

***INVITRO* REGENERATION OF *Echinops kebericho* FROM SHOOT  
EXPLANTS**

**M.Sc. THESIS**

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## **BIOGRAPHICAL SKETCH**

The author Habtamu Gebrewold was born on December 09, 1982 in Addis Ababa the capital city of Ethiopia from his father Gebrewold Tufa and his mother Tejitu Feyissa. He attended his elementary and junior school at Ethiopia Erimja and Abune Bassilios School in Addis Ababa respectively. He attended his secondary and high school education at Ayer Tena Senior Secondary School in Addis Ababa. He then joined Kotebe College of Teacher Education in 2002, graduated with a Diploma in Biology, and graduated with in B.Sc. Degree in Applied Biology at Addis Ababa University in February 2014.

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## ACRONYMS AND ABBREVIATIONS

2-4-D	2, 4 – dichlorophenoxy acetic acid
ANOVA	Analysis of Variance
AAU	Addis Ababa University
BA	6-benzyladenine
BAP	6-benzylaminopurine
CRD	Completely Randomized Design
EBI	Ethiopian Biodiversity Institute
EHNRI	Ethiopian Health and Nutrition Research Organization
EIARI	Ethiopian Institute of Agricultural Research
GA <sub>3</sub>	Gibberellic acid
IAA	Indole-3- acetic acid
IBA	Indole – 3- butyric acid
KN	Kinetin
LSD	Least Significant Difference
MS	Murashige and Skoog
NAA	1-Naphthalene Acetic Acid
PGR	Plant Growth Regulator
PTC	Plant Tissue Culture
TDZ	Thidiazuron

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# **INVITRO REGENERATION OF *Echinops kebericho* FROM SHOOT EXPLANTS**

## **ABSTRACT**

*Echinops kebericho* is an erect massive rootstock bearing perennial herbs or shrub belonging to the family Asteraceae. *Echinops kebericho*, endemic to Ethiopia is a critically endangered medicinal plant. It is among few important medicinal plants of the country valued primarily for root parts. The seeds of *Echinops kebericho* characterized by short viability period and lose their capacity to germinate very quickly. This study aimed to develop in vitro regeneration of *Echinops kebericho* from shoot explants. The study started with seeds germinations on culture jar for two different months. Seeds were sterilized 70% (v/v) alcohol 9 minute and 5.25% (w/v) sodium hypochlorite with added 1-2 drops of Tween 20, rinsed double distilled water and cultured Murashige and Skoog (MS) medium containing 30 g/L sucrose and 8 g/L agar. The shoot induction was MS medium supplemented with different concentrations of BAP and KN with combined 0.5 mg/l NAA. For regeneration and multiplication shoots were different concentration of BAP and KN .The, multiplied shoots were rooted on MS medium supplemented with concentration of IBA and IAA. The highest number of shoots induction was recorded 1.0 mg/l KN with combined 0.5 mg/l NAA (10.30±0.30) of shoot per explants and the shoot length (5.20 cm) were observed. The 1.0 mg/l KN showed maximum shoot regeneration on shoot regeneration media and shoot multiplication media respectively. The highest percentage of rooted plants were obtained from in 1/3 MS basal medium in while maximum root length were recorded on the medium supplemented with 0.5 mg/l IAA were recorded. Among 30 plantlets were hardened and successful acclimatized to the green houses with 80% survival. Overall results of the present study the effect on hormone and techniques of invitro regeneration. In order to see further achievement in vitro regeneration of *Echinops kebericho* use combination hormone cytokinin and auxin.

**Keywords:** *Echinop kebericho*, invitro regeneration, Shoot Explants, Plant Growth Regulator

# 1. INTRODUCTION

*Echinops kebericho* Mesfin is an erect massive rootstock bearing perennial herb or shrub that grows up to a height of 1.2 m with leafy stem (Begashaw and Tileye, 2014). The genus *Echinops* belongs to family Asteraceae and comprises over 120 species. *Echinops kebericho* and 11 other spp. are known to occur in Ethiopia and some are used in traditional herbal medicine (Yinebeb, 2008). The 12 species of *Echinops* that occur in Ethiopia are confined to the high lands of the country between 1700 and 2900 m.a.s.l. They grow in dry and stony lateritic soils. *E. kebericho* is different in the characteristics of its growth and in the dissection of its leaf blade. Thus, populations from dry, stony lateritic soils are perennial herbs, whereas those growing in deep vertisols are shrubs (Balcha *et al.*, 2009).

According to Belachew (1993), *kebericho* is among few the important medicinal plants of the country valued primarily for its root part. *E.Kebericho* medicinal value was documented in the ancient medico religious pharmacopoeia reports and some ethno botanical surveys show long traditional use of the plant for the preparation of medicines against migraine, mental illness, heart pain, lung TB, leprosy, kidney disease, malaria, bilharzias, syphilis, hemorrhoid, amoebic dysentery and other ailments. In laboratory, the toxic property of *E.kebericho* has been exploited in the control of insect pests of medical veterinary and agricultural importance. The essential oil of *E.kebericho* also possesses strong anti-leishmanial activity with moderate cytotoxicity and weak hemolytic effects and Bioactive extracts of the species have also shown anti microbial effects equal to or better than penicillin.

The genes *Echinops* species contain alkaloids, saponins, phytosterols, polyphenols, carotenoids, sesquiterpine (alcohol/lactones), lignans, acetylenic, thiophene compounds and essential oil (Yinebeb, 2008). In Ethiopia tissue culture technology started in 1980 at Addis Ababa University with focus on micropropagation of indigenous forest species like *Podocarpus falcatus*, *Cordia africana*, *Hagenia abyssinica* and *Annengria* that are either difficult to regenerate vegetatively or require long time or have seed viability problem. Moreover, in Ethiopia, tissue culture

technology is being used in the mass propagation of beverage crops, fruits, spices, root crops, industrial crops, and medicinal and indigenous trees including *Arabica coffee*, pineapple, Banana, cardamom, Vanilla, Garlic, Cassava, Sweet potato, Enset ,Grapevine, Sugarcane , Endod, *Arthimessia*, *Hygennia* and *Podocarpus* tree spp. and recently potato (Habtamu and Wassu, 2016). *In vitro* and *ex vitro* seed based propagation would be the promising option for multiplication and conservation of *Echinops kebericho* (Balcha, 2009). Thus, *In vitro* regenerations are the practices of rapidly multiplying stock plant material to produce large number of progeny plants and the basic nutritional requirement of *in vitro* cultured plant cells are very similar to those utilized by parts in nature. However, the nutritional requirement for better *in vitro* propagation varies depending on the type of protoplast cells, tissue, and organ of the plant species. Nutrient medium is defined by its composition of mineral salts, carbon sources, vitamins, plant growth regulators and other organic supplements. Particular medium is identified by its salt composition and other additives such as amino acids, vitamins, growth regulators and other organic supplements can be added to a given salt concentration to get the desired result (Balcha et al., 2009).

Optimization of *invitro* regeneration protocol for a given species, therefore, requires manipulation of the composition of growth media and selection of different explants to come up with better results. With this premise, this study was designed to develop *invitro* regeneration protocol for *E.kebericho* with the following general and specific objectives.

**General objective:**

- To develop *in vitro* regeneration protocol for *Echinops kebericho* from shoot explants.

**Specific objectives:**

- To optimize the appropriate plants growth regulators concentration for shoot initiation and multiplication
- To determines appropriate plants growth regulator concentration and combination for efficient rooting
- To evaluate the degree of acclimatization of plantlets under greenhouse condition

## 2. LITERATURE REVIEW

### 2.1. Ecology and Botanical description of *Echinops kebericho*

*Echinops kebericho* Mesfin belongs to the family Asteraceae and commonly known in Ethiopia as kebericho, taxonomically the genus *Echinops* comprises 120 species of which 12 are found in Ethiopia. *Echinops kebericho* is an erect perennial herb or shrub growing up to 1.2 m high and forms a massive rootstock. The 12 species that occur in Ethiopia are confined to the high lands of the country between 7° 30 'N and 38°45'E and at altitudes between 1700 and 2900 m.a.s.l .They grow in dry and stony lateritic soils. *E. kebericho* has variable growth habit and unique dissection of leaf blade. Thus, populations from dry, stony lateritic soils are perennial herbs, whereas those growing in deep vertisols are shrubs (Balcha *et al.*, 2009). The stem is leafy throughout, sparsely arachnoids, pubescent, sometimes mixed with dark purple uniseriate hairy leaves, elliptic, amplexicaul, and basal ones up to 25x15cm diameter wide (Balcha *et al.*, 2009).

