ADDIS ABABA UNIVERSITY

COLLEGE OF NATURAL AND COMPUTATIONAL SCIENCE

ZOOLOGICAL SCIENCES DEPARTMENT

GENERAL BIOLOGY PROGRAM

In vitro propagation of anchote (*Coccinia abyssinica*(Lam.) Cogn) from shoot explants

A Thesis Submitted to the School of Graduate Studies, Addis Ababa University, in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biology

By: Dejen Tekola

Advisor: Tileye Feyissa(PhD)

Addis Ababa, Ethiopia

September, 2018

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DECLARATION

I the undersigned, declare that this thesis is my original work and has not been presented by others. All sources of materials used have been acknowledged. This thesis was entitled; *In vitro* propagation of Anchote (*Coccinia abyssinica*(Lam.) Cogn) from shoot explants.

Dejen Tekola Signature_____ Date_____

I the undersigned, declare that this thesis has been submitted for examination with my approval as an advisor:

Tileye Feyissa (PhD) Signature_____ Date_____

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L IST OF ABBREVIATION

ANOVA	Analysis of Variance
BAP	6 Benzyl Amino Purine
CRD	Completely Randomized Design
EDTA	Ethylene Diaminetetraacetic Acid
IBA	Indol -3-Butyric Acid
LSD	Least Significance Difference
MS	Murashige and Skoog
NAA	αNaphthalene Acetic Acid
SD	Standard Error
SPSS	Statistical Processor for Social Science

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ABSTRACT

Among the major root and tuber crops, anchote (*Coccinia abyssinica (*Lam.) Cogn) *is a potencial tuber crop mostly cultivated in western and southern parts of Ethiopia. It serves as a food, social-cultural, medicin and economic plant for the farming communities. The plant is commonly propagated by seeds and as pollination may occur, it is difficult to obtain true-to-type plants. Therefore, in vitro propagation was used to overcome this problem. This study was conducted to determine the effect of: seed coat on germination, different concentration of NaOCl and varying exposure time on explant sterilization, different concentration of BAP on shoot initiation, different concentration of BAP alone and in combination with NAA on shoot multiplication, different types and concentration of rooting hormones on root induction, and to investigate the percentage of survival after acclimatization. The decoated seeds resulted in higher germination (67%) than coated seeds (43%) after three weeks of sowing. Ten percent NaOCl (v/v) resulted in 60% sterilized explants for 15 min exposure. The highest mean shoot number per explant (2.33±0.88) was obtained on MS medium containing 1.0 mg/l BAP during shoot culture initiation. During multiplication, the maximum shoot numbers per explant (4.40±1.73 and 4.40±1.30) were obtained on MS medium supplemented with 1.0 mg/l BAP in combination with 0.10 mg/l NAA and 1.50 mg/l BAP in combination with 0.10 mg/l NAA respectively. The maximum root numbers (12.27±3.58) and the maximum root length (5.98±1.13 cm) were obtained on the half strength MS medium containing 0.25 mg/l IAA. The percentage of survival after acclimatization was 78%. This protocol can be used to produce large number of disease free clean planting materials of anchote within short period of time. So that, this study could be used for agricultural, medicinan, industrial and commercial production of anchote in good quality and quantity.*

Key words: *Coccinia abyssinica (Lam.) Cogn*, *in vitro* propagation, MS medium, and Plant Growth Regulator

INTRODUCTION

Ethiopia is bio-diversity hot spot, because, it has highly diversified climate and soil types. There are about 23 recognized vegetation types; the number of flora species is estimated to be about 6,500 to 7000 of which 12% are endemic to Ethiopia (Biodiversity report, 2007). More than 30 indigenous starchy roots and tuber crops (wild and cultivated) from Ethiopia are identified as edible (Getahun, 1973). Anchote(*Coccinia abyssinica* (Lam.) Cogn.) is one of these indigenous tuberous crop belongs to Order: Cucurbitales; Family: Cucurbitaceae and Genus: Coccinia. The genus *Coccinia* is made up of 30 species. There are about 10 species of *Coccinia* in Ethiopia; however, only *C. abyssinica* is cultivated for human consumption (Bekele, 2007). It has different vernacular names in different languages; Ancoote in (Afaan Oromoo), Ushushu (Welayita), Shushe (Dawuro), and Ajjo (Kafigna) (Demel *et al.*, 2010). Among the major root and tuber crops, anchote is a potential crop produced in West Wollega zone of Ethiopia. It serves as a food, cultural, social and economic crop for the farming communities (Mengesha et al., 2012). Even though, anchote is a major root crop of the south and western regions of Ethiopia, it is less known to other parts of the country.

Anchote is both a tuber crop and leafy vegetable. The tuberous root is the principal component of the crop and it produces tender young leaves from the new growing bud (Abera and Gudeta, 2007). In terms of diversity, anchote has been found highly diversified in its characteristic (Yassin et al., 2013; Bekele et al., 2014; Wondimu et al., 2014). Based on the root colors; there are two anchote dimma (red pigmented) and addi (white flesh) (Yambo and Feyissa, 2013).

According to Abera and Gudeta (2007), anhote has enormous genetic diversity, as it is indigenous and long-stayed in the production systems. Due to the lower attention given to the research and development of anchote, there is no variety so far developed and released. There are traditional selection practices being practiced by farmers to have anchote types of desirable qualities, such as larger tuber size (Fikadu, 2011). Unlike many other crops, anchote can be grown with minimal inputs and is able to produce reasonable yields in conditions of low soil fertility, acidic soils or drought and in intercropping with cereals.

For a long time, the farming and utilization practices of anchote have been passed from generation to generation in the course of oral tradition, with very little recorded information (Gemeda, 2000). It is one of the many underutilized crops with less emphasis given in terms of investigation and preservation practices despite its nutritional, social- cultural and economic importance to the growers (Yassin et al., 2013).

The common way of anchote propagation is via seeds, and as both cross- and self-pollination may occur in anchote (Getahun, 1969; Hora, 1995; Jeffrey, 1995), it may be difficult to obtain true-to-type plants. Micropropagation is used as a means to improve crop varieties and it has been getting attention and being applied in agricultural research centers. It enables production of large number of plantlets in short period of time as well as maintenance of germplasm under controlled conditions in small spaces and with reduced labor requirement (Hartmann et al., 2002). Micropropagation relies on the phenomenon of cell totipotency (Thorpe, 2007). It is performed in a controlled environment. Surface sterilization by using different chemicals is necessary (Mineo, 1990). MS medium is mostly used for this purpose. Micropropagation offers a great potential to large scale multiplication for subsequent exploration (Boro et al., 1998).

There has been preliminary micropropagation research on anchote (Yambo and Feyissa, 2013). However, the explants of these previous researches were seeds whose genotype was not known. The present work reports on *in vitro* propagation of anchote from *ex vitro* shoot tip explants. The objectives of this study were to evaluate the effect of seed coat on the germination of seeds; and different concentration of sodium hypochlorite (NaOCl) and varying exposure time on explants sterilization; to investigate the effect of different BAP concentrations on initiation of culture; and different concentrations of BAP alone and in combination with multiplication of anchote shoots; to examine the effect of different IAA, NAA and IBA concentrations on half strength MS medium on root induction; and to investigate the percentage of survived plantlets after acclimatization. This will contribute for large quantity and healthy plant materials production for agricultural, medicinal and commercial purposes.

2. LITRATURE REVIEW

2.1. Description of Anchote (*Coccinia abyssinica* (Lam.) Cogn.)

Anchote produces one or two tubers per plant on average, and stems are vines which can grow up to two meter in height (Abera and Gudeta, 2007). Anchote usually requires staking for quality seed production. It produces heart shaped leaf, oval shape fruit and flat smooth whitish seed in the fruit ((Yassin et al., 2013). Its heart shaped leaves are palmate lobed with slightly toothed margins and arise singly at each node. The stems and leaf stalks are solid and not hollow like most other cultivated cucurbitaceous crops.

The tuberous root is the principal component of this crop (Abera and Gudeta, 2007). In terms of diversity, anchote has been found highly diversified in its characteristic (root length, root diameter, root yield per plant, marketable root yield and total root yield) (Yassin et al., 2013; Bekele et al., 2014; Wondimu et al., 2014). Based on the root colors; there are two anchote cultivars well known among the local farmers, called as dimma (red pigmented) and addi (white flesh) (Yambo and Feyissa, 2013). The length of fruits ranges from 5 to 9 cm, and diameter from 3 cm to 6 cm (Yassin et al., 2013). The verities anchote could be due to the genotype of the plant and environmental factors.

