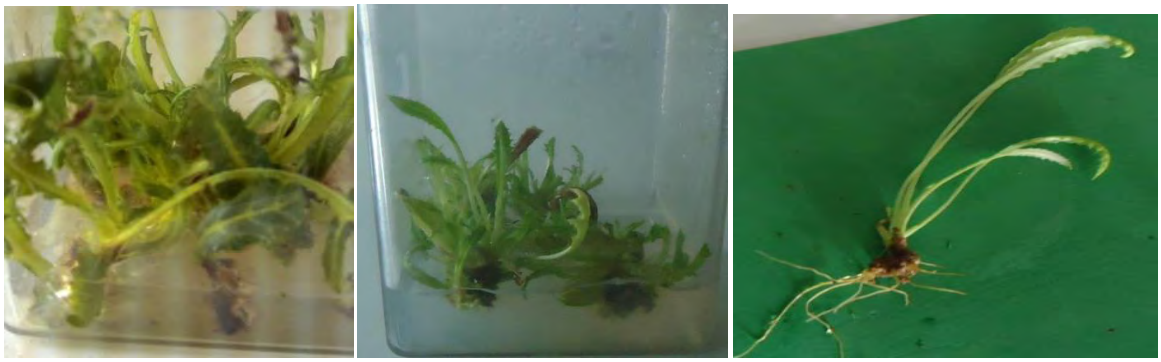
ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES
INSTITUTE OF BIOTECHNOLOGY

Protocol optimization of *in vitro* propagation of an endangered endemic medicinal plant, *Echinops kebericho,* through shoot tip culture



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

BY

Muluken Birara

Addis Ababa, Ethiopia

December, 2016

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PROTOCOL OPTIMIZATION OF *IN VITRO* PROPAGATION OF AN ENDANGERED ENDEMIC MEDICINAL PLANT, *ECHINOPS KEBERICHO*, THROUGH SHOOT TIP CULTURE

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ABSTRACT: Echinops kebericho *is the most important and critically endangered Ethiopian medicinal plant in Asteraceae family. This plant is threatened due to the commercial harvesting and sale of its roots. This study was aimed to develop efficient* in vitro *propagation protocol for* E. kebericho *using shoot tip explants. Shoots excised from* in vitro *germinated seedlings were used as explants for culture initiation on Murashige and Skoog (1962) (MS) medium supplemented with 1.0 mg/l kinetin (KIN). The* in vitro *initiated shoot tips were transferred onto solid MS medium supplemented with different concentrations of KIN in combination with BAP, KIN in combination with IBA and BAP in combination with IBA were used for shoot multiplication. For rooting, full, half and 1/3 strength MS medium supplemented with IAA was used. MS medium without PGRs was used as control. The result showed that highest mean number (14.44±0.29) of shoots per explant was obtained on a medium containing combination of 0.5 mg/l KIN with 0.5 mg/l IBA and the highest shoot height (6.50±0.28 cm) was obtained on multiplication medium fortified with 1.0 mg/l KIN in combination with 0.5 mg/l IBA. Maximum number of healthy roots (6.8±0.39) was observed on 1/3 strength MS medium containing 0.1mg/ IAA. Maximum root length of 4.68±0.22cm was achieved on 1/3 strength MS medium containing 0.5 mg/l IAA. Up on acclimatization, 85.5% plants survived. The results demonstrated that, this study is important for mass propagation and ultimate conservation of this endangered medicinal plant.*

Key words: ***Echinops kebericho, In vitro*** **propagation, Medicinal Plant, Plant Growth Regulator**

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LIST OF ABBREVIATIONS

BAP	6-Benzyl Amino Purine
IAA	Indol acetic acid
GA3	Gibberellic Acid
IBA	Indol-3-Butyric Acid
IBC	Institution of Biodiversity Conservation
EBI	Ethiopia Biodiversity Institute
KIN	Kinetin
MS	Murashige and Skoog basal media
PGR	Plant Growth Regulator

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1. INTRODUCTION

The use of herbal medicines continues to expand rapidly across the world with many people now resorting to these products for treatment of various health challenges in different national healthcare settings (WHO, 2004). This past decade has obviously witnessed a tremendous surge in acceptance and public interest in natural therapies both in developing and developed countries, with these herbal remedies being available not only in drug stores, but now also in food stores and supermarkets. It is estimated that up to four billion people of the world's population living in the developing world rely on herbal medicinal products as a primary source of healthcare and traditional medical practice which involves the use of herbs is viewed as an integral part of the culture in those communities (Mukherjee, 2002; Bodeker et al., 2005; Bandaranayake, 2006).

The list of medicinal plants in Ethiopia, which is documented for National Biodiversity Strategy and Action Plan by Tesema et al. (2002) shows that about 887 plant species were reported to be utilized in traditional medicine. Among these, 26 species are endemic and they are becoming increasingly rare and are at the verge of extinction. It is believed that the greater concentrations of these plants are found in the southern and south western parts of the country following the concentration of biological and cultural diversity (Yineger, 2005; UNEP, 1995).

In Ethiopia, 70-90% of the population relies on traditional medicine especially from medicinal plants for their primary health-care needs and these will not diminish in the future because modern health-care service is both limited and expensive (Vasisht and Kumar,2002, Lehoux *et al.,* 2012).

Echinops kebericho (Mesfin), belong to family Asteraceae/Compositeae, endemic to Ethiopia, is erect perennial herb or shrub up to 1.2 m high, commonly from a massive rootstock with leafy stems (Sebsebe Demissew, 1993). The root is used as a fumigant mainly after childbirth. It is also said that the smoke is effective against typhus and fever, and when chewed it reduces stomach ache. The root is also used to cure intestinal diseases in cattle. The Oromo tribes are known to use the smoke as a snake repellent in the house (Kloos, 1977). Ethnobotanical surveys also show that this plant has long been traditionally used to prepare medicines against migraine, mental illness, heart pain, lung TB, leprosy, kidney disease, malaria, bilharzia, syphilis and amoebic dysentery (Abebe and Ayehu, 1993; Desta, 1993; Abera, 2003).

Echinops kebericho is categorized as critically endangered Endemic Shrubs of Ethiopia which is a popular general purpose medicinal plant in Ethiopia. According to Getaneh (2011), *E. kebericho* is the scarcest medicinal plant in Mecha Wereda, West Gojjam Zone of Amhara region due to overexploitation for different purposes combined with medicinal uses. Because the roots of this species are used for medicine, it is approaching almost an extinction level in the area. In addition, since the natural regeneration of *E. kebericho* is restricted to a specific microclimate, the domestication of this threatened medicinal plant species is still not easy. *E. kebericho* propagates by seed which is incompetent due to lack of adequate seeds and seed 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.

It is important to develop effective propagation techniques for *Echinops kebericho.* In addition, the development of a rapid *in vitro* plant propagation method using the shoot tip explants of *Echinops kebericho*, promotes scientific activities including pharmacological studies and extraction of medicinally important compounds, commercial cultivation and sustainable use of the species.

The *in vitro* propagation method would be the promising option for multiplication and conservation of *E. kebericho*. Reports in this area, however, are limited. So far, the only report of the *in vitro* and *ex vitro* seed based propagation of *E. kebericho* (Balcha *et al.,* 2009) and micropropagation of *E. kebericho* from shoot tip explants was by Manahlie and Feyissa (2014). Considerable efforts are still required to find out efficient *in vitro* methods for the propagation of this critically endangered medicinal plant. Therefore, this study was designed to protocol optimization of *in vitro* propagation of endemic medicinal plant, *E. kebericho* using shoot tip explants

2. LITERATURE REVIEW

2.1. Taxonomy and Description of *Echinops kebericho*

E. kebericho Mesfin belongs to the Asteraceae family and it is commonly known as Kebericho. Taxonomically, the genus *Echinops* comprises 120 species, of which 12 are known to occur in Ethiopia. *E. kebericho* is an erect perennial herb or shrub, up to 1.2 m high, usually from a massive (c. 30 - 50 x 10 - 20 cm) rootstock; stem leafy throughout, sparsely arachnoid-pubescent, sometimes mixed with dark purple uniseriate hairs. Leaves elliptic, amplexicaul, basal ones up to 25 x 15 cm, pinnatisect with segments divided into triangular lobes all ending in sharp pungent spines, or bipinnatiset with linear-lanceolate segments; upper surface arachnoid-pubescent mainly along median vein, lower surface densely tomentose, dull, with uniseriate hairs along midvein. Head 3 - 4 cm in diameter, spineless. Peduncle up to 5.5 cm long, arachnoid-pubescent, 1 - 2 mm wide. Capitulum 2 - 2.5 cm long. Outer phyllaries 3 - 4-seriate, outermost row spathulate, 4 - 6 mm long, with a few white, soft, bristles along margins in the lower half, middle row spathulate with apiculate apex, 6 - 10 mm long, with several white, soft bristles on margins toward apex; innermost series lanceolate, tapered at base and at the apex, 9 - 14 x c. 1 mm, with bristles at margins in middle and toward tip; apex acute. Inner phyllaries oblong-linear, fused for up to 3/4 of their length, fimbriate at the apex, c. 10 - 13.5 x c. 1 mm, glabrous, ciliate at margins near the apex. Corolla white or bright blue, c. 12 - 15 mm long, tube glabrous except near base of limb where papillose; anthers light brown. Achenes oblong-elliptic, 7.5 - 10.5 x 1.5 - 2.5 mm, covered with appressed silvery white ciliolate bristles. Pappus of numerous, contiguous, narrow, ciliolate scales that are connate at base (Sebsebe Demissew, 1993).

