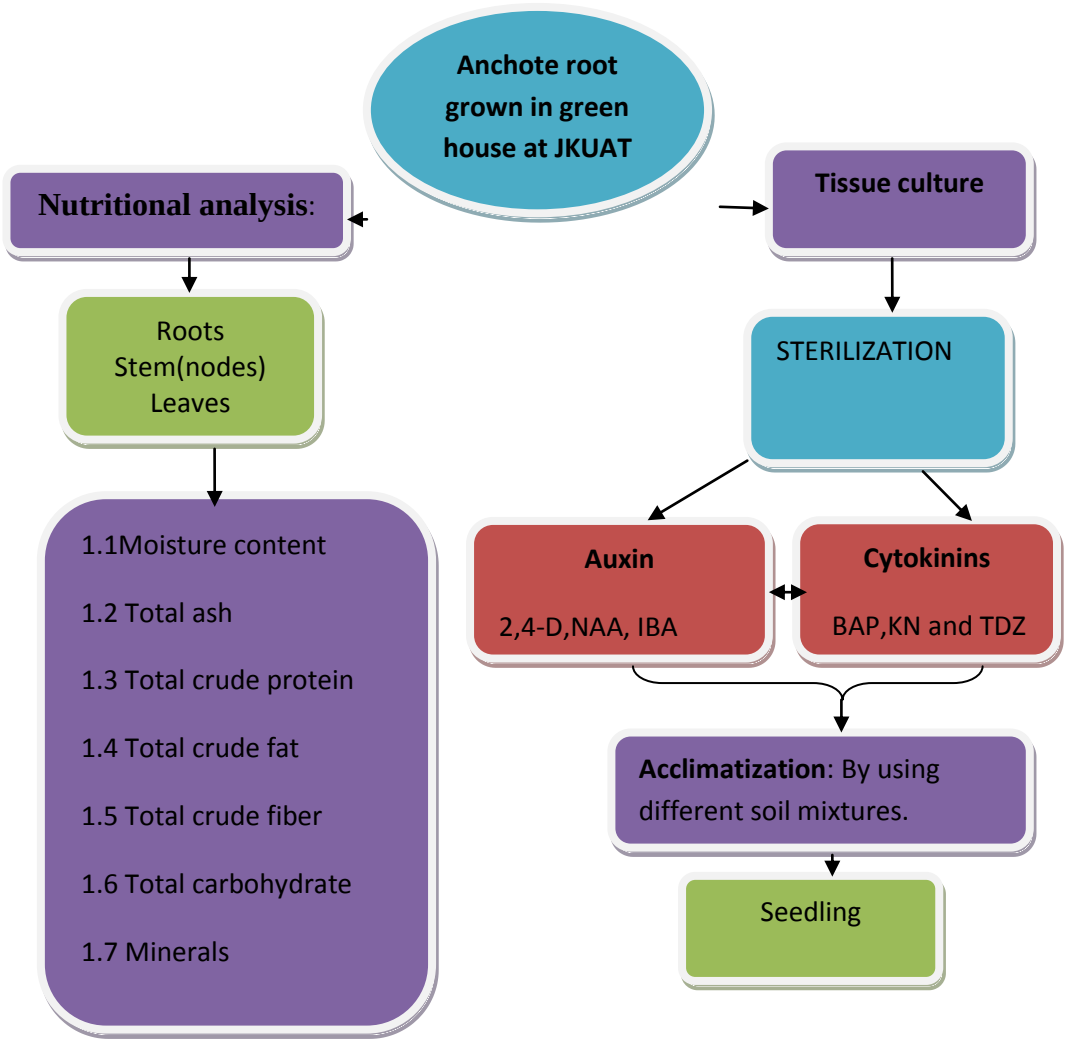


Figure 3. 1 A summary of the research design

3.3 Nutritional Parameters

3.3. 1 Sample Collection and Preparation

Fresh roots, stems and leaves of *C.abyssinica were* collected from three month old Anchote growing in the greenhouse at Juja and taken to the Food biochemistry Laboratory where they kept in cold room at 4 ^{0}C. The samples were thoroughly washed and rinsed with distilled water before being dried in the oven at 40°C. The dried samples were then ground into fine powder and stored in fresh plastic polythene bags for analysis.

3.3.2 Reagent and Solutions

All the chemicals and reagents used in this study were analytical grade. The following reagents and material were used :Copper sulfate penta hydrate ($CuSO_4.5H_2O$), Potassium sulfate(K_2SO_4), Sulfuric acid(H_2SO_4),Boric acid (H_3BO_3),Methyl red ($C_{15}H_{15}N_3O_2$), Methyl blue ($C_{16}H_{18}CIN_3S.3H_2O$), Bromcresol green ($C_{21}H_{14}Br_4O_5S$) ,Sodium hydroxide (NaOH) and Ethanol (C2H6O),95% (v/v).

3.3.3 Proximate Analysis

3.3.3.1 Moisture Determination

Moisture contents was estimated by drying the samples in an oven at 105±5°C till constant weight (AACC, 2000; Method No. 44-15.

$$\%\text{Moisture} = \frac{\text{weight of fresh samples} + \text{dish before drying} - \text{weight of dry samples} + \text{dish}}{Wt\ of\ dry\ samples} * 100$$

3.3.3.2 Determination of total Crude Fat

The crude fat of leaves, stems and roots was determined using hexane as a solvent in Soxhlec System according to the procedure given in AACC (2000) Method No. 30-25. About 150ml of an anhydrous diethyl ether (petroleum ether) was boiled point of 40-60ºC and placed in the flask. Five grams of the sample were weighed into a thimble and then thimble was plugged with cotton wool. The thimble with content was placed into the extractor; the ether in the flask was then heated. As the ether vapour reaches the condenser through the side arm of the extractor, it condenses to liquid form and drop back into the sample in the thimble, the ether soluble substances are dissolved and are carried into solution through the siphon tube back into the flask. The extraction continues for at least 4 hours. The thimble was removed and most of the solvent was distilled from the flask into the extractor. The flask was then disconnected and placed in an oven at 65ºC for 4 hours, cool in desiccator and weighed.

$$\%\text{Crude fat} = \frac{\text{weight of flask} + \text{extract} - \text{Weight of flask}}{\text{wt of sample}}$$

3.3.3.3 Determination of total Crude Fiber

Crude fiber of the samples was determined following the procedure in AACC (2000) Method No. 32-10.Crude fiber was estimated in fat free samples by treating with 1.25% sulfuric acid (H_2SO_4). Left over material was subjected to a further treatment with 1.25% Sodium hydroxide(NaOH)solutions. The solution was gently boiled for about 30mins, maintaining constant volume of acid by the addition of hot water. The Buckner flask

funnel fitted with whatman filter was pre-heated by pouring hot water into the funnel. The boiled acid sample mixture was then filtered hot through the funnel under sufficient suction. The residue was then washed several times with boiling water (until the residue was neutral to litmus paper) and transferred back into the beaker. Then 200 ml of pre-heated 1.25%Sodium sulfate ($Na_2SO_{4)}$ was added and boiled for another 30 min. Then it filtered under suction and washed thoroughly with hot water and twice with ethanol. The residue was dried at 65ºC for about 24hr and weighed. The residue was transferred into a crucible and placed in muffle furnace (400-600ºC) and ash for 4hours, then cooled in desiccator and weighed.

$$\%\text{Crude fibre } = \frac{\text{Dry wt of residue before ashing } - \text{ wt of residue after ashing}}{wt\ of\ sample} * 100$$

3.3.3.4 Determination Crude Protein

Crude protein was determined by kjeldahl method as described in AACC (2000) Method No. 46-30. The method involves: Digestion, Distillation and Titration.

Digestion: about 5g of the sample was weighed into kjeldahl flask and 25ml of concentrated sulphuric acid, 0.5g of copper sulphate, 5g of sodium sulphate and a speck of selenium tablet was added. Heat was applied in a fume cupboard slowly at first to prevent undue frothing, digestion continued for 45mins until the digesta became clear pale green. The digest was left until it cooled completely and 100mls of distilled water was rapidly added. The digestion flask was rinsed 2-3 times and the rinsing added to the bulk.

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

Titration: The alkaline ammonium borate formed was titrated directly with 0.1NHydrochloric acid (HCl). The titre value which is the volume of acid used was then recorded. The volume of acid used was fitted into the following formula.

$$\%\mathrm{N} = \frac{\{14 \text{ x VA x } 0.1 \text{ x w }\} * 100}{1000 \text{ x}100}$$

VA = volume of acid used w= weight of sample

$$\%\text{crude protein} = \%\mathrm{N} * 6.25$$

3.3.3.5 Determination of Total Ash

Ash was estimated by direct incineration of sample; igniting it in a muffle furnace at 550°C till grayish white residue (AACC, 2000; Method No. 08-01). The crucible was then placed in the desiccator and weighed. The following formula was used to calculate ash value.

$$\%\ \mathbf{Ash} = \frac{\mathbf{wt\ of\ crucible + ash - wt\ of\ crucible}}{\boldsymbol{wt\ of\ sample}} \mathbf{*100}$$

3.3.3.6 Determination of Total Carbohydrates

The fraction of carbohydrate was calculated AACC(2000): for each triplicates samples from total crude fat, protein ,moisture content, crude fiber and total ashes which were determined previously using the following formula:

% Carbohydrate= [1- (Total crude fat+ protein+ fiber+ moisture+ total ash)] x100

3.3.3.7 Minerals Analysis

The mineral elements comprising ,potassium, sodium ,copper, Manganese, phosphorous, iron, calcium, magnesium, and zinc were determined according to Adeyeye and Omotayo (2011) with slight modifications. For each of the sample (2g) was weighed and subjected to dry ashing in a well cleaned porcelain crucible at $550^{o}C$ in a muffle furnace. The resultant ash was dissolved in 5.0 ml of HNO_3/ HCl /H_2O (1:2:3) and heated gently on a hot plate until brown fumes disappeared. To the remaining material in each crucible, 5.0 ml of de – ionized water was added and heated until a colorless solution was obtained. The mineral solution in each crucible was transferred into a 100.0 ml volumetric flask by filtration through Whatman filter paper and the volume was made to the mark with de – ionized water. This solution was used for elemental analysis by atomic absorption spectrophotometer.

3.3.3.8 Experimental Design, Data Collection and Statistical Analysis

Samples were analyzed in triplicates .Results were expressed as mean ± Standard DEVIATION(SD). The differences between the different nutritional parameters was

determined by performing a one way analysis of variance (ANOVA).The mean differences were separated using the Student-Neuman -keul multiple comparison test.