The flower head of *E. kebericho* is 3-4 cm in diameter; spineless peduncle up to 5.5 cm long, arachnoids, pubescent, 1-2 mm wide, and capitulum 2-2.5 cm long. Its outer phyllaries are 3-4cm seriate, the outer most row spatulate, 4-6mm long, with a few white, soft bristles along margins in the lower half. The middle row is spatulate with apiculate apex is which 6-10 mm long with several white, soft bristles on arranging toward apex. The innermost series lancelet, tapered at base and at apex, is 9-14 mm long and 5 mm wide with bristles at margins in the middle and toward the tip. The apexes are as high as up to 3/4 of the length the acute criner phyllaries. The fimbriation at apex corolla is white or bright blue and 12-15 mm long where as the tube is glabrous, except near the base line, is light brown. The cypselas oblong –elliptic are 7.5-10.5 x 1.5-2.5cm diameters covered with appressed silvery, white ciliolate bristles Pappus of numerous, contiguous, narrow, ciliolate scales that are connate at base (Sebsebe , 1993).

## 2.2. Significance of *Echinops kebericho*

*Echinops kebericho* are known in Ethiopia as medicinal plant and varied medicinal applications, documented in the ancient medico religious pharmacopoeia (Belachew, 1993). Various ethno-botanical surveys report that this plant particularly the root is used traditionally for the preparation of medicines against migraine, mental illness, heart pain, lung TB, leprosy, kidney disease, malaria, bilharzias, syphilis, hemorrhoid, amoebic dysentery and other ailments infections and also has anti-helminthic, anti-tumor, anti-mutagenic, anti-bacterial and fungicidal properties (Yinebeb, 2008). In laboratory, the toxic properties of *E. kebericho* are exploited for the control of insect pests of medical veterinary and agricultural importance (Begashaw and Tileye, 2014). Contemporary specialists in botanical medicines emphasize the burning of plants in order to use smoke as may be inhaled or use to fumigate the whole house to get rid of insects and parasites. A few medicinal plants that are available on Ethiopian market are the root of *E. kebericho* whose smoke is usually to fumigate the body as a general medicine and to rid a house of pests and insects (Ermias, 1990). When inhaled, the smoke from burning of this plant helps to relieve of headache and “evil spirit” and the roots burns for smoke to ward off mosquitoes and as a snake repellent in the house (Teklehaymanot, 2007).

Yinebeb *et al.* (2011) reported dehydrocostus lactone (41.83%), and  $\beta$ -phellandrene (10.84%), germacrene  $\beta$  (5.38%),  $\alpha$ -selinene (4.13%),  $\alpha$ -pinene (3.63%),  $\beta$ -pinene (3.62%) as the main constituents of *Echinops kebericho*. *Echinops* species reported as to contain wide range chemical constituent including alkaloids, saponins, phytosterols, polyphenols, carotenoids, sesquiterpene (lactones/alcohols) lignans, acetylenic, thiophene compounds, and an essential oil (Hymete *et al.*, 2007). The roots of *E. kebericho* are extremely rich in sesquiterpenes amounting to about 10% of the dry weight of the roots (Abegaz, 1996). These sesquiterpenes are biologically active for antitumor, anti-mutagenic and cytotoxic properties. The species presents itself as commercially viable for the sesquiterpene lactones, if successful efforts are made to develop suitable means of using these compounds (Abegaz, 1996).

The essential oil of *Echinops kebericho* also possesses strong anti-leishmanial activity with moderate cytotoxicity and weak hemolytic effects. The Bioactive extract of the species also has shown to have anti-microbial effects equal to or better than penicillin, especially against *Streptococcus beta haemolyticus*, *Escherichia coli* and *klebsiela aereo* genes. Alkaloid extract of the root has a very strong lethal activity against earthworms (Begashaw and Tileye, 2014).

### **2.3. *Echinops kebericho* propagation and its challenges**

*Echinops kebericho* propagates through seeds; however, overlapped fruit walls cover, its seeds. The pubescent hairs found between these walls and on the surface of the cotyledons. This structure generally facilitates the incorporation of pathogens, which causes seed contamination during *in vitro* germination. Seeds of *E. kebericho* have very low *in vitro* germination percentage after 5 months of storage and its germination declines with longer storage time (Begashaw and Tileye, 2014). Seed viability in *Echinops kebericho* was reported to decrease faster when stored at room temperature (25°C), but even storing at 4°C did not help much as the viability of seed stored at 4°C was only about 70% after 10 months of storage (Balcha *et al.*, 2009). Moreover, seeds of *E. kebericho* could not uniformly germinate after 3 months storage time. Hence, seeds of this species characterized by short viability period, when stored at room temperature thus lose their capacity to germinate very quickly. Therefore, *Echinops kebericho* propagation through seeds poor, due to lack of adequate seeds and seeds viability loss after short period. Propagation through seeds is time consuming to accomplish large-scale production for conservation and cultivation of the species. Despite these problems, the local people destruct the whole plant before seed set as they use the root part for medicinal purpose (Begashaw and Tileye, 2014).

### **2.4. The Concept of Plant Tissue culture**

Plant tissue culture is the science of aseptically (free from microorganisms) growing of any plants part or explants (e.g., protoplast, cell, tissue and organ) from mother plants on artificial media (Singh, 2000 and George, 2008). Tissue culture is based on concept of totipotency, the ability of plant cells and tissues to develop in to completely new

plants (Taswar *et al.*, 2013). Hence, Plant tissue culture is the science or art of isolating plant cells, tissues and organs from another plant and growing an artificial media under aseptic condition. German botanist Gotten Haber land and Philip White originally proposed the idea of plant tissue culture in 1902. *In vitro* growth of tissues have been achieved successfully from tomato roots explants in 1934. The first whole plants regeneration through tissue culture was in carrots made by Stewart in 1958. Stewart also demonstrated the development of somatic embryos in liquid culture of carrot cells. Today, the number of plant species propagated through tissue culture techniques is large. Plant cell/ tissue culture is used for clonal propagation, production of disease free plants, germplasm conservation, secondary metabolite production, triploid production, invitro pollination, fertilization embryo rescue and somatic hybridization (Balcha, 2009).

A part from the conventional benefits, tissue culture research has expanded its scope in various aspects for the production of secondary metabolites and recombinant proteins. The use of tissue culture technology for secondary metabolites production is proved more efficiently as compared to conventional extraction from cultivated field. These commercially important secondary metabolites present in plants are flavonoids, alkaloids, steroids, tannins; shikonin, naphthoquinone pigment and terpenes. This produced using cell cultures in many plant species (Rao and Ravishankar, 2002). Large-scale production of recombinant proteins by transgenic plant cell cultures, making them integral parts of molecular farming, minimizes cost as compared to traditional pharmaceutical production. Therefore, plants cell cultures are also advantageous for molecular farming because of high level of containment that they offer as compared to the completely field-grown plants and the possibility of commercially producing recombinant proteins (Neal, 2008). So far, more than 20 pharmaceutical compounds like antibodies, interleukins, erythropoietin, human granulocyte macrophage colony stimulating factor (HGM-CSF), and hepatitis B antigen have been produced in cell suspension cultures (Shad wick and Doran, 2005).

### **2.4.1. Types of plant tissue culture**

(George *et al.* (2008) classified plant tissue culture into two; namely cultures of unorganized tissues and cultures of organized tissues. Cultures of unorganized tissues include callus cultures (any plant tissue or organ), cell-suspension cultures (friable callus), protoplast culture (protoplast) and microspore culture (anthers). Root cultures (tip of either primary or lateral roots), shoot culture (tips of shoots), meristem culture (meristem), nodal culture, embryo culture (immature and mature embryos), leaf or leaf sheath cultures, fruit culture and flower cultures are classified as cultures of organized tissues. The first four of this group are called cultures of indeterminate organs (growth is potentially unlimited) and the last four are termed as culture of determinate organ (destined to have only a defined size and shape).

### **2.4.2. Plant tissue culture research activities in Ethiopia**

The plant tissue culture activities first started in 1980 at Addis Ababa University with focus on micropropagation of indigenous forest species such as *Podocarpus falcatus*, *Cordia africana*, *Hagenia abyssinica* and *Annengria sp.* Because of that these species are either difficult to regenerate from seeds or require long time for regeneration. Now a day, tissue culture technology is being used in Ethiopia for mass propagation of beverage crops, fruits, spices, root crops, industrial crops, medicinal and indigenous trees, These includes *Arabica coffee*, pineapple, banana, Cardamom, Vanilla, Garlic, Cassava, Sweet potato, Enset, Grape vine, Sugarcane, Endod, *Arthimessia*, *Hygennia* and *Podocarpus falcatus* and recently potato (Habtamu and Wassu, 2016). A more comprehensive and concerted plant tissue culture research activities started at the EIAR since for 2000 with emphasis on protocol optimization for mass propagation disease cleansing and *in vitro* propagation for conservation of economically important crop species such as banana, hybrid coffee, pineapple and potato to farmers in various parts of the country (Adana, 2009).

