Evaluation of Carbon Sources and Plant Growth Regulators for Successful Callus Induction of two Cotton Cultivars (Worer 50 and Sisikuk 02) from Cotyledon and Hypocotyl Explants

By: Tizibt Yeshigeta

A Thesis Submitted to

The department of Applied Biology

School of Applied Natural Sciences

Presented in Partial Fulfillment of the Requirement for the Degree of Master's in Applied Biology (Specialization in Biotechnology)

Office of Graduate Studies

Adama Science and Technology University

June, 2021

Adama,Ethiopia

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DECLARATION

I hereby, declare that this Master Thesis entitled "Evaluation of Carbon Source and Plant Growth Regulator for Successful Callus Induction of two Cotton Cultivar (Worer 50 and Sisikuk 02) from Cotyledon and Hypocotyl Explants" is my original work. That is, it has not been submitted for the award of any academic degree, diploma or certificate in any other university. All sources of materials that are used for this thesis have been properly acknowledged through citation.

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RECOMMENDATION

I/we, the advisor(s) of this thesis, hereby certify that I/we have read the revised version of the thesis entitled “Evaluation of Carbon Source and Plant Growth Regulator for Successful Callus Induction of two Cotton Cultivar (Worer 50 and Sisikuk 02) from Cotyledon and Hypocotyl Explants” prepared under my/our guidance by Tizibt Yeshigeta submitted in partial fulfillment of the requirements for the degree of Master’s of Science in Applied Biology (Specialization in Biotechnology).

Therefore, I/we recommend the submission of revised version of the thesis to the department following the applicable procedures.

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Major Advisor	Signature	Date
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Co-advisor	Signature	Date

APPROVAL OF BOARD OF EXAMINERS

We, the advisors of the thesis entitled “Evaluation of Carbon Source and Plant Growth Regulator for Successful Callus Induction of two Cotton Cultivar (Worer 50 and Sisikuk 02) from Cotyledon and Hypocotyl Explants” and developed by Tizibt Yeshigeta, hereby certify that the recommendation and suggestions made by the board of examiners are appropriately incorporated into the final version of the thesis.

Major Advisor	Signature	Date
Co-advisor	Signature	Date

We, the undersigned, members of the Board of Examiners of the thesis by Tizibt Yeshigeta have read and evaluated the thesis entitled “Evaluation of Carbon Source and Plant Growth Regulator for Successful Callus Induction of two Cotton Cultivar (Worer 50 and Sisikuk 02) from Cotyledon and Hypocotyl Explants” and examined the candidate during open defense.This is, therefore, to certify that the thesis is accepted for partial fulfillment of the requirement of the degree of Master of Science in Applied Biology (Specialization in Biotechnology).

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LIST OF ABBRIVATIONS

2, 4-D	2, 4-dichlorophenoxy Acetic Acid
B5	Gamborg medium
CRD	Completely Randomized Design
KN	Kinetin
MARC	Melkassa Agricultural Research Center
MS	Murashige and Skoog medium
NAA	α-Naphtaline Acetic Acid
PGRs	Plant Growth Regulators
PTC	Plant tissue culture
SAS	Statistical Analyses System
TDZ	Thidiazuron
WARC	Worer Agricultural Research Center

ABSTRACT

Cotton (Gossypium hirsutum L) is one of the most important fiber crops, which play a key role in the world economy, and it is commercially grown in the tropical and sub-tropical region of more than 90 countries. However, the production of cotton is adversely affected by biotic and abiotic stress. To overcome such kind of problem the application of tissue culture techniques is very essential. Therefore, the objective of this study was to optimized carbon, growth regulator and explant sources for successful callus induction of two commercially grown cotton (Gossypium hirsutum L) cultivars ('Worer 50' and 'Sisikuk 02'). For this experiment cotyledon and hypocotyl with the size of 2-3mm^2 and 3-5mm respectively from seven days old, aseptically seeding cultured on MSB5 medium supplemented with three carbon sources (maltose, glucose and sucrose) at different concentrations to investigate their effect on callus initiation and phenol reduction. For callus induction, cotyledon and hypocotyl cultured on MSB5 medium containing different concentrations and combinations of KIN (kinetin), TDZ (thidiazuron), NAA (naphthalene acetic acid) and 2,4-D (2,4- dichloro phenoxy acetic acid), used. The results revealed that maltose (30 g/l and 40 g/l) and glucose 30 g/l showed callogenesis at hypocotyl explant within a week and both glucose and maltose at all concentration level was effective to control phenolic compound. Whereas sucrose with all concentration showed phenolic compound and relatively showed less callus response. Maximum percentage of callus with compact feature was recorded on MSB5 culture medium fortified with 0.5 mg/l KIN + 0.1 mg/l 2,4-D for Sisikuk 02 88 % and 72 % for Werer 50 . Friable callus with maximum percentage were recorded on MSB5 medium fortified with 0.1 mg/l TDZ in combination with 0.1 mg/l NAA. Hypocotyl explant showed 16 to 88% callus response whereas cotyledon explant gave from 4 to 76%. The results of this study provide useful information to go for the next stages of regeneration and of course better carbon sources, explant and hormone combination were identified for the cotton cultivars grown in Ethiopia.

Keywords: Callus, Cotton, Cotyledon,Ethiopia, Explant,Hypocotyl,Phenolic compound

1. INTRODUCTION

Cotton (Gossypium *hirsutum* L.) is one of the most important fiber crop, which plays a key role in the world economy, and it is commercially grown in the tropical and sub-tropical region of more than 90 countries (Sahito *et al*., 2015). It is the richest source of fiber and the backbone of the textile industry. Further, Cotton seed is used for oil extraction and seed cake is used as animal food. The word "cotton" is derived from the Arabic word "qutum" or "kutum", meaning a soft substance. Scientists searching caves in Mexico found bits of cotton bolls and pieces of cotton cloth that proved to be at least 7,000 years old. However, no one distinguishes perfectly how old cotton is (Sakhanokho and Rajasekaran, 2016).

According to the report of Juturu *et al*. (2015) China,the USA, India, Uzbekistan, Pakistan, the Middle East and Australia are the foremost cotton cultivator countries and together they covered about 80% of the world's cotton. In the continent of Africa, leading cotton-growing countries are Egypt, Tanzania, Mali, and Burkina Faso (Basal and Sezener, 2012). Ethiopia is one of the Sub-Saharan African countries that produce and export cotton. The country has an estimated area of 2,575,810 hectares that is suitable for the cultivation of cotton. However, the total production area is only about 100,000 hectares (Bosena Tegegne *et al.,* 2011).

The production of cotton is adversely affected by both biotic and abiotic stress, from which the biotic factors such as insect pests take the major of the production challenges. Survey results revealed that more than 68 species of insect and mite pests have been recorded on cotton in major cotton growing areas of Ethiopia. Among these insect pests, those belonging to bollworms namely African, Pink, Sudan, and Spiny bollworms caused 36 - 60% yield loss in which African bollworm called *Helicoverba armigera* are the dominant bollworm species (Alemayehu Refera and Ababu Demissie, 1985).

In Ethiopia, cotton consumes about 65% of the total pesticide purchase. This chemical is mainly used for the protection of cotton from major insect pests. This huge application of diverse chemistries of insecticides poses a lot of challenges to human, animals, and the environment through affecting beneficial non-target organisms. On top of this, these insect pests had developed resistance to several chemistries of insecticides around the globe including Ethiopia. To cope up with this problem, looking for sound and environmentally

friendly alternatives for the management of major insect pests are of paramount importance for maximizing cotton production and keeping safe the environment from hazardous insecticide chemicals (Yang *et al*., 2013).

Genetic improvement of cotton through conventional breeding is limited by incompatibility obstacle and consumes more time to improve variety. The limited genetic variability that existed among cotton germplasms in Ethiopia did not allow for further improvement of the cotton plant against many biotic and abiotic stresses. To overcome such kind of problem, tissue culture is the alternative technique with the capability to improve the production of cotton and obtain cotton plants with the desired trait within a short time of period. Normally, cotton is recalcitrant for *in vitro* culture in which regeneration is somewhat difficult (Hayta-smedley *et al*., 2018).

Several attempts have been made to obtain efficient cotton regeneration protocol all over the world. However, the main factors that influence the efficiency of *in-vitro* culture and determine the response of cultured tissue in cotton are carbon sources (Kouakou *et al* ., 2007; Zhang *et al.*,2001), explant sources (Hazra *et al*., 2000), type of plant growth regulators (sun *et al*., 2006) and culture medium (Sanghera *et al* .,2009). Therefore, it is quite important to investigate the effect of these factors on the development of efficient regeneration protocol in cotton cultivars, which is a prerequisite to get transgenic lines of the local cotton cultivar. In Ethiopia, *in-vitro* regeneration of cotton has not been yet reported. This study is the first attempt to optimize sources of carbons, explants, genotypes and plant growth hormones in tissue culture protocols for efficient regeneration of cotton (*Gossyium hirsutum*) cultivars (Werer 50 and Sisikuk 02).

1.2. Statement of the Problem

To meet the rising demand for superior quality cotton as the natural textile fiber and to limit injury of environment caused by the wide use of pesticides, there is a growing interest in the quantitative and qualitative improvement of cotton varieties. Recognizing the importance of plant tissue culture for genetic transformation and micropropagation, attempts have been made to regenerate callus and tissues in culture. However, cotton's *in-vitro* regeneration is limited due to a lack of shoot elongation, difficulties of rooting and browning which causes the death of tissues or explants. These problems are related to carbon sources, growth regulators, genotypes, explant sources and other culture conditions

such as medium composition or other physical culture conditions (Ganesan *et al*., 2005; Kamal *et al.*,2011; Ozyigit *et al*., 2007; Ghaemi *et al*., 2011; Zafar, 2004; Ikram-Ul-Haq ,2004). Hence, determining the optimum carbon sources, growth regulators and explant sources is important to develop an effective and efficient tissue culture protocol for such recalcitrant crops. Therefore, the present study has the following general and specific objectives.

1.3. Objectives of the study

1.3.1. General objective

- To optimize carbon source and growth regulator for successful callus induction of two commercially grown cotton (*Gossypium hirsutum* L.) cultivars ('Worer 50' and 'Sisikuk 02').