3.4 Tissue Culture

3. 4.1 Media Preparation

The Murashige and Skoog (1962) media was used for all the experiments (Appendix -1). Media was prepared by dissolving the organic and inorganic components in distilled water. The solution was stirred until dissolved and made up to final volume. The media pH was adjusted to between 5.7 and 5.8 by using either 1N HCl or 1N NaOH before the gelling agent (2.8g/l gelrite) was added. Media were then heated on a hot plate with continuous stirring using a magnetic stirrer until gel rite was dissolved and media dispensed in the culture vessels. The culture vessels were capped with lids and placed in trays and autoclaved. Autoclave was set at a temperature of $121^{o}C$ and a pressure of $1.1kg/cm^2$ for 20 minutes. All media was autoclaved within 12 hours of preparation and when possible freshly autoclaved media was used. However, when it was not possible to use the media immediately it was stored in a refrigerator at $4^{o}C$ for no longer than two weeks before use. Medium without growth regulators will used as a control.

3.4.2 Plant Growth Regulators

Plant growth regulators were weighed and stocks were prepared as the following; 0.5mg/ml 6Benzylaminopurine,0.5mg/mlThiadazuron(TDZ),1mg/mlKinetin(Kn),1Mg/ml Naphtylacetic acid acid(2,4-D) clearly labeled and stored in the refrigerator at

4^{o}C(Appendex-2). The volume of hormones required were calculated as the following.

$\mathbf{C_1V_1=C_2V_2}$ where :

$\mathbf{C_1}$=Concentration hormonal stock solution.

$\mathbf{v_1}$=Volume of hormonal stock solution required

$\mathbf{C_2}$=Desired hormone concentration.

$\mathbf{V_2}$=desired volume of medium.

However, the concentration of each hormonal stock solution was calculated by using molecular weight of respective hormones as the following:

$\frac{1M}{c1} = \frac{xg/l}{yg/l}$ where: x= is the molecular weight of the hormone in g/l.

y=mass of the hormone prepared as the stock solution per liter.

1M=1molar of the hormone.

BAP, NAA and 2,4-D were evaluated for callus induction at 0,5,10,20 and 40µM levels. BAP and KN were evaluated for direct regeneration at 0.1,0.2,0.3,0.5,1,1.5,2.5,5,10, 20 and 40 µM levels. Thidiazuron (TDZ) were evaluated at 0.01,0.025,0.05, 0.1, 0.5, 1, and 1.5 µM .Rooting of the regenerated microshoot was assessed using various concentration of NAA ,2,4-D and IBA at 0,0.1,0.2,0.3,0.5 and 1,2 µM levels. Medium without growth regulators were used as a control.

3.4.3 Aseptic Techniques

The process of sterilization and dissection of plant materials was carried out under sterile conditions in lamina flow cabinet. The cabinet was switched on and swabbed down with 70% ethanol using cotton wool or sterile towel and kept running for about 15 minutes before the work in the cabinet starts. All the plant materials were dissected on sterile papers. The lamina flow cabinet was frequently swabbed down with 70% alcohol. Hands were sprayed with 70% ethanol at suitable intervals while working for protracted periods in front of the cabinets. Personal hygienic precautions were observed by wearing a clean lab coat and gloves while working in the lamina flow cabinet.

3.4.4 Dissecting Tools

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

3.4.5 Washing of Glassware and Vessels

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

3.4.6 Surface Sterilization of Explants

Leaf and nodal explants were harvested and transported from the greenhouse in a beaker containing tap water to the laboratory. Once in the laboratory, they were cleaned with liquid soap and cotton wool and kept under running tap water for 2 hours. They were then dipped in 0.5% fungicide (Ridomil) for 1 hour. The explants were then transferred to the lamina flow cabinet, immersed in 70% (v/v) ethanol for 30 seconds and rinsed twice with sterile distilled water. The sterilization was carried out using JIK® which contains 0.2% NaOCl and 0.385% NaOCl for different time intervals 5, 10 and 15 minutes. They were then rinsed four times in sterile distilled water.

3.4.7 Incubation Conditions

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

3.4.8 Acclimatization

Four-week-old rooted micro-shoots transplanted into the plastic pots containing peat moss and perlite (3:1) and placed under shade to maintain the humidity. The plantlets were acclimatized in a controlled greenhouse condition at 24ºC under 16/8 hour (light/dark) photoperiod and irrigated regularly.

3.4.9 Experimental Design, Data Collection and Analysis

The experiments were laid out in completely randomized design (CRD) with each treatment repeated at least two times. Ten replicates per treatment were used at the outset of tissue culture experiments with five explants per treatments .The level of replication used per treatment combination varied depending upon the availability of experimental materials. For the sterilization experiment, percent clean explants were recorded. This was calculated as total number of contaminated explants / total number of explants*100. For the callus induction, data on percentage callus forming cultures (calculated as (total number of explants with callus / total number of cultured explants*100) were recorded. For the micropropagation experiment, percentage of explants that produced shoots, the number of microshoots and their lengths were recorded. All the data were subjected to one-way analysis of variance (ANOVA) and the significant differences between treatment means were assessed by using INSTAT GRAPH PAD Software. The results were expressed as percentage (%) and mean ± standard deviation (SD).

CHAPTER FOUR

4. 0 RESULT

4.1 Nutritional Properties of Anchote

4.1.1 Nutritional Composition

As shown in Table 4.1 the Anchote plant parts showed significant differences in nutritional composition ($p<0.05$) on dry weight basis. Anchote roots had significantly higher percent moisture (24.7±0.5%) and carbohydrate contents (46.9 ±1%). The leaves were significantly richer in protein content compared to the other parts, while the Anchote stems had significantly higher ash and fiber content (13.0±0.4% and 27.7±0.4%) respectively. However, the Anchote leaves and stems showed no significant differences in percentage of fat and carbohydrate content.

Table 4. 1 Mean percentage of nutritional composition of leaves, roots and stems of Anchote (*Coccinia abyssinica)for dry samples.*

Parameters	Leaf	Root	Stem
Moisture(Mean±SD)	21.4 ± 0.4^{b}	24.7 ± 0.5^{a}	15.7 ± 0.7^{c}
Protein%(Mean±SD)	18.3 ± 1^{a}	8.2 ± 0.1^{c}	12.4 ± 0.5^{b}
Fat%(Mean±SD)	4.3 ± 0.1^{a}	2.4 ± 0.2^{b}	3.0 ± 0.1^{b}
Fiber%(Mean±SD)	16.1 ± 0.8^{b}	12.4 ± 0.4^{c}	27.7 ± 0.4^{a}
Total Ash%(Mean±SD)	10.2 ± 0.4^{b}	5.4 ± 0.2^{c}	13.0 ± 0.4^{a}
Carbohydrate%(Mean±SD)	29.5 ± 0.7^{b}	46.9 ± 1^{a}	28.2 ± 1^{b}

The means followed by the same letters along the rows are not significantly different from each other ($p < 0.05$) Student-Newman-Keuls Multiple Comparisons Tests , SD=standard deviation. Results are mean of triplicate determinations .

Mean percentage of nutritional composition of leaves, roots and stems of Anchote (*Coccinia abyssinica)for fresh wet based samples g/100.*

PARAMETRE	**LEAF**	**ROOT**	**STEM**
MOISTURE	78.6	75.3	84.3
PROTEIN	4.5	2.7	2.2
Fat	1.2	0.2	0.69
Fiber	4.4	4.04	5.15
Total ash	2.8	1.8	2.42
Carbohydrate	8.03	15.68	5.24

4.1.2 Minerals

The minerals composition of Anchote parts are presented in Table 4.2. Among the three parts, the stem was the richest by minerals followed by leaves and roots respectively. The highest mineral element found in the stem was Magnesium (Mg) with mean average

336±0.4 Mg/100 followed by potassium(K)which was found with mean average 323±3 Mg/100 and Calcium(Ca) was found with mean average of 313.5±1 Mg/100.According to our current result the highest mean average (76.9±0.9) Mg/100 of Phosphorous was observed in stems followed by leaves (71.5±2 Mg/100 but the root had the lowest mean average (54.1± 0.8)Mg/100 phosphorous. Generally, the result showed that most minerals are concentrated in the stem followed by leaves except for Copper (Cu) which was observed in all parts in small amounts.

Table 4. 2 Mean percent mineral composition of leaf, root and stem of Anchote (C. *abyssinica*) in Mg/100g.

Parameters	Leaf	Root	Stem
Ca(Mean±SD)	303.8 ± 0.4^{b}	117.1 ± 0.9^{c}	313.5 ± 1^{a}
K(Mean±SD)	283.3 ± 2.0^{b}	192.1 ± 2^{c}	323 ± 3^{a}
Na(Mean±SD)	17.2 ± 0.2^{b}	13.9 ± 0.2^{c}	23.2 ± 0.1^{a}
Mg(Mean±SD)	328.1 ± 0.6^{b}	157.8 ± 0.4^{c}	336 ± 0.04^{a}
P(Mean±SD)	71.5 ± 2^{b}	54.1 ± 0.8^{c}	76.9 ± 0.9^{a}
Zn(Mean±SD)	106.1 ± 0.7^{b}	106.3 ± 0.5^{b}	148.4 ± 0.4^{a}
Fe(Mean±SD)	6.7 ± 0.1^{a}	3.5 ± 0.1^{b}	8.6 ± 0.3^{a}
Mn(Mean±SD)	295.8 ± 0.2^{a}	137.9 ± 0.2^{b}	174.7 ± 0.2^{c}
Cu(Mean±SD)	0.9 ± 0.1^{a}	$0.8\pm0.0.1^{a}$	1.7 ± 0.1^{a}

The means followed by the same letters along the row are not significantly different from each other(α=0.05) Student-Newman-Keuls Multiple Comparisons Tests , SD= Standard Deviation. Results are mean of triplicate determinations .

4.2.1 Effect of Concentration Of Commercial Bleach(JIK®) and Exposure Time on Sterilization

4.2.1.1 Leaf Explants

The mean percentage of clean surviving explants showed that the effectiveness of the sterilization procedure increases with increase in concentration of commercial bleach and exposure time. A concentration of 0.19% commercial bleach and an exposure time of 10 minutes gave significantly higher percentage surviving clean explants (82.5±0.6%) than the other concentration and exposure times (Table 4.3). Further increase in exposure time led to a significant decline in percentage clean surviving explants. A shorter exposure time resulted in explants death due to microbial contamination, while at prolonged exposure time death was due to scorching by the sterilants. However, as indicated in the table, at a concentration of 0.385% commercial bleach, there was no significant difference in mean percentage clean surviving explants among the various exposure times. Death was noted to be mainly due to scorching by the commercial bleach.