2.2. Origin and distribution of *Echinops kebericho*

The genus *Echinops* comprises 120 species in the family Asteraceae/Compositeae and is distributed in semi-humid zones of tropical and North Africa, Mediterranean basin, and temperate regions up to Central Asia (Getachew *et al.,* 2011 cited in Hedberg *et al.,* 2004; Teresa *et al.,* 2005). Twelve species occur in Ethiopia but confined to the highlands of the country between 7^{o} 30′ N and 38^{o} 45′ E and at altitudes between 1700 and 2900 m.a.s. l. (Tadesse and Abegaz, 1990; Erko, 2006). *E. kebericho* grows in dry and stony lateritic soils. It is variable in habit and in dissection of the leaf blade. Populations from dry, stony lateritic soils are perennial herbs whereas those growing in deep vertisols are low shrubs (Tadesse and Abegaz, 1990; Erko, 2006).

2.3. Importance of *Echinops kebericho*

E. kebericho is a well-known plant to the majority of the rural population due to mainly its traditionally medicinal use. The roots of *Echinops kebericho*, (Amh: QEBERICHO; Oromo: QEREBICHO), has been used as a fumigant, particularly after child birth, and as a medicinal plant to treat leprosy for centuries (Tadesse and Berhanu, 1990 cited in Mesfin and Brook, 2010). Ethno-botanical surveys have documented that *E. kebericho* has long been traditionally employed by the local healers to prepare medicines against migraine, mental illness, heart pain, lung TB, leprosy, kidney disease, malaria, bilharzia, syphilis and amoebic dysentery (Abebe and Ayehu, 1993; Desta, 1993; Abera, 2003). Rootstock of *E. kebericho* is one of the 10 medicinal plants sold throughout the markets of the country, which is used as a source of income for a large number of local people and traditional healers (Kloos, 1976). The large tuberous roots are sold

either cut up as small pieces or in whole in many open markets in Shewa, Gojjam and Wellega regions (Tadesse and Berhanu, 1990; Tadesse, 1994 in Mesfin and Brook, 2010). Contemporary specialists in botanical medicines emphasize the burning of plants in order to use smoke as a therapeutic vehicle. Smoke may be inhaled or used to fumigate the whole house to get rid of insects and parasites. Among smoking plants available in many medicinal plant markets of Ethiopia, the most common is the root of *E. kebericho* which is used usually to fumigate the body as a general medicine and also to rid a house of pests and insects (Ermias, 1990). It is also claimed that the smoke is effective against typhus fever, and when chewed it relieves stomach ache and as a taenicidal herb (to expel tapeworm) (Asfaw and Demissew, 2009). The smoke from burning the plant is inhaled to relieve headache and also as a cure for "evil eye" (possession by evil spirits in Ethiopian folk religion). The root is burned for smoke to ward off mosquitoes and as a snake repellant in the house (Teklehaymanot and Giday, 2007)**.**

Echinops species were reported to contain a wide range of chemical constituents comprising alkaloids, saponins, phytosterols, polyphenols, carotenoids, sesquiterpene lactones/alcohols, lignans, acetylenic and thiophene compounds, and essential oil (Hymete *et al.*, 2005; Hymete *et al.*, 2006). In the oil of *E. kebericho*, 43 compounds representing 92.85% of the total essential oil constituents were identified. Sesquiterpene lactones, monoterpene hydrocarbons, sesquiterpene hydrocarbons, and oxygenated monoterpene structures represented 41.83, 23.97, 14.90, and 2.80%, respectively, of the total essential oil. The main constituents of the oil were dehydrocostus lactone (41.83%), β-phellandrene (10.84%), germacrene B (5.38%), α-selinene (4.13%), α-pinene (3.63%), and β-pinene (3.62%) (Yinebeb *et al.*, 2011). The tubers are extremely rich in sesquiterpene amounting to about 10% of the dry weight of the tubers (Abegaz,

1996). It is a biologically active compound having antitumor, antimutagenic and cytotoxic properties. The species presents itself as commercially viable for the Sesquiterpene lactones and if successful efforts are made to develop suitable uses for these compounds (Abegaz, 1996). The bioactive extract of the species also has been shown to have antimicrobial effects equal to or better than penicillin, especially against *Stereptococcus beta-haemolyticus, Escherchia coli, Klebsiela aerogenes* (Debela, 1993; Belachew, 1993). Alkaloid extract of the roots of *E. kebericho* has been shown to have a very strong lethal activity against earthworm (Hymete and Kidane, 1991; Hymete and Afiffi, 1996; Erko, 2006). An enlarged rootstock of *E. kebericho* increases soil fertility, and also reduces soil erosion, especially when growing on mountainous slopes.

2.4. *E. kebericho* propagation and its challenges

E. kebericho is propagated by seed and seeds are covered by overlapped fruit wall. The pubescent hairs are found between these walls and also on the surface of the cotyledon. This is generally the case that such overlapped walls facilitate the incorporation of pathogens, which causes seed contamination during *in vitro* germination. Seeds have very low *in vitro* germination percentages after 5 months of storage and its germination declines with increased storage time (Manahlie and Feyissa 2014). Seed viability in *E. 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 about 70% after 10 months (Balcha *et al.,* 2009). Moreover, seeds of *E. kebericho* could not uniformly germinate after 3 months storage time. Hence, seeds of this species are characterized by short viability period when stored at room temperature, thus lososes its their capacity to germinate very quickly. *E. kebericho* propagates by seed which is

incompetent due to lack of adequate seeds and seed 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 (Manahlie and Feyissa, 2014).

2.5. Plant tissue culture and its application

Tissue culture is the *in vitro* aseptic culture of cells, tissues, organs or whole plant under controlled nutritional and environmental conditions often to produce the clones of plants (Thorpe, 2007).The technique depends mainly on the concept of totipotency of plant cells (Haberlandt, 1902) which refers to the ability of a single cell to express the full genome by cell division. Along with the totipotent potential of plant cell, the capacity of cells to alter their metabolism, growth and development is also equally important and crucial to regenerate the entire plant (Thorpe, 2007**).** Tissue culture techniques are being increasingly exploited for clonal multiplication and *in vitro* conservation of valuable indigenous germplasm threatened with extinction. Greater demand for plants especially for the purpose of food and medicine is one of the causes of their rapid depletion from primary habitats. Micropropagation offers a great potential for large scale multiplication of such useful species for subsequent exploitation (Boro *et al*., 1998**).**

In vitro cell and organ culture offers an alternative source for the conservation of endangered genotypes (Sengar *et al;* 2010). Germplasm conservation worldwide is increasingly becoming an essential activity due to the high rate of disappearance of plant species and the increased need for safeguarding the floristic patrimony of the countries (Filho *et al;* 2005). Tissue culture protocols

can be used for preservation of vegetative tissues when the targets for conservation 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 (Tyagi *et al;* 2007). The plant species which do not produce seeds (sterile plants) or which have 'recalcitrant' seeds that cannot be stored for long period of time can successfully be preserved via *in vitro* techniques for the maintenance of gene banks **(** Hussain *et al*.,2012**).**

Micropropagation is the process of vegetative growth and multiplication from plants tissues or seeds. It is carried out in aseptic and favourable conditions on growth media, using various plant tissue culture techniques (Zhou and Wu, 2006; Leifert et al; 1989; Bhojwani and Razdan, 1996).