## 2.5. Effect of Growth Media on Tissue Culture

The basic nutritional requirements of *invitro* cultured plant cells are very similar to those utilized by plants in nature. However, the nutritional composition used *in vitro* varies depending on the type of protoplasts, cells, tissues, organs and plants species. A nutrients medium is defined by its composition of mineral, salts, carbon sources, vitamins, plant growth regulators and other organic supplements. Its salts composition and other additives such as amino acids, vitamins, and growth regulators (Balcha, 2009). In case of solid medium identify a particular medium, some gelling agents are also required (Murashige and Skoog, 1962). The most commonly used medium is the formulation of Murashige and Skoog (1962). It consists of macronutrients including ions of nitrogen (N), potassium (K), calcium (Ca), phosphorus (P), magnesium (Mg), sulphur (S), micronutrients needed in small amount including ; iron (Fe), nickel (Ni), (Fe), chlorine (Cl), manganese (Mn), zinc (Zn), boron (B), copper (Cu), molybdenum (Mo); sugar (as energy source replacing carbon obtained from atmosphere by fixation through photosynthesis). Some organic compounds in trace amount needed in many media to improve growth are plant growth substances and vitamins ; (generally incorporated as well as media solidifying agent (Agar) for semi-solid medium preparations are also required (George *et al.*, 2008).

The sugar concentration is dependent on the type and age of the explants; very young embryos require a relatively high sugar concentration, hence growth and development increases with sugar concentration, until an optimum and then decreases at high concentrations. The common used source of carbon is sucrose at a concentration of 2-5%, however glucose and fructose are to known support good growth of some tissues (Bhojwani and Razdan, 2004), although an important role *in vitro* cultures as a carbon sources as well as an osmotic agent. In addition to carbohydrate-modulated gene expressions in plants are to known (Koch, 1996). Sugar is an important component in medium and its addition is essential for *in vitro* growth and development of plants because photosynthesis is insufficient, due to the growth-taking place in conditions unsuitable for photosynthesis or without photosynthesis (Pierik, 1997).

The sugar concentration chosen is dependent on the type and age of growth material; very young embryos require a relatively high sugar concentration. Generally growth and development increases with sugar concentration, until an optimum is reached and then decreases at high concentrations. Variation in shoots response is observed in different sugars and lower concentration of dextrose found to enhance the root and shoot growth in comparison to sucrose and maltose. Sucrose has been replaced by dextrose in rice grain culture, found to be more efficient, and can be used for further tissue culture experiments (Ruby et al., 2007). Four vitamins including Myo-inositol, thiamine, nicotinic acid, and pyridoxine are an ingredients of (Murashige and Skoog, 1962) medium and have been used in varying proportions for the culture of tissues of many plant species. The requirements of cells for added vitamins vary according to the nature of the plant and the type of culture (Welanders, 1977; Nitsch and Nitsch, 1965), vitamins were not necessary or were even inhibitory to direct shoot formation on petiole explants (Roest and Benkelman, 1975)

## **2.8. Plant Growth Regulators**

Hormones are organic compounds naturally synthesized in higher plant that affect the growth and development of plants by exerting a profound influence on physiological processes (Hopkins and Huner, 2009). These growth regulators are required in combination in the media as it is always the manipulation and variation of auxins and cytokinins levels that can successfully change the growth behavior of plant cultures (Gonzales, 1994). Apart from natural hormones, synthetic hormone has developed which act-like the natural ones. These synthetically produced hormones commonly called, plant growth regulators and have a Biological activity, which equals or exceeds that of the equivalent endogenous hormones (Gaspar *et al.*, 1996). In tissue culture plants, growth regulators are important media components in determining the developmental pathway of the plant cells. Growth regulators used in different proportions to break dormancy and enhance shoot formation, as the apical dormancy is under control of these growth regulators (Madhulatha *et al.*, 2004). The requirements for growth substance varies depending on the type and source of the explants and on their endogenous level ( Bhojwani and Razdan. 1996).

The cytokinins and auxins are of importance *in vitro* culture as the later are concerned with root formation, the former is mainly required in the media for shoot formation and growth of buds (North *et al.*, 2012). The irreversible degradation of cytokinins, catalyzed by cytokinin oxidase, is an important mechanism by which plants modulate their cytokinin levels (Brugiere *et al.*, 2003). Cytokinins incorporated mainly to initiate cell division and differentiation of adventitious shoots from callus and organs. These compounds are also used for shoot proliferation by the release of axillary buds from apical dominance (Bhojwani and Razdan, 1996). In tissue culture Furfurylamino purine (Kinetin), Thidiazuron (TDZ) and zeatin are alone and in combination with other cytokinin and auxins for proliferation and multiplication of different plant species (Gaspar *et al.*, 1996). Cytokinins hormone has derived from adenine (Aminopurine) and play an important role in the *invitro* manipulation of plant cells and tissues (Torres *et al.*, 2001). The most common cytokinins are kinetin, 6-Benzyl Amino urine (BAP), Thidiazuron (TDZ) and zeatin and 2iP (Pierik, 1997). These hormones are concerned with cell division, modification of apical dominance, shoot differentiation. In tissue culture cytokinin to initiate cell division and differentiation of adventitious shoots from callus and organs.

These compounds are also used for shoot proliferation by the release of axillary buds from apical dominance (Bhojwani and Razdan, 1996). Auxins are added to the culture medium to promote the growth of callus, cell suspensions and organs to regulate morphogenesis. The morphogenesis that occurs in a plant tissue culture depends on the ratio and concentration of auxins and cytokinins in the medium with this regard adventitious and auxiliary shoot proliferation occurs. When the ration of auxin to cytokinin is low whereas root induction of plantlets, embryogenesis and callus initiation occur when the ratio of auxin to cytokinin is high (George *et al.*, 2008).

## **2.6. Aseptic Environments for tissue culture**

Microbes compete adversely with plant tissue cultures for nutrients and their presence often results in increased culture mortality or can also result in variable growth, tissue necrosis, reduced shoot proliferation and reduced rooting (Oyebanji *et al.*, 2009). The

source of microbial contaminants can be the explants or materials used to operate tissue culture procedures. Therefore, it is necessary to sterilize explants and the equipment used for this process is avoided microbial contamination through effective surface sterilization of explants for *in vitro* culture initiation. Sterilization is the process of making explants contaminant free before establishment of cultures. Various sterilization agents are used to decontaminate plant tissue for *in vitro* cultures (Mineo, 1990, Aishwarya and Robinson, 2013). These sterilants may also be toxic to the plant tissue, hence proper concentration of sterilants and duration of exposing the explants to various sterilants are to minimize explants injury and achieve better survival. In a essence, requirements on the type concentration and time of exposure differs from one plant to another and for different parts of plants depending on their morphological characters for example, softness/hardness of the tissue (Srivastava et al., 2010; Singh *et al.*, 2011).

Most surface contaminants such as bacteria and fungi can be eliminated by sterilizing the plant material with a suitable sterilant agent. Surface sterilizing agents are commonly applied for 10-15 minutes. Under aseptic conditions, the plant material washed to three or four times for 5 minutes, that removes the sterilants (Rolando et al., 1986). Explants may also be surface sterilized with an aqueous solution of sodium or calcium hypochlorite. The calcium salt is preferred as it is less phytotoxic. Many laboratories use commercial products usually contain 5.25% NaOCl as the active agent when diluted with water, the final sterilizing solution contain not less than 0.5 % NaOCl (Rolando et al., 1986). Sterilization of laboratory instruments is carried out by autoclaving, alcohol washing, baking, radiations, flaming and fumigation (Rayne and Hunter, 1993). A considerable decrease in bacterial contamination has seen by using ultrasonic solicators (Hussey, 1982).

### **3. MATERIALS AND METHODS**

#### **3.1. Description of the study area**

The experiment was conducted at the Plant tissue culture Laboratory of the Institute of Biotechnology, Addis Ababa University. It is found at Latitude 8° 58' N and 38° 47' E and longitude with an elevation 2200 m.a.s.l. The average annual rainfall is 1000 mm to 2200 mm with maximum and minimum mean temperature of 22°C and 8°C respectively.