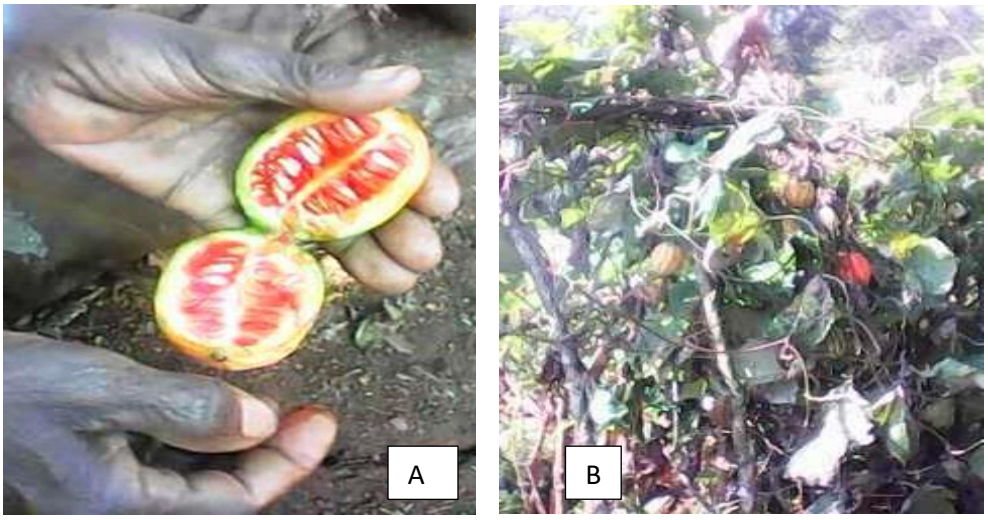

Figure 1. Anchote plant (A) seeds inside frute, and (B) shoot parts of the plant (Photo:Abose, 2017).

2.2. Origin and Ecological Distribution of Anchote

Ethiopia is the country in the world where crop domestication started, and considered as a primary gene center for several crop plants (Vavilov, 1951). Other scientists (Harlan, 1969; Frankel, 1973) reported the existence of many cultivated crops in Ethiopia which show considerable genetic diversity. According to IBCR (2001), at least 7000 vascular plant species occur in Ethiopia, of which 12% are believed to be endemic. It is also stated in ENBSAP (2005) that crops such as anchote (*Coccinia abyssinica* (Lam.) Cogn.), are believed to be originated in Ethiopia. According to Westphal (1974), anchote is one of several root and tuberous crops (Yam, Taro, Oromo Potato, Irish Potato, Sweet potato and Enset) grown in west and southwestern parts of the highlands. Anchote is endemic to the Western parts of Ethiopian highlands (Getahun, 1973; Westphal, 1974). According to Edward (2001), anchote is grown as a root crop only in the west, south west and southern regions of Ethiopia. This plant is endemic to the western part of Ethiopian highlands, which consist of East and West Wollega, Kelam Wollega and Matu regions where it can be found in wild and cultivated forms (Fekadu et al., 2013, 2014). Yassin et al. (2013) also stated some of the Western and Southwestern provinces which are well known for the production are Wollega, Kaffa, Sidamo and Illubabor, where other tuberous species of *Colocosia*, *Dioscorea* and *Musa* are also cultivated.

Ethiopia has about 18 agro-ecological zones which are endowed with suitable climatic and edaphic conditions for quality and quantity production of various kinds of root and tuber crops (EIAR, 2008). Anchote is cultivated over an extensive range of environments from 1300 to 2800 meter above sea level, with an annual rainfall of 762 to 1016 mm, with sporadic distribution (Getahun, 1973; Abera and Gudeta, 2007; Yassin et al., 2013; SFF, 2014). It occurs in many parts of Ethiopia including the western, southern and northern parts (Edward, 1991; Yambo and Feyissa, 2013). Oromia regional state is well known for its anchote production in Ethiopia. However, the cultivation is also scattered in southeastern, central and eastern Ethiopia (Bekele et al., 2014). It is adapted well to south and western parts of the country. Westphal (1974) classified the country into many agro ecological regions based on climate, altitude and soils. Of which two regions have special connections with anchote. The south eastern part of the Ethiopian high lands; these areas are situated at

altitude of 1800 meter above sea level, and have afisoils as a major soil type. They receive 950 to 1500 mm average annual rainfall. The south western parts of the Ethiopian high lands including Wollega, Illubabor and Jimma have oxisols, ultisols and vertisoils as major soil types. The southwestern parts of the Ethiopian highlands are situated at 1500 to 2400 meter above sea level and receive an annual rainfall of about 1500 mm to over 2000 mm per year. Anchote is adapted to many ecological conditions. It is popular for being drought resistant, can be grown in bad soil conditions (Getahun, 1973; Wondimu et al., 2014). A suitable temperature for root's proper growth and development range from 12 to 28°C with a soil pH of 4.5 to 7.5 (Wondimu et al., 2014).

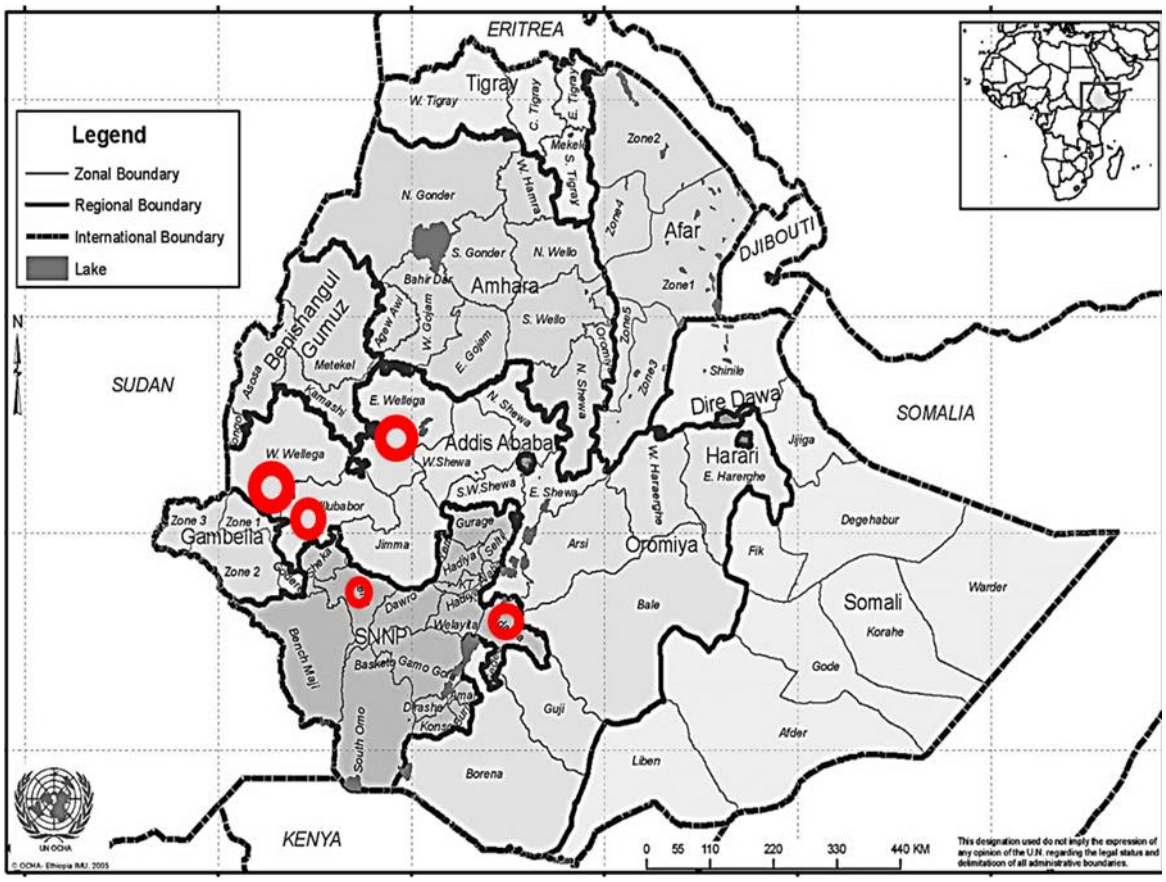

Figure 2. Map of Ethiopia showing the major areas of production and consumption of Anchote as indicated by red spots (Getahun, 1973).

2.3. Taxonomy and Name of Anchote

The scientific classification of Anchote (*Coccinia abyssinica* (Lam.) Cogn) according to Candolle (2007) is Domain: Eukaryota; Kingdom: Plantae; Subkingdom: Viridaeplantae; Phylum:Tracheophyta; Subphylum: Euphyllophytina; Class: Magnoliopsida; Subclass: Dilleniidae; Superorder: Violanae; Order: Cucurbitales; Family: Cucurbitaceae; Subfamily: Cucurbitoideae;Tribe: Benincaseae; Genus:Coccinia; Specific epithet: abyssinica Cogn. Botanical name: *Coccinia abyssinica* (Lam.) Cogn.