2.6. Source of Explants and Aseptic Techniques

Tissue culture success mainly depends on the age, types and position of explants (Gamborg *et al*; 1976) because not all plant cells have the same ability to express totipotency (Sasikumar et al; 2009; Murashige and Skoog, 1962; Staba and Seabrook, 1980). The most commonly used explants are shoot tips, nodal buds and root tips. Large explants can increase chances of contamination and small explants like merisems can sometimes show less growth (Fowler, 1993; Staba and Seabrook, 1980)**.**

Most surface contaminants such as bacteria and fungi can be eliminated by surface sterilizing the plant material with a suitable sterilizing agent. Surface sterilizing agents are normally applied for 10-15 minutes. Under aseptic conditions, the sterilizing solutions are then removed and the plant material washed 3 or 4 times for 5 minutes each time by agitation in sterile distilled water.

Washing is important to remove excess sterilizing agent which inhibits plant growth (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 a household's bleach such as Clorox. These commercial products usually contain 5.25% NaOCl as the active agent, when diluted with water (1 part bleach: 9 parts 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 (Rayns and Hunter, 1993). A considerable decrease in bacterial contamination was seen by using ultrasonic sonicator (Hussey, 1982).

2.7. Composition of Culture Medium

Tissues from different parts of a plant may have different requirements for satisfactory growth. The composition of culture medium is a major determinant of *in vitro* growth of plants. Plant tissue culture medium contains all the nutrients required for the normal growth and development of plants. It is mainly composed of macronutrients, micronutrients, vitamins, other organic components, plant growth regulators, carbon source and some gelling agents in case of solid medium (Murashige and Skoog, 1962).

According to the recommendations of the International Association for Plant Physiologists, the elements required by plants in concentrations greater than 0.5 mmol 1^{-1} are referred to as

macroelements, relatively large amounts of some inorganic elements (the so-called major plant nutrients): ions of nitrogen (N), potassium (K), calcium (Ca), phosphorus (P), magnesium (Mg) and sulphur (S); and those in concentrations less than 0.5 mmol 1^{-1} are microelements, small quantities of other elements (minor plant nutrients or trace elements): iron (Fe), nickel (Ni), chlorine (Cl), manganese (Mn), zinc (Zn), boron (B), copper (Cu), and molybdenum (Mo), (De Fo ssard, 1976).

Carbohydrates play an important role in *in vitro* cultures as an energy and carbon source, as well as an osmotic agent. In addition, carbohydrate-modulated gene expression in plants is 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. The most commonly used source of carbon is sucrose at a concentration of 2-5%. However, glucose and fructose are also known to support good growth of some tissues (Bhojwani and Razdan, 2004). Variation in shoot response was observed in different sugars and lower concentration of dextrose is found to enhance the root and shoot growth in comparison to sucrose and maltose. Sucrose has been replaced by dextrose in rice grain culture and found to be more efficient and can be used for further tissue culture experiments (Ruby et al, 2007).

Four vitamins; *myo*-inositol, thiamine, nicotinic acid, and pyridoxine are 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. Welander (1977) found that Nitsch and Nitsch (1965) vitamins were not necessary, or were even inhibitory to direct shoot formation on petiole explants of *Begonia* x *hiemalis*. Roest and Bokelmann (1975) on the other hand, obtained increased shoot formation on *Chrysanthemum* pedicels when MS vitamins were present.

2.8. Plant Growth Regulators

The highest rate of micropropagation often depends not only on the selection of the most suitable explant, but also on the discovery of the correct combination of growth regulators, and/or the best nutritional composition of the medium (Mathews, 1987) for particular explants. The growth regulators are required in very minute quantities. There are many synthetic substances having growth regulatory activity, with differences in activity and species specificity. It often requires testing of various types, concentrations and mixtures of growth substances during the development of a tissue culture protocol for a new plant species (Bhojwani, 1996).

Auxins (IAA, IBA, NAA or 2, 4-D) are involved in the regulation of several physiological processes, for example, apical dominance and formation of lateral and adventitious roots. These growth regulators generally cause cell elongation and swelling of tissues, cell division, callus formation and the formation of adventitious roots as well as the inhibition of adventitious and axillary shoot formation (Pierik, 1997). Also auxins are often added to the culture medium to promote the growth of callus, cell suspensions or organs, and to regulate morphogenesis,

especially in combination with cytokinin (George, 1993). IBA and IAA are widely used for rooting and, in interaction with a cytokinin, for shoot proliferation. 2, 4-D and 2, 4, 5-T are very effective for the induction and growth of callus. 2, 4-D is also an important factor for the induction of somatic embryogenesis and usually used after dissolved in ethanol or dilute NaOH (Bhojwani, 1996).

Cytokinins are derived from adenine (aminopurine) and play an important role in the *in vitro* manipulation of plant cells and tissues (Torres *et al*., 2001). The most common cytokinins used are kinetin, 6-BenzylAminoPurine (BAP), thidiazuron (TDZ) and zeatin and 2iP (Pierik, 1997). These hormones are concerned with cell division, modification of apical dominance, shoot differentiation, etc. In tissue culture media, cytokinins are 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, 1996).

Gibberellins are a group of compounds that are not necessarily used in the *in vitro* culture of higher plants. In some species, these growth regulators are required to enhance and in others to inhibit growth (Razdan, 1993). Gibberellic acid (GA3) is the most common gibberellin used. It induces the elongation of internodes and the growth of meristems or buds *in vitro* (Pierik, 1997).
All kinds of plant tissue cultures produce ethylene, and the rate of production increases under stress conditions. In cultures, ethylene is also produced when the organic constituents of the medium are subjected to heat, oxidation, sunlight or ionizing radiation (Matthys *et al.,* 1995).

Abscisic acid is most often required for normal growth and development of somatic embryos and only in its presence do they closely resemble zygotic embryos (Ammirato, 1988). It is also known to promote morphogenesis in Begonia cultures. There has been some interest in the application of growth retardants, such as paclobutrazol, during the acclimatization stage of micropropagation to reduce hyperhydricity and regulate leaf growth and function in relation to control of water stress (Smith and Krikorian, 1990).

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3. OBJECTIVE

3.1. General objective

To determine the optimum combination of plant growth regulator concentrations for *in vitro* propagation of an endemic medicinal plant *E. kebericho* using shoot tip explants

3.2. Specific objectives

- To evaluate the combinations of different concentration of plant growth regulators (KIN, BAP, IBA) on shoot multiplication of *E. kebericho*;
- To evaluate the combinations of different concentration IAA and MS medium strength on root induction;
- To asses survival rate of acclimatized plants in greenhouse.

4. MATERIALS AND METHODS

4.1. Plant material

Mature *E. kebericho* seeds were obtained from The Ethiopian Public Health Institute, Medicinal Plant Garden, Addis Ababa, Ethiopia.

4.2. Stock Solutions and medium preparations

4.2.1. MS medium stock solution preparation

Murashige and Skoog (1962) (MS) medium was used throughout the experiments. Full strength stock solution of macronutrients, micronutrients, Fe-Na-EDTA and FeSO4 mixture and vitamins were prepared separately. To do so, appropriate amount of each nutrient was weighed in grams per litter (appendix 1) and dissolved in double distilled water consecutively 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 magnetic stirrer, finally the solution was dispensed in to plastic bottles and stored at -20°C until used.

4.2.2. Plant growth regulators stock solution preparation

Plant growth regulators, 6-benzyl amino-purine (BAP), kinetin (KIN) and IBA for shoot induction and indole acetic acid (IAA) for rooting were used in this study. All of the plant growth regulators stock solutions were prepared by weighing and dissolving the powder in double distilled water at the ratio of 1:1 (1mg/ml) and dissolved by 2-3 drops of 1N NaOH and/or 1N HCl based on the requirement of the plant growth regulators. Then, the volume was

adjusted by adding double distilled water and stirred using magnetic stirrer. Finally, growth regulator stock solutions were stored in a refrigerator at +4°C for short term use.

4.2.3. Culture medium preparation

Culture medium was prepared by taking 100 ml/l macronutrient, 10 ml/l micronutrient and 10 ml/l vitamin stock solution of MS medium. Then, 30 g/l of sucrose was dissolved and different concentrations of BAP, KIN and IBA for shoot induction and IAA for rooting were added. The pH of the culture medium was adjusted to 5.8 using 1N NaOH and/or 1N HCl. Then 7 g/l agar was added. Then gently mixed medium was heated in microwave oven until the agar was melted followed by dispensing 50 ml of the prepared medium in to Magenta GA-7 culture vessels. The culture vessels were covered with caps immediately after dispensing the medium and autoclaved at a temperature of 121°C and pressure of 105 KPa for 15 minutes.