Table4. 3:Effect of various concentrations of Commercial Bleach (3.85%NaOCl) and time exposure on leaf explants sterilization of Anchote**.**

Treatments		**M%ODE**	**M%OFC**	**M%OBCE**	**M%OCSE**
NaOCl %	**Time**	**±SD**	**±SD**	**±SD**	**± SD**
0	5	3 ± 0.6^{f}	42 ± 0.5^{a}	30 ± 0.4^{a}	25 ± 0.9^{e}
0.19	5	4 ± 0.2^{e}	14 ± 0.1^{b}	10.6 ± 0.7^{b}	71 ± 0.3^{bcd}
0.19	10	6.3 ± 0.2^{d}	5.0 ± 0.3^{c}	6.2 ± 0.3^{c}	82.5 ± 0.6^{a}
0.19	15	$21.5\pm1b^{c}$	2.7 ± 0.5^{d}	1.5 ± 1^{c}	74.3 ± 0.6^{bc}
0.385	5	23.1 ± 0.5^{b}	3.6 ± 0.6^{c}	5.2±0.2c	68.0 ± 0.7^{cd}
0.385	10	25.5 ± 0.6^{b}	2.5 ± 0.5^{c}	2.6 ± 0.2^{c}	69.6 ± 0.7^{cd}
0.385	15	30.8 ± 0.9^{a}	1.1 ± 0.3^{c}	1.0 ± 0^{c}	67.4 ± 1^{d}

The means followed by the same letters along the columns are not significantly different from each other(P-Value ≤0.05) Student-Newman-Keuls Multiple Comparisons Tests , SD= Standard **Key**: M%ODE=Mean percentage of dead explants, M%OFCE= Mean percentage of fungal contamination explants, M%OBCE= Mean percentage of bacterial contamination explants. M%OCSE = Mean percentage of Cleaning surviving explants.

4.2.1.2 Nodal Explants

As shown in Table 4.4 a concentration of 0.19% commercial bleach and an exposure time of 15 minutes gave significantly higher percentage surviving clean explants (84.5±0.6)

than the other levels and exposure times . Further increase in exposure time led to a significant decline in percentage clean surviving nodal explants. At shorter exposure time explant death was due to microbial contamination, while at prolonged exposure time death was due to scorching by the sterilants.

Table 4.4:Effect of various concentrations of Commercial Bleach(3.85%NaOCl) with different time exposure on nodal explants sterilization of Anchote(C. *abyssinica*)**.**

Treatments		M%ODE±SD	M%OFCE±SD	M%OBCE ±SD	M%OCSE ±SD
NaOCl%	TIME				
0	5	2.5 ± 0.4^{f}	46.5 ± 4^{a}	37.5 ± 3^{a}	16.0 ± 0.1^{e}
0.19	5	4.0 ± 0.6^{e}	18.2 ± 0.8^{b}	16.5 ± 2^{b}	63.9 ± 1.0^{d}
0.19	10	6.8 ± 0.3^{c}	8.5 ± 0.6^{c}	7.7 ± 0.5^{c}	76.7 ± 0.4^{b}
0.19	15	9.3 ± 0.6^{c}	3.2 ± 0.3^{d}	3.0 ± 0.3^{d}	$84.5.0\pm0.5^{a}$
0.385	5	21.5 ± 1^{b}	15.9 ± 2^{c}	10.9 ± 2^{c}	67.3 ± 0.7^{c}
0.385	10	23.7 ± 1^{b}	3.1 ± 0.2^{d}	3.1 ± 0.9^{d}	72.4 ± 1.0^{c}
0.385	15	32.2 ± 1^{a}	2 ± 0.2^{d}	0 ± 0^{e}	68.8 ± 0.8^{c}

The means followed by the same letters along the column are not significantly different from each other($p<0.05$) Student-Newman-Keuls Multiple Comparisons Tests , SD= Standard One-way Analysis of Variance (ANOVA. SD= Standard Deviation, M%ODE=Mean percentage of died explants, M%OFCE= Mean percentage of fugal contaminated explants, M%OBCE= Mean percentage of bacterial contaminated explants.

4.2.2 Effect of Hormone Concentration on Callus Induction

In our present investigation calli induction were directly initiated from young nodal and leaf explants of Anchote (*Coccinia abyssinica)* after successful sterilization.

The young leaf explants were transferred to fresh MS medium supplemented with different concentration of auxins and cytokinins including (2,4-D, BAP, NAA) of which the most effective one's are given in (Table 4-5 and Table 4-6). On MS basal medium supplemented with 2,4-D (5µM) leaf explants showed callus initiation after 12^{th} day of inoculation with mean percentage 65±3 of the cultures. The calli were formed at basal cut portion of leaf explants. On MS basal fortified with 5µM NAA and 5µM 2,4-D, the leaf explants exhibited callus initiation with mean percentage of 76±3 cultures after to 12^{th} days of inoculation (Table4.6 and Plate4.1B). For this particular concentration the calli increased in size in the same media composition up to 4 weeks of inoculation for leaf explants.

MS medium supplemented with BAP and 2,4-D in combination or alone induced callus from leaf explants in different cultures after 12^{th} day of inoculation as shown in (Table4-5). On other hand , MS medium supplemented with BAP and 2,4-D , the percentage of callusing increased with an increase in concentration up to 5 µM and then declined with further increase in the concentration of 20µM BAP and 40µM 2,4-D. This gave minimum mean percentage (67±3)callusing and it was significantly different at $P \leq 0.05$.The optimum concentration of BAP and 2,4-D in terms of maximum mean percentage of

callusing (80±2) was observed from leaf explants at 5.0 µM when BAP and 2,4-D were used in combination (Table 4.5 and Plate 4.1A).

Table 4. 5: Effect of various concentrations of BAP and 2,4-D on callus induction from leaf explants of Anchote(*Cocinia abyssinica)*

Hormones concentration in µM		**percentage of callus responded**
BAP	**2,4-D**	**(Mean ±SD)**
0	0	0.0 ± 0.0^{e}
0	5	65 ± 3.0^{c}
5	5	80 ± 2^{a}
5	10	77 ± 2^{ab}
5	20	73 ± 2^{b}
0	10	54.3 ± 3^{d}
20	40	67 ± 3^{c}

One-way Analysis of Variance (ANOVA .The means followed by the same letters among the column are not significantly different from each other, Student-Newman-Keuls Multiple Comparisons Tests,p-Value≤0.05 ,SD= Standard Deviation .

Table 4. 5: Effect of various concentrations of NAA and 2,4-D on callus induction from leaf explants of Anchote(*Cocinia abyssinica).*

Hormones concentration in µM		**percentage of callus responded (Mean ±SD)**
NAA	**2,4-D**	
0	0	0.0 ± 0^{e}
0	5	65.0 ± 3^{c}
5	5	76.0 ± 3^{a}
5	10	71.4 ± 2^{ab}
5	20	69.5±2b
10	0	43.9 ± 2^{d}
0	40	46.4 ± 1^{d}
20	40	61.3 ± 2^{c}

One-way Analysis of Variance (ANOVA .The means followed by the same letters among the column are not significantly different from each other, Student-Newman-Keuls Multiple Comparisons Tests ,p-Value≤0.05 ,SD= Standard Deviation .

Similarly, On MS basal media fortified with 5 µM BAP and 5µM 2,4-D, the nodal explants exhibited callus initiation with highest mean percentage of 78±2 cultures after 12 days of inoculation. The callus responding also increased in size in the same media composition up to 4 weeks of inoculation (Table4.7 and Plate4.1C). MS basal medium supplemented with NAA and 2,4-D induced callus in different cultures after 13th day of inoculation .The highest mean percentage (65.5±2) callus was obtained from the media fortified with 5 µM (NAA and 2,4-D) for nodal explantsTable4.8 and Plate4.1D) .But the

lower mean percentage of callus was obtained from highest concentration of the hormones supplied in combination for nodal explants .On other hand ,the lowest mean percentage of callus was obtained from the media fortified with 5µM2,4-D alone (Table4.7 and Table4.8).

Table 4. 6**:**Effect of various concentrations of BAP and 2,4-D on callus induction from nodal explants of Anchote(*Cocinia abyssinica)***.*

Hormones concentration in µM		**percentage of callus responded (Mean ±SD)**
BAP	**2,4-D**	
0	0	0.0 ± 0^{e}
0	5	53.3 ± 2^{d}
5	5	78.4 ± 2^{a}
5	10	70.3 ± 3^{b}
5	20	65 ± 2^{c}
20	40	54 ± 2^{d}

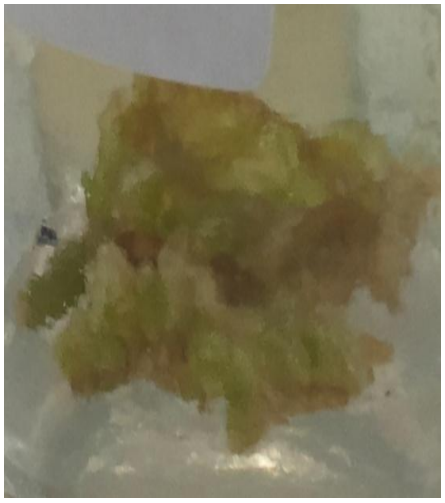
One-way Analysis of Variance (ANOVA .The means followed by the same letters among the column are not significantly different from each other, Student-Newman-Keuls Multiple Comparisons Tests:p-Value≤0.05,SD= Standard Deviation ,n=not recorded.

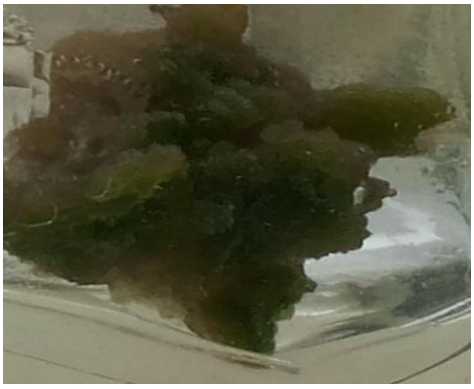
Table 4. 7: Effect of various concentrations of BAP and 2,4-D on callus induction from nodal explants of Anchote(*Cocinia abyssinica).*

Hormones concentration in µM		**percentage of explants with calli (Mean ±SD)**
NAA	**2 ,4-D**	
0	0	0.0 ± 0^{d}
0	5	43.3 ± 2^{c}
5	5	65.5 ± 1^{a}
5	10	$57+3^{b}$
10	20	51.5 ± 2^{c}
20	40	47.5 ± 2^{c}

One-way Analysis of Variance (ANOVA .The means followed by the same letters along the column are not significantly different from each other, Student-Newman-Keuls Multiple Comparisons Tests , p-value ≤0.05 ,SD= Standard Deviation

A 

B 

C 

D 

Plate4. 1:Effects of different concentration of auxins and cytokinins on nodal and leaf explants of Anchote callusing: (A) callus induction from leaf explants with 5µM (BAP+2,4-D) ;(B) callus induction from leaf explants with 5µM(NAA and 2,4-D);(C) callus induction from nodal explants with 5µM(BAPand2,4-D) and (D): callus induction from nodal explants with 5µM(NAA and 2,4-D).