#### **3.2. Culture medium preparation**

The culture medium used for this experiment was MS medium (Murashige Skoog, 1962), which was prepared from mixture of macronutrient, micronutrient, Fe-Na-EDTA and vitamins. Appropriate amount of each nutrient was weighed in grams per liter (appendix 1). It was then dissolved in double distilled water sequentially making sure that it dissolved completely one after the other. After all the components fully dissolved by the help of using magnetic stirrer, the solution was finally dispensed into plastic bottles and stored at 20 °C until used.

Stock solutions of plant growth regulators were prepared separately for shooting and rooting experiments. For shoot formation, cytokinins: 6-benzyl amino-purine (BAP) and Kinetin (KN) were combined to 1- Naphthalene Acetic acid (NAA) were used and for rooting experiment, auxins: Indole-3- butyric acid (IBA) and Indole -3- acetic acid (IAA) were used. After all the plant growth regulators were prepared by weighed and dissolved its powders in double distilled water at a ratio of 1:1(1mg/mL), it was dissolved by mixed with 2-3 drops of 1N NaOH or 1N HCl based on the requirement of the plant growth regulators. Then, the volume was adjusted by adding double distilled water and it was stirred using magnetic stirrer, and then growth regulators stock solutions were stored in a refrigerator at +4°C for short-term use. Finally, the culture medium was prepared by taking 50 ml/L macronutrient, 5ml/L micronutrient, 5ml/L vitamins stock solution of MS medium, and sucrose (30g/L). The pH of the culture medium was adjusted to 5.8 using 1N NaOH and 1N HCl. Subsequently, 8 g/l

agar was added, heated for two to three minutes and it placed in microwave oven until the agar melted. Finally, 50 ml of the prepared medium dispensed in to culture vessels and the culture vessels covered immediately with caps, then autoclaved at a temperature of 121°C and pressure of 105 KPa for 15 minutes.

### **3.3. Source of explants**

In order to raise mother plants that help as source of explants (600-800) fresh seeds of *Echinops kebericho* were harvested from plants grown in the Medicinal Plant Garden of Ethiopia Health and Nutrition Research Institutes (EHNRI) in Addis Ababa. The seeds were then washed thoroughly in tap water for 10 minutes. The seed surface sterilized with 70 % ( v/v) alcohol for 9 minute and then in 5.25 % ( w/v) sodium hypochlorite for 5 minutes. Tween 20 (1-2 drops) was used as a surfactant in the sterilants. Then the seeds were rinsed 4-5 times with sterilized double distilled water to remove traces of sterilants. Thereafter, seedlings were raised from sterilized seeds in sterilized culture vessels of hormone free MS medium. Totally the experiment consisted of twenty culture vessels which contained five seeds each and the experiments were repeated twice.

### **3.4. Culture conditions**

This study, (Murashige and Skooge, 1962) media were solidified with 0.8 g/L agar. The pH of the medium was adjusted to 5.8 using 1N NaOH or 1N HCl and autoclaved at 121° for 15 minutes under 105-kpa pressure. All cultures were incubated under a light intensity of  $22\mu\text{mol m}^{-2} \text{s}^{-1}$  and 16 h photoperiod provided by white fluorescent lamps at  $25\pm 2^\circ\text{C}$ .

### **3.5. Culture initiations**

Shoots excised from three-week-old *in vitro* grown seedlings were cultured on MS medium containing 50 ml medium supplemented with concentrations of KN or BAP 0.0, 0.5, 1.0, 1.5, 3.0 mg/L and combined with 0.5 mg/L of NAA and 30 g/L sucrose (w/v) in order to get young shoot explants for shoot regeneration. Ten treatments were

employed that contained three-culture vessels each with five shoots were used and the experiment was repeated twice.

### **3.6.Shoot regeneration from initiated shoots**

The above initiated shoots were harvested after 30 days of culturing. Then cut into small pieces (0.5-1.0 cm) and cultured on MS medium supplemented with various concentrations of KN or BAP (0.0, 0.5, 1.0, 1.5, 2.5 mg/L.). The explants placed with a biaxial surface in contact with the medium. Total ten treatments were employed each of which contained three petridishes that consisted of ten explants were used and the experiments was repeated twice.

### **3.7.Shoot Multiplication**

Regenerated shoots from the above experiments were taken from the medium and cultured on full strength MS medium supplemented with 0.0, 0.5, 1.0, 2.0, 3.0 mg/L concentration of KN or BAP and medium solidified with 0.8 g/L agar. Ten treatments were employed which contained three-culture vessels with five shoots each and the experiment was repeated twice.

### **3.8.Root induction**

For root induction, multiplied shoots from multiplication medium were excised, and transferred on to 1/3 strength MS medium supplemented with 0.0, 0.5, 1.0, 1.5, 2.5 mg/L of IBA or IAA and were fortified 10 g/L sucrose (w/v) with solidified 8 g/L agar, total ten treatment, each three culture vessels, each with three shoots were used. The numbers of root per shoot and length were recorded after four weeks.

### **3.9.Acclimatization**

After proper roots, grown the plantlets were taken out from the culture vessels, gently washed under tap water to remove the adhering medium and the plantlets were transferred to plastic pots that contained a sterilized soil and then covered with polyethylene bags to prevent humidity losses. The Plantlets were maintained at 25±

2°C temperatures and under bright light. After 40 days, the numbers of plantlets that survive was recorded.

### **3.10. Data analysis**

Data were analyzed for variance by Duncan's multiple range test (DMRT) by using the SAS software version 9.1.3 (SAS institute, 2002) and mean differences were analyzed by least significant difference (LCD) test using at  $P < 0.05$  confidence level.

## 4. RESULTS AND DISCUSSION

### 4.1. *In vitro* seed Germination

The presents study, *in vitro* fresh seed germination was uniform (100%) cumulative germination. It was recorded in 30 days of culturing (Appendix figure 1). In agreement with this result, Begashaw and Tileye (2014) who reported of the seed, germination was slow and did not begin within the initial 10 days for five-month-old seeds. In contrast, seeds stored for 3 months began germinating within the first 10 days though percentage of seed germination was low. Over the 30 days study, the highest and uniform (100%) cumulative germination was recorded in the fresh seeds. Seed viability in *Echinops kebericho* was reported to decrease fast when stored at room temperature (25°C) but even storing at 4°C did not help much (Balcha et. al., 2009).

### 4.2. Culture initiation

The shoots excised from three weeks old *in vitro* germinated seedlings were used in order to get young shoots. A well-developed young shoots were observed after four weeks culture. Cultured on MS medium with no BAP and KN, 60% of shoots /explants were observed. This value was significantly ( $P < 0.05$ ) lower than values observed cultured on a medium that contained BAP or KN combined with 0.5 mg/L of NAA (Table 1).

Hundred percent shoot induction was observed on MS medium supplemented with 1.5 mg/L of BAP combined with 0.5 mg/L of NAA ,whereas the same result was observed at 1.5 mg/L of KN combined with 0.5 mg/L NAA. The number of shoots /explants and shoots length showed similar trend with the highest results obtained in a medium supplemented with 1.0 mg/L of KN combined with 0.5 mg/L of NAA (Table1). In agreement with (Ali *et.al.*, 2010; Yan *et.al.*, 2010). KN is much more effective than BAP. This suggests that KN induces better shoot formation than BAP at lower concentration. Therefore, BAP at a relatively optimum concentration would be 1.5 mg/L combined with 0.5 mg/l of NAA (Table 1) but further increase in concentration of BAP and KN above this concentration was found to decrease the responses. Begashaw

and Tileye (2014), who reported that maximum shoot proliferation, took place on shoot induction media containing 1.0 mg/L KN in *E.kebericho* made similar observations.

Table 1. Effect of various concentrations of KN and BAP (in MS medium) shoot induction from shoot explants of *E. kebericho* Values are mean  $\pm$ SE, n=3

PGR <sub>s</sub> (mg/L)		Shoot induction (%)	Shoot number /explants	Shoot length(cm)
BAP	NAA			
0.0		60	2.97 $\pm$ 0.22 <sup>d</sup>	3.00 $\pm$ 0.15 <sup>d</sup>
0.5	0.5	93.2	5.83 $\pm$ 0.20 <sup>c</sup>	3.17 $\pm$ 0.17 <sup>d</sup>
1.0	0.5	92.3	3.80 $\pm$ 0.40 <sup>d</sup>	3.00 $\pm$ 0.15 <sup>d</sup>
1.5	0.5	100	7.75 $\pm$ 0.05 <sup>b</sup>	4.48 $\pm$ 0.09 <sup>b</sup>
3.0	0.5	73.3	3.20 $\pm$ 0.20 <sup>d</sup>	2.87 $\pm$ 0.14 <sup>b</sup>
<b>KN</b>				
0.0		60	2.97 $\pm$ 0.22 <sup>d</sup>	3.00 $\pm$ 0.15 <sup>d</sup>
0.5	0.5	94.8	6.00 $\pm$ 0.15 <sup>c</sup>	4.17 $\pm$ 0.15 <sup>c</sup>
1.0	0.5	100	10.30 $\pm$ 0.30 <sup>a</sup>	5.20 $\pm$ 0.14 <sup>a</sup>
1.5	0.5	100	8.10 $\pm$ 0.28 <sup>b</sup>	5.17 $\pm$ 0.13 <sup>b</sup>
3.0	0.5	91.2	3.67 $\pm$ 0.45 <sup>d</sup>	3.90 $\pm$ 0.14 <sup>d</sup>

The mean for the same letters within the same column are not statically different ( $p > 0.05$ ), whereas those represented by different letters are significantly different from each other.