There are seven species recorded in flora of Ethiopia since 1995, *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* and *C. schliebenii* Harms. The remaining three species have not yet been described (Hora, 1995).

Anchote is the vernacular name of *C. abyssinica* (Lam.) Cogn. Other names which exist are 'Ushushu' (Welayita), 'Shudhe' (Dawuro) and 'Ajjo' (Kafinga). However, anchote is the most common name, which comes from Afan Oromo origin, as it is found primarily in Oromia region (Fekadu et al., 2013, 2014).

2.3. Propagation of Anchote

Anchote can be propagated by vegetative method (planting the tubers) or seed propagation (Bekele et al., 2014), however, the most common way for propagation is via seeds (Hora, 1995; Yambo and Feyissa, 2013). Seeds are extracted from fully mature red-ripe fruits, which are harvested before they start rooting. Such fruits are macerated or sliced to separate the seeds from the fleshy juicy part (Olika, 2006). The seeds are then mixed with an equal quantity of wood ash and dried in sun. The moisture content of the seeds for storage is based on the desired level. During this storage period, the seeds are usually kept in either clay or wooden pots or wrapped in a sheet of cloth (Hora, 1995; Olika, 2006). Vegetatively, it is achieved by planting either the whole tuber or by slicing it in two or more pieces, each pieces, having rootlets and an external covering. This is usually done to establish "Mother" plants, called "Guboo" to serve as a seed source for further plantings.

2.4. Uses of Anchote

2.4.1. Food and Nutritional Uses of Anchote

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 (EIAR, 2008). The low agricultural productivity, recurrent drought and socio-political factors have greatly contributed to critical food shortages in Ethiopia coupled with over-dependence on few cereal crops; thus, integration of root and tuber crops into the food system of the people should be given a serious attention. Anchote can be safely stored under the ground, which thus gives added food security to the population in times of main crop failures.

Anchote is cultivated for its starchy roots and young shoots as vegetables (Bekele et al., 2014, Fekadu et al., 2014). Anchote is a valuable food source (Bekele, 2007). It is a subsistence crop commonly produced to fill food security gaps during summer season (June to September) (Fufa and Urga, 1997). As a food, it is a rich source of carbohydrate, vitamins, minerals, protein and calcium as compared to other root crops (Nebiyu et al., 2008; Fekadu, 2011). It is also rich in minerals such as calcium, magnesium, iron and low levels of anti-nutrients such as phytate, oxalate, tannin and cyanide (Fekadu et al., 2013, 2014; Yassin et al., 2013). Root and tuber crops have good nutritive value and phytochemical contents which are beneficial to the human health. In addition to major food staff (proteins, carbohydrates, fats, mineral and vitamins), anchote contains phytochemicals such as alkaloids, tannins, phenols, flavonoids and saponins (Okwu and Ukanwa, 2007).

Nutritionally, 100 gram of anchote contains moisture 74.93g, crude protein 3.25g, CHO 92.11g, crude fat 0.19g, total ash 2.19g and crude fiber 2.58g with a total gross energy of 382.78 Kcal/100g. In addition, anchote contains 119.5 mg Ca, 5.49 mg Fe, 34.61 mg P, 79.73 mg Mg and 2.23 mg of Zn in 100g of anchote (Bradbury and Holloway, 1988; EHNRI, 1997; USDA, 2002; Ayele, 2009). Habitually, its storage root serves as a side dish with cereals as; kitifo, lankata (finely grounded tuber), ‘wot’, soup and murmura (boiled tuber cut in pieces). Similarly, the leaf also primed as ‘wot’ and serves as a side dish with bread or injera (Hora, 1995). Apart from daily sources of calories and another essential

nutrient, it is prepared for special ceremonies and guests; it is sometimes also used for animal fattening (Bekele et al., 2014).

Preparation of Anchote Food: Like many other root, and tuber crops, anchote is rarely eaten raw (Fufa, and Urga, 1997). It undergoes some form of processing and cooking before consumption. The roots are cleaned superficially, or peeled with knife and then cooked in boiling water or grated. The washed tuber was boiled for about three to three and half hours, peeled and sliced to uniform thickness of about 5 mm using a stainless steel knife.

The simplest preparation is boiling the harvested roots and peeling them before eating with some salt and grounded pepper. Other more complex preparations involved the addition of many spices and good quantity of butter. Butter and spiced boiled anchote is made into a paste and eaten alone or with local bread (Getahun, 1973). Traditionally, boiling could be after peeling or before peeling. Such processing can have both detrimental and beneficial effect to the nutrient content of food. The purpose of such processing is to make anchote more palatable, digestible, to inactivate enzyme inhibitors, and other anti-nutrient to qualify it for human consumption (Dawit and Hagos, 1991).

Cooked roots are usually served with kochkocha (a fermented side-dish prepared from ground green pepper with green leafy varieties of spices like coriander (*Coriandrum sativum*), sweet basil (*Ocimum basilium*), ginger (*Zingiber officinale*), garlic and salt. Anchote sauce locally known “ittoo anchote” prepared by cooking sliced roots with butter and a traditional dish called “lanqaxaa” are both made for special occasions of wedding, engagements, birthdays and religious ceremonies (SFF, 2014). Stew prepared from anchote flavored with spices and butter is eaten with the main dish in the Wollega region of Ethiopia (Umeta et al., 2005). In a study by Fekadu et al. (2013, 2014) on the effect on nutritional and sensory qualities of traditional preparation/cooking methods, it was found that the local methods of preparation were helping in increasing crude fiber content, the bioavailability of many minerals and resulted in the reduction of anti- nutritional factors. In the sensory analysis, most preferred cooking practices were boiling the roots before peeling. As anchote leaves are also edible, they are cooked like a vegetable and served with other foods (Mengesha et al., 2012). The tender leaves and top growing buds are plucked together like

tea leaves, and cooked to be served with other especial food. The sliced, sun dried and ground (flour) of anchote remains in good conditions for a long time. The flour is used to prepare a soup when boiled with bone-marrow from animals.

2.4.2. Medicinal Uses of Anchote

The juice prepared from tubers of anchote has saponin as an active substance and is used to treat gonorrhea, tuberculosis, and tumor cancer (Dawit and Hagos, 1991). According to Getahun (1973) also, traditional practitioners use anchote to treat different types of diseases such as diabetes, gonorrhea, tuberculosis, asthma and cholesterol lowering.

Traditionally, in anchote birth place, Wollega, people use over stayed or over matured anchote tuber when they face a problem of bone-fracture and makes lactating mothers healthier and stronger, due to the fact that anchote is supposed to contain high Ca, Fe and proteins than other common and widespread root and tuber crops (Hora, 1995; Abera and Gudeta, 2007; Fekadu, 2013, and Yambo and Feyissa, 2013). In other words, the local people believe that anchote has medicinal value in healing many maladies.

2.4.3. Economic and Socio-cultural Uses of Anchote

Anchote is not only grown for home consumption but also for sale, apart from roots and leaves of food, seeds and seedling for propagation are some of the items which are marketed (Hora,1995). The price for anchote tuber varies with tuber size, time of the year, supplies amount and market location.

In a comparative study by Abera and Gudeta (2007), anchote root yield presented a higher response to organic fertilizer than inorganic ones, which is important in the case of poor farmers who can produce their farm yard manure at low or no cost to enhance the root yield.

The production of anchote has strong cultural ties with Oromo Nation. Because of its attachment to customs and tradition of the people in the region, the revival in the society is affecting and will continue to influence anchote culture (Fekadu, 2011). In the western parts of Ethiopia, tubers of anchote are boiled and prepared with local butter for Meskel holiday in September, which is celebrated for the finding of the true cross.

The role played by Oromo women in anchote culture is a lot. They work as: Breeders by selecting a desirable quality of anchote and growing it, growers as they plan, plant and weed the plants, harvesters as they determine the maturity stage that gives anchote of the type and how to harvest it properly, processor, as they prepare, process and taste for its quality both physical and behavioral; and finally as marketers and distributors (Hora, 1995). The benefits that the Oromo women derive from anchote the existing experience is that they can decide or convince their husbands in family forums, to reach decisions on the many affairs concerning anchote. This includes site selection (the decision on where to grow), what size of land area to use for anchote, variety selection, buying or selling of anchote products and for what purpose to use the money in return. That is encouraging and should be appreciated is that besides their right to decide on the many affairs of anchote, Oromo women have experience in exercising this right. They have stood on their own feet and decided freely and fairly what they think is should be done in cultivating and using anchote. It is the advantage that exceeds the monetary benefits (Olika, 2006). Women are at the center of production and processing of this crop, which makes it more valuable regarding social and gender perspective (Abera and Gudeta, 2007; Mengesha et al., 2012). As women are responsible for the marketing of the crop, an increase in popularity of crop can enhance the income generation and power of women in the community.