4.3 Determination of The Optimum Cytokinin Levels for Multiple Shoot Production of *Coccinia Abyssinica.*

This experiment was undertaken with an objective of optimizing cytokinins levels for Anchote (*Coccinia abyssinica*) regeneration. 6-Benzyl aminopurine (BAP), Thiadizuron (TDZ) and Kinetin (Kn) were used at different concentration and evaluated either alone or in combination on the different parameter.

4.3.1 The Effect of TDZ on Shooting

The effect of Thiadizuron (TDZ) alone and in combination with 6-Benzyl aminopurine (BAP) were studied on the regeneration potential of nodal explants at 0.01 ,0.025,0.05,0.1,0.5,1 and 1.5µM .In my study ,TDZ at concentration of 1 and 1.5µM resulted in callus induction instead of regenerating shoot. However, some shoots were produced on media supplemented with 0.1and o.5µM TDZ. Some of the explants inoculated in this media also produced leaflets and calli.

Table 4. 8**:**Effect of various concentrations of TDZ on shoot proliferations from nodal explants of Anchote(Cocinia abyssinica).

Hormones Concentration in µM TDZ	**%response ±SD**	**Mean No. of shoots/ explant ±SD**	**Mean Length (cm)of shoots/ explant ±SD**	**Mean No .of leaves/ explant ±SD**
0.01	75±0.7[b]	2.5±0.2[d]	1.56±0.4[a]	3±0.1[e]
0.025	84±0.4[a]	3.4±0.1[a]	1.53±0.4[b]	6±0.7[c]
0.05	75±0.5[b]	3.20±0.3[b]	1.5±0.2[b]	8±0.6[a]
0.1	55±0.5[c]	2.67±0.3[c]	0.9±0.2[d]	7±0.1[b]
0.5	25±0.2[d]	2.0±1.0[d]	0.5±0.1[e]	5±0.2[d]
1	17±0.[4e]	1.2±0.4[e]	0.4±0.3[e]	n(Callusing)
1.5	8±0.7[f]	1.0±1.0[e]	0.3±0.5[e]	n(callusing)

One-way Analysis of Variance (ANOVA .The means followed by the same letters along the column are not significantly different from each other, Student-Newman-Keuls Multiple Comparisons Tests ,pValue≤0.05,SD= Standard Deviation ,n=not recorded.

However, the highest number of shoot regeneration was observed on MS basal medium supplemented with 0.025 µM TDZ(3.4±0.1) alone (table-4.9). It demonstrates that TDZ might effective at very low concentration for Anchote regeneration as compared to other cytokines like BAP and kinetin that were used at relatively high concentration for shoot regeneration .

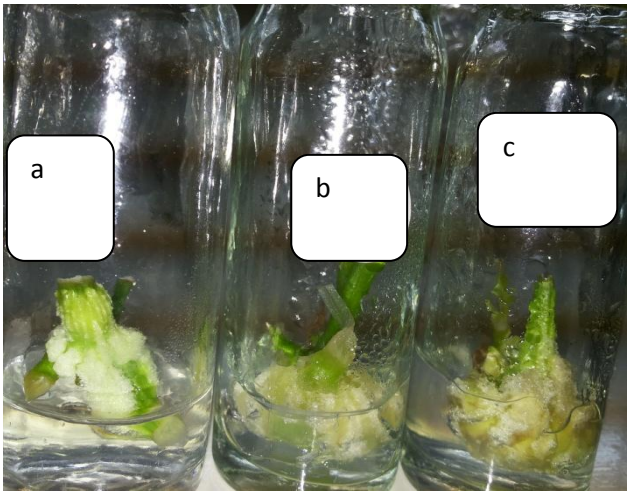


Plate 4. 2:Effects of TDZ at higher concentration:(a) 0.5µM;(b): 1µM and 1.5 µM on direct shoot generation of Anchote.

Combinations of different concentration of 0.025µM TDZ with 2.5µM BAP induced shoot buds in all treatments but the percentage response and the number of shoots per regenerating leaf segments were very low as compared to BAP used alone .

4.3.2 Effect of BAP on Number and Length of Shoots

Regeneration percentage, mean number of leaves ,length and number of shoots per explant were recorded after 21 days treatment with BAP to evaluate the effect of hormonal concentrations. There were some statistically significant differences among the concentrations of BAP tested (Table4 .10).The highest mean percentage of shooting response was obtained on the medium supplemented with 0.5 µM of BAP (85.0±0.5). The lowest mean percentage of shooting response was obtained on the medium supplemented with 40 µM (27.5±0.5) .Similarly, the highest number of shoots and the lowest number of shoots were obtained on the medium supplemented with 2.5 µM and 40

µM of BAP (3.4±0.5 ±0.5 and1.4±1) shoots/explant, respectively. The highest shoot length (3.2±0.2) cm was also noticed on media supplemented with 2.5µM.

Table4. 9**:** Effects various concentrations of BAP on shoot proliferations from nodal explants of Anchote .(Cocinia abyssinica).

Hormone Concentrations in µM BAP	**%response ±SD**	**Mean No. of shoots/ explant ±SD**	**Mean Length of shoots(cm)/ explant ±SD**	**Mean No .of leaves/ explant ±SD**
0	40±0.2^{e}	1.0±0.6^{df}	1.54±0.3^{c}	1.5±1^{f}
0.1	47±0.9^{f}	1.2±0.1^{d}	1.56±0.1^{c}	3.2±0.4^{e}
0.2	65.5±0.5^{d}	1.6±0.5^{cd}	1.71±0.2^{c}	3.4±0.9^{e}
0.3	80±0.3^{b}	1.9±0.2^{c}	1.90±0.2^{c}	4.2±0.7^{d}
0.5	85±0.5^{a}	2.2±0.1^{bc}	2.1±0.4^{c}	5.5±0.8c
1.5	80±0.7^{b}	2.5±0.4^{b}	2.6±0.1^{b}	6.20±0.8^{b}
2.5	75±0.1^{c}	3.4±0.5^{a}	3.2±0.2^{a}	8.5±0.9^{a}
5	55±0.4^{e}	2.0±0.3^{c}	2.5±0.2^{b}	6.8±0.5^{b}
10	45±0.5^{f}	1.8±0.7^{c}	1.8±0.2^{c}	5.67±0.6^{bc}
20	35±0.9^{g}	1.4±0.2^{d}	1.70±0.4^{c}	5.65±0.3^{bc}
40	27.5±1^{h}	1.2±0.8^{d}	1.52±0.2^{c}	5.52±0.5^{c}

The means followed by the same letters along the column are not significantly different from each other; Student-Newman-Keuls Multiple Comparisons Tests , p value≤0.05SD=Standard Deviation.

Elongation and multiplication of shoots were obtained on Murashige and Skoog (MS) basal medium, containing 2.5µMBAP (Plate4.3E).

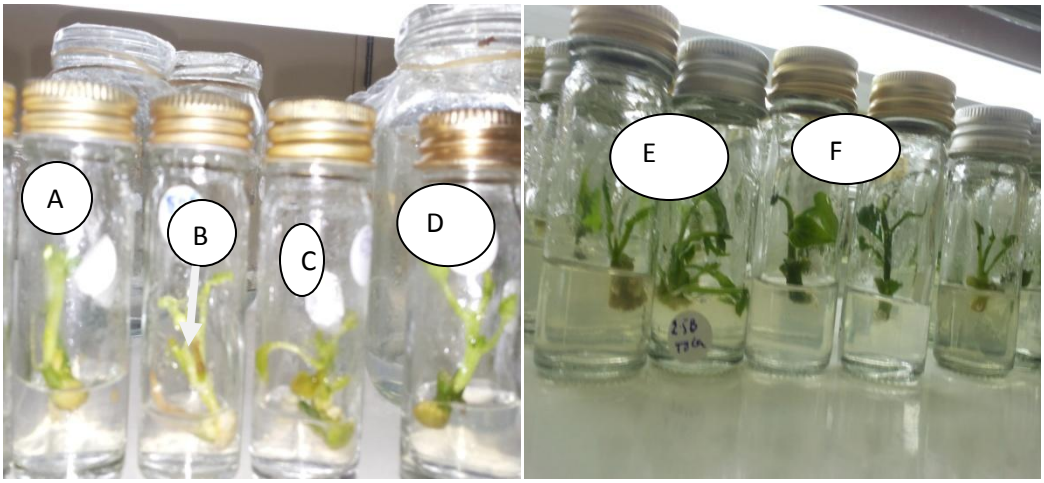


Plate4.3:Effects of BAP Concentration:(A)= 10µM,(B)=40µM), (C)=20µM, D)=5µM, (E)=2.5 µM and (F)= 0.5µM.

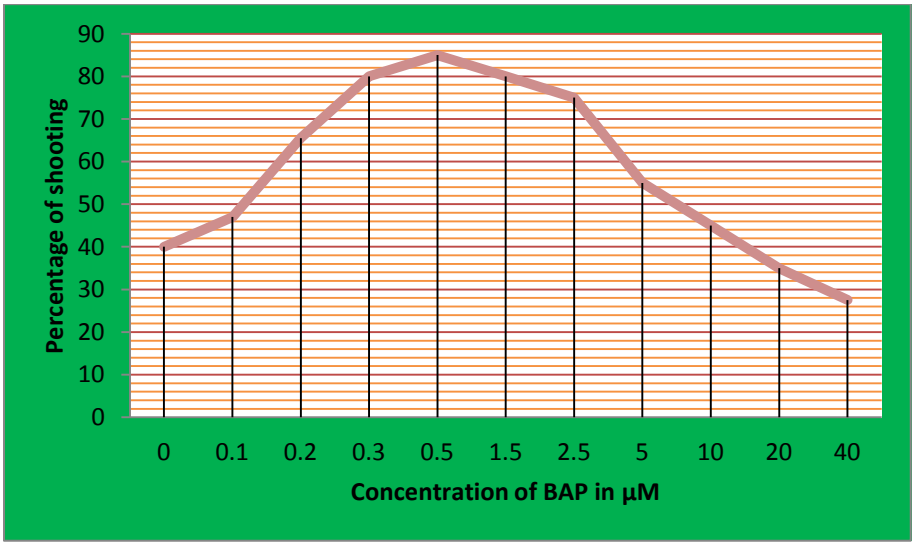


Figure4. 1: Effects of various concentration of BAP on direct shoot induction of Anchote

4.3.3 The Effect of BAP And TDZ in Combination on Shooting

In this experiment ,regeneration percentage, mean number of leaves , length and number of shoots per explant were recorded after treatment with different concentrations of BAP

and TDZ supplemented on MS basal media .There were statistically significant differences among the concentrations of BAP and TDZ tested in combination. (Table 4 .11).The highest mean percentage of shooting response (81±0.2) was obtained on the medium supplemented with 0.3 μM BAP and 0.025 μM TDZ .On other hand, the lowest mean percentage of shooting response (43.8±0.1) was obtained on the medium supplemented with and 5μM BAP and 0.025 μM TDZ . Similarly, the highest number of shoots and the lowest number of shoots were obtained on the medium supplemented with 0.3μMBAP and 0.025μM TDZ ,and 0.3μM BAP and 0.01 TDZ (4.2±0.1) and1.8±0.3) shoots/explant, respectively .