1.3.2. Specific objectives

- To evaluate the effect of different carbon sources on phenol secretion and callus initiation.
- To evaluate the effect of differences in genotype on the success of callus induction of two cotton cultivar.
- To evaluate the effect of explant sources on the success of callus induction of two cultivars of cotton.
- To optimize growth regulators for successful callus induction of two cotton cultivars.

1.4. Significant of the study

- Offer a solution to control phenolic secretion in cotton callus culture by adjusting carbon sources concentration.
- Importance to identify better explant sources and cultivar for further cotton investigated.
- Contribute to other researchers for further study in cotton associated with tissue culture.

2. LITERATURE REVIEW

2.1. Origin, taxonomy and morphology of cotton

Cotton, in the genus *Gossypium* belongs to the tribe Gossypiae, in member of Malvaceae family. The genus arises as a separate evolutionary lineage around 11-14 million year ago and it has three main center of biological diversity those are Africa, Asia and Mesoamerica (Mexico, Central America and South America). The genus *Gossypium* contain about 50 recognized species globally, out of these only four are cultivated for their spinnable fiber and the remaining 46 species are distributed throughout the tropics and sub tropics in wild forms (Li *et al*., 2015; Senchina *et al*., 2003).

Cotton has four cultivated species two diploids (2n=26) namely *G. arboreum* L. *and G. herbaceum* L. which belongs to the old world cotton while the other two species *G. barbadense, and G. hirsutum* are tetraploid (2n=52) known as New world cotton. Among those species, *G.hirsutum* L species is one of the most commonly cultivated species in the world followed by *G. barbadense.* More than 80% of the world's cotton area covered by tetraploids, which play a vital role in cotton agriculture across the world. On the other hand, diploid cotton is mostly cultivated in Asia and the Middle East. India is the only country where all the cultivated species and some of their hybrid combination are commercially grown. Each of these species has a unique domestication history, diversification and use that have exhibited their genetic structure through the management and artificial selection of the variation caused by evolutionary processes over millions of years (Brubaker *et al*., 1999).

Cotton is a warm-weather crop and the cultivated species are not tolerant of freezing temperatures. Climate generally sets the geographic limits for cotton across the world. The temperature could influence the growth of cotton such as a minimum temperature for better germination at field conditions requires 15^{o}C, for vegetative growth is 21^{o}C - 27^{o}C and it can tolerate temperature to the extent of 43^{o}C but temperature below 21^{o}C is detrimental to the cotton crop. Cotton becomes inactive at a temperature below 15^{o}c (Williams *et al*., 2015). Cotton can grow on any soil type if temperature and moisture are favourable. However, the most desirable soils, in terms of moisture relationships with the growing plant are the silt loams. The cotton crop requires at least 500mm of water during its growing season (Waddle, 1984).

2.2. Importance of cotton

Cotton is one of the most important sources of natural fibers and well known for textiles and all the activities surrounding its production and commerce (Wegier *et al*.,2016). Cotton fiber or lint is the most important product, which is used in making cotton cloth and many other products obtained from cotton namely oil for food, raw material for biodiesel, cosmetic, pharmaceutical sectors and edible oil (Carvalho *et al*., 2017). The short fuzz on the seed provides cellulose that is used in Plastics, explosives, quality paper, flat-panel television screens, padding mattresses, furniture automobile cushions, and computer chip boards. The cottonseed is used in shortening, margarine, cooking oil, salad dressing and cosmetics. Less refined grades are used to manufacture soap, candles, detergents, artificial leather, oilcloth, and many other commodities. In addition meal from crushed seed used for livestock, poultry and fish feed. Furthermore, hulls are used for fertilizer, fuel and packing. The stalks and leaves are also used for fertilisation to enrich the soil during ploughing and fibers from the stalks are used for pressed paper and cardboard (Rogers *et al*., 2002).

Its importance extends to medicinal use through utilizing their extracts for cough; constipation and gonorrhoea. Furthermore, the mixtures of cottonseeds and leaves used to treat snakebites and scorpion sting and leaves of cotton used against parasites, dysentery, and plaster for gouty joints and eliminate lymphatic filariasis (Hammad *et al*. 2014). Syrup of cotton flowers is certain treatment for hypochondria. Cotton medicinal use through utilizing their extracts including for treating hypertension, anticancer activity and as a male contraceptive (Chaturvedi and Nag, 2015).

2.3. Cotton production in Ethiopia

In Ethiopia, cotton is grown in varied soils, climates and agricultural practices under irrigated and rain-fed conditions by private commercial and smallholder farmers. Approximately, 60% and 40% of Ethiopia cotton are produced under irrigated rain-fed conditions, respectively. In Ethiopia, cotton production is practiced under different agro-ecologies ranging from 300-1800 meters above sea level (EIA, 2012).

Total agro-ecological potential area for cotton production is estimated to be 2,575,810 hectares and actual production was estimated to be 42,371 hectares. The wide potential

areas of each region are reported as follow in Tigray 269,130ha, in Amhara 678,710 ha, in South nations, nationalities and people region (SNNPR) 600,900 ha, in Oromia 407,420 ha, Gambella 316,450 ha, Benshangul 303,170ha, Afar 200,000 ha and Somali 225,000ha. Most of the areas are low land and at river basins. Omo-Ghibe, Wabi Shebelle, Awash, Baro-Akobo, Blue Nile, and Tekeze river basins are the major potential areas for cotton growing (Alebel Bayrau *et al*., 2014).

According to the report of Bosena Tegegne, (2008) Ethiopia have about 2.6 million hectares of land that suitable for cotton production, among this about 1.7 million hectares or 65% is found in 38 high potential for cotton production whereas the remaining 0.9 million hectares or 35% in 75 medium potential districts. From this land, Ethiopia produced approximately 120,000 tons of cotton annually. Still, the amount of cotton produced in the country is small and the current domestic cotton production is much lower than the potential of the country. The national yield average is very low, only 11q/ha although actual yield under different production systems varies. The average yield on irrigated farms ranged from 20 to 30 q/ha, while the potential in research farm is 35 to 40 q/ha. The major markets for Ethiopian cotton are Africa, Asia, and Europe, with Asia alone accounting for 67% of the total exports (Partzsch and Kemper, 2019; Melesse Zeleke, 2019).

2.4. Factor affecting cotton production

Many factors significantly affect cotton production both in quantity and quality wise across the world. These factors include both biotic (Insect pests, weeds and pathogens) and abiotic (drought and salinity) stress especially insect pests play a significant role in affecting cotton productions. Lepidopteran insect pests namely, *Helicoverpa armigera* and *Pectinophora gossypiella* are worldwide pests of cotton (Wilkins *et al*., 2000; Yang *et al*., 2013).The major insect pest species infesting cotton in Ethiopia are boll worms, cotton mealy bug, thrips, aphid, whitefly, jassid, cotton leaf worm, the red spider mite, lesser army worm, flea beetle and strainers. Among these, boll worms are the most important pests of cotton, with African boll worm (ABW) being on top. This pest alone could cause about 36-60% yield loss. Their economic importance is due to their concealed feeding habit, which hindered them from any contact with spraying insecticides. Following this difficult feeding nature of the pest, the huge application of diverse chemistries of

insecticides poses many challenges to the environment by affecting beneficial non-target organisms. On top of this, these insect pests had developed resistance to several chemistries of insecticides globally including Ethiopia (Geremew Terefe *et al*., 2004).

2.5. Plant tissue culture and its application

Plant biotechnology standing for the development of advantageous plant by eliminating major limitations of traditional plant breeding. Tissue culture is *in vitro* aseptic culture of cells, tissues, organs or whole plant under controlled nutritional and environmental conditions by isolating them from healthy mother plant. It is important tool for both basic and applied study as well as for commercial application (Omamor *et al*., 2007).

The concept of tissue culture technique was develop by a German botanist Haberlandt Gottlieb (1902), who recognized as father of tissue culture and the first man to isolate completely differentiated cells in a nutrient medium. This technique is the process to produce thousands of clone plant from small pieces (that is explants) for well-known desired agricultural qualities relatively within short period, regardless of the season and weather in a year round basis. According to the finding of Hoy *et al.* (2003) depending on the species and the objective of the study the original (explant), tissue pieces may be taken from shoot tip, leaf, lateral bud, stem and root tissue of the mother plant. The plantlets that produced by tissue culture are a true copy of the mother plant and show identical characteristics to the mother plant (Idowu *et al*., 2009; Chaturvedi and Chaturvedi, 2017).

The development of tissue culture techniques is based on the fact of plant cell totipotency that refers to the ability of a single cell to express the full genome (capable to form tissues, organs, organ systems and complete individuals) by cell division and differentiation processes. Equivalent to totipotent of plant cell, the capacity of cells to alter their metabolism, growth and development are essential and crucial to regenerate the entire plant (García *et al*., 2010).

Tissue culture techniques used to multiply plant and *in vitro* conservation of valuable indigenous germplasm threatened with extinction. *In vitro* cell and organ culture offers an alternate source for the conservation of endangered genotypes. Germplasm conservation is vital activity due to high rate of disappearance in plant species and extend the interest of protection floristic patrimony in the world. Tissue culture protocols used for preservation

of vegetative tissues when the targets to protect are clones instead of seeds, to keep the genetic background of a crop and to avoid the loss of the conserved patrimony due to natural disasters, biotic or abiotic stress (Chandana *et al*., 2018; Oseni *et al*., 2018).

Besides to that, plant tissue culture also helps to conserve plant species which do not produce seeds or which have recalcitrant seeds that cannot be stored for long period can successfully preserved via *in vitro* techniques for the maintenance of gene banks. As reported by Cruz-Cruz *et al*. (2013) micro propagation is another application of tissue culture technique that allows the production of huge number of plants from small pieces of mother plant comparatively in short period of time. It is an aseptic process requires sophisticated laboratory procedure with unique facilities and special skills and have five well-defined multistep process contains, each with its specific requirements. Begin with the preparation of mother plant to offer quality explants, initiation of aseptic cultures, multiplication, rooting of *in vitro* formed shoots and transfer of plants to greenhouse or field conditions consistently (Iliev *et al*., 2010; Saini and Jaiwal, 2002).

It has many advantages over conventional methods of plant propagation such as creating of pathogen free plants, used for studies in genetic transformation, possible to produce haploid organisms and allow to production a large number of plants in a short time from a selected number of genotypes. Where traditional methods of multiplication are either not available or ineffective in largescale multiplication systems (Al Amin *et al*., 2009).