### 4.3.Shoot regeneration from initiated shoots

The shoots of *E.kebericho* were inoculated on MS solid medium supplemented with varying level of KN or BAP and shoot initiation started after 2 week of culture from explants. The percentage of direct shoot regeneration from shoot explants and shoots numbers/explants were significantly different( $p < 0.05$ ).

The regeneration of explant higher when the explants were cultured on MS medium supplemented with KN than when supplemented with BAP(Tabel 2).When KN alone was used, shooting responses were in order of 1>0.5>1.5>2.5>0mg/L, suggesting that 1.0 mg/L better responses than other concentration level. The result goes along with

Tajo.A and Smrithi.PK (2016) who reported it belongs to the family Asterraceae *Steviare baudiana* that KN treated explants achieved higher shoot regeneration than those treated with other concentration. BAP supplemented , however percentage of shoot regeneration and number of shoot/explants was better under 0.5 mg/L (Tabel 2). The highest number of shoot per explants was obtained from the medium containing 1.0 mg/L KN.

Table 2.Effect of various concentration of KN and BAP (in MS medium) on *in vitro* regeneration of shoots from shoot explants of *E.kebericho* Values are mean $\pm$  SE, n=3.

PGR <sub>s</sub> ( mg/L)	Shoot regeneration %	Shoot number /leaf explants
<b>KN</b>		
0.0	13.3	0.67 $\pm$ 0.33 <sup>c</sup>
0.5	65	5.83 $\pm$ 0.48 <sup>b</sup>
1.0	86.7	6.00 $\pm$ 0.97 <sup>a</sup>
1.5	51.7	4.33 $\pm$ 0.61 <sup>bc</sup>
2.5	60	3.00 $\pm$ 0.37 <sup>c</sup>
<b>BAP</b>		
0.0	13.3	0.67 $\pm$ 0.33 <sup>b</sup>
0.5	65	3.8 $\pm$ 0.48 <sup>a</sup>
1.0	46.7	2.50 $\pm$ 0.22 <sup>b</sup>
1.5	50	3.50 $\pm$ 0.22 <sup>b</sup>
2.5	50	3.17 $\pm$ 0.31 <sup>b</sup>

The mean for the same letter within the same column were not statically different (p =0.05) whose values represent mean  $\pm$ S.E. Thirty explants per treatment were carried out.

#### **4.4. Effect of KN and BAP on shoot multiplication of *E.kebericho***

Multiplication of shoots occurred in all concentration of growth regulators, but the number and the length of shoots per explants varied. The explants that were cultured on MS medium without growth regulators (control), showed less shoot proliferation.

The mean for the number of shoots was  $1.50 \pm 0.22$  and the mean for the shoot length was  $1.33 \pm 0.21$ .

When the regenerated shoots were cultured on medium containing 1.0 mg/L KN, maximum shoot proliferation and shoot length were observed, similar observations were made by Muluken (2016) who reported that maximum mean number of shoots per explants ( $11.32 \pm 1.05$ ) was attended on medium containing 1.0 mg/L KN. The growth in the number of shoot and shoot length was higher when they were cultured on MS medium containing KN than on a medium containing BAP (Table 3). Hence, overall shoot proliferation was in order of  $1 > 0.5 > 2 > 3 > 0$  mg/l KN. With reference to Begashaw and Tileye (2014), BAP combined with NAA was less effective for shoot multiplication. In contrast, KN is more effective for shoot multiplication as it demonstrated in *E.kebericho*. Therefore, 1.0 mg/L KN is better than other levels in shoot proliferation.

The effects of KN in combination with NAA for shoots multiplications were observed in *E.kebericho* and other members of the Asteraceae family using a variety of explants (Wildi et.al., 1998 and Begashaw and Tileye, 2014). *Artemisia amygdalina* (Rasool et al., 2013), leaf explants of *Brassica* sp. (Dunwell, 1998) and *Centella asiatica* (Mohapatra et al., 2008)

Table 3. Effect of various concentrations of KN, BAP on shoots multiplication of *E.kebericho*. Values are mean  $\pm$ SE, n=3.

PGR <sub>s</sub> (mg/l)	No. of shoot /explants Mean $\pm$ SE	Shoot length(cm) Mean $\pm$ SE
<b>KN</b>		
0.0	1.50 $\pm$ 0.22 <sup>d</sup>	1.33 $\pm$ 0.21 <sup>c</sup>
0.5	4.17 $\pm$ 0.31 <sup>b</sup>	3.17 $\pm$ 0.31 <sup>b</sup>
1.0	5.00 $\pm$ 0.00 <sup>a</sup>	4.33 $\pm$ 0.21 <sup>a</sup>
2.0	2.17 $\pm$ 0.48 <sup>c</sup>	1.50 $\pm$ 0.22 <sup>c</sup>
3.0	1.67 $\pm$ 0.21 <sup>c</sup>	1.50 $\pm$ 0.22 <sup>c</sup>
<b>BAP</b>		
0.0	1.50 $\pm$ 0.22 <sup>d</sup>	1.33 $\pm$ 0.21 <sup>c</sup>
0.5	2.50 $\pm$ 0.43 <sup>c</sup>	2.50 $\pm$ 0.22 <sup>b</sup>
1.0	3.83 $\pm$ 0.17 <sup>b</sup>	4.00 $\pm$ 0.37 <sup>a</sup>
2.0	2.17 $\pm$ 0.31 <sup>c</sup>	1.50 $\pm$ 0.22 <sup>c</sup>
3.0	1.50 $\pm$ 0.22 <sup>d</sup>	1.50 $\pm$ 0.22 <sup>c</sup>

The means for the same letters within a column were not statically different ( $p>0.05$ ). Whereas those represented by different letters were significantly different from each other.

#### 4.5. Rooting induction

For root induction, individual shoots were placed on the medium supplemented with different concentrations of IAA and IBA. As a result, roots induction were noticed in all the concentrations but with varied response with reference to the number and length of the roots per shoots. *In vitro* derived shoots were transferred to 1/3 strength MS medium containing different concentrations of IAA or IBA (0.0, 0.5, 1.0, 1.5 2.5 mg/l) and significantly affected the root length and number at  $p< 0.05$  (Table 5).

A maximum mean number of roots per shoot (5.33 $\pm$ 0.33) was obtained on 1/3 strength MS rooting medium containing 0.5 mg /L IAA (Table 4). The highest mean length of root per explants (5.83 $\pm$ 0.31cm )was obtained on 1/3 strength MS rooting medium 0.5 mg/l IAA( Table 4). This result complies with a conclusion reached by Mirna et.al (2008) that the highest rooting from shoot explants of *Inula verbascifolia* (Wild) were

found on MS medium supplemented with 0.5 mg/L IAA. On 1/3 strength MS rooting medium containing 0.5 mg/l IAA, 85.5% survival rate was recorded in the green house which is in conformity with the previous report of *E.keberichio* Muluken (2016)

Table 4. Effect of different concentration of IAA, IBA (1/3 MS basal media strength) on rooting of *in vitro* regenerated shoots of *E.keberichio*.

PGR <sub>s</sub> (mg/l)	No. of root /explants Mean ±SE	Root length(cm) Mean ±SE
IAA		
0.0	0.67±0.21 <sup>c</sup>	0.50±0.22 <sup>d</sup>
0.5	5.33±0.33 <sup>a</sup>	5.83±0.31 <sup>a</sup>
1.0	4.67±0.21 <sup>a</sup>	4.33±0.33 <sup>b</sup>
1.5	3.67±0.21 <sup>b</sup>	4.00±0.37 <sup>b</sup>
2.5	3.67±0.21 <sup>b</sup>	2.67±0.21 <sup>c</sup>
IBA		
0.0	0.67±0.21 <sup>cd</sup>	0.50±0.22 <sup>c</sup>
0.5	1.33±0.21 <sup>c</sup>	0.67±0.21 <sup>c</sup>
1.0	0.33±0.21 <sup>d</sup>	0.50±0.22 <sup>c</sup>
1.5	3.67±0.21 <sup>a</sup>	3.83±0.40 <sup>a</sup>
2.5	2.17±0.31 <sup>b</sup>	2.33±0.21 <sup>b</sup>

Mean for with the same superscripts in the same column were not significantly different ( $p < 0.05$ ). The values represented the mean ±S.E. Thirty shoots per treatments were conducted.