Table 4. 10:Effects of various concentration of BAP and TDZ in combination on direct shooting regeneration of Anchote.

Hormones and concentration in μM		% of Shooting (Mean±SD)	No. of shoots/explant (Mean±SD)	Length of shoots(cm)/explant (Mean±SD)
BAP	TDZ			
0	0	41±0.7^{c}	1.3±0.1^{d}	1.54±0.3^{c}
0.2	0.01	80.5±0.2^{a}	2.8±0.3^{c}	2.9±0.7^{a}
2.5	0.01	76.5±0.1^{b}	3.4±0.6^{b}	2.4±0.8^{ab}
5	0.01	45±0.9^{c}	2.4±0.2^{c}	2.2±0.1^{b}
0.3	0.025	81±0.5^{a}	3.6±0.8^{b}	2.8±0.2^{a}
2.5	0.025	75.8±0.7^{b}	4.2±0.1^{a}	2.4±0.8^{ab}
5	0.025	43.8±0.1^{c}	2.2±.01^{c}	2.0±0.3^{bc}

The means followed by the same letters along the columns are not significantly different from each other, Student-Newman-Keuls Multiple Comparisons Tests , p Value ≤0.05;SD= Standard Deviation.

4.3.4 The Effects of Kinetin

The effect of hormonal concentration of kinetin was significant (P≤ 0.05) on shoot number of sprouted plantlets at (0.3,0.5,1, 2.5 and 5μM(Table 4.12). The highest mean value for shoot number of 2.6±0.7 was obtained in full MS media supplemented with 2.5 μM kinetin . The lowest mean value for shoot number of 1.30±0.1 was obtained in MS media supplemented with 0.1 μM Kinetin. There were shoot sprouts in media without hormones (control experiment).

There were some significantly different in mean shoot length values of sprouted plantlets on full Ms media supplemented with different concentration of kinetin. The highest and lowest mean value for shoot length (2.76±0.8 cm and 1.56±0.4 cm) was obtained in full MS media supplemented with 0.5 µM and 0.1 µM kinetin respectively. The result showed that deep green color was observed in leaves and stems at lower concentrations (0.1 µM, 0.2 µM and 0.3 µM) which is slightly different from normal growth. On the other hand , kinetin at high concentration (5 µM or above) resulted in similar effect just like those observed with BAP on *in vitro* plantlets.

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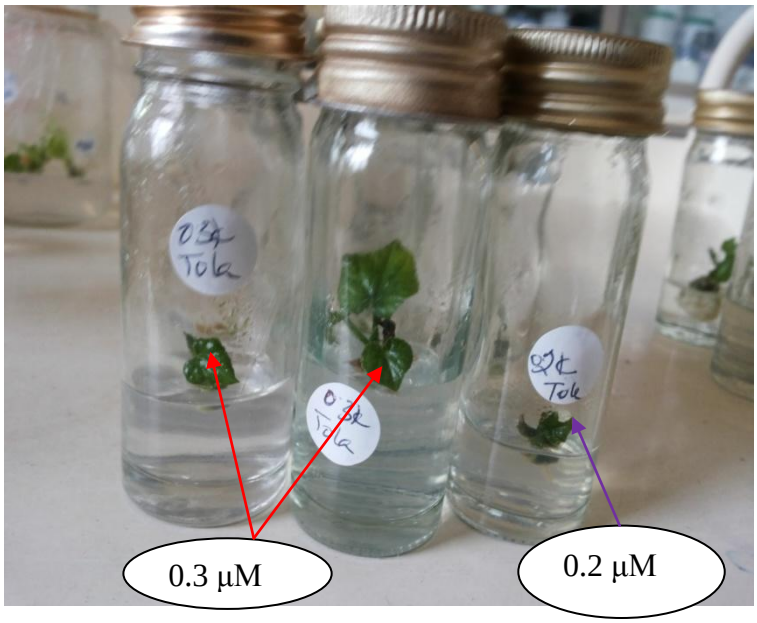


Plate 4. 4:Effects of Kinetin at lower concentration(0.2 and 0.3 µM).

Table 4. 11:Effect of various concentrations of kinetin on shoot regeneration from nodal explants of Anchote(*Cocinia abyssinica*).

Kinetin Concentration μM	%Response (Mean±SD)	No. of shoots/ explant (Mean±SD)	Length(cm) of shoots/explant (Mean±SD)
0	42 ± 0.3^{fe}	1.20 ± 0.2^{c}	1.54 ± 0.3^{c}
0.1	45 ± 0.5^{e}	1.30 ± 0.1^{c}	1.56 ± 0.4^{c}
0.2	49 ± 0.2^{ed}	1.42 ± 0.8^{bc}	1.57 ± 0.5^{c}
0.3	58 ± 0.1^{c}	1.54 ± 0.2^{bc}	1.70 ± 0.50^{bc}
0.5	65 ± 0.2^{b}	1.80 ± 0.4^{bc}	2.76 ± 0.8^{a}
1	67 ± 0.3^{b}	1.94 ± 1^{b}	2.70 ± 0.2^{ab}
2.5	72 ± 0.2^{a}	2.6 ± 0.7^{a}	2.4 ± 0.4^{b}
5	48 ± 0.9^{c}	2.0 ± 0.5^{ab}	2.20 ± 0.5^{b}
10	39 ± 0.5^{f}	1.5 ± 0.9^{bc}	1.8 ± 0.3^{bc}
20	21 ± 0.9^{g}	1.3 ± 0.3^{c}	1.78 ± 0.4^{bc}
40	19 ± 0.4^{h}	1.0 ± 0.6^{c}	1.7 ± 0.8^{bc}

The means followed by the same letters along the column are not significantly different from each other Student-Newman-Keuls Multiple Comparisons Tests $p\leq0.05$,SD =Standard Deviation.

Plate4. 5: Shoot multiplication on media supplemented 2.5µM BAP**.**

4.4 Determination of The Optimum Auxins Levels for Rooting of *In Vitro* Plantlets

For the production of complete plantlets the *in vitro* raised shoots were transferred to MS basal medium supplemented with various concentrations of auxins (NAA and IBA). In MS media supplemented with 0.5µM NAA (Table 4.13 and Plate 4.6), the cultures showed highest root initiation percentage response (86±0.3) after 12 days of culture from basal cut portion of the shoot explants . Within 2 weeks roots were developed in each responsive culture.

Table 4. 12: Effect of various concentrations of NAA on rooting r of Anchote in vitro shoots.

Hormones concentration in µM NAA	%Root response/ explant (Mean±SD)	No. of roots/explant (Mean±SD)	Root length(cm)/ explant (Mean±SD)	Shoot height(cm)/ explant (Mean±SD)
0	40±0.5^{g}	2.1±1^{d}	2.5±0.6^{a}	1.5±0.2^{d}
0.1	55±0.3^{f}	2.5±0.2^{d}	2.4±0.8^{a}	2.0±0.4^{c}
0.2	65±0.2^{e}	2.7±0.2^{d}	2.1±0.6^{ab}	2.2±0.1^{c}
0.3	70±0.3^{d}	3.3±0.4c	1.6±0.7^{b}	2.3±0.7^{bc}
0.5	86±0.3^{a}	4.6±0.6^{b}	1.3±0.5^{b}	2.5±0.4^{b}
1	80±0.2^{b}	5.5±0.5^{a}	1.20±0.6^{b}	3.4±0.3^{a}
2	76±0.7^{c}	5.7±0.6^{a}	1.0±0.7^{b}	2.8±0.2^{b}

The means followed by the same letters along the column are not significantly different from each other; Student-Newman-Keuls Multiple Comparisons Tests , p value≤0.05 SD=Standard Deviation.

The lowest mean percentage of rooting response also recorded when the micro shoots transferred onto MS full media fortified with 0.1µNAA exhibited root initiation after 12 days of culture. The sprouted roots increased in size in the same medium up to 3 weeks of culture. The highest root number (5.7±0.6) was noticed in the medium contain highest concentration (2µM) of NAA. Similarly, this treatment also resulted in shortest (1.0±0.7) cm root length. But the longest (2.5±0.6) cm root length was observe on the free hormone

media. This was not significantly different from the Ms full media with lower concentration (o.1 μM and 0.2μM) of NAA.

Table 4.13: Effects of various concentration of IBAs on direct rooting regeneration of Anchote.

Hormone concentration in μM IBA	%Root response/ explants (Mean±SD)	No. of root/explant (Mean±SD)	Root length(cm)/explants (Mean±SD)	Shoot length (cm)/ explants (Mean±SD)
0.1	55 ± 0.3^{d}	2.4 ± 1^{bc}	1.7 ± 0.6^{b}	1.9 ± 0.2^{b}
0.2	64 ± 0.5^{c}	2.6 ± 0.4^{ab}	1.9 ± 0.7^{b}	2.1 ± 0.4^{ab}
0.3	75 ± 0.1^{b}	2.7 ± 0.5^{ab}	2.1 ± 0.4^{ab}	2.2 ± 0.1^{ab}
0.5	84 ± 1^{a}	3.4 ± 0.8^{a}	2.3 ± 0.3^{a}	2.4 ± 0.2^{ab}
1	78 ± 0.4^{b}	3.2 ± 0.6^{a}	2.4 ± 0.9^{a}	2.5 ± 0.1^{a}
2	50 ± 0.5^{e}	3 ± 0.9^{a}	2.43 ± 0.2^{a}	1.9 ± 0.8^{b}

The means followed by the same letters along the column are not significantly different from each other; Student-Newman-Keuls Multiple Comparisons Tests , p value ≤0.05; SD=Standard Deviation.

MS full media supplemented with IBA (0.5μM) exhibited highest root mean percentage initiation (84±1) in cultures after 12^{th} day of inoculation. Further, roots developed in this medium were comparatively thinner compared to those under NAA. The main root also produced lateral roots within 2 weeks of inoculation (Table 4.14 and Plate 4.6A).

Plate 4. 6: *In vitro* roots under different auxins concentration on full strength of MS media :(a) with 0.5µM IBA;(b) with 0.5µM NAA;(c) Plantlets prepared for hardening on different soil mixture.