2.6. Factors affecting *in-vitro* propagation

In the process of plant tissues culture (PTC), there are several aspects to be considered before conducting the research. These include choosing of explants, culture media, plant growth regulator and problems during culture, such as contamination, which is the most common challenge of PTC, browning and proliferation difficulty have a direct effect on the results (Kakuei and Salehi, 2015).

Plant tissue culture media consists of major elements, trace elements, source of carbon, growth regulators, vitamins, water, gelling agent in the case of solid media and some organic material like amino acids. All the indicated components have a special effect on the growth and morphogenesis of cultured plant (George *et al*., 2008; Saad and Elshahed, 2012). pH of culture media is another major factor that adversely affects both the growth

of plants and the activity of plant growth regulators when it is highly alkaline or acidic. Commonly pH of culture media adjusted between 5.5 - 6 using HC1 and NaOH before adding agar (Skirvin *et al*., 1986). Additionally genotype, source and position of explant and other physical factors including light hour, temperature, humidity also affects *in-vitro* propagation of plants.

2.6.1. Source of explant and aseptic condition

Explant is a material used as an initial source for tissue culture. Tissue culture success mainly depends on the age, types and position of explants as the ability to express totipotency differs among the plant cells. The most commonly used explants are leaf, petiole, hypocotyl, epicotyl, cotyledon, embryo, internode and root (Hussain *et al*., 2012). Large explants can increase chances of contamination and small explants like meristems can sometimes display less growth. Commercial tissue culture laboratories normally use lateral shoots, which contain meristems. Meristems are usually used as explant of choice as they are made up of actively dividing cells in an organized manner. However, explants should be chosen from typical, healthy, disease-free, well-tested mother plants that should be ex-vivo cultivated under optimal conditions to reduce contamination and promote the growth of tissues to be cultured (Strosse *et al*., 2004; Rani and Kumar, 2017).

The most important earlier treatment to make tissue culture fruitful is surface-sterilization of explant aims to eliminate all microorganisms that can easily grow under *in-vitro* conditions. Since *in-vitro* conditions provide bacteria and fungi with an optimal growth environment, contamination can be created either through carryover of microorganism on the surface of the tissues of explants or through faulty processes in the laboratory (Daud *et al*., 2012).

Chemicals such as Bleach, ethanol, calcium hypochlorite, mercuric chloride, hydrogen Peroxide, silver nitrate and bromine water are commonly used sterilizing agent that can help to disinfect many plant materials. In the process of sterilization surfactants like Tween 20 is frequently added to the sterilizing solution. Culture media also sterilized usually by autoclaving at the temperature ranging from 115ºC - 135ºC. Ineffective sterilization interrupts the progress of tissue culture studies (Oyebanji *et al*., 2009).

2.6.2. Composition of culture medium

The composition of the culture medium is one of the most important factors that govern the growth and morphogenesis of cultured tissue. Scientists have developed numerous media formulations after the formulation of Murashige and Skoog's medium (MS), Nitsch and Nitsch medium, White medium and Gamborg medium for micro propagation. However, Murashige and Skoog's medium (MS) has been most widely used, because most plants respond to it favourably (Kumar, 2011).

The specific composition of the culture medium will depend on what is required for the growth of expant. Type, strength and combination of media are also one of significant parameter for optimizing the regeneration protocol of *in-vitro* propagation (Patel and Krishnamurthy, 2013). Culture media holds all the nutrients required for the normal growth and development of plants. It's mainly composed of mineral elements such as potassium, calcium, magnesium, nitrogen, phosphorous and Sulphur, which are required in large quantities (macronutrients) white others like iron, manganese, copper, zinc, boron and molybdenum, are required in small quantities (micronutrients). Vitamins, other organic components, plant growth regulators, carbon source and some gelling agents in the case of solid medium (Ramage and Williams , 2002; Molnár *et al*., 2011).

Sugar is added as one of the component of tissue culture media considered as it serves as a sole source of carbon for *in-vitro* growth and development of plants. Apart from this, sugar also serves as signalling molecules repressing or activating plant gene involved in many essential processes such as respiration, defense response, metabolism and transformation of energy, which are required for the growth of the cell. Optimum sugar concentration increases the growth and development of cultured plant (Alina *et al*., 2006; Mazinga *et al*., 2014; Gauchan, 2012).

Vitamins are supplemented with medium to achieve the best growth of the tissues. Four vitamins; Myo-inositol, thiamine, nicotinic acid, and pyridoxine are ingredients of Murashige and Skoog (1962) medium and have been used in different amount for the culture of tissues of many plant species. Among the vitamins, only thiamine seems to be universally required. Requirements for other vitamins are specific to each plant and differs with the nature of the plant of species and the type of culture (Saad and Elshahed, 2012).

The pH of plant tissue culture media is usually adjusted by adding diluted HCl or NaOH solution with a medicine dropper for both solid and liquid medium while keeping the medium stirred. Most tissue cultures are grown at pH ranging from 5.2 to 5.8. The medium is adjusted to optimum pH depending on the plant species used and the purpose of the study before the addition of agar (Skirvin *et al*., 1986).

2.6.3. Plant growth regulators

Plant growth regulators (PGRs) play a vital role to determine the development of plant cells and tissues in a culture medium. It required in very small quantity in the media. The requirement for these substances significantly differs with the tissue and it depends on their endogenous level. There are many synthetic substances having growth regulatory activity, with different activity and species specificity. It needs to test various types, concentrations and combinations of growth substances during the development of a tissue culture protocol for a new species (Suman *et al*., 2017; Madhulatha *et al*., 2004).

Nutritional factors is unspecific with respect to growth and differentiation predominately recognizable in quantitative terms. However, PGRs apply for rather specific influences usually at low concentrations in the medium. There are several classes of PGRs namely cytokinins, auxins, gibberellins, ethylene and abscisic acid (Bisht *et al*., 2018). The main one that used frequency in plant tissue culture experiment are presented below.

Auxins are involved in many developmental processes with plants, including elongation of stem and internodes, tropisms, apical dominance, abscission and rooting. In tissue cultures, auxins used to cause cell elongation, swelling of tissues, cell division, callus formation and the formation of adventitious roots (Tivendale and Cohen, 2015).The most commonly used auxins are indole-3- acetic acid (IAA), indole-3- butyric acid (IBA), naphthalene acetic acid (NAA),and 2,4- dichloro phenoxy acetic acid (2,4-D). IAA and IBA are naturally occurring auxins whereas NAA and 2, 4-D are synthetic. IBA, NAA and IAA are widely used for rooting (Dąbski and Parzymies, 2007; Kumar and Reddy, 2011).

Cytokinins are another most important group of PGRs which are adenine derivatives that modulate a number of physiological and biochemical processes. These hormones are concerned with cell division, modification of apical dominance, shoot differentiation. In plant tissue culture medium, cytokinins are incorporated mainly for cell division,

differentiation of adventitious shoots from callus and organ and shoot proliferation. Most commonly used cytokinins are kinetin(KIN), 6 Benzyl Amino Purine (BAP), thidiazuron (TDZ), 2-isopentyl adenine(2ip) and zeatin among these KIN,TDZ and BAP are used more frequently whereas zeatin and 2iP are not widespread as they are expensive (specially zeatin) in addition to that they are also relatively unstable (Piotrowska *et al*., 2005).

High auxin to cytokinins ratio commonly favors root formation, whereas high cytokinins to auxin ratio favors shoot formation. Balance of both auxin and cytokinins will often produce an unorganized growth of cells, or callus. However, morphology of the outgrowth will subjective to plant species as well as the medium composition. (Jayaraman *et al*., 2014) 2, 4 dichlorophenoxyacetic acid (2,4-D), naphthalene acetic acid (NAA), 6-benzylaminnopurine (BAP), and thidiazuron (TDZ) are among the most PGRs that used for callus induction (Sharma and Nautiyal, 2009). Only a few of the gibberellins are used in plant tissue culture media, GA3 being the most common (Overvoorde *et al*., 2010; Rout, 2004).

2.7. *In vitro* propagation of cotton

Improving cotton through modern biotechnological approaches is quite important to obtain improved characters. In this regard, the application of genetic engineering could play significant role in improving several agronomic characteristics of cotton by transferring desirable traits using various methods of gene transfer. In this case, the application of tissue culture techniques is very essential for successful transformation of the target genes (Kalbande, and Patil, 2016).

Comprehensive studies have been conducted on callogenesis in cotton from several cultivar using various explant and different growth regulator combination. Since the first attempts of somatic embryogenesis in cotton using the variety Coker 310 by Price and Smith (1979) several researcher have worked extensively for getting efficient callus culture and regeneration of cotton plant for different cotton cultivars all over the world (Shoemaker *et al*.,1986; Mishra *et al*., 2003; Aydin *et al*.,2004; Wu *et al*., 2004). As studied by Han *et al*. (2009), plant regeneration from cotton cells was achieved from two year old callus of G. *hirsutum* Coker310 variety. For the successful application of the tissue culture technique in crop breeding, callus growth and plant regeneration potential of each crop must be determined (Khaleda and Al-Forkan, 2006).

3. MATERIALS AND METHODS

Seeds of two cotton cultivars namely ‘Worer 50’ and ‘Sisikuk 02’ were taken as plant materials for this study. Both cultivars were collected from Worer Agricultural Research Center (WARC) found in the Afar region, Ethiopia. The study was conducted at Plant Tissue Culture Laboratory, Melkassa Agricultural Research Center (MARC).

3.1. Seed surface sterilization and germination

Acid delinted seeds of cotton were surface sterilized by 70% ethanol for 5 minutes and rinsed three times with sterilized distilled water followed by 15% commercial bleach (5.25% (v/v) NaOCl solution with two drops of Tween 20 for 15 min. After that, the seeds were washed three times with sterile distilled water and further disinfected using 3g of fungicide(redomen) for 10 minutes followed by rinsing five times with sterile distilled water. Then the sterilized seeds were aseptically inoculated on PGRs free MSB5 basal medium for germination. To certify aseptic condition under *in-vitro* conditions, instruments used for sterilization were autoclaved at a temperature of 121°C and 105 KPa pressure for 45 minutes. All sterilization activities were performed under a laminar flow cabinet.