4.3. *In vitro* seed germination

Seeds of *E. kebericho* were released from the thin layers of fruit and washed with detergent under running tap water to remove the pubescent hairs. Seeds were surface sterilized in 70% ethanol for 9 min and rinsed 3 to 4 times by sterile distilled water, to lower the toxic effect of ethanol, followed by 25% Clorox solution for 5 min and rinsed 3 to 4 times using sterile distilled water. The sterilized seeds were aseptically planted on 50 mL growth regulators-free MS (Murashige and Skoog, 1962) medium in culture vessel (10 x 6 cm, height, width, respectively) containing 30 g/L sucrose (w/v) and solidified by 7 g/L agar (w/v). Before addition of agar, pH of the medium was adjusted to 5.8 with 0.1 N HCl or 0.1 N NaOH and autoclaved at 121 ºC for

15 min under 105 Kpa pressure. Cultures were incubated under light intensity of 22 µmol m^{-2} s^{-1} and 16 h photoperiod provided by white fluorescent lamps at 25±2ºC.

4.4. Culture Initiation

Shoot tips excised from two-week-old germinated seedlings were used for culture initiation on MS medium containing 1.0 mg/L kinetin, 30 g/l sucrose (w/v) and 8 g/l agar (w/v). Before addition of agar, the pH of the medium was adjusted to 5.8. Cultures were incubated under light intensity of 22 µmol m^{-2} s^{-1} and 16 h photoperiod provided by white fluorescent lamps at 25±2ºC.

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4.5. Shoot Multiplication

Shoot tips from *in vitro* cultured seedlings were transferred onto MS agar medium supplemented with different concentrations of BAP (0.0, 0.5, 1.0, 1.5, 2.0, 2.5 mg/l) in combination with IBA (0.0, 0.05, 0.1, 0.5 mg/l), KIN alone (0.0, 0.5, 1.0, 1.5, 2.0, 2.5 mg/l) and BAP alone(0.0, 0.5, 1.0, 1.5, 2.0, 2.5 mg/l), KIN (0.0, 0.5, 1.0, 1.5, 2.0, 2.5 mg/l) in combination with BAP (0.0, 0.05, 0.1, 0.5 mg/l) and KIN (0.0, 0.5, 1.0, 1.5, 2.0, 2.5 mg/l) combination with IBA (0.0, 0.05, 0.1, 0.5 mg/l) for shoot multiplication. A total of 59 treatments were employed and for each treatment three culture vessels, each with ten shoot tips, were used. Cultures were incubated under light intensity of 22 µmol m^{-2} s^{-1} and 16 h photoperiod provided by white fluorescent lamps at 25±2ºC for 30 days and the number of shoots per explant and shoot length were recorded after four weeks.

4.6. Rooting and acclimatization

Shoots multiplied on multiplication medium were excised and transferred on to full, half and one third strength MS media, either without plant growth regulators or supplemented with different concentrations of IAA (0.0, 0.05, 0.1, 0.5, 1.0, 1.5, 2.0, 2.5 mg/L) for rooting. A total of 8 treatments were employed and for each treatment three culture vessels, each with ten shoot tips, were used. Cultures were incubated under light intensity of 22 μmol m^{-2} s^{-1} and 16 h photoperiod provided by white fluorescent lamps at 25±2ºC for 30 days and number of roots per shoot and root length were recorded after four weeks.

The *in vitro*-derived rooted shoots were transplanted into 10 cm in diameter pots containing sterilized mixture of soil, sand and peat in the ratio of 2:1:1 respectively and placed in the greenhouse at 26 ± 2°C, natural light). To ensure high humidity, the plants were covered with plastic bags, which were removed after 14 days of acclimatization. Forty days after transplanting the plantlets to the pots, the survival rate was assessed.

4.7. Experimental design

A completely randomized design (CRD) was used for all experiments. The study was conducted at Plant Tissue Culture and Molecular Biology Laboratory, Addis Ababa University. Data were subjected to analysis of variance (ANOVA) and least significant difference (LSD) test using statistical data analysis software SPSS 20.0 version at 0.05 probability level.

5. RESULTS

5.1. Shoot multiplication

Effect of BAP and IBA on shoot multiplication of *E. kebericho*

The response of explants cultured on MS medium supplemented with different concentrations of BAP in combination with IBA is presented in Table 1. After two weeks of culture on MS medium containing different concentrations of BAP in combination with IBA, the shoots were emerged from the explants (Fig. 1). The MS medium containing various concentrations of BAP in combination with low level of IBA produced shoots that are not significantly different at P = 0.05 after 4 weeks (Table 1). Maximum number of regenerated shoots (2.33±0.33) was observed in MS medium supplemented with 2.0 mg/l BAP +0.5 mg/l IBA. Maximum shoot length (3.33±0.17 cm) was observed in MS medium supplemented with 1.5 mg/l BAP+0.05 mg/l IBA

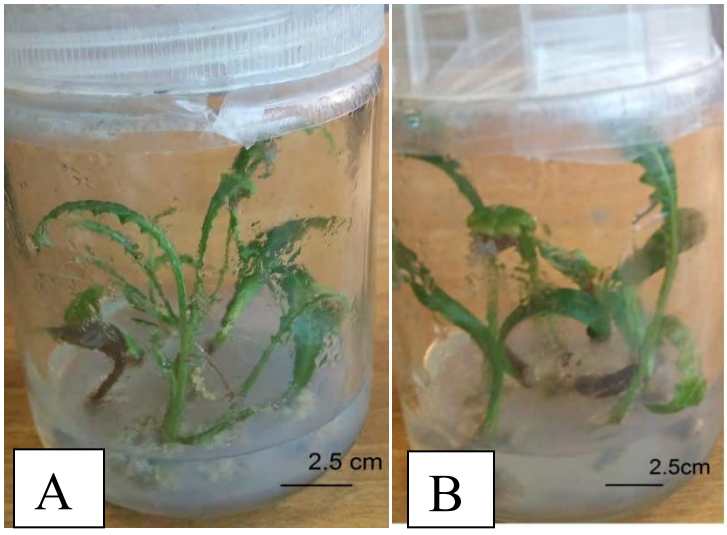

Figure1: Shoot multiplication from shoot tip explants of *E. kebericho* on MS medium containing BAP in combination with IBA. A) 2.0 mg/l BAP +0.5 mg/l IBA, B) 1.5 mg/l BAP+0.05 mg/l IBA

Table 1: Effect of different concentrations of BAP in combination with IBA on shoot multiplication of *E. kebericho*

PGRs (mg/l)		No. of shoots/ explant Mean±SE	Shoot length(cm) Mean±SE
BAP	IBA		
0	0	3.33±0.88^{a}	1.88±0.28^{cd}
0.5	0.05	1.83±.44^{bcd}	1.30±0.15^{c}
0.5	0.1	1.00±0.00^{d}	2.66±0.20^{abc}
0.5	0.5	1.29±0.15^{abc}	2.46±0.50^{abc}
1.0	0.05	1.67±0.33^{abc}	2.86±0.40^{ab}
1.0	0.1	1.00±0.00^{d}	2.99±0.32^{ab}
1.0	0.5	1.00±0.00^{d}	2.36±0.06^{abc}
1.5	0.05	1.33±0.33^{abc}	3.33±0.17^{a}
1.5	0.1	1.00±0.00^{d}	2.35±0.02^{abc}
1.5	0.5	1.00±0.00^{d}	1.96±0.33^{abc}
2.0	0.05	1.40±0.40^{cd}	1.80±0.15^{bc}
2.0	0.1	1.00±0.00^{d}	2.10±0.10^{abc}
2.0	0.5	2.33±0.33^{ab}	2.90±0.20^{ab}
2.5	0.05	1.00±0.00^{d}	2.10±0.10^{abc}
2.5	0.1	1.27±0.15^{abc}	2.16±0.52^{abc}
2.5	0.5	1.73±0.37^{bcd}	2.03±0.20^{abc}

Means with the same letter within the same column are not statistically different ($P < 0.05$). The values represent mean ± S.E. Thirty shoots per treatment were used.