#### 4.6. Acclimatization

Plantlets were allowed to grow on rooting media for about one month after root initiation. Plantlets with developed root were carefully and systematically removed from culture vessels. In addition, they were washed by running tap water then they immediately planted in potted soils. Totally 30 plants transferred to greenhouse on five pots each containing six plants from these transferred plantlets 80% of them survived (Fig. 7).

## 5. SUMMARY AND CONCLUSIONS

*Echinops kebericho* is among few prioritized medicinal plants of Ethiopia that needs urgent conservation. Developing *invitro* regeneration protocol for *E.kebericho* is very useful to preserve germplasm of this critically endangered species of high medicinal importance, to promote scientific activities (pharmacological studies and extraction of medicinally important compounds), commercial cultivation and sustainable usage of the species. The presents study was conducted to establish a protocol for *in vitro* regeneration of *Echinops kebericho* from shoot explants and effect of hormonal treatment via cytokinins (BAP and KN) and auxin (IBA and IAA) were observed for *in vitro* shoot regeneration and root induction respectively. The study started that the fresh seeds were washed thoroughly in tap water for 10 minutes, surface sterilized with 70 % ( v/v) alcohol for 9 minute and then in 5.25 % ( w/v) sodium hypochlorite for 5 minute with tween 20 (1-2 drops) and were cultured hormone free MS medium. As a resulted, *in vitro* fresh seed germination was uniform (100%) cumulative germination at 30 days of culturing.

The best shooting responses in terms shoot numbers and shoots length per explants were obtained in MS medium supplemented with 1.0 mg/L KN with combined 0.5 mg/L NAA for shoot inductions while the medium supplemented with 1.5 mg/L BAP combined with 0.5 mg/L of NAA were to be used a relatively optimum concentration. Further increases in concentration of BAP and KN above these concentrations found to decreases the responses. The highest multiplication of shoot was obtained at 1.0 mg/L, when KN alone was used, the shoots responded higher than others concentration, when KN alone was used, shooting responses were iorder of 1>0.5>1.5>2.5>0mg/L, this suggested that 1.0 mg/L better responses than other concentration. Depending on results observed in the present study can be concluded that MS medium supplemented with 1.0 mg/L KN and 0.5 mg/L BAP can be effective used for shoot regeneration of *E.kebericho*. The number of root induced and root length with the highest result obtained in a medium supplemented with 0.5 mg/L IAA. Totally 30 plants transferred to greenhouse on five pots each consisted six plantlets from these transferred plantlets 80% of them survived.

## 6. RECOMMENDATIONS

The following recommendations should focus on based on the present study.

- Further studies may be needed using combination of hormone such as KN with BAP and KN with IBA and BAP with IAA for shoot regeneration and multiplication.
- The regeneration and multiplication plantlet was intervening callus, therefore, the effect on cytokinin and auxin that form callus on the regeneration and multiplication should be further studied.

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## 8. APPENDIX

**Appendix table 1.** Stock solution for MS (Murashige and Skoog 1962)

Components	Concentration (g/L)	mL/L during media preparation
<b>Micronutrients</b>		
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	1.72	
H <sub>3</sub> BO <sub>3</sub>	1.124	
MnSO <sub>2</sub> ·4H <sub>2</sub> O	3.38	
MnSO <sub>4</sub> ·H <sub>2</sub> O	0.05	5ml/l
KI	0.166	
NaMoO <sub>4</sub> ·2H <sub>2</sub> O	0.05	
CoCl <sub>2</sub> ·6H <sub>2</sub> O	0.05	
Na <sub>2</sub> EDTA	7.472	
FeSO <sub>4</sub> ·7H <sub>2</sub> O	5.56	
<b>Macronutrients</b>		
NH <sub>4</sub> NO <sub>3</sub>	33	
KNO <sub>3</sub>	38	
CaCl <sub>2</sub> ·2H <sub>2</sub> O	8.8	50ml/l
MgSO <sub>4</sub> ·7H <sub>2</sub> O	7.4	
KH <sub>2</sub> PO <sub>4</sub>	3.4	
<b>Vitamins</b>		
Myo-inositol	20	
Glycin (glycocoll)	0.4	
Nicotinic acid (NaOH)	0.1	5ml/l
Pyridoxin (B <sub>6</sub> )	0.1	
Thiamin (B <sub>1</sub> )	0.02	

\* Alternatives

□ Na<sub>2</sub>EDTA and FeSO<sub>4</sub>·7H<sub>2</sub>O prepared alone

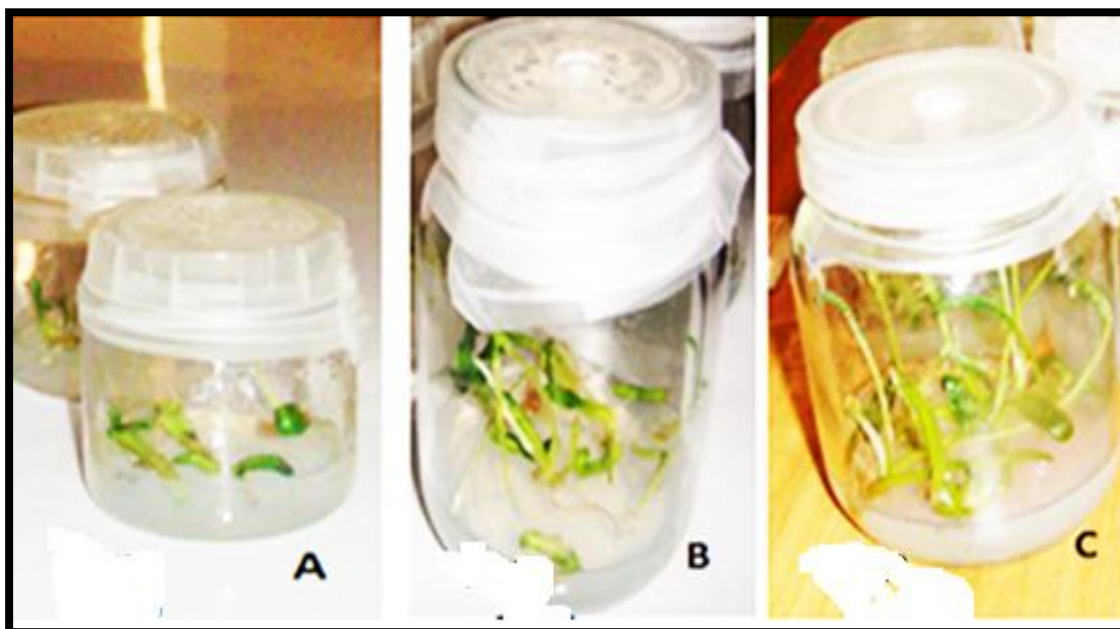
**Appendix figure**

Figure 1. (A, B, C) *In vitro* fresh seeds germination of *E.kebericho* in 10 d, 20 d and 30 d respectively

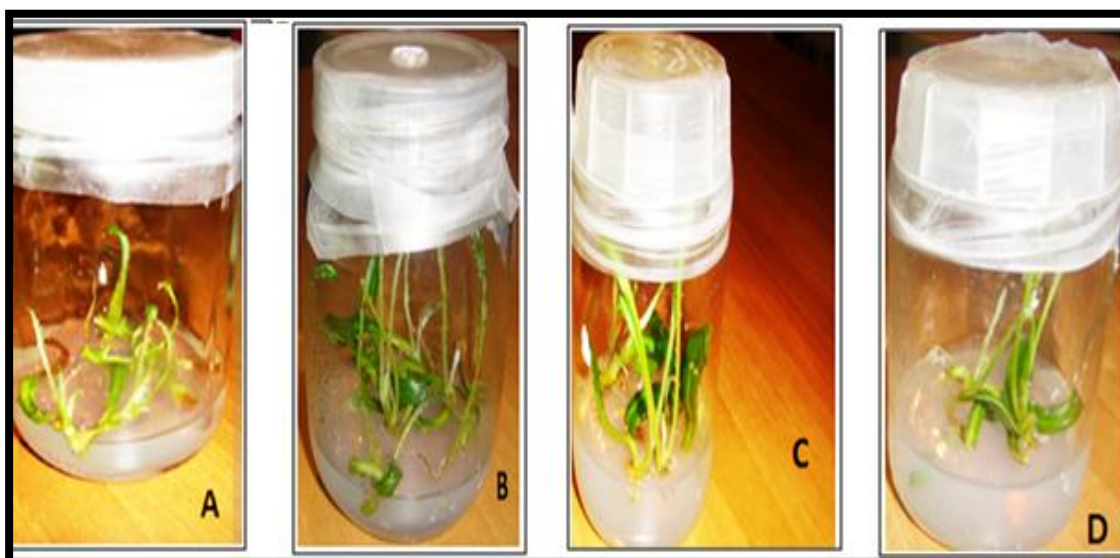


Figure 2. Effect of different concentrations of KN and BAP on shoot induction of *E. kebericho* (A) control, (B) 1.0 mg/L KN + 0.5 mg/L NAA, (C) 0.5 mg/L KN + 0.5 mg/L NAA (D) 1.5 mg/L BAP + 0.5 mg/L NAA