4.5 Acclimatization

In this study, the rooted plantlets were removed from MS full media, washed thoroughly and placed in different soil mixtures with different ratio in different pots after 2 weeks for acclimatization. The developed plantlets were maintained in the greenhouse in the following acclimatization conditions: Soil: manure(1:1) which gave the best results with 83.83±1 % survival rates, followed by Soil: Peat Fertilizer/ADP (3:1:1) with 82.7±0.9 % survival rates , Soil: Sand: Manure (3:1:2) with 80.83±2 % survival rates and treatment with soil only gave (65.83±3)% survival rates (Table 4-15 and Plate 4.7A).In this experiment we observed that plantlets established from *in vitro* regeneration were morphologically identical to the mother plant and developed normally, and also started bearing new shoot and root after two weeks in the green house (Table 4.15 and Plate 4.7B).

Table 4. 14: Survival of Anchote plantlets after hardening with different soil ratio

TREATMENTS	Ratio	No. of platelets	%Survival(Mean±SD)
Soil+ Sand +Manure	3:1:2	20	80.83 ± 2^{a}
Soil+ Manure	1:1	20	83.33 ± 1^{a}
Soil+ Peat+ Fertilizer/ADP	3:1:1	20	82.7 ± 0.9^{a}
Soil only	0:1:0	20	65.83 ± 3^{b}

The means followed by the same letters along the column are not significantly different from each other(α=0.05) Student-Newman-Keuls Multiple Comparisons Tests , SD=standard Deviation.

a.  b  . c.

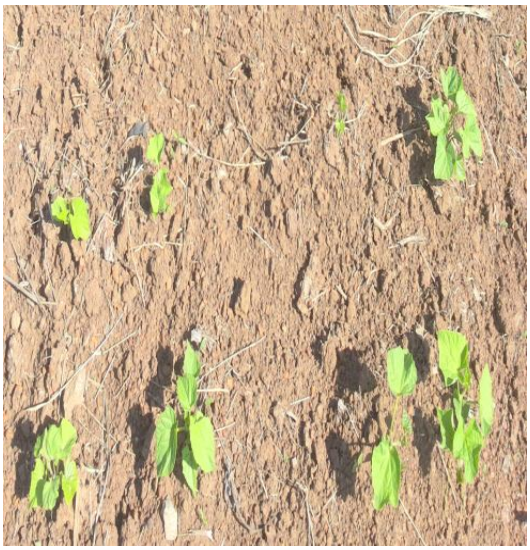
 d 

Plate 4.7**:**Survival of plantlets after hardening :(a) One day old plantlet (b) two weeks old plantlets (c)Three weeks old plantlet.(d) Mother plant grown field**.**

CHAPTER FIVE

5.0 DISCUSSIONS

From above result ,the Anchote plant parts showed significant differences in nutritional composition ($p<0.05$) on dry weight basis. Anchote roots had significantly higher percent moisture(24.7±0.5%) and carbohydrate contents(46.9 ±1%).The leaves were significantly richer in protein content compared to the other parts, while the Anchote stems had significantly higher ash and fiber content. However, the Anchote leaves and stems showed no significant differences in percentage of fat and carbohydrate content.

The results revealed that the plant was a good source of carbohydrate and to some extent fibers and proteins .The high proximate component of biochemical observed the in root was carbohydrate and compared to carbohydrate in the leaf and stem parts of Anchote ;it showed that the root is rich in carbohydrates .Carbohydrates are the main source of energy and serves as the most basic fuel source for life forms on this planet including people. Carbohydrates are the main source of energy for human brain and nervous system, keep the digestive system in functional.

As the result showed that the highest protein was observed in leaf (18.3±1) and this indicate that the leaf is good source of protein compared to the protein found in the stem and root parts .The result of ANOVA showed that protein in leaves significantly different at $\alpha=0.05$. However the lowest protein was observed in roots (8.2±0.1).The current results do not agree with (Fekadu . *et al.*, 2013). This difference might be accounted by various factors. The amount of nutrients in plant tissues varies seasonally with age,

maturity, plant part submitted for analysis, crop load, rootstock, and sometimes even cultivar., all the above factors, can bring their positive or negative effects on the nutrient contents (Pettinelli ,2009).

Proteins are synthesized by plants and animals to play a role in their physiology. The functions of protein include communication (e.g., insulin), structural (e.g., collagen in skin or keratin in hair),biochemical catalysts (e.g. enzymes), transportation (e.g., hemoglobin to transport oxygen in the blood), defense (e.g., antibodies), and storage (e.g., globulins in seeds).Hence proteins play a central role in biological systems (Fenna,1996).

The highest percentage fiber was observed in stem, followed by leaves and root respectively. This revealed that the stem has the lowest energy compared to root and leaf. As (Wise M.,2004) indicated that some foods are energy-rich as they contain little or no water, fiber or other material which does not yield energy and foods with much water and dietary fiber are usually energy-poor. However this does not mean that dietary fibers has no health benefit. Dietary fiber intake provides many health benefits. A generous intake of dietary fiber reduces risk for developing the following diseases: coronary heart disease, stroke, hypertension, diabetes, obesity and certain gastrointestinal disorders (NRC ,1989*)* .

The results of the mineral constituents of the Anchote (*Coccinia abyssinica*) are revealed that some of the macro and micro elements present in the three parts of *C. abyssinica* were in different concentrations. The elements detected are classified among the essential

ones for plants. It is obvious that the elements detected were translocated after their absorption by the root from the soils. In effect, the amount detected in theme was dependent on the amount in the soil, the efficient mechanism of absorption of the nutrients by the plant and the need of the plant for the nutrients. The above factors suggest why varied concentrations of the mineral elements were detected in the three different parts of the *C.abyysnica*.

Among the elements detected, Magnesium had the highest concentration, followed by Potassium, Calcium, manganese, Zinc ,Phosphorus Iron, and Copper, respectively in all three parts of *C. abyssinica*. But high concentration of the minerals are concentrated in stem compared to the root and leaves. On other hand leaves are richer in minerals than the roots. This result is agree with (Dereje and Girma,2013) who reported that leaf nutrient contents is higher than that is found in Anchote tuberous root .

In plant nutrition, potassium is considered as the most important cation with regard to its content in plant tissues, physiological and biochemical functions. Minerals are important for vital body functions such as acid base and water balance (Abd *et al.*,2012). Calcium and Phosphorus are the minerals present in the largest quantity in the structure of the body and in the bones. Fe is an important constituent of Hb while plants contribute these minerals and enhance their availability in daily life. Sodium and potassium present in the intracellular and extracellular fluid help in maintaining the body's electrolyte balance and membrane fluidity . Phosphorus is in the skeleton with the remainder in the muscle, skin, nerves and other organ (Pravina *et al.*, 2013). Additionally Phosphate is involved in the

metabolism of carbohydrate, lipids and protein, and helps to regulate the acid-base balance in the body and functions as a cofactor in many enzyme systems. Phosphorus is involved in high energy systems where phosphate bonds capture and then release vital energy in the cells of many tissues, especially those involved in muscle contractions.

Copper is also a vital dietary nutrient, although only small amounts of the metal are needed for well-being Although copper is the third most abundant trace metal in the body the total amount of copper in the body is only 75-100 milligrams Copper is present in every tissue of the body, but is stored primarily in the liver, with fewer amounts found in the brain, heart, kidney and muscles. inc is involved in numerous aspects of cellular metabolism .It was estimated that about 10% of human proteins potentially bind zinc, in addition to hundreds which transport and traffic zinc. It is required for the catalytic activity of more than 200 enzymes and it plays a role in immune function , wound healing , protein synthesis, DNA synthesis and cell division . Zinc is required for proper sense of taste and smell and supports normal growth and development during pregnancy, childhood, and adolescence .

It is believed to possess antioxidant properties, which may protect against accelerated aging and helps speed up the healing process after an injury; however, studies differ as to its effectiveness (Araya *et al*,2006) .Zinc ions are effective antimicrobial agents even at low concentrations. Cells in the salivary gland, prostate, immune system and intestine use Zn signaling as one way to communicate with other cells .In the brain, zinc is stored in specific synaptic vesicles by glutamatergic neurons and can modulate brain excitability .It

plays a key role in synaptic plasticity and so in learning .It also can be a neurotoxin, suggesting zinc homeostasis plays a critical role in normal functioning of the brain and central nervous system (Osredkar and Sustar, 2011).

The current result highlighted that all the three Anchote parts can be source of food and minerals (Endashaw , 2007).It is a valuable food source and according to local farmers, it helps in fast mending of broken/ fracture bones and displaced joints, as it contains high calcium, and proteins than other common and wide spread root and tuber crops) . Traditionally, it is also believed that, Anchote makes lactating mothers healthier and stronger (Abera *et al* , 1995). Dawit and Estifanos reported that the juice prepared from tubers of Anchote has saponin as an active substance and is used to treat Gonorrhea, Tuberculosis, and Tumor Cancer .Similarly other research has found that the fruits, leaves, flowers and roots of the cucurbitaceae members are consumed as food, and they are known to have medicinal properties as such as diuretics, alexiterics, laxatives, hepato protectives, antivenumous, anti-hypertension, anti-diabetic, astringent, antihelminthic, aphrodisiac, antiseptic and is capable of curing pimples or acne (Chunduri, 2013).

In general our results showed that leaves, roots and stems parts of *Coccinia abyssinica* have appreciable nutrients and minerals. The established superior nutritional composition of these Anchote parts highlighted their usefulness in terms of addressing nutritional deficiency particularly in Ethiopia and should therefore be promoted due to their high nutritional value.

Sterilization is the process of making explants contamination free before establishment of cultures. Various sterilization agents are used to decontaminate the tissues. These sterilants are also toxic to the plant tissues, hence proper concentration of sterilants, duration of exposing the explant to the various sterilants, the sequences of using these sterilants has to be standardized to minimize explants injury and achieve better survival (CPRI, 1992).

The present result showed that the two explants (leaf and nodal) of *Coccinia abyssinica* have shown different response for different concentration of bleach at different time duration. This shows that different tissue types requires different concentration of sterilants and time of exposures. The result agreed with that of (Rezadost *et al.*,2013) who reported that the surface sterility chosen for an experiment typically depend on the explants and also plant species.

The present study also demonstrates that commercial bleach is effective and can remove surface contaminants from Anchote explants. Various research work have shown that different explants are commonly surface-sterilized using sodium hypochlorite (household bleach), ethanol, and fungicides when using field-grown tissues. The time of sterilization is dependent on the type of tissue; for example, leaf tissue require a shorter sterilization time than seeds with a tough seed coat (Funguomali *et al.*, 2013, Sharma and Nautiya, 2009). The result for the present sterilization study also conform that both leaves and nodal explants dependent to different concentrations of commercial Jik® and duration time. Leaf explants showed that they sterile when treated at 0.19% of NaOCl

with10Minute duration of time. Further increasing jik concentration and duration of time was resulted in the death of more explants .On other hand. concentration of 0.19% commercial bleach and an exposure time of 15 minutes gave significantly higher percentage surviving clean explants (84.5±0.6) than the other levels and exposure times for nodal explants .Further increase in exposure time led to a significant decline in percentage clean surviving nodal in both explants. At shorter exposure time, explants death were due to microbial contamination, while at prolonged exposure time death was due to scorching by the sterilants.