3.2. MSB5 stock preparation

MS (Murashige and Skoog, 1962) salt with Gamborg vitamins B5 (Gamborg *et al*., 1968) medium was used throughout the experiments. Nitrate, sulfate, halide, PBMO (Potassium Boric acid molybdate), FeEDTA (Ferrous sulfate Ethylene diamine teraacetic acid), vitamins and thiamine were prepared separately (Table1). To do so, an appropriate amount of each nutrient was weighed in grams per litter and dissolved in double distilled water sequentially, in such a way that the next nutrient was added after the first one was completely dissolved. After all the components were fully dissolved using a magnetic stirrer, finally the solution was dispensed into labelled plastic bottles and stored at +4°C until used.

Table 1:Macro and micro nurtrient 100* MS stock solution

Stock (100*)	Component	Amount/L
Nitrate	Ammonium nitrate (NH_4NO_3) Potassium nitrate (KNO_3)	165g 190g
Sulfate	Magnesium sulfate($MgSO_4.7H_2O$) Manganous sulfate ($MnSO_4.4H_2O$) Zinc sulfate ($ZnSO_4.7H_2O$) Cupric sulfate ($CuSO_4.5H_2O$)	37g 2.2g 0.86g 2.5mg
Halide	Calcium chloride ($CaCl_2.2H_2O$) Potassium iodide (KI) Cobalt chloride ($CoCl_2$. $6H_2O$)	44g 83mg 2.5mg
PBMO	Potassium phosphate (KH_2PO_4) Boric acid (H_3BO_3) Sodium molybdate (Na_2MoO_4)	17g 620mg 25mg
NaFeEDTA	Ferrous sulfate ($FeSO_4.7H_2O$) Ethylene diamine teraacetic acid, disodium salt (Na_2EDTA)	2.78g 3.72g

3.3. Plant growth regulators stock preparation

In this study, all plant growth regulators (PGRs) stock solutions were prepared by weighing and dissolving the powder in distilled water at 1:1 (1mg/ml) ratio. The cytokinins used for the study were Thidiazurone (TDZ), kinetin (KIN) whereas the auxins were 2, 4-Dicholorophenoxyacetic acid (2,4-D) and α-naphthalene acetic acid (NAA). The powdered crystal of the PGRs were first weighed and dissolved in 3-4 drops of NaOH and HCl , using a magnetic stirrer. After complete dissolution, the volume was adjusted by adding double-distilled water. Finally, PGRs solutions were stored in a refrigerator at a temperature of $+4^0$ C.

3.4. Evaluation of the effect of carbon sources on callus initiation and phenol reduction

To evaluate different carbon source in phenolic reduction and callus initiation the culture medium was prepared by taking 10 ml from each of the stock solutions namely nitrate, sulfate, halide, PBMO, FeEDTA, vitamin and thiamine, and then different concentration (10 - 40g/l) of maltose, sucrose and glucose were added (Table 2). All the MSB5 culture medium supplemented with 0.5mg/l KIN with the combination 0.1mg/l 2,4-D.The pH was adjusted to 5.7 - 5.8 using NaOH or HCl. Finally, 8 g/L of agar added and the medium was boiled on a stove until the agar melted. Then, 30 ml of the prepared medium was poured into culture vessels. The culture vessels were covered with caps immediately after dispensing the medium and autoclaved by steam sterilization at a temperature of 121°C and 105 KPa pressure for 15 minutes. The medium was then stored in the culture room for at least 72 hours before inoculation. The experiment has twelve treatments plus one control group. Each treatment was replicated six times. Each replication was cultured with six explants. The experiment was conducted using two different explant sources (hypocotyl and Cotyledon) separately. The size of cultured hypocotyl and cotyledon had approximately 3-5 mm and 2 - 3 mm2 respectively.

Table 2: Carbon sources and concentration used callus initiation and phenol secretion

Carbon sources	Concentration (g/l)
Maltose	10
	20
	30
	40
Sucrose	10
	20
	30
	40
Glucose	
	10
	20
	30
	40

3.5. Effect of hormones on callus induction

Callus induction culture medium was prepared by taking 10 ml from each of the stock solutions namely nitrate, sulfate, halide, PBMO, FeEDTA, vitamin and thiamine from prepared stock, then 30 g/l maltose were added sequentially. After mixing all the components the volume was adjusted to the required level of 1 liter. P^H was adjusted to 5.7-5.8 using NaOH or HCl. Finally, 8g/L of agar was added and the medium was boiled on a stove until the agar melted. Then, 30 ml of the prepared medium was dispensed into culture vessels. The culture vessels were covered with caps immediately after dispensing the medium and autoclaved by steam sterilization at a temperature of 121°C and 105 KPa pressure for 15 minutes. The experiment has 13 treatments, 12 with different concentrations and a combination of plant growth hormones (KN, TDZ, NAA and 2, 4-D) and the control without hormone (Table 3). Each treatment was replicated five times. Two different explant sources hypocotyl and cotyledon from two cotton cultivar (Worer 50 and Sisikuk 02) were used with the sizes of 3-5 mm size and 2 - 3 mm^2 respectively. The experiment was conducted separately for each of the explants. Five explants were cultured per each replication.

Table 3: List of growth regulator, concentration and their combination for callus induction

Treatment	growth regulator (mg/l)			
	KIN	TDZ	NAA	2,4-D
T1	0.1			0.1
T2	0.1			0.5
T3	0.5			0.1
T4	0.5			0.5
T5		0.1	0.1	
T6		0.5	0.1	
T7		1	0.1	
T8		0.1	0.5	
T9		0.5	0.5	
T10		1	0.5	
T11	0.1		0.5	
T12	0.5		0.5	
T13				

3.6. Experiment design and culture conditions

A completely randomized design (CRD) was used for all experiments. All cultures were maintained at 25±2ºC under cool fluorescent light with a 16/8 h light/dark cycle. Subculture was carried out every four weeks.

3.7. Data analysis

The statistical significance of the data obtained from this study for carbon sources in callus initiation was determined by one-way analysis of variance (SAS version 2009). The mean values are compared by least significant difference (LSD) ($p < 0.05$) and the percentages of phenolic secretion and callus induction were calculated as follow were calculated.

$$\%\ \text{of phenolic secretion} = \frac{\text{number of explants showing phenolic secretion}}{\text{Total number of explants}}$$

$$\%\ \text{of callus induction} = \frac{\text{number of explants showing callus induction}}{\text{Total number of explants}}$$

4. RESULT

4.1. Seed germination

Surface sterilized seed of two cultivars (‘Worer 50 ’and ‘Sisikuk 02’) cultured on growth regulator free MSB5 medium showed germination after 4 days of culturing. Both cultivars showed elongated root and hypocotyl of similar length.

4.2. Effect of different carbon sources on callus initiation

This experiment was conducted with three sugars (glucose, maltose and sucrose) and tested at four different concentration (10, 20, 30 and 40g/l) as carbon sources on MSB5 callus initiation medium to evaluate their effect on callus initiation and phenol secretion. Significant differences were observed among carbon sources (glucose, maltose and sucrose) at each level of concentration within each week. In the first week, MSB5 medium supplemented with sucrose showed no callus initiation at all level of concentrations. On the other hand, those MSB5 medium supplemented with glucose at 30 g/l showed 46.67 %, whereas maltose at 30 g/l and 40 g/l showed 76.67 % and 60 % callus induction, respectively. During this first week of culturing significant differences at (P=0.05) was observed among glucose, maltose and sucrose at 30 g/l and 40 g/l concentration levels (Table 4). During the second week, MSB5 medium supplemented with glucose and maltose showed a response for callus induction at all levels of concentration. While MSB5 medium supplemented with sucrose showed callus initiation response only at 30 g/l concentration. During the second week of culturing significant differences were observed among glucose, maltose and sucrose (Table 4). The highest percentage of callus induction was observed by maltose at 30 g/l (80%) and 40 g/l (73.33%) followed by glucose at 20 g/l (70%). During the third week, except MSB5 medium supplemented with 10 g/l and 20 g/l of sucrose, all the other medium showed response for callus initiation. At 30 g/l and 40 g/l maltose revealed a significant difference as compared with glucose and sucrose. While at 20 g/l glucose revealed a significant difference as compared with maltose and sucrose. The highest percentage of callus induction was observed by maltose at 30 g/l (86.67%) and 40 g/l (83.33%) followed by glucose at 20 g/l (80%).

4.2.1. Effect of carbon sources concentrations on Callus initiation

Significant differences were observed among concentration levels for glucose, maltose and sucrose. During the first week at the different level of concentrations, both glucose and maltose showed significant differences. Glucose revealed significant difference in percentage of callus induction at 30 g/l (46.67 %) as compared with 10 g/l (0 %), 20 g/l (0 %) and 40 g/l (0 %) of concentration. Similarly, maltose revealed significant difference of percentage callus induction at 30 g/l (76.67 %) as compared with 10 g/l (0 %), 20 g/l (0 %) and 40 g/l (60 %) of concentration (Table 4). During the second week of post culturing, glucose at 20 g/l (70 %) showed a significant difference in the percentage of callus induction as compared with 10 g/l and 40 g/l of concentrations. While maltose at 30 g/l and 40 g/l showed a significant difference in percentage callus induction (80% and 73.33 %) as compared with 10 g/l and 20 g/l of concentrations, respectively. In the second week, among MSB5 medium supplemented with sucrose only at 30 g/l concentration showed callus induction 33.33 % as compared with 10 g/l, 20 g/l and 40 g/l of concentrations (Table 4). During the third week of post culturing, glucose at 20 g/l (70 %) and 30 g/l (60 %) showed a significant difference in the percentage of callus induction as compared with 10 g/l and 40 g/l of concentrations. While maltose at 30 g/l and 40 g/l showed a significant difference of percentage callus induction (86.67 % and 83.33 %) as compared with 10 g/l and 20 g/l of concentrations, respectively (Table 4). In this third week, sucrose gave 40 % callus induction both at 30 g/l and 40 g/l of concentration. Otherwise, callus initiation did not respond to sucrose both at 10 and 20 g/l of concentrations in the MSB5 medium.