2.5. Cultivation and Harvesting of Anchote

Anchote is found both cultivated and wild (Edwards, 1991). The crop is harvested for young shoots as vegetables, seeds for propagation and tubers for the preparation of different anchote dishes (Yambo and Feyissa, 2013). Among the major root and tuber crops, anchote is a potential crop produced on nearly 3000 ha of land in West Wollega zone with a yield of about 25,000 tonnes (Anonymous, 2011). Hora (1995) reported that a farmer in Western parts of Wollega usually allocate 400 to 600 square meters of land for anchote production mainly for home consumption. In West Oromia region, it is produced on several hundred hectares with an average yield of 10 to 15 tonnes per ha (Gemeda, 2000). Annual total production has been estimated about 25000 tons in the whole country (Bekele et al., 2013). According to a report (Hora, 1995), farmers have their own experience by which they maintain seeds for the next planting. Among the quality attributes the farmers take in to

account are cooking quality, durable quality and time taken for tuber formation. There are traditional selection practices being followed by farmers to have anchote types of desirable qualities, such as larger tuber size (Fikadu, 2011). Women usually do the selection and maintenance of good quality anchote root and discard of the undesirable ones.

More than 98.62 % of farmers selected to grow the white type. They said this type has more advantage like less fiber content, quickly prepared, soft, attractive and less out ward rootlets than red type. On the other hand, red type has medicinal value than white one (Elias, 2018).

Its productivity shows a difference based on genotypes, soil fertility level, location and cultural practices used. Under farmers condition, it can yield 20 to 30 tonnes per ha (Hora, 1995; BARC, 2004). However, under research condition it has a potential to yield up to 73 tonnes per ha (Fikadu, 2011) and 76.45 tonnes per ha (Mengesha et al., 2012). Harvesting of fruits and roots is done 4 to 5 months after planting, whereas the leaves can be plucked at any time when they are available in adequate amounts (Yassin et al., 2013).

Wondimu et al. (2014) mentioned that genotypes which produce more above ground biomass (fruits and leaves) tend to produce lower root yields. This could be attributed to higher assimilate competition among shoots and roots. Yassin et al. (2013) investigated the effects of flower bud removal on root yields; the study was conducted at research site under irrigation conditions. The highest root yield was found in Kuwe accession which was 94.37tonnes/ha, and the lowest was 37tonnes/ha for the accession 240407B. In all the treatments flower bud removal increased the root yield by about 15% regardless of the cultivar; this is due to the strong competition between generative and root part of the plant. Highest root weight per plant was also observed for Kuwe variety. Significant variations were found among the different accessions of anchote regarding root yield and other yield component parameters, the presence of developing fruit negatively affect the yield components of the crop. It was recommended that accessions Kuwe should be used as it has the highest productivity among all the accessions, and removal of flower bud would help in gaining more root yield.

Traditionally, farmers use *in situ* stored anchote tuber with the assumption of nutrient concentration increases over time. This was approved that yield and nutrient concentration

of anchote is affected by harvesting dates and *in situ* storage. Extending the harvesting dates and *in situ* storage from 4 to 7 month significantly increased fresh and dry tuber yield by an average of 450%. The results confirmed that delayed harvesting dates and *in situ* storage improved anchote tuber nutrient concentrations, largely calcium and iron (Abera and Haile, 2016).

Pit Storage is practiced by farmers when the harvest is more than consumption as roots kept in the open can lose moisture and susceptible to rotting and pest attack. No studies were found on the post-harvest physiology, storage, and decay of anchote (Hora, 1995). Traditionally, roots are harvested manually with traditional tools when needed which helps in reducing post-harvest losses. Roots can be kept underground up to two years without a significant loss in quality (Wondimu et al., 2014).

Therefore, Yambo and Feyissa (2013) recommended fruits should be collected on time, formal seed supply systems should be developed, better way for fruit collection, seed extraction, processing, storage and distribution of seeds should be developed. Ripened anchote fruits should be collected on time before they start rotting and attacked by insects, formal seed supply systems should be developed for such marginalized root crops and better way for fruit collection, seed extraction, processing, storage and distribution of seeds should be developed. Research work is required to study the effect of post-harvest processing, packaging, and storage of seeds on germination quality.

Usually farmers sow anchote seeds in April or May and harvest in July or August. Anchote harvesting date often depends on farmers' wealth condition. At the time of food shortage, farmers are forced to harvest anchote as early as possible. It is interesting to note that early harvest may result in low yield and poor quality produce. Anchote produces one or two roots per plant, which is usually harvested after 4 to 5 months of planting when the leaves turn yellow (Hora, 1995; Abera, 1997). Remarkably, anchote has special merits in that the tubers can be maintained on the farm (stored in soils) for considerable number of months after attained harvestable maturity (Abera and Gudeta, 2007).

Sowing Method: Anchote is commonly propagated by seeds. The existing practice is to sow the seeds by broadcasting. After broadcasting the seeds are covered either by ploughing with oxen or more commonly using a digging hoe (Qonfaro) with or without metal (Ayele, 2018). During broadcasting farmers prefer a narrow spacing of about 20 cm than a wider spacing. According to (Hora, 1995), intra-row spacing highly affect root yield while inter-row spacing affect root yield and average root weight per plant. The reduction of intra-row spacing from 30 cm to 10 cm resulted in increase of total tuberous root yield by 137%. Reduction of inter-row spacing from 100 cm to 40 cm resulted in high total tuberous root yield by 37.4%. Therefore, 40cm to 60cm inter-row and 10 cm intra-row spacing are recommended for the western sub-humid zones of Oromia. Yambo and Feyissa (2013) mentioned that a very low germination rate (9%) was observed in the seeds which were purchased from the market. This could be due to prolonged seed storage, seeds from unripe fruits.

2.6. Threats and Protection of Anchote

Anchote is believed to be low input crop with minimal occurrences of diseases and pest attack (Hora, 1995). It is not seriously attacked by disease and pests, but fruit decay result prematurely due to certain kind of wasp and fruit fly effect. Cholera (*Vibrio cholera*) has an effect on the mother plant result premature falling down of fruit. Grass hopper affects seedling. The tubers are rarely attacked by rodents, if and only if, other root crops are harvested early (Elias, 2018). There has been an incident of wild animals digging the roots and eating them, other than that any form of disease incident on root are rare and thus protection from disease and pest is not common (Hora, 1995). It remains minimally affected by pests such as termites (Getahun, 1973 and Wondimu et al., 2014).

Farmers usually grow anchote near dwelling areas. This land gives an equal opportunity for weeds to grow luxuriously. Hence, anchote needs early weeding and better land preparation (Getahun, 1973). Fruit fly bores into the fruit and pre-disposes it to decay. Wild animals, particularly wild pig, Porcupine and Wart-hog; domestic animals; sheep, goat, cattle and donkeys are on the list. The wild animals eat the anchote tuber by digging into the soil and are terrible pests of anchote. The roots have to be protected by fencing the anchote field

properly and also by guarding the crop at night when these pests come out and attack the crop (Getahun, 1973 and Wondimu et al., 2014).

Rotting and pest attack on matured fruits can take place. Seeds should be harvested immediately after fruits are matured and sun dried and packed in indigenous packages such as bamboo baskets or wooden boxes (Yambo and Feyissa, 2013). However, more studies are required on pre and post-harvest problems which occur in fruits and affect the seed quality.

2.7. Micropropagation of Anchote

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. The addition of suitable hormones can then induce root growth, and the plants can 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 relies on the phenomenon of cell totipotency (Thorpe, 2007), the ability of single cell to divide, to produce all the differentiated cells characteristic of organs, and to regenerate into a whole plant. The different techniques of culturing plant tissues may offer certain advantages over traditional methods of propagation. Growing plants *in vitro* in a controlled environment, with in-depth knowledge of the culture conditions and the nature of the plant material, ensures effective clonal propagation of genetically superior genotypes of important plants.

Tissue cultures represent the major experimental systems used for plant genetic engineering, as well as for studying the regulation of growth and organized development through examination of structural, physiological, biochemical and molecular bases underlying developmental processes. Micropropagation has become an important part of the commercial propagation of many plants (Thorpe, 2007) because of its advantages as a

multiplication system (Razdan, 2003). Different techniques in plant tissue culture may offer certain advantages over traditional methods of propagation which include: The production of exact copies of plants; Regeneration of whole plants from plant cells that have been genetically modified; Production of plants from seeds that have very low chances of germinating and growing.