Effect of KIN alone and BAP alone on shoot multiplication of *E. kebericho*

A significant difference was observed from culture on various concentrations of KIN and BAP at P = 0.05 after 4 weeks (Table 2). Maximum mean number of shoots per explant (11.32±1.15) was attained on medium containing 1.0 mg/l KIN. The next highest mean number (5.10±0.60) of shoot was obtained in the presence of 2.0 mg/l KIN. Shoots on all culture media had healthy appearance and grew vigorously (Fig. 2). On MS medium containing BAP alone, the poorest

shoot proliferation rate with poor shoot quality was observed. Mean number of shoots per explant in this treatment was lower than plant growth regulators-free MS medium. Maximum mean shoot length (4.30±0.00 cm) was achieved on MS medium supplemented with 1.0 mg/l KIN.

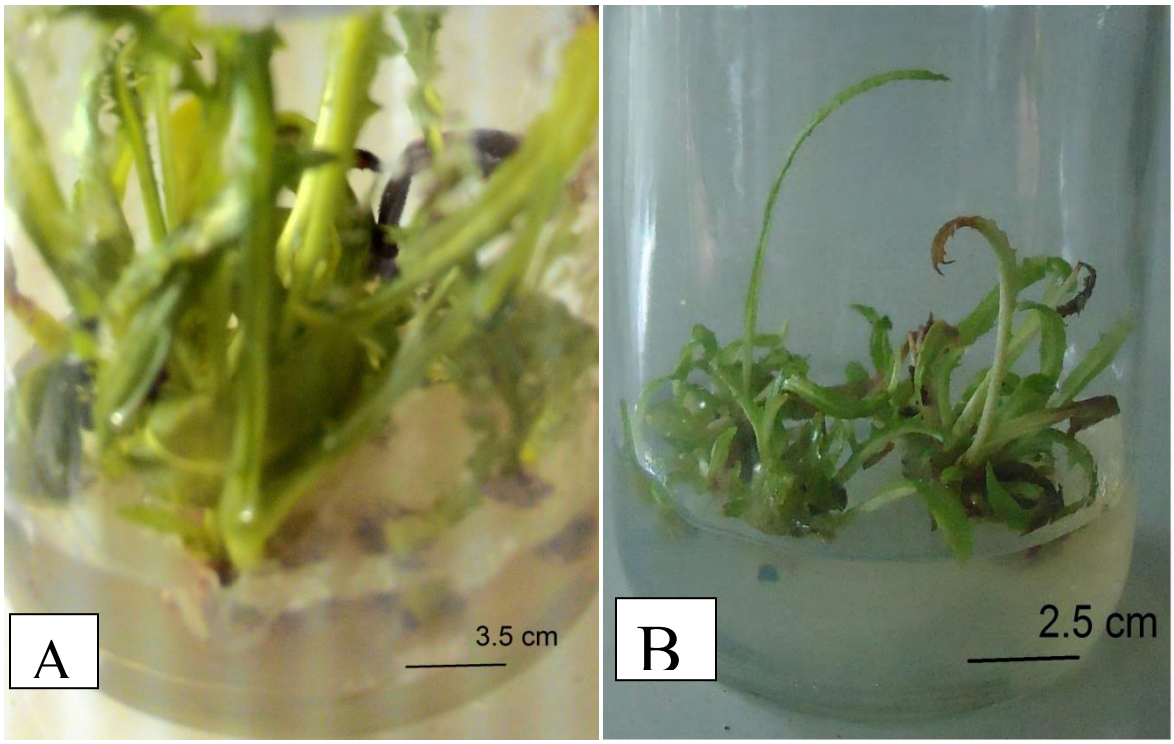

Figure 2: Shoot multiplication from shoot tip explants of *E. kebericho* on MS medium containing KIN and BAP alone. A) 1.0 mg/l KIN, B) 0.5 mg/l BAP

Table 2: Effect of different concentrations of KIN alone and BAP alone on shoot multiplication of *E. kebericho*

PGRs (mg/l)	No. of shoots/ explant Mean±SE	Shoot length(cm) Mean±SE
KIN		
0	3.33±0.88^{bc}	1.88±0.28^{d}
0.5	3.17±0.63^{bcd}	3.50±0.28^{ab}
1	11.32±1.15^{a}	4.30±0.00^{a}
1.5	3.66±1.20^{bc}	3.33±0.33^{abc}
2.0	5.10±0.60^{b}	2.50±0.00^{bcd}
2.5	1.72±0.32^{cd}	4.10±0.33^{a}
BAP		
0.5	2.70±0.15^{bcd}	1.64±0.22^{d}
1.0	1.40±0.13^{cd}	2.28±0.14^{cd}
1.5	1.00±0.00^{d}	1.54±0.07^{d}
2.0	1.39±0.12^{cd}	2.66±0.26^{bcd}
2.5	1.00±1.00^{d}	1.96±0.14^{d}

Means with the same letter within the same column are not statistically different ($P < 0.05$). The values represent mean ± S.E. Thirty shoots per treatment were used.

Effect of KIN in combination with BAP on shoot multiplication of *E. kebericho*

Among the various combinations of BAP and KIN used, the MS medium containing 0.5 mg/l KIN + 0.05 mg/l BAP produced maximum mean number of shoots per explant (5.22±1.48). There was no significance difference among plant growth regulators-free MS medium, medium containing 2.0 mg/l KIN + 0.1 BAP, 1.0 mg/l KIN + 0.01 BAP and 1.0 mg/l KIN + 0.1 BAP in number of shoots per explants at p = 0.05 (Table 3). Maximum shoot length (5.33±0.16 cm) was observed when KIN (0.5 mg/l) was used along with 0.05 mg/l BAP. Cultures under this combination appeared healthy but poor in proliferation rate as compared to BAP and KIN alone.

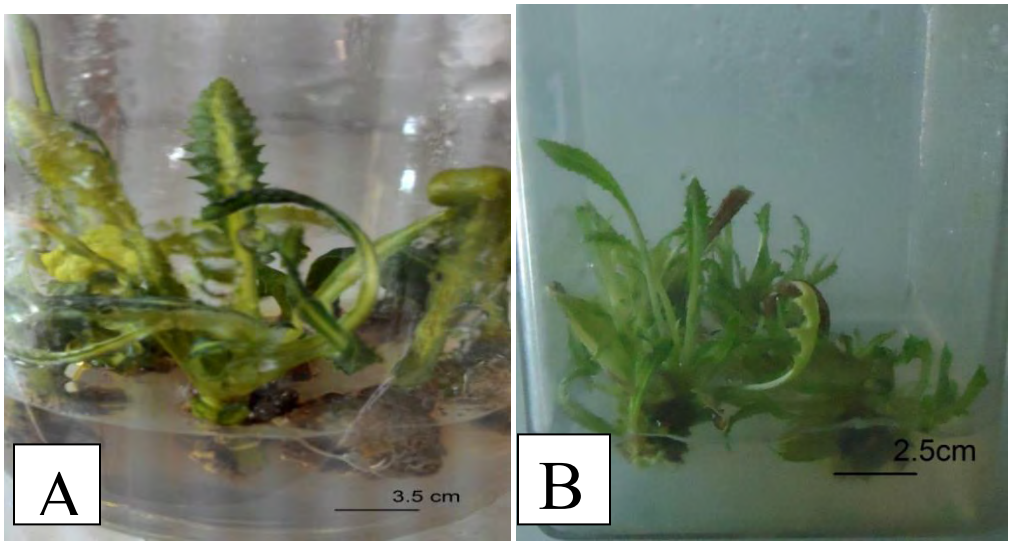

Figure 3: Shoot multiplication from shoot tip explants of *E. kebericho* on MS medium containing KIN in combination with BAP. A) 0.5 mg/l KIN + 0.05 mg/l BAP, B) 2.0 mg/l KIN + 0.1 BAP

Table 3: Effect of different concentrations of KIN in combination with BAP on shoot multiplication of *E. kebericho*

PGRs (mg/l)		No. of shoots/ explant Mean±SE	Shoot length (cm) Mean±SE
KIN	BAP		
0	0	3.33±0.88^{ab}	1.88±0.28^{cd}
0.5	0.05	5.22±1.48^{6a}	5.33±0.167^{a}
0.5	0.1	1.60±0.37^{c}	1.60±0.20^{cd}
0.5	0.5	1.20±0.11^{c}	2.86±0.36^{bcd}
1.0	0.05	3.20±0.46^{ab}	3.40±0.05^{bc}
1.0	0.1	3.13±1.08^{ab}	3.37±0.709^{bc}
1.0	0.5	1.69±0.17^{c}	2.46±0.66^{cd}
1.5	0.05	1.76±0.39^{c}	1.80±0.30^{cd}
1.5	0.1	1.65±0.23^{c}	1.78±0.28^{cd}
1.5	0.5	1.74±0.37^{c}	1.35±0.07^{d}
2.0	0.05	2.04±0.39^{ab}	1.93±0.23^{cd}
2.0	0.1	3.84±1.29^{ab}	4.66±0.44^{ab}
2.0	0.5	1.41±0.30^{c}	2.23±0.12^{cd}
2.5	0.05	3.26±0.16^{ab}	2.93±0.46^{bcd}
2.5	0.1	2.15±0.104^{ab}	2.76±0.37^{bcd}
2.5	0.5	2.05±0.35^{ab}	2.00±0.25^{cd}

Means with the same letter within the same column are not statistically different ($P < 0.05$). Thirty shoots per treatment were used.