Table 4: Percentage callus initiation for Worer 50 cotton cultivar among carbohydrates at different level of carbon at each week in MSB5 medium

Carbon Level	[a]% Callus Initiation ± SE								
	Week1			Week2			Week3		
	Glucose	Maltose	Sucrose	Glucose	Maltose	Sucrose	Glucose	Maltose	Sucrose
10g/l	0.00±0.00Ba	0.00±0.00Ca	0.00±0.00Aa	46.67±6.7Ba	6.67±6.7Cb	0.00±0.00Bb	53.33±6.7Ba	6.67±6.7Cb	0.00±0.00Bb
20g/l	0.00±0.00Ba	0.00±0.00Ca	0.00±0.00Aa	70.00±6.8Aa	53.33±6.6Bb	0.00±0.00Bc	80.00±7.3Aa	53.33±6.6Bb	0.00±0.00Bc
30g/l	46.67±4.2Ab	76.67±8.0Aa	0.00±0.00Ac	56.67±6.1ABb	80.00±7.3Aa	33.33±8.4Ac	60.00±7.3ABb	86.67±6.7Aa	40.00±7.3Ab
40g/l	0.00±0.00Bb	60.00±7.3Ba	0.00±0.00Ab	43.33±9.5Bb	73.33±6.7ABa	0.00±0.00Bc	53.33±6.7Bb	83.33±6.7Aa	40.00±7.3Ac

[a] Values for each parameter followed by a different letter within each column for capital letters (among concentrations) and within each row for small letters (among carbon sources) independently at each week are significantly different at P = 0.05. The data represent percentage means from six replicates ± standard error (SE) .

4.2.2.Effect of carbon sources types and concentrations on timings of callus induction

Significant differences were observed among weeks for glucose, maltose and sucrose. MSB5 medium supplemented with maltose revealed a significant difference of callus induction among the weeks at 20 g/l and 40 g/l concentrations (Table 4). The highest callus induction 86.67 % and 83.33 % was observed in the third week at a concentration level of 30 and 40 g/l, respectively and this is followed by 80 % and 76.67 % callus induction in the second and the first week at a concentration level of 30 g/l, respectively. On the other hand,MSB5 medium supplemented with 10 g/l, 20 g/l and 40 g/l of glucose concentrations revealed a significant difference in callus induction among weeks (Table 4). The highest callus induction 80 % was observed in the third week at a concentration level of 20 g/l followed by 70 % callus induction in the second week at the same concentration level. Concerning MSB5 medium supplemented with sucrose, a significant difference of callus induction was observed among the weeks at 30 g/l and 40 g/l concentrations. The highest percentage of callus induction 40 % was observed in the third week both at 30 and 40 g/l sucrose concentrations (Table 4). Callus formation for Cotyledon explants failed to respond to all carbon sources up to the second week. However, it responded to glucose (30 g/l) and maltose (30 and 40 g/l) within three weeks.

4.2.3. Effect of carbon sources on phenol secretion

Maltose and glucose were effective to control tissue browning in all concentration for both explants. However, phenolic observed in the second week for cotyledon explant with less amount on MSB5 medium supplemented by sucrose at all concentration. In the third week, an increase in phenolic was observed both for cotyledon and hypocotyl explant. Browning of explants was noticed first at the cut end then gradually diffused into the medium. Based on the result, phenol secretion was comparatively higher in cotyledon than hypocotyl explant. MSB5 supplemented with sucrose at 30mg/l and 40g/l concentration showed callus induction with considerable phenolic secretion as shown by the browning of the medium (Figure 1).

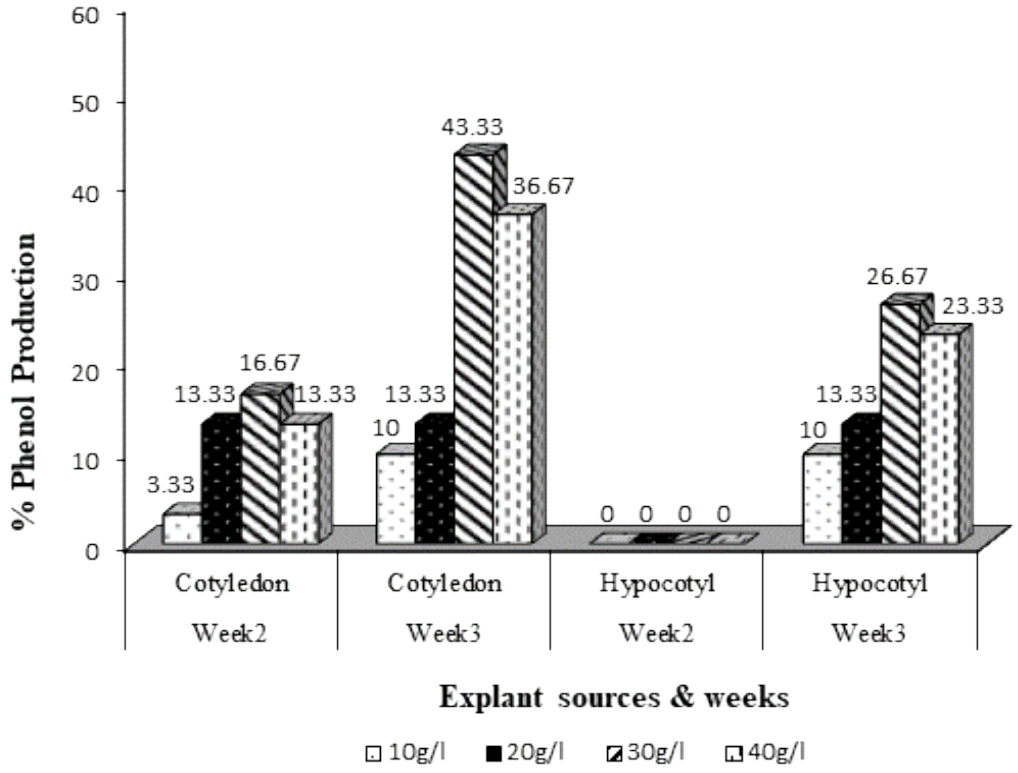

Figure 1: Effect of different level of sucrose on phenol production on two explant sources of Worer 50 cotton cultivar on MSB5 medium

4.3. Effect of Plant growth regulators on callus induction

For optimizing the right hormone combination for callus induction, different auxin-cytokinin combinations were evalvated. For this experiment, two cotton cultivars: Sisikuk 02 and Worer 50 were used to evaluate the effect of plant growth regulators for callus induction. Cotyledon and hypocotyl from the two cotton cultivars were used as a source of explant.

4.3.1. Effect of different PGRs on callus induction in different explant sources and cultivars

In the present study, different concentrations and combination of (2,4 D, TDZ, KN and NAA) had been used for callus induction using hypocotyl and cotyledon as explant in 'Worer 50' and 'Sisikuk 02'cultivar. Responses of both cotyledon and hypocotyl explants to callus indication were quite different. The two cultivars also showed variable responses to callus induction. The better quality callus responses were observed for Sisikuk 02 with 84% friable callus on MSB5 medium supplemented with 0.1 mg/l TDZ in combination with 0.1 mg/l NAA (T5, Table 5) as compared with Worer 50 with 64% friable callus on MSB5 medium supplemented with 0.1mg/l TDZ in combination with 0.1mg/l NAA and on MSB5 medium supplemented with 0.1mg/l TDZ in combination with 0.5mg/l NAA and (T5 and T8, Table 6) using hypocotyl as explant sources. Likewise, better quality callus responses were observed for Sisikuk 02 with 44 % friable callus on MSB5 medium supplemented with 0.5 mg/l KIN in combination with 0.1 mg/l 2,4-D (T3, Table 7) as compared with Worer 50 with 32 % friable callus on MSB5 medium supplemented with 1 mg/l TDZ in combination with 0.1 mg/l NAA (T7, Table 8) using cotyledon as explant sources. Based on these observations, MSB5 medium supplemented with plant growth regulator promoted callus induction except for the MSB5 medium supplemented with 0.5 mg/l KIN in combination with 0.5 mg/l 2,4 D and 1mg/l TDZ in combination with 0.5mg/l NAA that failed to induced callus formation from either hypocotyl or cotyledon explant for Worer 50 cultivar. Both explants that cultured on MSB5 medium without growth regulator hormones (control) did not form callus.

Table 5: Effect of different concentration and combination of growth regulators on callus induction percentage, callus colour and callus features at Sisikuk 02 using hypocotyl as explant.

Tre't	Plant Growth Regulators (mg-1)				Callus morphology		
	KN	TDZ	NAA	2,4-D	% of explant IC	Callus color	Callus features
T1	0.1			0.1	56	Green	Compact
T2	0.1			0.5	32	Creamy yellow	Compact
T3	0.5			0.1	88	Whitish green	Compact
T4	0.5			0.5	68	White	compact
T5		0.1	0.1		84	Green	Friable
T6		0.5	0.1		80	Green	Compact
T7		1	0.1		68	Yellow	Friable
T8		0.1	0.5		48	Light yellow	Compact
T9		0.5	0.5		60	Creamy	Friable
T10		1	0.5		80	Whitish	Friable
T11	0.1		0.5		60	Brown	Compact
T12	0.5		0.5		76	Green	Friable
Control	-	-	-	-	0		

Data were collected after two months of culture on MSB5 medium supplemented with different concentration and a combination of auxins and cytokinins for callus induction. Each value represent the means of five. KIN (kinetin);TDZ (thidiazuron); 2,4-D (2,4-dichloro phenoxy acetic acid) ;NAA (naphthalene acetic acid): Tre't (treatment) ; IC (inducing callus); NC(no callus formed)

Table 6: effect of different concentration and combination of growth regulator on callus induction percentage, callus color and callus features at Worer 50 using hypocotyl as explant

	Plant Growth Regulators (mg-1)				Callus morphology		
Tre't	KIN	TDZ	NAA	2,4-D	% of explant IC	Callus color	Callus features
T1	0.1			0.1	48	Creamy white	compact
T2	0.1			0.5	16	Yellow	compact
T3	0.5			0.1	72	Dark yellow	Loose compact
T4	0.5			0.5	NC		
T5		0.1	0.1		64	Yellow	Friable
T6		0.5	0.1		20	Greenish	Compact
T7		1	0.1		48	Yellow	Friable
T8		0.1	0.5		64	Light green	Friable
T9		0.5	0.5		32	Yellowish	Friable
T10		1	0.5		NC		
T11	0.1		0.5		24	Green	Friable
T12	0.5		0.5		64	Yellow	Compact
Control	-	-	-	-	NC		