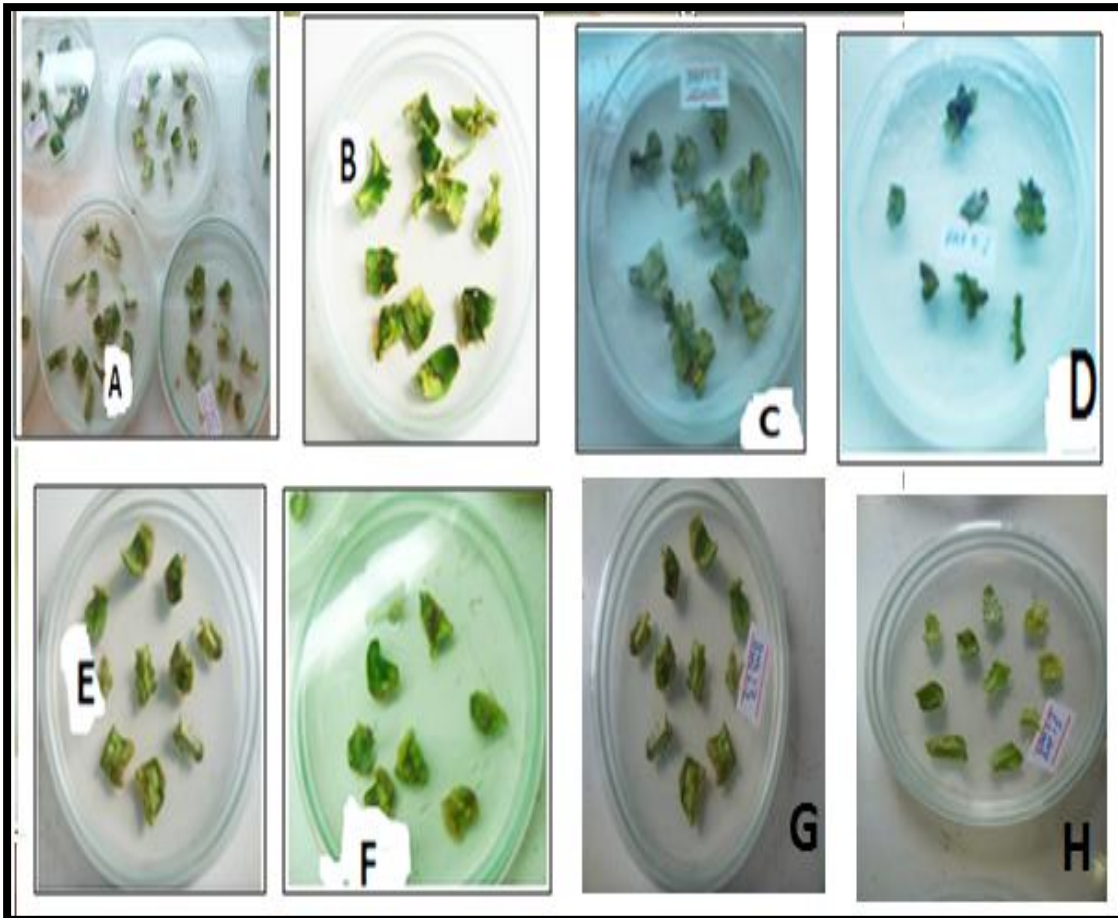


Figure 3. (A, B, C, D) explants inoculation on MS medium containing 0.5 mg/L and 1.5 mg/L BAP respectively and (E, F,G,H) leaf inculcation on MS medium 1.0 mg/L and 0.5 mg/L KN respectively

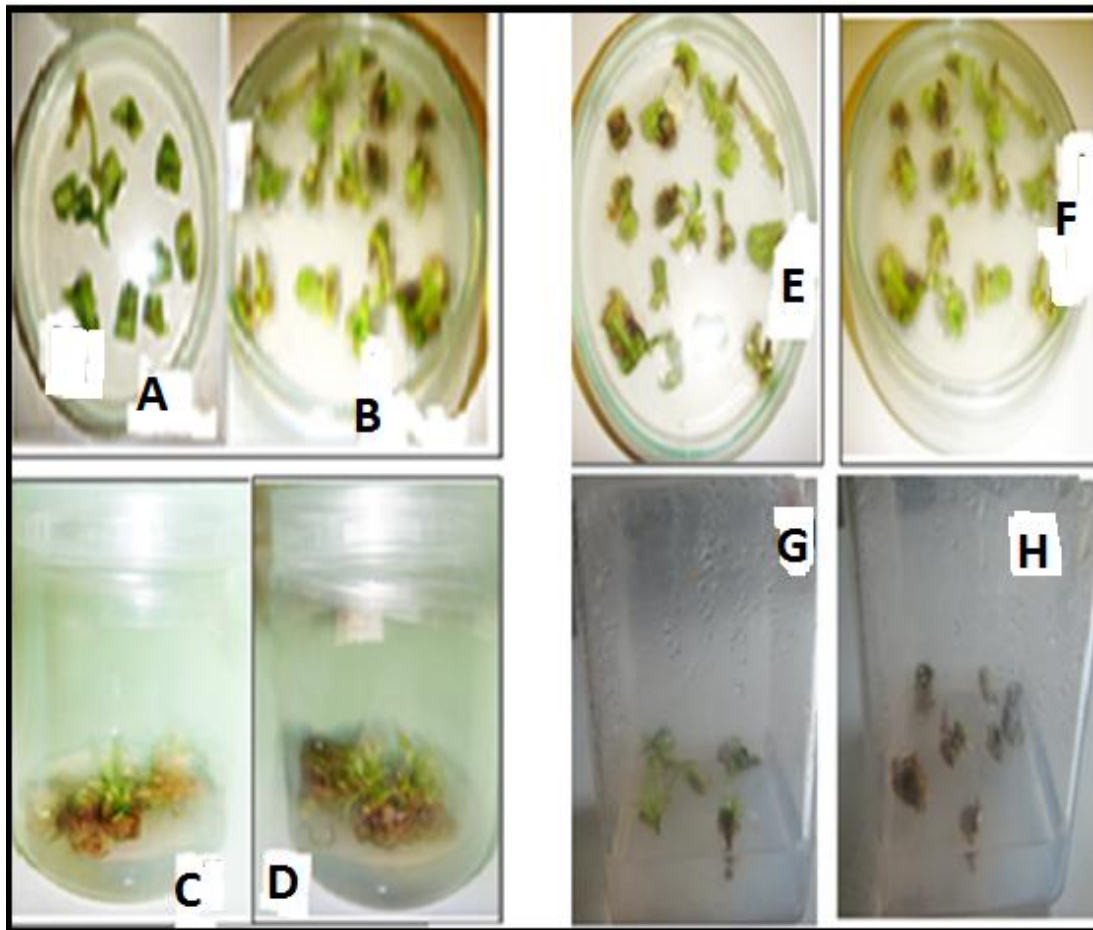


Figure 4. (A,B,C,D) shoot regeneration from shoot explants on MS medium containing 1.5 mg/L and 0.5 mg/L BAP and (E,F,G,H) shoot regeneration from shoot explants on MS medium containing 1.0 mg/L and 0.5 mg/L KN respectively