In modern plant tissue culture, it is performed under aseptic conditions. Living plant materials from the environment are naturally contaminated on their surfaces (and sometimes interiors) with microorganisms, so surface sterilization of starting material (explants) in chemical solutions usually alcohol sterilization followed by sodium or calcium hypochlorite or mercuric chloride are needed. Mercuric chloride is seldom utilized as a vegetation sterilant today, except other sterilizing agencies are discovered to be ineffective, as it is unsafe to use, and is tough to dispose of (Mineo, 1990). Explants are then usually placed on the surface of a solid culture medium, but are sometimes placed directly into a liquid medium particularly when cell suspension cultures are desired. Solid and liquid media are generally composed of inorganic salts and few organic nutrients, vitamins and plant hormones. Solid media are prepared from liquid media with the addition of gelling agent, usually purified agar.

The composition of the medium, particularly the plant hormones and the nitrogen source (nitrate versus ammonium salts or amino acids) have profound effects on the morphology of the tissues that grow from the initial explants. Forexample, an optimal concentration of auxin (Indole acetic acid, αNaphthalene acetic acid) by stimulating cell expansion, particularly cell elongation promote the proliferation of roots, while an optimal concentration of cytokinins (Zeatin, Kinetin) may yield shoots by producing two immediate effects on undifferentiated cells: the stimulation of DNA synthesis and increased cell division (Bakare, et al., 2010).

A balance of both auxin and cytokinin will often produce an organized growth of cells, or callus because both cell division and cell expansion occur in actively dividing tissue, but morphology of the outgrowth will depend on the plant species as well as medium composition. As cultures grow, pieces are typically sliced off and transferred to

new media (sub-cultured) to allow for growth or to alter the morphology of the culture. As shoots emerge from a culture, they may be sliced off and rooted with auxin to produce plantlet, when matured can be transferred to potting soil for further growth in the greenhouse as normal plants (Barceto et al., 2001).

3. OBJECTIVES

3.1. General objective

The general objective of this study was to develop *in vitro* propagation protocol of anchote (*Coconia abyssinica* (lam.) (cogn.) from *ex vitro* shoot explants.

3.2. Specific objectives

- To evaluate the effect of decoating seed coat on the germination of seeds
- To evaluate the effect of different concentration of sodium hypochlorite (NaOCl) and varying time exposure on explants sterilization
- To investigate the effect of different BAP concentrations on the initiation of the culture
- To investigate the effect of different concentrations of BAP alone and in combination with NAA on shoot multiplication of anchote shoots
- To examine the effect of different IAA, NAA and IBA concentrations on half strength MS medium on root induction and,
- To evaluate the percentage of survived plantlets after acclimatization.

4. MATERIALS AND METHODS

4.1. Plant Materials

The seeds of white anchote variety were taken from West Wollega, Laliwa Sabi district. These seeds were sown in pots that contain loam soil. The shoots from grown plants were used as explant.

4.2. Medium Preparation

4.2.1. Stock Solution Preparation

MS (Murashige and Skoog, 1962) medium was used. The stock solutions of the medium were prepared separately by weighting the recommended amount of macronutrients, micronutrients, vitamins, and EDTA. The solutions were poured into plastic bottles and stored at – 20 °C until used.

4.2.2. Plant Growth Regulators Preparation

Plant growth regulators: Indole-3-Butyric Acid (IBA), 6-Benzyl Amino Purine (BAP), αNaphthalene Acetic Acid (NAA) and Indole Acetic Acid (IAA) were prepared in 1.0 mg/ml concentration as stock solution. To prepare PGRs stock solution: 0.10mg powdered of each hormone was weighted and dissolved in 3-4 drops of NaOH by stirrer in small glass bottle. Upon complete dissolution, 10 ml of double distilled water was added to it and stirred gently and stored at a temperature of +4 °C.

4.2.3. Culture Media Preparation

For shoot initiation, to prepare one liter of MS medium, 800 ml of double distilled water was added into a volumetric flask, 30g sucrose, 50 ml macronutrients, 5 ml micronutrients, 5 ml iron EDTA, 5 ml vitamins, and different concentration of BAP were added and allowed to dissolve. Distilled water was added to make the total volume up to 1 liter. Then the pH of the medium was adjusted to 5.6 to 5.8 using either 1M HCl or 1M NaOH. After that the solution was 7.0 g agar powder was added dissolved gently by using magnetic stirrer until

the entire agar was dissolved and wait up to the solution was boiled. The medium (50 ml) was dispensed into each culture vessels and autoclaved at 121°C for 15 min.

For shoot multiplication, the same procedures as that of shoot initiation was used except using additional growth regulators (BAP alone and in combination NAA in different concentrations).

For rooting, the same procedures as that of shoot multiplication were used except using half amount of sucrose, macro and micronutrients, and different concentration of auxins (IBA, IAA, and NAA). The different types and concentrations of growth regulators for initiation, multiplication, and rooting were shown in **Tables 3, 4, 5 and 6** respectively.

4.3. Seed Germination

A total of 120 seeds were used for germination. Sixty seeds were decoated and the remaining sixty were not decoated. Thirty seeds were planted in each of four pots, which mean decoated seeds were planted in two pots and coated seeds were planted in other two pots. At the first, second and third weeks, the numbers of germinated seeds were recorded.

4.4. Shoot Initiation

Three-month-old healthy plants were used as a source of explant. About two to three centimeter long shoot tip explants were taken and placed in a beaker containing tap water. Once in the laboratory, they were washed in running tap water. Then, the explants were taken in to a sterile laminar air flow cabinet. Then, the explants were rinsed with distilled water two to three times. The explants were surface sterilized in 70% alcohol for about 10 seconds, followed by washing in sterile distilled water three to four times followed by sterilization in NaOCl 10% for 15 min and washed in sterile distilled water three to four times. At the end, the sterilized shoot explants were cultured on shoot multiplication culture media (thirty explants were used on each treatments) and kept in culture room at 16 hours photoperiod under light intensity of 22µmol $m^{-2}s^{-1}$and temperature of 25 ± 2°C. After a month, the effects of different concentration of BAP (0, 0.25.0.50 and 1.00) were recorded (**Table3**).

4.5. Shoot Multiplication

The initiated shoot explants were cultured on MS medium containing different concentrations of BAP alone (0.1, 0.25, 0.5. 1, 1.5, 2) and BAP (0.25, 0.5, 1, 1.5, 2) in combination with NAA (0.1, 0.5, 1, 1.5) and growth regulator free for shoot multiplication (**Tables 4 and 5**). Thirty shoot explants per treatment were used. After a month of culture, the number of shoots and their height from each treatment was recorded.

4.6. Rooting and Acclimatization

About three to five centimeter long shoots were taken from the multiplied shoots and cultured on growth regulators free half strength MS medium for three days. Then cultured on a half strength MS medium supplemented with different types and concentrations of auxin, IAA (0.10 and 0.25 mg/l), 0.5 mg/l IBA, NAA (0.25, and 0.5 mg/l), and 15 g/l sucrose. Growth regulators free medium was used as a control. After three weeks on rooting medium, the plantlets were taken from the culture vessels and the roots were washed under tap water. The root length and the number of roots per plantlet were recorded. The plantlets were transferred to pot containing sterilized soil. The pots were covered with transparent plastic bags with random holes from underside for aeration and watered twice every day. The plastic cover were partially removed after a week and completely removed after two weeks. Then finally, the number of survived plants was recorded after a month.

4.9. Data analysis

Primary data were collected from experments. The experments were done on the effect of seed coat on seed germination, effect of different concentration of NaOCl and different exposure time for shoot explants sterilization; effect of different concentrations of growth regulators for shoot initiation, multiplication and rooting and percentage of survival after acclimatization.

The data were subjected to analysis of variance (ANOVA) using SPSS computer software, version 16.0. The mean separation method (LSD) was used to compare means between

treatments. Mean homogenicity analysis was carried out using Tukey's B homogeneity test. For all probability level 0.05 (p=0.05) was used considering for statistical significance. All the data were manipulated and presented in tables.

5. RESULTS

5.1. Seed Germination

Decoating the seeds of anchote seed during *ex situ* germination showed significant effect. The decoated seeds have germinated faster than the coated ones. The germination of the decoated seeds resulted in high germination (67%) after three weeks whereas the coated seeds of anchote resulted in low germination (43%) at the same time interval (**Table 1**).