Data were collected after two months of culture on MSB5 medium supplemented with different concentration and a combination of auxins and cytokinins for callus induction. Each value represents the means of five. KIN (kinetin);TDZ (thidiazuron); 2,4-D (2,4-dichloro phenoxy acetic acid) ;NAA (naphthalene acetic acid): Tre't (treatment) ; IC (inducing callus); NC(no callus formed)

Table 7: Effect of different concentration and combination of growth regulator on callus induction percentage, callus color and callus features at Sisikuk 02 using cotyledons as explant

	Plant Growth Regulators (mg-1)				Callus morphology		
Tre't	KN	TDZ	NAA	2,4-D	% of explant IC	Callus color	Callus features
T1	0.1			0.1	76	Yellow	Compact
T2	0.1			0.5	68	Brown	Compact
T3	0.5			0.1	44	Whitish	Friable
T4	0.5			0.5	28	White	Compact
T5		0.1	0.1		20	yellow	Friable
T6		0.5	0.1		4	Yellow	Friable
T7		1	0.1		4	Brown	Friable
T8		0.1	0.5		NC		
T9		0.5	0.5		NC		
T10		1	0.5		NC		
T11	0.1		0.5		NC		
T12	0.5		0.5		40	Greenish	Friable
Control	-	-	-	-	NC	-	-

Data were collected after 2 months of culture on MSB5 medium supplemented with different concentrations and a combination of auxins and cytokinins for callus induction. Each value represents the means of five. KIN(kinetin);TDZ (thidiazuron); 2,4-D (2,4-dichlorophenoxy acetic acid); NAA (naphthalene acetic acid);Tre't (treatment);IC(inducing callus); NC(no callus formed)

Table 8: Effect of different concentration and combination of growth regulator on callus induction percentage, callus color and callus features at Worer 50 using cotyledons as explant

	Plant Growth Regulators (mg-1)				Callus morphology		
Tre't	KIN	TDZ	NAA	2,4-D	% of explant IC	Callus color	Callus features
T1	0.1			0.1	NC		
T2	0.1			0.5	NC		
T3	0.5			0.1	20	Brownish	Loose compact
T4	0.5			0.5	NC		
T5		0.1	0.1		12	Greenish yellow	Friable
T6		0.5	0.1		NC		
T7		1	0.1		32	Yellow	Friable
T8		0.1	0.5		NC		
T9		0.5	0.5		NC		
T10		1	0.5		NC		
T11	0.1		0.5		NC		
T12	0.5		0.5		NC		
Control	-	-	-	-	NC		

Data were collected after 2 months of culture on MSB5 medium supplemented with different concentrations and a combination of auxins and cytokinins for callus induction. Each value represents the means of five. KIN(kinetin);TDZ (thidiazuron); 2,4-D (2,4-dichlorop henoxyacetic acid); NAA (naphthalene acetic acid);Tre't (treatment);IC(inducin g callus); NC(no callus formed)

4.3.2. Effect of PGRs combinations on callus nature using different explant sources and cultivars

The results of this study revealed that some PGRs combination gave better friable callus type than other PGRs for both Sisikuk 02 and Worer 50 cotton cultivars using the two different explant sources. For Sisikuk 02 cotton cultivars with hypocotyl as explant sources, all the twelve different combinations of PGRs gave both types of callus (i.e friable and compact), whereas for Worer 50 cotton cultivars with the same explant sources only 83.33 % of PGRs combinations gave both types of callus (i.e friable and compact). In terms of friable callus induction with the cultivar Sisikuk 02, only 41.67 % of the PGRs combinations gave friable callus for both hypocotyl (T5, T7, T9, T10 and T12, Table 5) and cotyledon (T3, T5, T6, T7 and T12, Table 7) explant sources, while for Worer 50 only 33.33 % (T5, T7, T8, T9 and T11, Table 6) and 16.67 % (T5 and T7, Table 8) of the PGRs combinations gave friable callus for hypocotyl and cotyledon, respectively. Hypocotyl explant showed more friable calluses for Worer 50 cotton cultivar as compared with cotyledon explant. The other observation was the variability of the type of callus colour observed among the different hormone combination, cultivar type and explant sources (Table 5 - 8). The entire friable callus obtained from Sisikuk 02 and Worer 50 cultivars on both types of the explant sources showed similarity in their color types which is green, yellowish, white/creamy white (Table 5 - 8). No specific color unique to each cultivar and explant sources was observed. Both hypocotyl and cotyledon showed more or less similar colour type, except that callus from cotyledon had shown whitish and brown colour. Furthermore, both hypocotyl and cotyledon showed both friable and compact callus features (Figure 2 – 5).

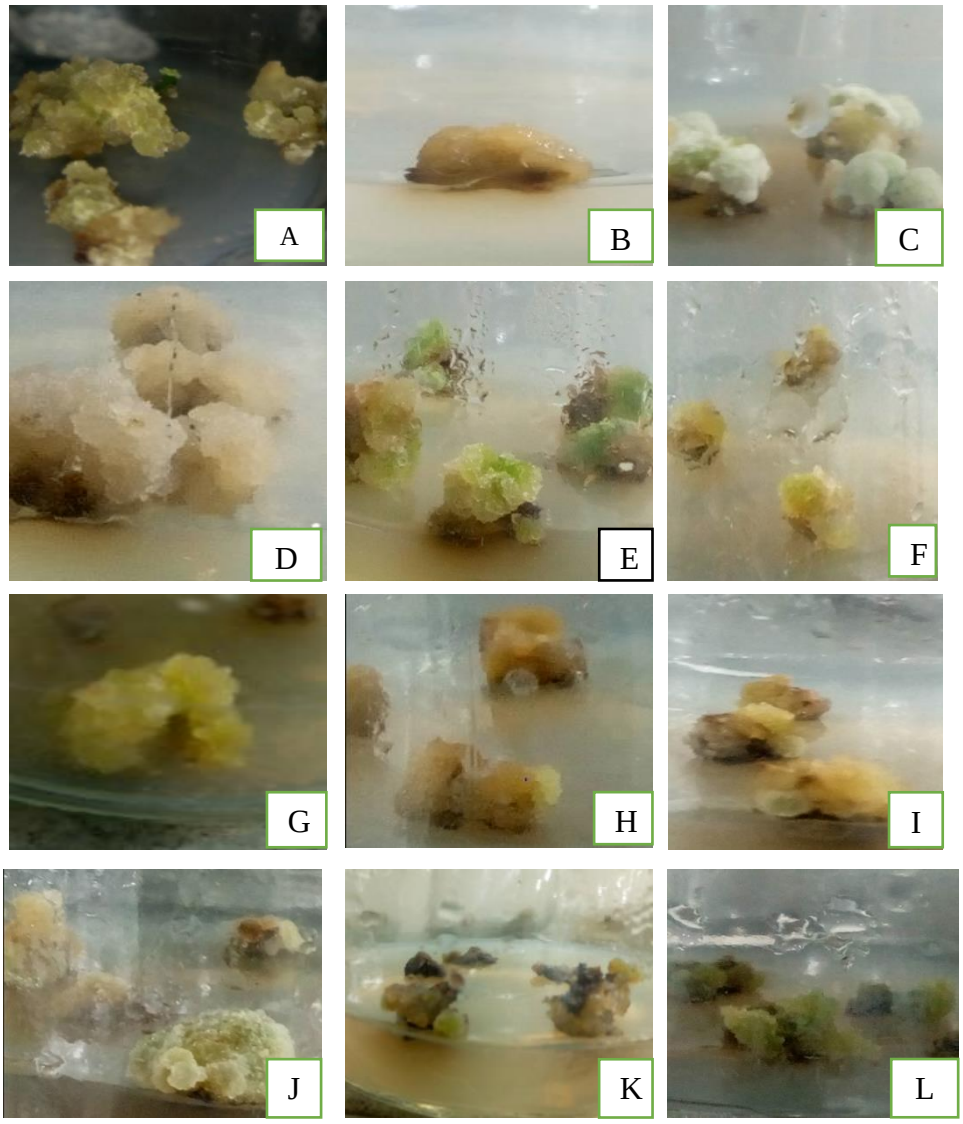

Figure 2: Induced callus from hypocotyl explants on Sisikuk 02 with different concentration and combination of PGRs after 8 weeks of growth