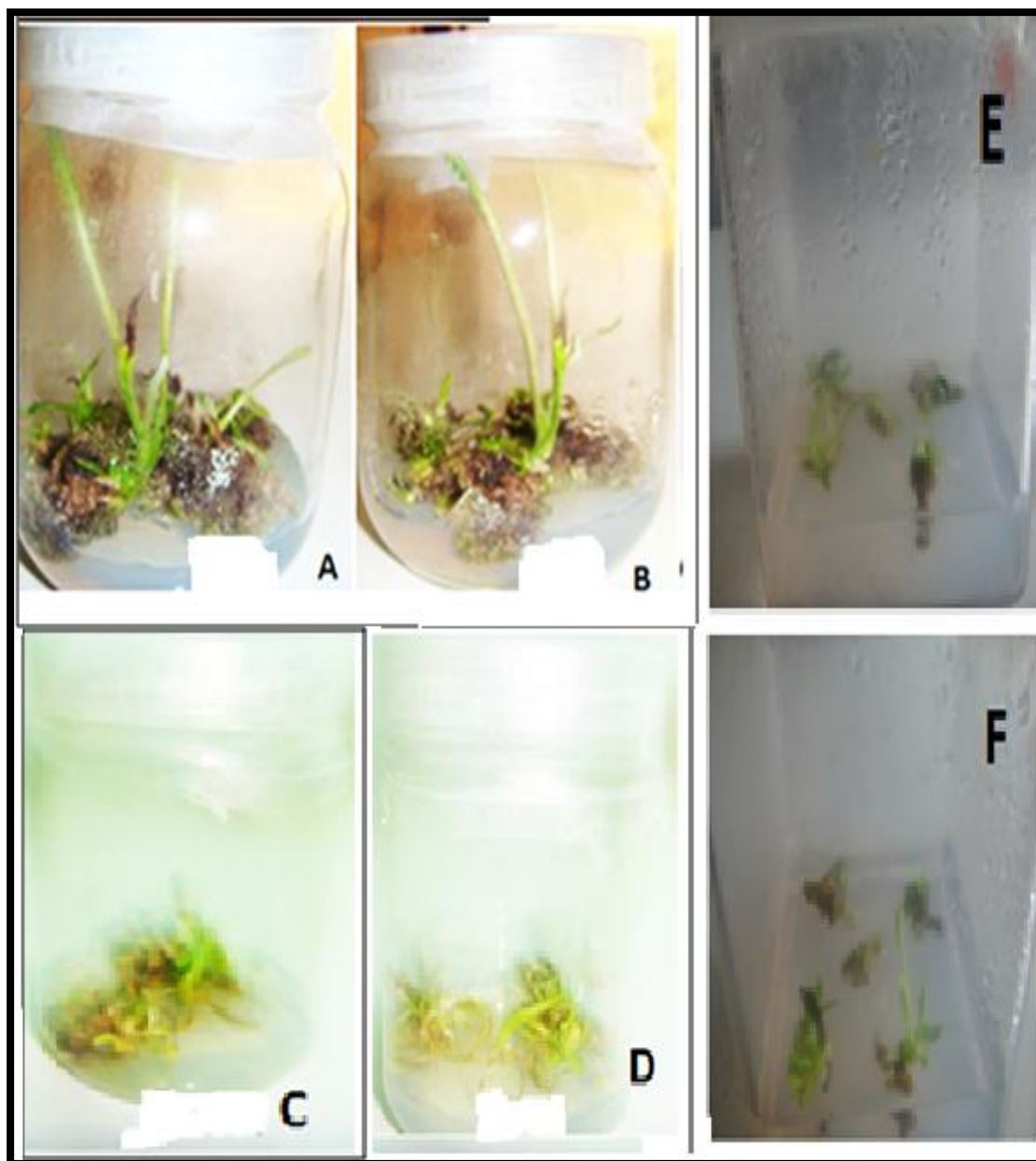


Figure 5. (A, B) shoot multiplication with some callus like form from shoot explants on MS medium supplemented with 1.0 mg/L BAP and 0.5 mg/LBAP (C , D, E,F) 0.5 mg/L and 1.0 mg/L KN respectively.

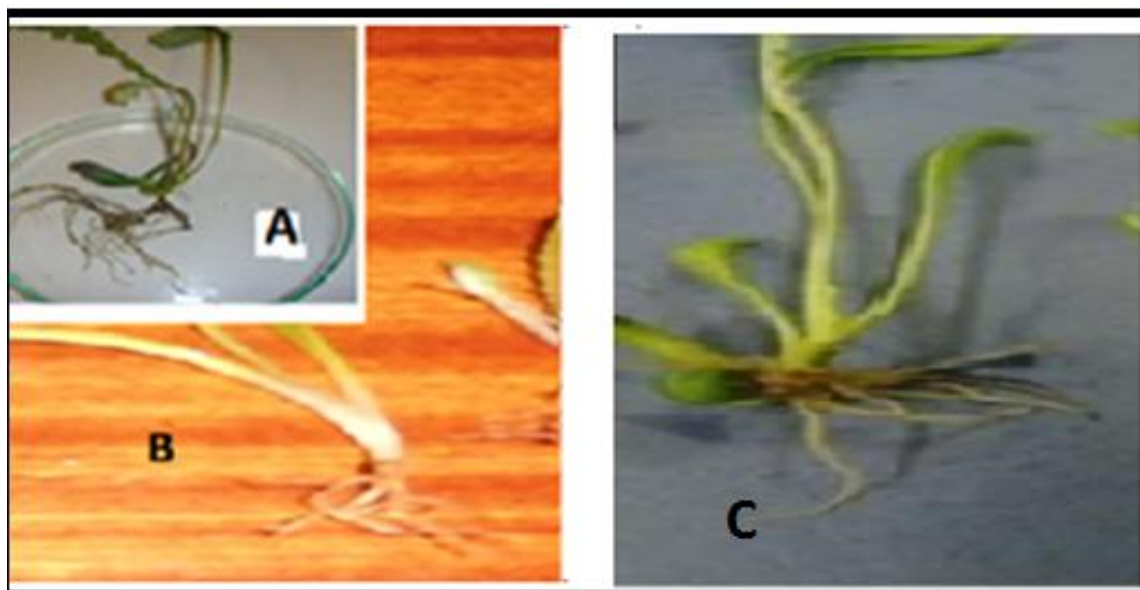


Figure 6. (A, B, C) *In vitro* rooting of *E. kebericho* shoots on MS medium 0.5 mg/L IAA

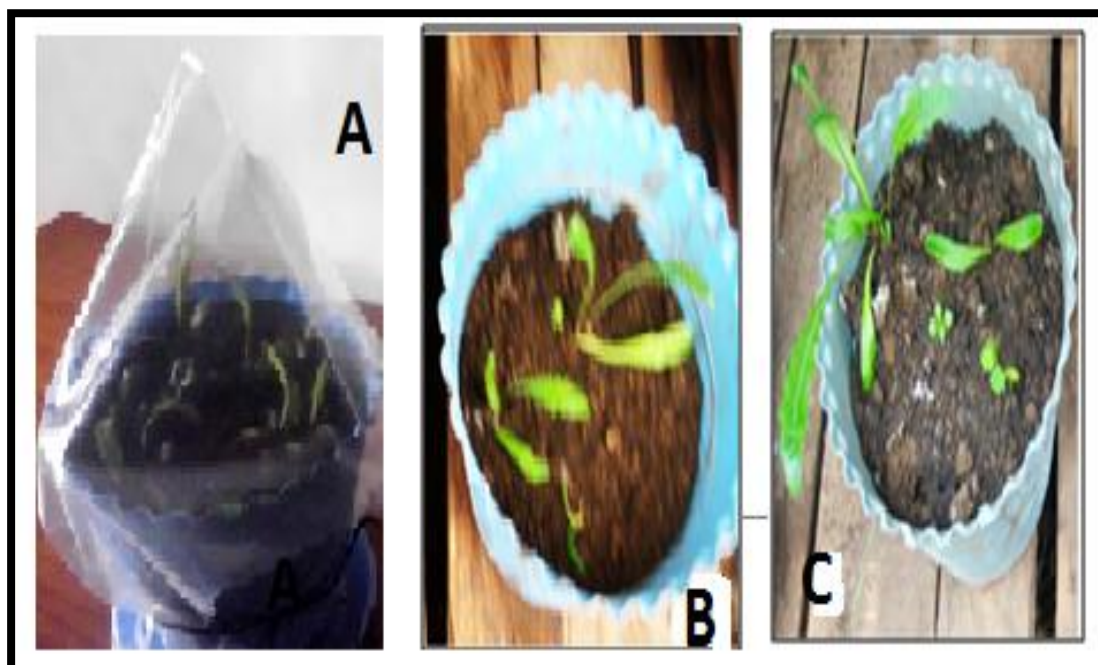


Figure 7. Acclimatization of *in vitro* rooted shoots of *E. kebericho* in green house. (A) Plants covered in polyethylene bag (B) After three weeks (C) After 40 days.