Table 1. Effect of seed coat on seed germination of anchote with different length of time

Time (Weeks)	Germination percentage	
	Coated seed	Decoated seed
1	0	10
2	15	48
3	43	67

Figure 3. Three to four weeks old anchote plants. Seedlings from coated seeds and (A and B); Seedlings from decoated seeds (C and D)

5.2. Surface Sterilization of Shoot Explants

The shoot explants that were surface sterilized with 10% NaOCl for 15 min resulted in the highest percentage (60%) contamination-free explants. It was also observed that the surface sterilized explants with the lowest concentration of NaOCl (5%) for 15 min resulted in the highest percentage contamination (53%) (**Table 2**).

Table 2. Effect of different concentrations of NaOCl (5% and10%) with different time length (15 and 20 min)

Concentration of NaOCl % (v/v)	Time (min)	Percentage of dead explants	Percentage of contaminated explants	Percentage of normal explants
10	15	13	27	60
10	20	25	20	55
5	15	0.5	53	46.5
5	20	14	33	53

5.3. Shoot Initiation of Anchote

Initiation difference was observed during shoot initiation of anchote from *ex vitro* shoot explants on different concentrations of BAP (0.00, 0.25, 0.5, and 1.00 mg/L) after a month (**Table 3**).The maximum mean number of shoots per explant (2.82±0.73) were obtained on MS medium containing 1.0 mg/l BAP and the maximum mean shoot length of 3.70 ± 0.58 cm was obtained on MS medium containing 0.25 mg/l BAP.

Table 3. Effect of different concentrations of BAP alone on shoot initiation of anchote

BAP (mg/l)	shoot number per explant mean ± SD	Shoot length (cm) Mean ± SD
0.00	1.31±0.48[b]	2.77±O.59[b]
0.25	2.35±0.70[a]	3.70±0.58[a]
0.50	2.53±0.84[a]	3.06±0.59[b]
1.00	2.82±0.73[a]	3.03±0.55[b]

Means with the same letter within the same column are not statistically different at p≤0.05. Thirty shoot explants per treatment were used.

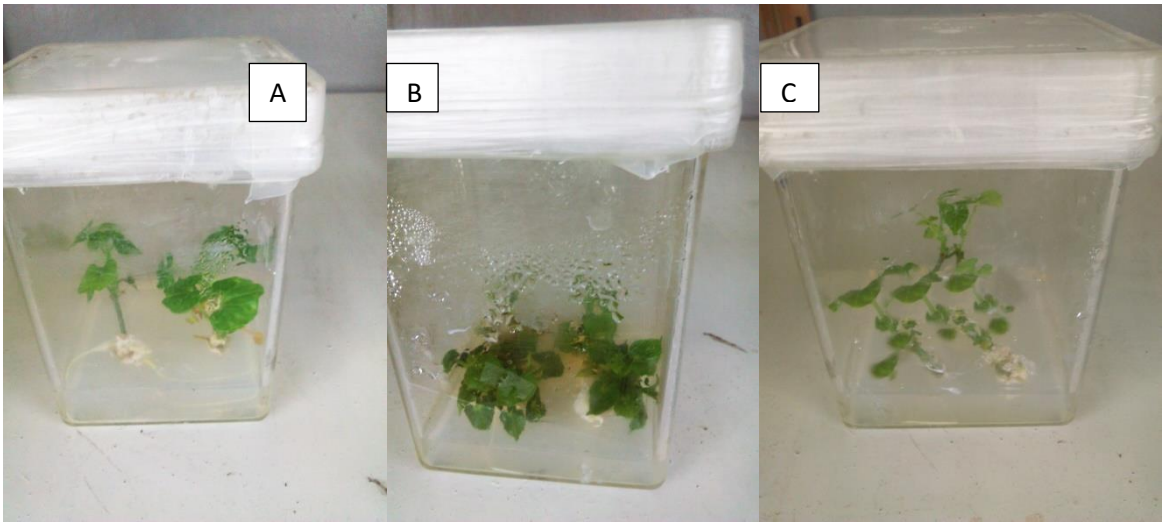

Figure 4. Shoot initiation of anchote from *ex situ* shoot explants on MS medium containing different concentrations of BAP alone.(A) Shoots initiated on growth regulators free MS medium (B) Shoots initiated on MS medium containing 1.0 mg/l BAP and (C) Shoots initiated on MS medium containing 0.25 mg/l BAP.

5.4. Shoot Multiplication

From various concentration of BAP used, the medium containing 1.50 mg/l BAP produced maximum mean number of shoots per explant (4.07±1.62). Maximum shoot length (3.56±0.51 cm) was observed when the medium contained 0.25 mg/l BAP (**Table 4**). The response of explants cultured on MS medium supplemented with different concentrations of BAP in combination with NAA is presented in **Table 5**. There was significant difference in the number of shoots per explant on MS medium containing various concentrations of BAP in combination with NAA at $p \leq 0.05$. Maximum numbers of shoots per explant (4.40±1.73 and 4.40±1.30) were observed in MS medium containing 1.0 mg/l BAP in combination with 0.10 mg/l NAA and 1.50 mg/l BAP in combination with 0.10 mg/L NAA respectively. Maximum shoot length (3.69±0.75 cm) was observed in MS medium supplemented with 0.25 mg/l BAP in combination with 0.10 mg/l NAA.

Table 4. Effect of different concentrations of BAP alone on shoot multiplication

BAP (mg/l)	Shoot number per explant Mean ± SD	Shoot length (cm) Mean ± SD
0.00	1.13 ± 0.43^{d}	2.14 ± 0.46^{e}
0.10	2.73 ± 1.26^{c}	2.34 ± 0.50^{e}
0.25	3.33 ± 1.29^{abc}	3.56 ± 0.51^{ab}
0.50	3.70 ± 1.62^{abc}	3.36 ± 0.51^{ab}
1.00	3.87 ± 1.60^{abc}	3.30 ± 0.56^{abc}
1.50	4.07 ± 1.62^{ab}	3.11 ± 0.73^{abcd}
2.00	3.90 ± 1.18^{abc}	3.06 ± 0.64^{abcd}

Means with the same letter within the same column are not statistically different at $p \leq 0.05$. Thirty shoot explants per treatment were used.

Table 5. Effect of different concentrations of BAP in combination with different concentration NAA on shoot multiplication

PGRs(mg/l)		Shoot number per explant	Shoot length (cm)
BAP	NAA	Mean ± SD	Mean ± SD
0.25	0.10	3.33±1.52^{abc}	3.69±0.75^{ab}
0.50	0.10	3.47±1.43^{abc}	3.35±0.71^{ab}
0.50	0.50	3.30±1.15^{abc}	2.36±0.71^{e}
1.00	0.10	4.40±1.73^{a}	3.33±0.63^{ab}
1.00	0.50	3.87±1.70^{abc}	2.00±0.64^{bcd}
1.00	1.00	3.43±0.90^{abc}	2.23±0.65^{e}
1.50	0.10	4.40±1.30^{a}	3.46±0.75^{ab}
1.50	0.50	3.93±1.26^{ab}	3.41±0.79^{ab}
1.50	1.00	3.77±1.07^{abc}	2.69±0.75^{cde}
1.50	1.50	2.97±0.67^{bc}	2.66±0.71^{de}
2.00	0.10	4.17±1.29^{a}	3.43±0.83^{ab}
2.00	0.50	3.40±0.93^{abc}	3.23±0.84^{abcd}
2.00	1.00	3.67±0.84^{abc}	3.10±0.84^{abcd}

Means with the same letter within the same column are not statistically different at $p \leq 0.05$. Thirty shoot explants per treatment were used.

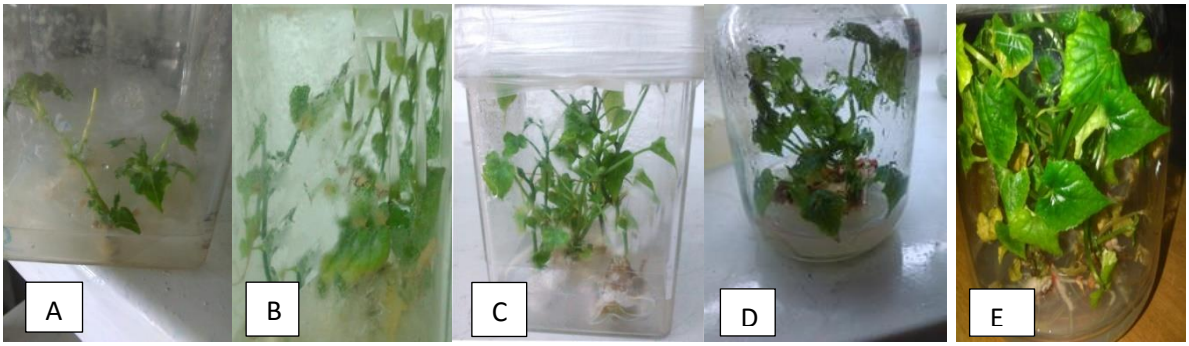

Figure 5. Shoot multiplication of anchote from shoot explant on MS medium containing. (A) Growth regulators free medium, (B) 0.25 mg/l BAP alone, (C) 1.0 mg/l BAP and 0.10 mg/l NAA, (D) 1.5 mg/l BAP and 0.10 mg/l NAA, and (E) 2.0 mg/l BAP and 1.0 NAA.