A= 0.1mg/l KIN+0.1mg/l 2,4-D

B= 0 .1mg/l KIN+0.5mg/l 2,4-D

C= 0.5mg/l KIN+0.1mg/l 2,4-D

D= 0.5mg/l KIN+0.5mg/l 2,4-D

E= 0.1mg/l TDZ+ 0.1mg/l NAA

F= 0.5mg/l TDZ+ 0.1mg/l NAA

G= 1.0mg/l TDZ+ 0.1mg/l NAA

H= 0.1mg/l TDZ+0.5mg/l NAA

I= 0.5mg/LTDZ+ 0.5mg/l NAA

J= 1.0mg/l TDZ+ 0.5mg/l NAA

K= 0.1mg/l KN+0.5mg/l NAA

L= 0.5mg/l KN+0.5mg/l NAA

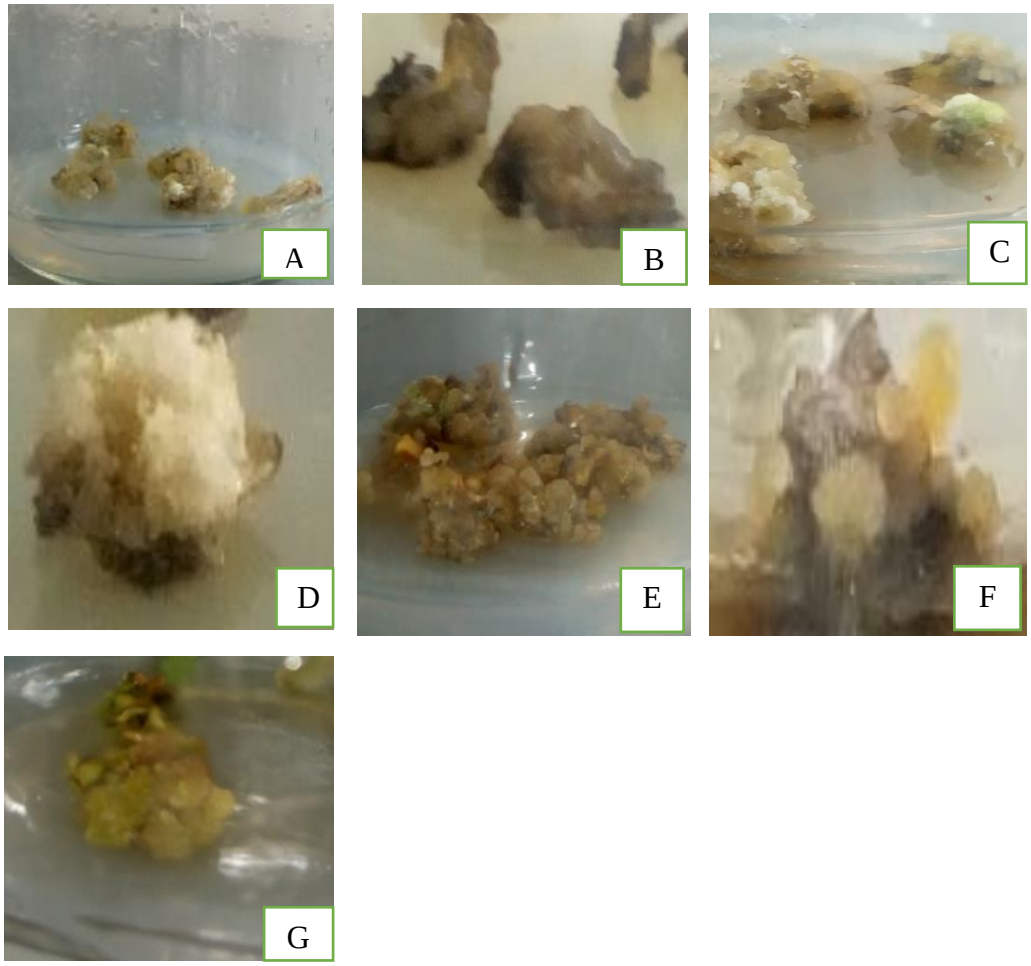

Figure 3: Induced callus from cotyledons explants on Sisikuk 02 with different concentration and combination of PGRs after 8 weeks of growth

A=0.1mg/lKIN+0.1mg/l 2,4-D B=0.1mg/l KIN+0.5 mg/l 2,4-D

C=0.5mg/l KIN +0.1mg/l 2,4-D D=0.5gm/l KIN+0.5mg/l 2,4-D

E= 0.1mg/l TDZ+0.1mg/l NAA F =0.5mg/l TDZ +0.1mg/l NAA

G=0.5mg/l KIN +0.5mg/l NAA

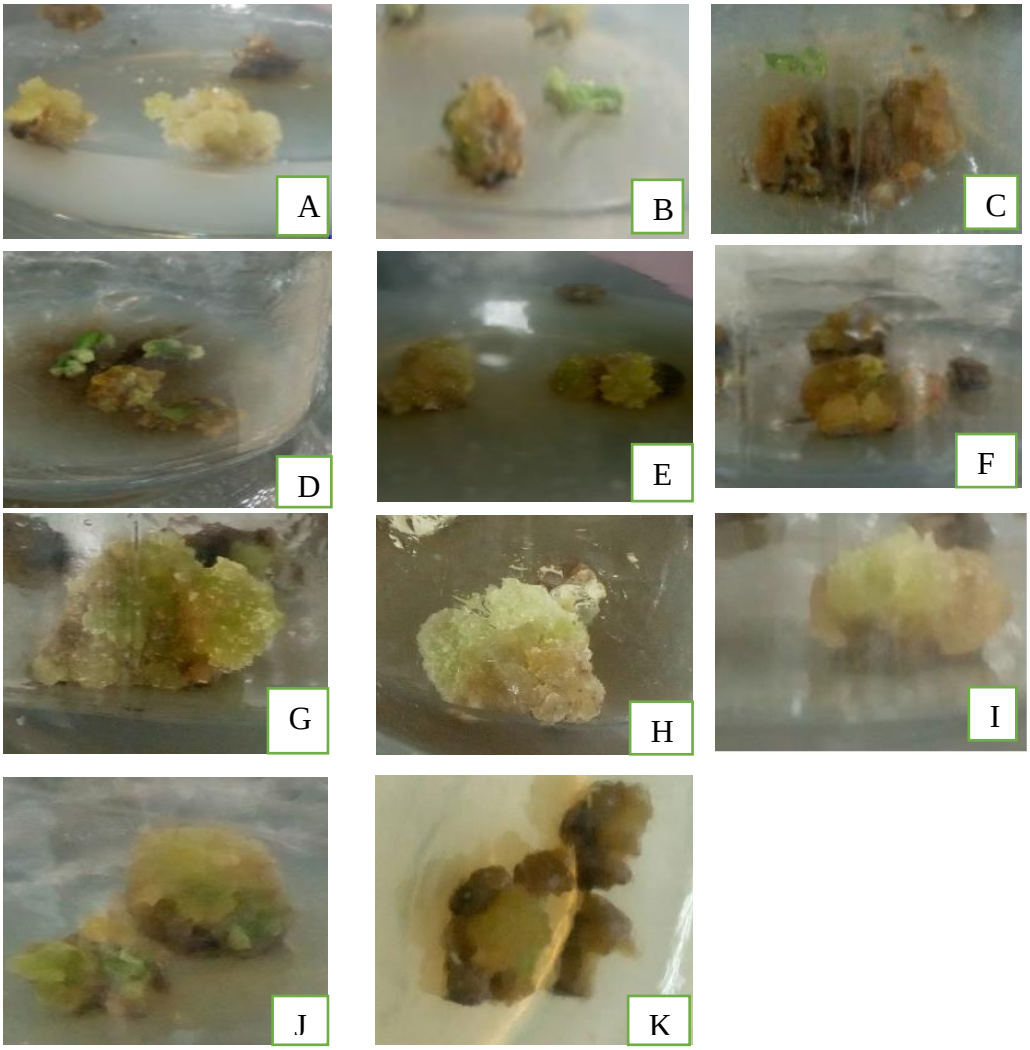

Figure 4: Induced callus from hypocotyl explants of Worer 50 on different concentration and combination of PGRs after 8 weeks of growth

A= 0.1mg/l KIN+0.1mg/l 2, 4-D

B= 0.1mg/l KIN+0.5mg/l 2, 4-D

C= 0.1mg/l TDZ+ 0.1mg/l NAA

D= 0.1mg/l TDZ+ 0.1mg/l NAA

E= 0.5mg/l TDZ+ 0.1mg/l NAA

F= 1.0mg/l TDZ+ 0.1mg/l NAA

G= 0.1mg/l TDZ+0.5mg/l NAA.

H= 0.5mg/l TDZ+ 0.5mg/l NAA

I= 1.0mg/l TDZ+ 0.5mg/l NAA

J=0.1mg/l KN+0.5mg/l NAA

K= 0.5mg/l KN+0.5mg/l NAA

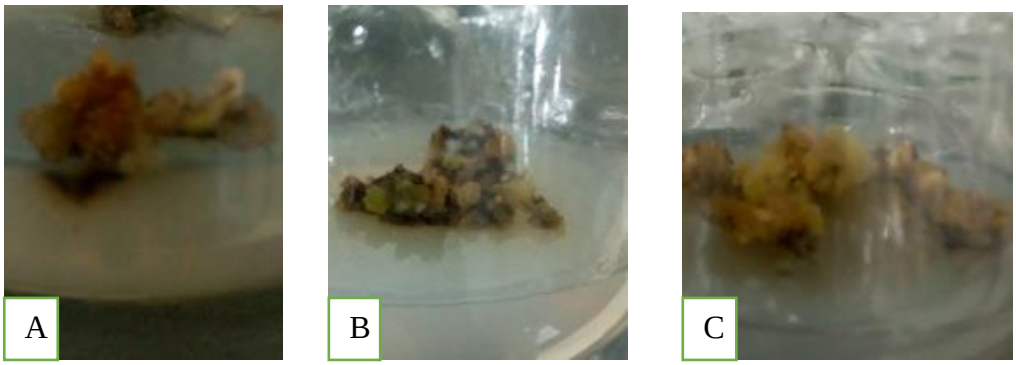

Figure 5: Induced callus from cotyledon explants of Worer 50 on different concentration and combination of PGRs after 8 weeks of growth

A= 0.5mg/l KIN+0.1mg/l 2, 4-D B= 0.1mg/l TDZ +0.1mg/l NAA

C= 1mg/l TDZ+0.1mg/l NAA

5. DISCUSSION

5.1. Effect of carbon sources on callus initiation and phenol secretion

The present study was undertaken to evaluate the influence of three different carbohydrates (glucose, maltose and sucrose) at different concentration levels for callus initiation and control of phenol compound secretion. Phenolic compound secretion adversely affects the callus culture of cotton and finally leads to the death of the explants (Kumar *et a*l., 2015). It is well known that cotton is naturally rich in phenolic compounds, therefore those factors that affect phenolic compound secretion, callus induction and proliferation need to be optimized for getting a high frequency of callus culture (Chamandoosti, 2010). Furthermore, regeneration capability can also be influenced by the type of carbon sources and hence the study focuses its target to find out suitable carbon sources which are highly required to avoid excessive phenolic secretion in culture medium and to speed up the frequency of callus culture.

5.1.1. Effect of carbon sources in callus induction

Cotyledon and hypocotyl explants were taken from aseptically raised seven days old *in-vitro* seedlings and tested on different carbon sources for callus induction. The result revealed that medium supplemented with maltose at 40 g/l, 30 g/l and glucose at 30 g/l gave early callus initiation after a week of post culturing using hypocotyl as explant, whereas it took three weeks for callus initiation using cotyledon as explant sources. This implies that callus initiation is highly affected by both explant sources and carbon sources. The result of this study is in agreement with Divya *et al*. (2008) who reported that a quick response of callus initiation for hypocotyl was obtained as compared with cotyledons. The study also revealed that different carbon sources used in this study gave a slow response of callus using cotyledon explant.