Effect of KIN in combination with IBA on shoot multiplication of *E. kebericho*

A significant difference was observed from culture on various concentrations of KIN in combination with IBA at P = 0.05 after 4 weeks (Table 4). High-quality, attractive culture and maximum mean number of shoots per explant (14.44±0.29) was attained on medium containing 0.5 mg/l KIN in combination with 0.5 mg/l IBA. The next highest mean number of shoots per explant (10.35±0.60) was obtained in the presence of 1.0 mg/l KIN in combination with 0.1 mg/l IBA. Shoots on all culture media had healthy appearance and grew vigorously (Figure 4). Maximum shoot length (6.66±0.16 cm) was observed when 2.0 mg/l KIN was used along with 0.1 mg/l IBA.

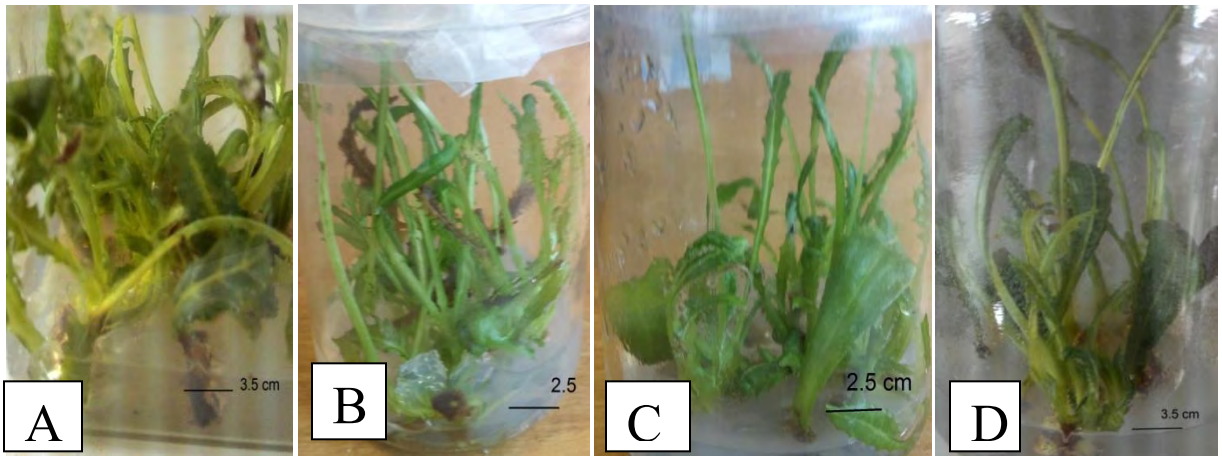

Figure 4: Shoot multiplication from shoot tip explants of *E. kebericho* on MS medium containing KIN in combination with IBA.(A) 0.5 mg/l KIN + 0.5 mg/l IBA; (B) 1.0 mg/l KIN + 0.1 IBA ; (C) 1.0 mg/l KIN + 0.5 IBA; (D) 2.0 mg/l KIN + 0.1 mg/l IBA

Table 4: Effect of different concentrations of KIN in combination with IBA on shoot multiplication of *E. kebericho*

PGRs (mg/l)		No. of shoots/ explants Mean±SE	Shoot length(cm) Mean±SE
KIN	IBA		
0	0	3.33±0.88^{def}	1.88±0.28^{cd}
0.5	0.05	2.90±.29^{ef}	2.83±0.44^{def}
0.5	0.1	6.77±1.85^{bcd}	2.660±.16^{def}
0.5	0.5	14.44±0.29^{a}	4.13±0.13^{bcd}
1.0	0.05	4.10±0.06^{ef}	3.33±0.16^{bcde}
1.0	0.1	10.35±0.60^{b}	3.66±0.16^{bcde}
1.0	0.5	8.31±0.64^{bc}	6.50±0.28^{a}
1.5	0.05	3.13±0.06^{ef}	4.16±0.16^{bcd}
1.5	0.1	1.54±0.28^{f}	1.50±0.50^{f}
1.5	0.5	3.20±0.63^{def}	3.00±0.28^{cdef}
2.0	0.05	2.66±0.33^{ef}	3.43±0.52^{bcde}
2.0	0.1	8.28±1.38^{bc}	6.66±0.16^{a}
2.0	0.5	2.14±0.45^{f}	2.40±0.05^{ef}
2.5	0.05	1.32±0.09^{f}	3.35±0.57^{bcde}
2.5	0.1	6.24±0.17^{cde}	4.73±0.25^{b}
2.5	0.5	4.80±0.25^{cdef}	4.62±0.32^{bc}

Means with the same letter within the same column are not statistically different at $P < 0.05$. Thirty shoots per treatment were used

5.2. Rooting and acclimatization

In vitro-derived shoots were transferred to full-strength, half-strength and 1/3 strength MS medium containing different concentrations of IAA (0.0, 0.05, 0.1, 0.5, 1, 1.5, 2, 2.5 mg/l) for root induction. Growth regulators free MS medium was used as control.

Full-strength, half-strength and 1/3 strength MS medium and different concentrations of IAA significantly affected the root length and root number at $P < 0.05$ (Fig.5). A maximum mean number of roots per shoot (6.81±0.39) was obtained on 1/3 strength MS rooting medium containing 0.1 mg/l IAA (Table 5). The highest mean length of root per explant (4.68±0.22cm) was obtained on 1/3 strength MS rooting medium containing 0.5 mg/l IAA.

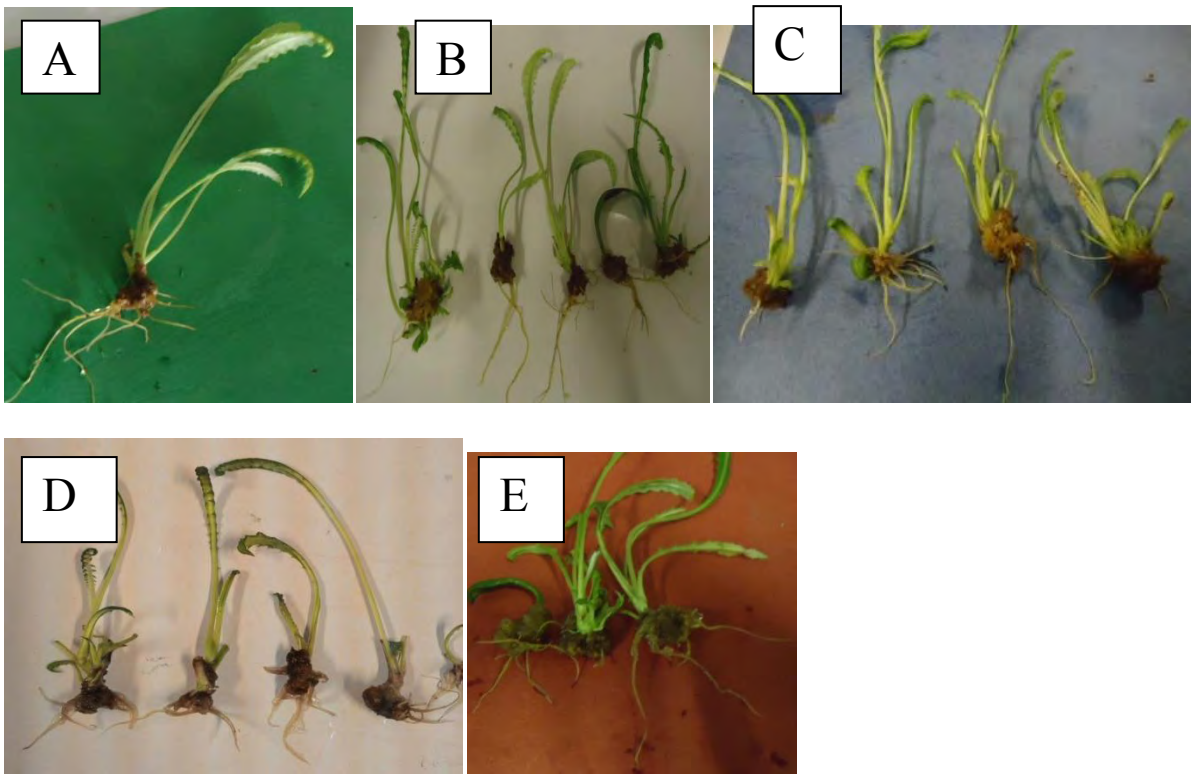

Figure 5: *In vitro* rooting of *E. kebericho* shoots on MS medium (full, Half and 1/3 strength) containing different concentrations of IAA. (A&B) 1/3 strength MS medium containing 0.1 mg/l IAA, (C&D) Half strength MS medium containing 1.0 mg/l IAA, (E) Full strength MS medium containing 0.5 mg/l IAA.