Sterilization should therefore guarantee the explants sterility and regeneration capacity which are known to be affected by disinfectant concentration and sterilization period. The *in vitro* protocol reported in this study can be used for sterilization of explants from vegetative tissues ,rapid multiplication of disease free plants, through leaf and nodal explants of Anchote(*Coccinia abyssinica*) and other related species.

Plant hormones (phytohormones) are small organic molecules that affect diverse developmental processes. Alterations in hormone responses have been responsible for several important agricultural advances, such as the breeding of semi-dwarf varieties and increased grain production (Ashikari *et al*., 2005).Virtually every aspect of plant development from embryogenesis to senescence is under hormonal control. In general, this developmental control is exerted by controlling cell division, expansion, differentiation and cell death. In this manner, diverse developmental processes can be

controlled, including formation of the apical-basal and radial pattern, seed germination ,shedding (Silverstone and Sun, 2000).

The basal medium (e.g., MS) is designed to keep plant tissues alive and thriving. Plant growth regulators or hormones are needed to manipulate the developmental program of tissues—say, to make callus tissue proliferate, or produce roots from shoots (Ali *et al.*, 2007). Growth regulators are the items most often manipulated as experimental factors to enhance tissue culture conditions.

In the present investigation callus in induction were also directly initiated from young nodal and leaf explants of Anchote (*Coccinia abyssinica)* after successful sterilization. The young leaf and nodal explants were transferred to fresh MS medium supplemented with different concentration of auxins and cytokinins including (2,4-D, BAP, NAA) of which the most effective one's are given in results part. On MS basal medium supplemented with 5µM 2,4-D leaf explants showed callus initiation after 12^{th} day of inoculation from of leafs with mean percentage 65±3 cultures. On MS basal media fortified with 5 µM NAA and5µM 2,4-D, the leaf explants exhibited callus initiation with highest mean percentage of 76±3 cultures after 12^{th} days of inoculation. The callus responding increased in size in the same media composition up to 4 weeks of inoculation . MS basal medium supplemented with BAP and 2,4-D induced callus in different cultures after 12^{th} day of inoculation .The highest mean percentage (80±2) callus was obtained the media fortified with 5 µMBAP and 5µM 2,4-D) .But the lowest mean

percentage of calli were obtained from highest concentration the hormones for both nodal explants.

Similarly, On MS basal media fortified with 5 µM BAP and 5µM 2,4-D, the nodal explants exhibited callus initiation with highest mean percentage of 78±2 cultures after 12 days of inoculation. The callus responding also increased in size in the same media composition up to 4 weeks of inoculation . MS basal medium supplemented with NAA and 2,4-D induced callus in different cultures after 13th day of inoculation .The highest mean percentage (65.5±2) callus was obtained the media fortified with 5µM (NAA and 2,4-D) for nodal explants .But the lower mean percentage of callus was obtained from highest concentration of the hormones supplied in combination for nodal explants .On other hand ,the lowest mean percentage of callus was obtained from the media fortified with 5µM2,4-D alone.

As the result showed that the leaf explants more responded for callusing compared to nodal explants .That is higher percentage of callusing was observed from leaf explants and this also showed that the leaf explants are more sensitive for callusing than the nodal explants.

Auxins have been reported to induce callus formation in tissue culture of plants (Tisserat, 1985).NAA and IAA promoted excessive callus formation in water melon (Compton and Gray, 1993). A high degree of callusing was reported in single node explants of fluted pumpkin cultured in medium containing both kinetin and NAA (Balogun *et al*., 2002). This study has shown, that for good callus formation, BAP

combined with 2,4-D is optimum for leaf explants. This two hormones in combination can be used as initiation medium for plant regeneration via somatic embryogenesis which will be useful in plant transformation. Similarly, the results are supported by (Devendra *et al.*,2009) who found that combination of BAP with 2,4-D or NAA produce callus in cucumber which is relative species for the Anchote.

In order to develop a protocol for production of regeneration of plants, the nodal segments were also sub cultured on MS medium supplemented with different cytokinins concentrations. Of the different concentration used, nodal segments responded very well in the combinations.MS basal medium fortified with TDZ, Kn and BAP induced shoot formation within 10-12 days of inoculation in cultures. When sub cultured in the same media composition, the shoots elongated which were later maintained in the same medium up to a period of 3 weeks. Regeneration percentage, mean number of shoots, length and number of shoots per explants were also recorded after 21 days treatment with BAP to see effects of hormonal concentration. There were some significant differences among the concentrations of BAP tested .

Anchote*(Coccinia abyssinica)* young nodal cultured on full MS medium supplemented with 0.1 and 0.5μM TDZ revealed that multiplication of shoots was very slow in all treatments. There were callus production and regeneration of adventitious shoots was in some treatments.

The effect of TDZ, a substituted phenyl-urea as a plant growth regulator has been also reported for many plant species including several recalcitrant woody species . In

Coccinia abyssinica highest shoot length (1.56cm) was observed on MS full strength medium after 21 days of initial culture . This was followed by MS supplemented with TDZ alone (0.01µM) .Lowest shoot length (0.5cm) was observed at 0.5µM TDZ on MS full strength medium . It is evident from these results that TDZ has influence on micro propagation even at very low concentrations. Maximum number of shoots (3.4) per explants was obtained on MS + 0.025µM TDZ and the lowest number of shoots(1) were obtained on Ms supplemented with 1.5µMTDZ.This might be due to the high concentration of TDZ as it modifies the endogenous cytokinins metabolism.

The effect of Thiadizuron (TDZ) alone and in combination with 6-Benzyl amino purine (BAP) were also studied on the regeneration potential of nodal explants at 0.01 ,0.025,0.05,0.1,0.5,1 and 1.5µM levels. In my study TDZ at 1µM and 1.5µM concentration resulted callus induction instead of regenerating shoot .However after a 4-weeks period, the medium supplemented with 0.1 and 0.5 TDZ had the highest shoot number (1.2 and 1 shoots/explants), However this was not significantly different from other medium with control group.

The High efficiency of shoot regeneration was observed in nodal explants on MS basal medium supplemented with 0.025 µM TDZ(84±0.4)% alone and 0.01µM TDZ with 0.3µM BAP. However ,maximum shoot number was obtained from media supplemented with 0.025µM TDZ + 2.5µM BAP. It demonstrates that TDZ might be used at very low concentration for Anchote regeneration as compared to other cytokines like BAP and kinetin that were used at relatively high concentration for shoot regeneration .

TDZ is relatively costly, a very low concentration of TDZ used in our studies circumvents its price consideration and seems to be quite cost-effective. Moreover, its influence on all the growth parameters under study necessitates further work using TDZ as a growth regulator to better understand its role in Anchote (*Coccinia abyssinica*) tissue culture. Furthermore ,the result is agree with (Mok *et al*., 1987) who reported that TDZ is stable and more active at lower concentrations than the adenine-type cytokinins.

Many researchers have shown that Thidiazuron (TDZ) has been considered one of the most active phenylureas having cytokinin-like activities (Huettman and Preece ,1993).However, according to (Mok *et al.*, 1987) compared to other active compounds ,extremely low for the stimulates maxillary shoot proliferation.

TDZ increases shoot formation of many woody plant species more efficiently than purine adenine derivatives, but it is ineffective for the proliferation in some species (Huetteman and Preece ,1993). On the other hand, TDZ may inhibit shoot elongation and it induces formation of shortened internodes in apple cv.(Van Nieuwkerk *et al*., 1986). In the present work, TDZ has also an effect on reduction of multiplied shoot length ,especially when high concentrations used, decreased in length of shoots was observed.

The highest and lowest mean percentage of shooting response were obtained on the medium supplemented with 0.5 μM and 40 μM of BAP (85.0±0.5 and 27.5±0.5) shoot/explants, respectively. Similarly, the highest number of shoots and the lowest number of shoots were obtained on the medium supplemented with 2.5 μM and 40 μM of BAP(3.4±0.5±0.5and1.4±1) shoot/explants respectively. Furthermore, high

concentrations of BAP (5µM or above) resulted in vitrification, callus formation and yellow color formation in stem and leaves .However, no change was observed in the appearance of shoots obtained from low concentrations of BAP (0.1-2.5µM).Elongation and multiplication of shoots were obtained on MS basal medium, containing 2.5µM/ Benzyl amino purine (BAP). These plantlets were finally maintained on the same medium composition up to a time period of 4 weeks.

Complete plantlet development was achieved when explants were cultured on MS basal medium fortified with BAP 0.1- 2.5µM. These results are similar to that of (Sunitibala and Kishor ,2009) who also reported similar work that BAP showed best response for multiple shoot induction of *Dendrobium transpare* L. However ,at 5µM or above the plantlets exhibited stagnant growth ,leaf wilting ,thinly and pale yellow appearances and callus induction were observed. But on a media supplemented with BAP0.1- 2.5µM the explants exhibited best morphogenetic potential as in cultures shoot differentiation started after7-8 days of inoculation. In the next 4 weeks these cultures showed enhanced growth along with the production of shoots. In summary, we noticed that BAP is capable of regenerating multiple shoot from nodal explants of Anchote. This is agreed with (Silva , 2010) who reported that Cytokinins promote cell division and shoot growth .

Regeneration percentage, mean number , length and number of shoots per explants were recorded after all hormones experiments. There were statistically significant differences among the concentrations of BAP and TDZ tested in combination.

The highest and lowest mean percentage of shooting response were obtained on the medium supplemented with 0.3µMBAP and 0.01µM DTZ, and 5µM BAPand0.025µM TDZ (82.5±0.2 and43.8±0.1) shoot/explants, respectively. Similarly, the highest number of shoots and the lowest number of shoots were obtained on the medium supplemented with0.3µMBAP-0.025µMTDZand0.3µMBAP-0.01µMTDZ(4.2±0.1and1.8±0.3) shoot /explants, respectively.

The effect of hormonal concentration of kinetin was significant (P≤ 0.05) on shoot number of sprouted plantlets at(0.3,0.5,1, 2.5and 5µM . The highest mean value for shoot number of 2.6±0.7 was obtained in full MS media supplemented with 2.5 µM kinetin . The lowest mean value for shoot number of 1.30±0.1 was obtained in MS media supplemented with 0.1 µM Kinetin. There were shoot sprouts in control MS media or without hormones.