5.1.2. Effect of carbon sources concentration in callus initiation

In terms of concentration level differences, maltose was found to be the best carbon source at 30 and 40 g/l followed by glucose at 20 and 30 g/l for efficient callus induction, which is in line with the study conducted by Sairam *et al*. (2003) who reported that maltose is the best source of carbon to stimulate callus compared to glucose at higher concentrations. Another study reported by Swankar *et al*. (1986) showed that sugar plays a significant role

in influencing cells proliferation and differentiation, albeit it does not have similar effects on the callus for all different cultivars and explants. In this regard, contrary to this finding, many other researchers also found that glucose and sucrose at different concentration levels gave better callogenesis as compared with maltose for different cotton cultivars (Burbulis *et al* 2007; Kouakou *et al*. 2007). This implies that other than differences in sugar type, the concentration of sugar and explant sources might influence on the callogenesis.

5.1.3. Effect of carbon sources types and concentrations on timings of callus induction

From the application point of view, in this study maltose minimizes time to obtained callus from hypocotyl explant sources. This of considerable importance for effective tissue culture application since it can avoid frequent medium refreshment. Early callus induction and growth could help to just go for the next stage of the regeneration process. In this respect, both maltose and glucose showed callogenesis on hypocotyl explant in the first week of culture at 76.67 % and 46.67 %, respectively. Whereas MSB5 medium fortified with sucrose took two weeks to give 33.33 % callus initiation. Among the carbon sources tested in this study, sucrose took more time and showed a minimal callus percentage compared to maltose and glucose. With respect to the explant sources, glucose at 30 g/l took three weeks for callogenesis using cotyledons as compared with hypocotyl explant which took a week time to get callus. However, the percentage of callus induction was lower compared to maltose at the same concentration. This is in line with Wu *et al*. (2004) and Smith *et al*. (1977) who described that 30% glucose was the best carbon source for callus culture initiation. Study conducted by Michel *et al*. (2008) similarly reported that glucose with 30% is best carbon sources for callus initiation.

5.1.4. Effect of carbon sources on phenol secretion

Phenolic compounds inhibit enzyme activity in the culture medium, which finally result in the darkening of the culture medium and subsequent lethal browning of explants (López *et al*., 2001; Ozyigit *et al*., 2007). Phenolic compounds synthesized by the explants in any periods of organogenesis have an important role in browning, rooting problems or even killing the explant sometime after culturing. In this study, maltose had shown a significant impact on phenolic secretion both by hypocotyl and cotyledon explants.The reason for this is the high and constant osmolarity of maltose in the culture medium was due to its slow

hydrolysis compared to sucrose and glucose this means maltose has low chemical breakdown to react with water. The result of this study is in agreements with the finding of Khan *et al*. (2010) who reported that using maltose as the sole source of carbon during callus induction, proliferation and somatic embryogenesis in cotton significantly reduce the secretion of excessive phenolic at any stage of the callus culture.

On the other hand, medium supplemented with all concentrations of sucrose produced phenolic compound starting from the second week with cotyledon explant. In this study, phenolic secretion was observed in the third week both in cotyledon and hypocotyl explants. Just like maltose, glucose is also effective in controlling the secretion of phenolic compounds at all concentration both in hypocotyl and cotyledon explants. Phenolic secretion was relatively higher in cotyledons explants than hypocotyl explants with a maximum phenolic concentration on medium fortified with sucrose (30 g/l and 40 g/l). The result of this study revealed the direct relationship between sucrose concentrations and explant sources for phenolic secretion. The is in agreement with studies conducted by Kumar *et al*. (2015) who reported that increasing the sucrose concentration of the culture medium leads to cell death due to the secretion of phenolic compounds in cotton. Similarly, Curtis and Shetty (1995) who reported that an increase in the concentration of sucrose in the culture medium resulted in the rise of phenol oxidation. This finding of this study is also supported by the study of Kadota *et al*. (2001) and Yildiz *et al*. (2007) who reported that high concentration of sucrose cause phenolic secretion and increase tissue necrosis leading tissue death.

5.2. Effects of plant growth regulators on callus induction

In this experimental study, we compared the effect of the combinations of different growth regulators on callus induction potential of two explants hypocotyls and cotyledons from seven day old seedlings for Worer 50 and Sisikuk 02 cultivars.

5.2.1. Effect of PGRs on callus induction in different explant sources and cultivars

The result of this study revealed that the percentage of callus induction from hypocotyl explants was higher than cotyledon explants both in Sisikuk 02 and Worer 50 cultivars. Percentage of callus induction from hypocotyl explant ranged from 16 to 88%. Whereas percentage of callus induction from cotyledon explant ranged from 4 to 76%. The results

of the present study showed the existence of large inter-explant difference in callusing responses. Therefore, selections of appropriate explant would significantly affect callus induction and regeneration plants and should be considered in a cotton tissue culture procedures. Reports from previous studies also showed superior performance of hypocotyl explants to cotyledon explants of different cotton cultivars (Han *et al*., 2009; Rajeswari *et al*., 2010; Kamal *et al*., 2011). Differences in callus forming ability of different explant types have also been reported in many others plants (Durrani *et al*., 2017; Mahmood and Razzaq, 2017). Savita *et al*, (2010) reported better performance of nodal explants compared to leaf and root explants for successful regeneration of *Citrus jambhiri* (rough lemon) Lush. Srivastava (2013) compared cotyledonary node, epicotyl, hypocotyl and leaf explants and reported better performance from cotyledonary node explant for successful regeneration of Pigeon pea (*Cajanus cajan*),.

Genotype is one of the most prominent factors that influence the response of explant in callus induction of cotton. The two cotton cultivars used in this study showed differences in their response to callus induction where Sisikuk 02 cultivar showed the highest response both for hypocotyl and cotyledon explants than Worer 50 cultivar. This variation is associated with differential sensitivity of tissue to the callus culture medium. The result of this study was in consistent with the previous callus induction study conducted by Michel *et al*. (2008) who reported that from 10 genotype investigated; only R405-2000 genotype gave high percentage of callus response as compared with others. Similarly, Rao *et al*. (2006), Jin *et al*. (2006) and Kouadio *et al*. (2007) suggested that genotypic differences result in differences in callus induction of cotton. This genotypic difference with respect to callus initiation was also observed in many other plants, such as flax (*Linum usitatissimum L.*, Burbulis *et al*., 2007), coffee (*Coffea arabica* L., Mwaniki *et al.*, 2019), sugarcane (*Saccharum* sp., Gandonou *et al*., 2005).

The MSB5 medium supplemented with 0.5 mg/l KIN in with combination of 0.5 mg/l 2,4-D and the MSB5 medium supplemented with 1 mg/l TDZ with the combination of 0.5 mg/l NAA failed for induction callus on Worer 50 cultivar. This result provide strong evidence that growth regulator requirements for callus induction vary depending on the cultivar. Both explant that inoculated without plant growth regulator did not showed any callus induction. This indicate that exogenous application of auxin and cytokinins are vital for callus induction in cotton.

5.2.2.Effect of different PGRs combinations on callus nature using different explant sources and cultivars

The present study showed that hypocotyl explant gave both friable and compact callus with different colors. Many other studies are in accordance with this result that different types of callus color and features found associated with explant type and cultivars (Ganesan and Jayabalan, 2005; Finer, 1988; Smith *et al*., 1977). This variation might be associated with the ability of explant to express totipotency of plant cell and different reaction with the culture medium.

Both cultivar gave maximum percentage of callus induction on MSB5 medium supplemented with 0.5 mg/l KIN in the combination of 0.1mg/l 2,4- D for Sisikuk 02 (88%) with whitish green compact feature and Worer 50 (72%) with dark yellow loose compact feature using hypocotyl explant. This results are in agreement with the findings of Kouakou *et al*.(2007); Zouzou *et al*.(2000); Rajeswari *et al*.(2010) who reported that culture medium fortified with 0.1mg/l 2,4-D + 0.5 mg/l KIN was the best combination of hormone to induce callus in cotton. This result also partially in line with the study reported by Sanghera *et al*. (2009) who reported that the culture medium supplemented with KIN showed compact callus.

MSB5 medium namely 1mg/l TDZ with 0.5 mg/l NAA gave 80% whitish friable callus using hypocotyl from Sisikuk 02 cultivar. Whereas MSB5 medium supplemented with the combination of (0.1mg/l TDZ +0.1mg/l NAA) ,(0.1mg/l TDZ +0.5mg/l NAA) and (0.5mg/l KIN+0.5mg/l NAA) obtained (64%) with yellow friable, light green friable and yellow compact features respectively at hypocotyl explant in the case of 'Werer 50' cultivar. In general the result revealed that the combination of TDZ with NAA at all concentration respond friable callus that considered as the most suitable hormone for efficient *in vitro* regeneration of cotton cultivars of this study. These findings are in line with Erisen *et al*.(2010) who reported that callus cultured medium fortified with the combination of NAA and TDZ were respond friable callus in other species called *Astragalus nezaketae*.

6. CONCLUSIONS AND RECOMMENDATIONS

6.1. Conclusions

Based on the result, it can be concluded that maltose (30 g/l) is found to be the best carbon source for callus initiation. Glucose and maltose at all concentration level has effective to control phenol secretion. Hypocotyl explant source is more suitable for callus induction at all hormone combination and respond best friable callus.Cotyledon explant displayed slow callus response with all hormonal combination. From the two cultivars, relatively 'Sisikuk 02' is best for friable callus indication. Among the different tested hormone combination, the maximum callus percentage was found in combination of 0.5 mg/l KIN with 0.1 mg/l 2, 4-D at both cultivar with compact features. The more effective friable callus formation was observed in MSB5 medium supplemented with the combination of 0.1 mg/l NAA with the 0.1 mg/l TDZ. This implies that this combination is favorable for further cotton *in vitro* culture.

6.2. Recommendations

The study provides a lot of information regarding callus induction of cotton in MSB5 medium, however a lot more works need to be done. Due to the nature of the crop, which is very recalcitrant for tissue culture, optimization of protocols for propagation is time consuming and needs a lot of efforts with several troubleshooting. Some of the research gaps identified in this study is listed down.

- Further studies on the performance of plant growth regulator toward best shoot induction.
- Further studies to find best plant growth regulator for rooting.
- Explants other than hypocotyl and cotyledon for *in vitro* propagation of cotton should be considered to investigate if they can offer better and efficient responses.
- Further studies using other cultivar is required to have a reliable regeneration for *in vitro* propagation of cotton and the need to evaluate the survival rate of plantlet in the field condition.

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