5.5. Rooting and Acclimatization

The maximum mean root number (12.27±3.58) and maximum mean root length (5.98±1.13 cm) were obtained on the half-strength MS medium containing 0.25 mg/l IAA.

Table 6. Effect of different concentrations of IAA, IBA and NAA on rooting of anchote

PGRs(mg/l)			Root number	Root length (cm)
IAA	NAA	IBA	Mean ± SD	Mean ± SD
0.00	0.00	0.00	1.60±1.10^{d}	2.09±1.15^{c}
0.10	0.00	0.00	6.97±3.07^{b}	4.43±1.51^{b}
0.25	0.00	0.00	12.27±3.58^{a}	5.98±1.13^{a}
0.00	0.25	0.00	3.43±1.25^{c}	1.88±0.70^{c}
0.00	0.50	0.00	6.60±1.48^{b}	2.16±0.77^{c}
0.00	0.00	0.50	0.60±1.13^{d}	0.19±0.38^{d}

Means with the same letter within the same column are not statistically different at $p \leq 0.05$. Thirty shoot explants per treatment were used.

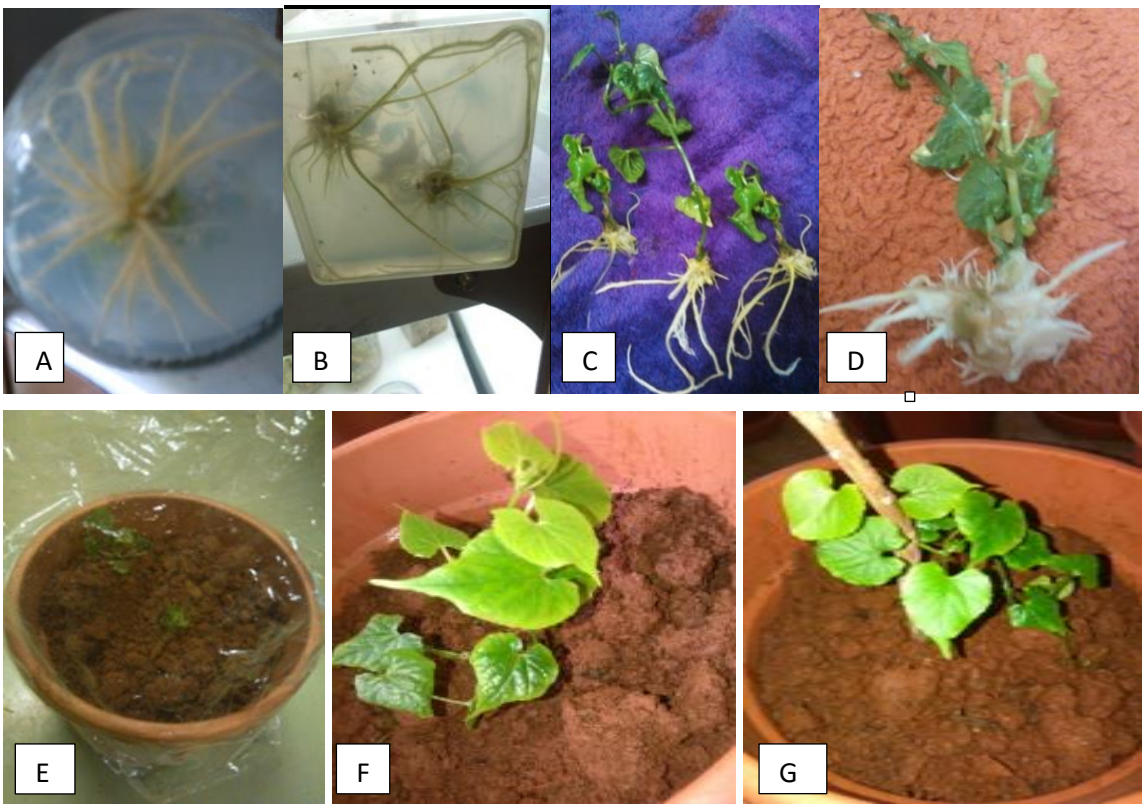

Figure 6. *In vitro* rooting of anchote on half strength MS medium containing (A) 0.25 mg/l IAA, (B) 0.25 mg/l NAA, (C) 0.10 mg/l IAA, and (D) 0.50 mg/l IBA, and acclimatization (E) Established plantlets after a week and (F&G) acclimatized and survived plantlets after a month.

6. DISCUSSION

6.1. Seed Germination

Seeds of anchote were planted to evaluate the effect of seed coat during seed germination. Decoating the seeds of anchote seed during seed germination has significant effect. Decoated seeds were germinated faster than the coated ones. The germination of the decoated seeds resulted in high germination (67%) after three weeks whereas the coated seeds of anchote resulted in low germination (43%) at the same time interval. This could be due to that seed coat prevents the exchange of gases and water that induce seed germination. Hartmann et al. (2002) also stated the presence of seed coat prevents the exchange of gases and water that induce germination. To increase the chance of germination, the seeds were water-soaked and the floating seeds were removed. But low germination of seeds (67%) were gained as compared to Abera and Haile, (2016) reported that average germination of seed was noted to be about 75% under laboratory condition and is presumed to be lower under field condition. There are different factors mentioned by Negash (1993) that affect the percentage of germination. These are duration of seeds storage, status of seeds and maturation of seeds. The quality of seed affects the growth and development of plants even after germination. According to Yambo and Feyissa (2013), prolonged seed storage, extracting seeds from unripe fruits as well as seed extraction from fruits dropped to ground may be some of the factors that reduce germination rate of anchote seeds. These could be the reasons that lower seed germination in this experiment. Therefore proper seed harvest and storage should be carried out.

6.2. Shoot Explant Sterilization

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 and affect the growth of desired plant tissue culture by competing with the plant tissue for nutrition, thus depriving the plant tissue of nutrients. Various sterilization agents are used to decontaminate explants. The surface sterility chosen for an experiment typically depend on the type of explants and also plant species (Rezadost et al., 2013). In this study, 5% and 10% concentrations of NaOCl with exposure time of 15

and 20 minutes were used for surface sterilization of shoot explant taken from *ex vitro* plants. Explants are commonly surface-sterilized using sodium and calcium hypochlorite (household bleach), ethanol, and fungicides when using field grown tissues. The shoot explants that were surface sterilized with 10% NaOCl for 15 minutes resulted in high (60%) contamination free explants. It was also observed that shoots that were surface sterilized with 5% NaOCl for 15 minutes led to high contamination of explants (53%) because of microbes could resist the effect of lower concentration of this stirilant. The same concentration of NaOCl (10%) applied for 20 min led to higher death of explants (25%). This could be due to the nature the explants which is shoot tip tissue. Shoot tip explants are highly affected by length of exposure time for stiralization. According to Guma et al. (2015) the time of sterilization is dependent on the type of tissue; for example, leaf tissue and shoot tissue requires a shorter sterilization time than seeds with a tough seed coat. Over-exposing tissues to decontaminating chemicals can also kill tissues, so there should be a balancing act between sterilizing explants and killing the explants themselves (Qin et al., 2012; Olewet et al., 2014). CPRI, (1992) also reported chemical sterilants are toxic to the plant tissues at higher concentrations, hence proper concentration of the sterilants, duration of exposure of the explant to various sterilants, the sequences of using these sterilants has to be standardized to minimize explant injury and achieve better survival.

6.3. Shoot Initiation

Depending on the type of explant, shoot formation may be initiated from apical and axillary buds. In this study, the explants were taken from shoot tip grown *ex vitro*. The highest mean number of shoots per explant (2.82±0.73) was produced on MS medium supplemented with 1.0 mg/l BAP with significant difference with PGR-free medium(1.31±0.48), but with no significant difference with other concentrations of BAP (0.100, 0.25, 0.50 mg/l) at p=0.05. When the concentration of BAP increased from 0.10 to 1.0 mg/l, the number of shoot increased because of cytocinin (BAP) hormone is used to increase shoot initiation and shoot proliferation.so not only shoot number, shoot length also increased as compared to PGR-free media.