There were some a differences in mean shoot length values of sprouted plantlets on full Ms media supplemented with different concentration of kinetin. The highest and lowest mean value for shoot length (2.76±0.8cm and1.56±0.4 cm) was obtained in full MS media supplemented with 0.5 µM and 0.1 µM kinetin respectively. The result showed that deep green color was observed in leaves and stems at lower concentration (0.1 0.2 and 0.3 µM) which is slightly different from normal growth. On other hand , kinetin at high concentration(5 µM or above) resulted in somewhat similar affects on in vitro plantlet as BAP .

The most commonly used auxins are indoleacetic acid (IAA), indole butyric acid (IBA), NAA (naphthalene acetic acid), and 2,4- dichlorophenoxy acetic acid (2, 4-D). For the production of complete plantlets the *in vitro* raised shoots were transferred on MS basal medium supplemented with various concentrations of auxins (NAA and IBA. In MS basal medium supplemented with (0.5μM)NAA, the cultures showed highest root initiation percentage response (86±0.3) after 12 day of inoculation from basal cut portion of shoot explants in cultures and within 2 weeks roots developed in each responsive culture. The lowest mean percentages of explants responded also recorded when the micro shoots transferred onto MS basal medium fortified with NAA (0.1μM) exhibited root initiation after 12 days of inoculation at the same time showing progress shooting. The sprouted roots increased in size in the same medium up to 3 weeks of inoculation.

The lowest mean percentages of explants responded also recorded when the micro shoots transferred onto MS basal medium fortified with NAA (0.1μM) exhibited root initiation after 12 days of inoculation. The sprouted roots increased in size in the same medium up to 3 weeks of inoculation. The present study reveals that, NAA gave better root induction than IBA on full MS Media. Efficient rooting was achieved for Anchote (*Coccinia abyssinica*) at different concentration with IBA (0.5 μM) and NAA (0.5μM) . From the literature , auxins are an important factor involved in rooting because they promote adventitious root formation in the vast majority of species (De Klerk, 2002). They are acts to promote cell division and growth, it does so mainly by increasing the expression of genes that encode required proteins for these processes.

In general the analysis of the effect of the two cytokinins (BAP and kinetin) on multiplication phase of Anchote infers that BAP gave the best results. Similarly best results for maximum shoot number was achieved in Anchote when BAP and TDZ were used in combination as shown .NAA proved to be better rooting hormone for Anchote nodal explants in terms of rooting and shooting percentage, number of roots per rooted explants and root length as compared to IBA .

The *ex-vitro* acclimation of *in vitro*-cultured plants is, in most cases, a difficult phase in the technology of micropropagation. For *ex-vitro* acclimation it is necessary to provide some optimal environmental factors in order to accomplish the gradual transition of the plantlets from the *in vitro* environment to the conditions of the normal, natural environment. These factors are: an adequate substrate (perlite, peat or various mixes), air humidity, adequate pH and ventilation. In most cases, the substrate used for acclimation is a solid one, and the humidity of the air is provided by artificial mist or fog installations or by protecting the cultures with transparent caps, transparent plastic sheets or wet tents.

It is important to recognize and understand the differences between an in vitro and a greenhouse environment. By that have greater tolerance to water stress and are photo synthetically competent can be developed as part of the acclimatization process in preparing plantlets for transferring out of culture. Roots formed in culture can be beneficial for enhancing early growth following transfer from culture. The optimum growth rate of deflasked plantlets frequently does not occur until new leaves and roots develop in the greenhouse environment. However, as species differ greatly in their

requirements there is no universal acclimatizing protocol as discussed by(Seelye *et al.*,2003).

Hence acclimatization work has great application possibilities for obtaining seedlings with high survival percentages and growth with the purpose of accomplish a sustainable management of indigenous plant. A substantial number of micro propagated plants do not survive transfer from *in vitro* conditions to greenhouse or field environment. The greenhouse and field have substantially lower relative humidity, higher light level and septic environment that are stressful to micro propagated plants compared to *in vitro* conditions. The benefit of any micro propagation system can, however, only be fully realized by the successful transfer of plantlets from tissue-culture vessels to the ambient conditions found *ex vitro.* Most species grown *in vitro* require an acclimatization process in order to ensure that sufficient number of plants survive and grow vigorously when transferred to soil.(Ilova's *et al*,1998)

From the above experiment, it is evident that, this study has established *in vitro* regeneration protocol and has revealed the effectiveness of various plant growth regulators on all the leaf and nodal explants of Anchote for callus induction and direct regeneration..

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATION

6.1 CONCLUSION

Result of this study showed that leaves, roots and stems parts of *Coccinia abyssinica* contain high nutrients and minerals. Anchote roots had significantly higher percent moisture (24.7±0.5%) and carbohydrate contents (46.9 ±1%). The leaves were significantly richer in protein content compared to the other parts, while the Anchote stems had significantly higher ash and fiber content (13.0±0.4% and 27.7±0.4%) respectively. The highest mineral element found in the stem was Magnesium (Mg) with mean average 336±0.4 Mg/100 followed by potassium(K)which was found with mean average 323±3 Mg/100 and Calcium(Ca) was found with mean average of 313.5±1 Mg/100.According to our current result the highest mean average (76.9±0.9) Mg/100 of Phosphorous was observed in stems followed by leaves (71.5±2 Mg/100 but the root had the lowest mean average (54.1± 0.8)Mg/100 phosphorous.

A concentration of 0.19% commercial bleach and an exposure time of 10 minutes gave significantly higher percentage surviving clean explants (82.5±0.6%) than the other concentration and exposure time for leaf explants and a concentration of 0.19% commercial bleach and an exposure time of 15minutes gave significantly higher percentage surviving clean explants (84.5±0.6) than the other levels and exposure times for the nodal explants .The efficient Sterilization protocol developed for the leaf and nodal explants and also established *in vitro* regeneration protocol and has revealed the

effectiveness of various plant growth regulators on all the leaf and nodal explants of Anchote for callus induction and direct regeneration.

On MS basal media fortified with 5 µM NAA and5µM 2,4-D, the leaf explants exhibited callus initiation with highest mean percentage of 76±3 cultures after 12th days of culture. The optimum concentration of BAP and 2,4-D in terms of maximum mean percentage of callusing (80±2) was observed from leaf explants at 5.0 µM when BAP and 2,4-D12th days of cultures.

The highest mean percentage (65.5±2) callus was obtained from the media fortified with 5 µM (NAA and 2,4-D) for nodal explants. On MS basal media fortified with 5 µM BAP and 5µM 2,4-D, the nodal explants exhibited callus initiation with highest mean percentage of 78±2 cultures after 12th days of cultures.

The highest number of shoots were observed on MS basal medium supplemented with 0.025 µM TDZ(3.4±0.1) alone . The High efficiency of shoot regeneration was observed from nodal explants on MS basal medium supplemented with 0.025 µM TDZ(84±0.4)% alone. The highest mean percentage of shooting response was obtained on the medium supplemented with 0.5µM of BAP (85.0±0.5)alone. The highest shoot length (3.2±0.2)cm was also noticed on media supplemented with 2.5µM BAP. The highest mean percentage of shooting response (81±0.2) was obtained on the medium supplemented with 0.3 µM BAP and 0.025 µM TDZ .

The Obtained results undoubtedly suggest that the cytokines type and concentration suitable for micro propagation of this plants is hormonal concentration level dependent

In general the analysis of the effect of the two cytokines (BAP and kinetin) on multiplication phase of Anchote infers that BAP gave the best results. Similarly best results for maximum shoot number was achieved in Anchote when BAP and TDZ were used in combination as shown .

In MS media supplemented with 0.5µM NAA , the cultures showed highest root initiation percentage response (86±0.3) after 12^{th} days of culture from basal cut portion of the shoot explants . The highest root number (5.7±0.6) was noticed in the medium contain highest concentration (2µM) of NAA. MS full media supplemented with IBA (0.5µM) exhibited highest root mean percentage initiation (84±1) in cultures after 12^{th} day of inoculation .From the auxins tested ,NAA at 0.5µM proved to be better rooting hormone for Anchote platelets in terms of rooting and shooting percentage, number of roots per rooted platelets and root length as compared to IBA.

Soil: manure(1:1) which gave the best results with 83.83±1 % survival rates, followed by Soil: Peat Fertilizer/ADP (3:1:1) with 82.7±0.9 % survival rates , Soil: Sand: Manure (3:1:2) with 80.83±2 % survival rates and treatment with soil only gave (65.83±3)% survival rates .Acclimatization work has great application possibilities for obtaining seedlings with high survival percentages and growth with the purpose of accomplish a sustainable management of indigenous plant like Anchote.

6.2 RECOMMENDATION

1. The biochemicals and small amounts of micro and macro elements detected in the three parts of Anchote (*Coccinia abyssinica*) could be regarded as important in being part of the cumulative intake of these mineral elements in human nutrition.
2. Further research should be undertaken, to compare nutritional values of Anchote from all the growing parts or zones of western Oromia, since soil nutrients varies from one ecological zone to the other.
3. The *in vitro* protocol reported in this study can be used for sterilization of explants from leaf and nodal explants, rapid multiplication of disease free Anchote(*Coccinia abyssinica*) and other related species.
4. This study will help to undertake further research on the Anchote for further improvement by using genetic engineering and Molecular techniques to produce nutritionally improved Anchote.
5. Further research *in vitro* propagation via embryogenesis and somatogenesis suggested.

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Appendixes

Appendix 1: Composition of Murashige and Skoog's Medium

Stock solution	Constituents	Concentration in stock solution g/l	Volume of stock solution in final medium ml/l	Final concentration in medium mg/l
A	NH_4NO_3	82.5	20	1650
B	KNO_3	95.0	20	1900
C	H_3BO_3	1.24	5	6.2
	KH_2PO_4	34		170
	KI	0.166		0.83
	$Na_2MoO_4.2H_2O$	0.05		0.25
	$COcl_2.6H_2O$	0.005		0.025
D	$Cacl_2.2H_2O$	88.0	5	440.0
E	$MgSO_4.7\ H_2O$	74.0	5	370.0
	$MnSO_4.4\ H_2O$	4.46		22.3
	$ZnSO_4.7\ H_2O$	1.72		8.6
	$CuSO_4.5\ H_2O$	0.005		0.025
F	Na_2.EDTA	7.45	5	37.35
	$FeSO_4.7\ H_2O$	5.57		27.85
G	Thiamine HCL	0.02	5	0.1
	Nicotinic Acid	0.1		0.5
	Pyridoxine HCL	0.1		0.5
	Glycine	0.4		2.0

Appendix 2:PLANT GROWTH SUBSTANCES

PLANT GROWTH SUBSTANCES	SOLVENT
BAP	0.1N NaOH and heat if required
Kinetin	0.1N NaOH
IAA	Absolute alcohol
IBA	Absolute alcohol
NAA	90% alcohol
2, 4-D	Absolute alcohol
TDZ	NaOH
2iP	Acidified water