Table 5. Effect of full, half and 1/3 strength MS media containing different concentrations of IAA on *in vitro* rooting of *E. kebericho*

PGR	MS Salt Strength					
	Full MS		Half MS		1/3 MS	
	No. of roots/ explant Mean±SE	Root length (cm) Mean±SE	No. of roots/ explant Mean±SE	Root length(cm) Mean±SE	No. of roots/ explant Mean±SE	Root length(cm) Mean±SE
IAA						
0	2.43±0.28^{bc}	2.23±0.17^{bc}	2.43±0.28^{cd}	2.23±0.17^{a}	2.43±0.28^{e}	2.23±0.17^{c}
0.05	0.70±0.35^{c}	1.26±0.63^{c}	4.03±0.14^{bc}	3.28±0.06^{a}	2.43±0.08^{e}	4.14±0.95^{abc}
0.1	1.23±0.12^{c}	2.21±0.16^{bc}	6.47±0.28^{a}	3.16±0.32^{a}	6.81±0.39^{a}	3.00±0.25^{abc}
0.5	5.60±.73^{a}	3.65±0.17^{a}	1.28±0.15^{d}	2.48±0.4867^{a}	6.13±0.27^{ab}	4.68±0.22^{a}
1.0	4.06±0.58^{ab}	3.33±0.06^{ab}	4.80±0.85^{ba}	3.46±0.12^{a}	4.66±0.88^{bcd}	3.75±0.29^{abc}
1.5	1.93±0.47^{bc}	2.09±0.12^{bc}	2.27±0.14^{d}	3.61±0.31^{a}	5.66±0.17^{abc}	3.72±0.42^{abc}
2.0	2.98±0.21^{bc}	2.46±0.07^{abc}	2.48±0.04^{cd}	2.67±0.42^{a}	2.97±0.33^{de}	4.31±0.25^{ab}
2.5	4.20±0.9^{ab}	2.86±0.28^{ab}	4.70±0.15^{b}	2.20±0.07^{a}	3.73±0.29^{cde}	2.44±0.28^{bc}

Means with the same letter within the same column are not statistically different at $P < 0.05$. Thirty shoots per treatment were used.

Plantlets with developed roots were carefully and systematically removed from culture vessels and washed by running tap water, then immediately planted in potted soils.

Among a total of 100 plants transferred to glasshouse, 85.5% survived (Fig. 6).

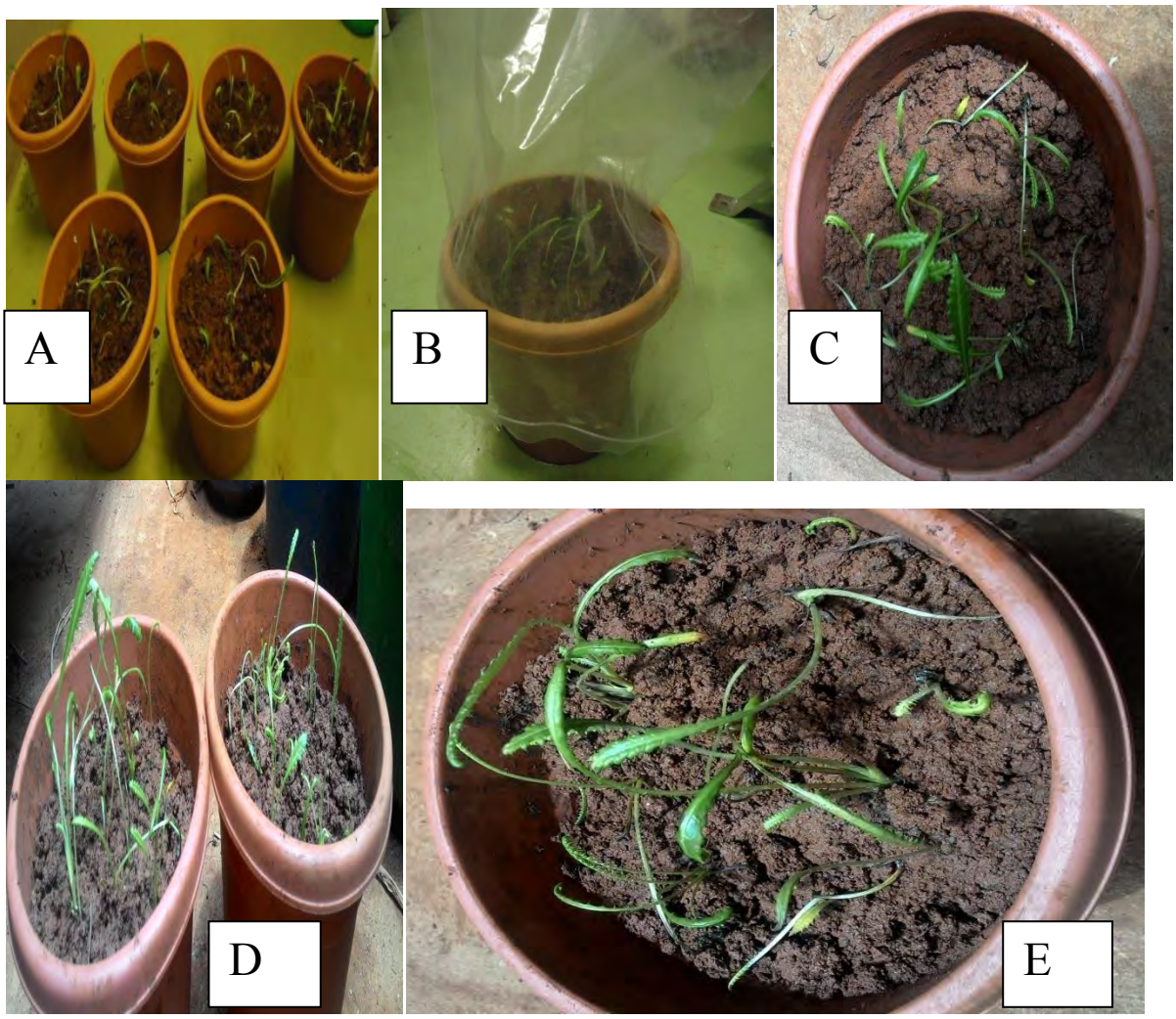

Figure 6: Acclimatization of *in vitro* rooted shoots of *E. kebericho* in glasshouse. (A) Plants transferred from the medium to the pots; (B) Plants covered in polyethylene bag after two weeks. (C) After three weeks (D and E) after 40 days

6. DISCUSSION

6.1. Shoot Multiplication

In this study, 59 different concentrations of KIN in combination with BAP, KIN and BAP alone and each of them in combination with IBA were used for shoot multiplication. KIN alone or in combination with IBA was found to be the most suitable plant growth regulators for mass micropropagation of *E. kebericho*. Maximum shoot proliferation (14.44±0.29) was observed on MS medium augmented with 0.5 mg/l KIN + 0.5 mg/l IBA followed by 11.32±1.15 shoots per explant on MS medium supplemented with 1.0 mg/l KIN. In the earlier study on *E. kebericho* (Manahlie and Feyissa, 2014) reported that highest number of shoots (11.07 ± 0.47) and (8.37 ± 0.50) was recorded on MS medium containing 0.5 mg/l KIN and 0.1NAA mg/l, and1.0 mg/l KIN respectively. Similarly, Gopal *et al.* (2013) reported 7.33 mean number of shoots per explant of *Artemisia amygdalina* on medium containing 1.0 mg/l KIN and 0.5 mg/l NAA. Therefore, the combination effect of KIN with IBA is better than combination effect of KIN with NAA for shoot proliferation *of E. kebericho.* In the other case, maximum shoot proliferation (3.17±0.63) was obtained on a medium containing 0.5 mg/l KIN. However, Manahlie and Feyissa (2014) reported that the highest number of shoots per explant (10.73 ± 0.56) was recorded on MS medium containing 0.5 mg/l KIN and 0.1 mg/l NAA. This difference might be due to the concentration of PGR, age of explants, source of explants, genotype and position of explants that may affects shoot multiplication.