The highest mean shoot length (3.70±0.58 cm) was produced on MS medium supplemented with 0.25 mg/l BAP with significant difference from PGR-free medium and other concentration of BAP (0.50 and 1.0 mg/l). When the number of shoots increased per explant, the length and thickness of shoots partial decreased because of nutrient computation of the proliferated shoots. In other study by Jane et al. (2016), the highest (3.40 ± 0.5) mean number of shoots/explant was obtained on the medium supplemented with 2.5 µM BAP.

When the concentration of BAP increased (0.00 to 1.0 mg/l), there were formations of unwanted callus at the base of shoot proliferation. This showed that the medium was conducive for high cells formation but without organogenesis. Jane et al., (2016) stated callus formation and hyper-hydrated shoot formation occurred with higher concentration of BAP (5 µM and above) and with lower concentration of BAP produced normal vigorous growing, green micro-shoots. There were also shoot formation on growth regulators free MS medium. This is in lined with the present study. There were about 1.31±0.48 mean numbers of shoot formation on growth regulators free medium.

6.4. Shoot Multiplication

When the concentration of BAP alone increased from 0.10 to 2.00, callus formation was increased. While lower concentrations of auxin (NAA) were added to the same concentration of BAP, better shoot formations were obtained. This situation agreed with Yambo and Feyissa, (2013) result, With increasing BAP concentration alone from 0.08 to 1.0 mg/l, callus induction was promoted whereas when lower concentration of IBA (0.05, 0.075 or 0.1 mg/L) was combined with the BAP concentrations within this range, well multiplied normal shoots were obtained. In the present study, maximum mean shoot numbers per explant (4.40±1.30 and 4.07±1.62) were produced on MS medium supplemented with 1.50 mg/l BAP in combination with 0.1 mg/l NAA and 1.50 mg/l BAP alone respectively. The number of shoot proliferated increased with increasing of the concentration of BAP from 0.1 to 1.50 mg/l. But, Yambo and Feyissa (2013) stated the concentration of BAP beyond 0.75 mg/l was super optimal. The maximum shoot length (3.69±0.75 and 3.56±0.51 cm) was obtained on the medium containing 0.25 mg/l BAP in combination with 0.1 mg/l NAA and 0.25 mg/l BAP alone respectively. The minimum shoot

number (1.13±0.43, 2.73±1.26 and 2.97±0.67) were produced on MS medium containing growth regulators free medium, 0.1 mg/l BAP alone and 1.5 mg/l BAP in combination with 1.5 mg/l NAA respectively. When the concentration of auxin (NAA) that combine with BAP increased, the number of shoots as well as the shoot length decreased in comparison with lower concentration of NAA that combine with BAP as showed in Table 5. When the concentration of NAA increased from 0.1 to 1.5 in combination with BAP, there were callus and root formation. This could be the reason that decreased shoot number and shoot length. The callus induction and inhibition of growth with increasing BAP concentration agrees with the micropropagation works of other tuber crops such as cassava (Beyene et al., 2010). 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. 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.

6.5. Rooting and Acclimatization

In the present study, data of root number and main root length per explant was recorded after three weeks of culturing on the rooting media. The maximum root number (12.27±3.58) and the highest root length (5.98±1.13 cm) were obtained from half strength MS medium containing 0.25 mg/l IAA with significant difference from other treatments used. In this work, 0.50 mg/l IBA produced the shortest roots, and produced callus at the base and the shoot parts became necroteic and died gradually. But, according to Beyene et al. (2010) as IBA concentration in rooting medium increased, root number per explant in cassava also increased, but the roots became shorter and devoid of branching as the present study.

Sammaiah et al. (2014) reported on bitter melon which belongs to the same family Cuccurbitaceae, highest numbers of roots were produced at 2.0 mg/l IBA and 1.5 mg/l IAA. When exposed to high concentration (above 3.0 mg/l) IBA/ IAA shoots become necrotic, lost leaves and the shoot tips died gradually. While at lower (below 1.0 mg/l) concentration of IBA and IAA, lower number of roots was induced. But for anchote, lower concentrations of rooting hormones are preferable. In the present study, IAA is better than NAA and IBA in

inducing rooting. NAA (0.50 mg/l) produced better root numbers (6.60±1.48) and root length (2.16±0.77 cm) as compared to IBA (0.50 mg/l) which gave less root number (0.60±1.13) and shorter root length (0.19±0.38 cm). As Jane et al., (2016) reported NAA is better rooting hormone for anchote micro shoots compared with IBA. A possible explanation for this could be because the stability of the two auxins is different: IBA is slowly oxidized (10%), while NAA is very stable (Dunlap et al., 1986). Another reason for the different effectiveness observed among the two auxins could be due to possible different affinities for auxin receptors, differences in uptake, transport, and metabolism (De Klerk et al., 1997). Variation in rooting response also may be a result of genotype or culture conditions.

The ultimate success of *in vitro* propagation lies in the successful establishment of plants in the soil (Saxena and Dhawan, 1999). Transplantation of *in vitro*-derived plants to soil is often characterized by lower survival rates. Before transfer of soil-rooted plants to their final environment, they must be acclimatized in a controlled environment room or in the glasshouse (Preece et al., 1991). Plants transferred from *in vitro* to *ex vitro* conditions, undergo gradual modification of leaf anatomy and morphology, and their stomata begin to function (the stomata are usually open when the plants are in culture). Plants also form a protective *epicuticular* wax layer over the surface of their leaves. Regenerated plants gradually become adapted to survival in their new environment (Donelly et al., 1993). During the current study, 78% of the regenerated plantlets were survived and developed new branches, and were ready for planting in the field for further growth. Higher (83%) survival rates were reported by Jane et al, (2016) in the greenhouse while lower survival (68%) was reported by Yambo and Feyissa (2013). The survival rates could be affected by the soil content, temperature, moisture and plant roots status. The regenerated plants did not show any variation in the morphology and growth when compared to the mother plant.

7. CONCLUSION

The common way of anchote propagation is via seeds, and as both cross- and self-pollination may occur in anchote, it may be difficult to obtain true-to-type plants. Therefore, *in-vitro* propagation technique is very important in providing many true-to-type copies of the desired plants, quick production of mature plants, regenerate genetically modified individuals, reduced chances of transmitting diseases and pathogens. Decoated seeds had better germination rate than coated seeds. 60% normal sterilized explants were obtained from 10%v/v NaOCl sterilization that expose for 15 min. The maximum shoot numbers regenerated were 4.40±1.73 and 4.40±1.30 shoots per explant on MS medium supplemented with 1.00mg/l BAP with 0.10mg/l NAA and 1.50 mg/l BAP with 0.10mg/l NAA respectively. The maximum root numbers (12.27±3.58) and the maximum root length (5.98±1.13 cm) were obtained on the half strength MS medium containing 0.25 mg/l IAA. The percentage of survival after acclimatization was 78%. Therefore, this protocol is used to produce many true-to-type copies and disease free anchote products which is used to fulfill food security gap in agriculture and it could be also used for commercial production of anchote products.

8. RECOMMENDATION

Even though, anchote is highly useful, there are few *in vitro* propagation studies conducted on it. So, more studies should be conducted on it.

The shoot sterilization should be optimized to get better normal explants.

When the concentration of BAP increased from 0.1 to 1.0, the number of shoots increased. So, the effect of higher concentration of BAP should be examined.

The genotypes of anchote explant should be predetermined before conducting *in-vitro* propagation.

For better improvement of the anchote by modern biotechnological techniques such as genetic transformation, more efficient regeneration protocol should be developed.

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10. APPENDEX

Components	Concentration(g/L)	ml/L during media preparation
Micronutrients		5ml/L
$ZnSO_4.7H_2O$	1.72	
H_3BO_3	1.124	
$MnSO_2.4H_2O$	3.38	
$MnSO_4.H_2O$	0.05	
KI	0.166	
$NaMoO_4.2H_2O$	0.05	
$CoCl_2.6H_2O$	0.05	
Na_2EDTA	7.472	
$FeSO_4.7H_2O$	5.56	
Macronutrients		50ml/L
NH_4NO_3	33	
KNO_3	38	
$CaCl_2.2H_2O$	8.8	
$MgSO_4.7H_2O$	7.4	
KH_2PO_4	3.4	
Vitamins		5ml/L
Myo-inositol	20	
Glycin (glycocoll)	0.4	
Nicotinic acid (NaOH)	0.1	
Pyridoxin (B6)	0.1	
Thiamin (B1)	0.02	

Appendix 1 stock solution for MS (Murashige and Skoog 1962)