BAP alone or in combination with IBA gave significantly ($P<0.05$) less number of shoots compared to the other , where a maximum of 2.33±0.33 shoots per explant was obtained on MS

medium augmented with 2.0 mg/l BAP and 0.5 mg/l IBA. A similar result was reported on *E. kebercho* (Manahlie and Feyissa, 2014) where maximum shoot number (5.73 ± 0.47) was obtained on a medium containing 2.5 mg/l BAP and 0.5 mg/l NAA. However, BAP was superior over KIN in propagation of *Artemisia amygdalina* where 19.67 ± 7.0 shoots were obtained from shoot tip explants on MS medium containing 0.2 mg/l BAP + 0.4 mg/l NAA (Rasool *et al*., 2013). BAP has been used in preference to other cytokinins to induce multiple shoots in *Artemisia pallens* (Sharief and Chandra, 1991), in *Artemisia vulgaris* (Sujatha and Kumari, 2007). It is stated that BAP is the most effective cytokinin for meristem, shoot tip and bud cultures (Nair et al., 1979). Dhaka and Kothari (2005) also reported that medium containing 0.5 mg/l BAP was sufficient for the multiplication of *Eclipta alba.*

The combination of KIN with BAP was found to be better in shoot formation than combination of BAP with IBA, maximum shoot number (5.22±1.48) was obtained on a medium containing 0.5 mg/l KIN + 0.05 mg/l BAP. The lowest mean number of shoots/explant (1.74±.37, 1.20±0.115, and 1.4100±.30238) and mean length of shoots (2.23±0.12, 2.86±0.36 and 1.35±0.07 cm) were obtained on MS medium supplemented with different concentrations of KIN in combination with BAP (1.5 mg/ KIN l + 0.5mg/l BAP, 0.5 mg/l KIN + 0.55mg/l BAP and 2.0 mg/ KIN + 0.5 5mg/l BAP) respectively.

In this investigation, higher concentrations of cytokinin reduced the shoot number as well as shoot length. The height of shoot increased when the number of the shoot increased which is in agreement with the report on *Eclipta alba* (Baskaran and Jayabalan, 2005). Hu and Wang (1983) reported that higher concentrations of cytokinin reduced the number of micropropagated shoots.

The highest mean shoot length (6.50±0.28 cm) was obtained on medium containing 1.0 mg/L KIN + 0.5 IBA and the number of shoots in this treatment was highest.

6.2. Rooting and acclimatization

Auxins are mainly used in root induction and their effect varies with type and concentrations used in different plant species (Swamy and Singh, 2002). IAA was reported as potential auxin for rooting in several plants including *Arachis stenosperma* and *A. villosa* (Vijayalakshmi and Giri, 2003). Significant difference on supplementation of different strength MS medium and concentration of IAA on rooting was observed. A maximum mean number of roots (6.81±0.39 were obtained in a 1/3 strength MS rooting medium containing 0.1 mg/l IAA. This was not significantly different ($P < 0.05$) from 6.47±0.28 roots produced on half strength MS medium supplemented with 0.1 mg/l IAA, (Table 5), which is in agreement with Mhatre *et al*. (2000) who observed satisfactory root development of more than 80 % on half strength MS medium containing 0.5 mg /l IAA in all three varieties of *Vitis vinifera.* At equal concentration of IAA, the number of roots was higher in 1/3 strength medium than that of half and full strength MS medium. This result reveals that reduction in MS salt concentration increases the growth and formation of roots. High salt content is not necessarily always optimal for growth and development of plants *in vitro* (Pierik, 1997). For that reason, the use of dilute media formulations has generally promoted better formation of roots, since high concentration of salts may inhibit root growth (Patel and Shah, 2009) even in presence of auxins in the culture medium (Grattapaglia and Machado, 1998).

Further raising or lowering IAA concentrations other than 0.1 mg/l did not raise mean number of roots per shoot including PGR free MS medium. However, Sujatha and Kumari (2007) reported that the rooting was higher in the presence of 2.85–17.13 mg/l IAA and increasing the concentration of IAA above 8.56 mg/l gradually led to a decrease in the frequency of root regeneration as it was also reported in *Sesbania drummondii* (Cheepala et al. 2004). The average number of roots per shoot ranged from 16–25 and the maximum root length observed was 15.5 cm. In other case, full strength MS medium containing 0.05 mg/l and 0.1 mg/l IAA produced callus at the cut end and resulted in smaller number of roots (0.70±0.35 and 1.23±0.12) respectively. These might be due to callus formation usually hinders rooting (Spethmann, 2000; Spethmann and Hamzah, 1988) and it can be the consequence of unusual hormonal proportion due to higher exogenous auxin treatment. Maximum mean length of root (4.68±0.22cm) was obtained on a 1/3 strength MS rooting medium containing 0.5 mg/l IAA.

The survival rate after two to four weeks of acclimatization decreased to 97% and 85.5%, respectively. There is the highest survival rate (85.5 %) of the acclimatized plantlets and the plantlets have grown normally. This result showed better acclimatization percentage than previously reported on *E.kebericho* by Manahlie and Feyissa (2014) where 83% survived after being transferred to the greenhouse.

7. CONCLUSION

In conclusion, *E. kebericho* propagates by seed which is incompetent due to lack of adequate seeds, seed viability loss after short period and time consuming for large scale production and conservation of this species. This *in vitro* propagation technique is very promising in providing genetically uniform planting material, for conservation and building up populations of this critically endangered plant species. Kinetin combined with IBA is the most important cytokine for the multiplication of *E.kebericho*. The highest mean number of shoots produced was 14.44 shoots per explant, on MS medium supplemented with 0.5 mg/l KIN+ 0.5 mg/l IBA, and the highest shoot height (6.50±0.28 cm) was obtained on multiplication medium containing 1.0 mg/l KIN in combination with 0.5 mg/l IBA. Highest mean number of roots produced was 6.81 roots per explant on 1/3 strength MS rooting medium containing 0.1 mg/l IAA.

8. RECOMMENDATIONS

Future perspectives, based on the present study, should focus on the following area:

- Explants other than shoot tips culture such as leaf, steam, and root for *in vitro* propagation of *E. kebericho* should be considered to investigate if they can offer better and efficient responses.
- The effect of sub-culturing on the multiplication of shoots should be further studied, to determine whether the sub-culturing increases or decreases shoot multiplication to a significant extent.
- The effect of different concentrations of auxins and cytokinins on callus induction and regeneration of shoots and roots should be further studied.
- The effect of different concentrations of auxins and cytokinins on direct regeneration of shoots and roots should be further studied.
- Low cost tissue culture methods should be further studied.

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10. APPENDIX

APPENDIX 1 Stock solution for MS (Murashige and Skoog1962**)**

Components	Concentration (g/L)	mL/L during media preparation
Micronutrients		
ZnSO4.7H2O	1.72	
H3BO3	1.124	
*** MnSO2.4H2O**	3.38	
*** MnSO4.H2O**	0.05	5 ml/l
KI	0.166	
NaMoO4.2H2O	0.05	
CoCl2.6h2O	0.05	
Na2EDTA	7.472	
FeSO4.7H2O	5.56	
Macronutrients		
NH4NO3	33	
KNO3	38	
Cacl2.2H2O	8.8	50ml/l
MgSO4.7H2O	7.4	
KH2PO4	3.4	
Vitamins		
Myo-inositol	20	
Glycin (glycocoll)	0.4	
Nicotinic acid (NaOH)	0.1	5ml/l
Pyridoxin (B6)	0.1	
Thiamin (B1)	0.02	

***** Alternatives

 Na2EDTA and FeSO4.7H2O prepared alone