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GENERAL BIOLOGY PROGRAM

MICROPROPAGATION OF *ANDRACHNE ASPERA* SPRENG FROM SHOOT TIP AND NODAL SEGMENTS

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

BY

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LIST OF ABBRIVATON

ANOVA………………………………….Analysis of Variance

BAP……………………………………….6-Benzyle Amino Purine

EDTA…………………………………….Ethylenediaminetetraacetic acid

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

IBA……………………………………….Indole-3-Butyric Acid

MS………………………………………..Murahige and Skoogbasal medium

PGR………………………………………Plant Growth Regulator

Micropropagation of *Andrachne asper* Spreng from shoot tip and nodal segments

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Abstract

Andrachne aspera belongs to Euphorbiacea family and mostly grows in dray woody land or bush land and in dry sandy or stony cover bed at elevation from sea level up to (2000m-2400m). This plant is mostly used as antibiotic and to treat poison of snake and malaria. The seeds were washed under running tap water followed by washing in 70% ethanol for 2 to 3minutes. These seeds were surface sterilized using 20% Clorox for 10 minute and washed using sterile distilled water. The sterilized seeds were then cultured on growth regulator free Murashige and Skoog (MS) medium. After germination, shoot tips were excised and cultured on MS medium supplemented with different concentrations BAP or kinetin for culture initiation. The initiated shoot tips were transferred to solid MS medium supplemented with different concentrations of BAP or kinetin in combination with NAA. On shoot initiation medium, MS medium containing 1.5 mg/l BAP resulted in highest mean shoot number per explants (5.03±0.29). On shoot multiplication medium, the highest mean shoot number per explants (5.12±0.29 from shoot tip), 5.23±0.22 from node explants and highest mean shot length (4.30±0.20 cm) were obtained on medium containing 0.5mg/l BAP in combination with 0.25 mg/l NAA. The highest mean number of roots per shoot (6.00± 0.77) and mean root length (6.00±0.55 cm) were obtained on half strength MS medium containing 0.25 mg/l IAA. After acclimatization, 90% plants survived in green house. BAP (1.5mg/l) was best for shoot induction and BAP in combined with NAA(0.5mg/l BAP +0.25 NAA) produced best result for shoot multiplication. Rooting of microshoots was successful in 0.25 mg/l of IAA. Cultivation of the propagated plant to provided medicine and reduce the endanger status of the plant.

Key words: - Acclimatization, *Andrachne aspera*, growth regulators, *in vitro* propagation, shoot multiplication, rooting.

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 nations health care setting (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 also in food stores and supermarkets. It is estimated that up to 4 billion people of the world's population living in developing world rely on herbal medicinal products as primary source of health care and traditional practice which involves the use of herb viewed as an integral part of the culture in those communities (Mukherjee, 2002; Bodeker *et al*, 2005;Bandaranayke, 2006).

Currently there are many well established herbal and plant medicine practice which are popular in many parts of the world .The world health organization (WHO) reported that 80% of people in the developing world uses medical plants for their primary health care (Vines,2004).

In Ethiopia, the list of medical plants 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 on 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).

Majority of Ethiopians rely on traditional medicine as their primary form of health care, yet they are endangered of losing both their knowledge and the plants they have used as medicines for millennia (Avigdro *et al.*, 2014) .

Ethiopia has been described as one of the most unusual and important sources of biodiversity in the world (USAID, 2008), yet is perilously close to losing much of this rich diversity due to deforestation, land degradation, lack of documentation of species in some areas as well as of traditional culture and potential acculturation (Teketay and

Bekele, 1995; Kassaye *et al.,* 2006; Giday*et al.,* 2009; Lulekal *et al.,* 2008). Intertwined with the irretrievable loss of important species of plants and animals is risk of traditional herbal medicine knowledge. An estimated 80 to 90% of Ethiopians use herbal medicine as a primary form of health care (WHO, 2009).

In Ethiopia, the majority of the rural populations traditionally use many plants as sources of medicine for humans and livestock (Tesfaye *et al,* 2009). Generally, traditional healers use roots, barks and other parts of the plants to prepare phytotherapies (Alexiads, 1996) and in the process they have developed their own local knowledge.

Medically, *Andrachne aspra* is rare species following Tackhom (1974).The aerial and root plants are used in local medicine in Pakistan and UAE to improve eye sight and to treat eye sores (Satiyanati1975). The plant is also used as expectorant (Kamal, 2001). There are contradicting reports in its use as a substitution of Polyaiasenga (used as expectorant) (Qzilbash,, 1949; Shah and Kahann, 1959). It is used in Ethiopia to relieve pain and reduce fever (Debebe *et al*., 2006), snake bite and malaria (Hunde et al, 2006). A crude alkaloidal mixture has been reported to show various biological activities (Hoppe and Khalsa., 1999).

For many years the traditional medicines was made by wildly grown local plant. Phytochemicals extracted from different parts of plant is used for the treatment of various diseases, for supplementing nutrition in food and cosmetic industries has a great potential. Most valuable phytochemicals are products of secondary metabolism and possess sufficient chemical or structural complexity, so that artificial synthesis is difficult (Leung, 1980).

A.aspera is propagated by a seed which is incompetent due to lack of seed and seed viability loss after short period. Propagation through seeds is time consuming and useful to produce a large mass plantlets.

Though the conventional breeding techniques have considerably increased the productivity of modern crops, the application of biotechnology could speed up further crop improvement. It over comes the barriers in conventional vegetative propagation and fulfills the demand for large scale cultivation in a short period by rapid multiplication.

Plant tissue culture technique now plays an important role in the micropropagation and improvement of plants. Medical plants are the richest sources of drugs for traditional medicine, neutraceutical, food supplements, folk medicines; pharmaceutical intermediates, etc. (Hammer et al., 1999). For many years tissue culture techniques are being applied for germplasm conservation mass propagation and disease free plan production of medicinal plants. With the help of tissue culture it is possible to produce a huge number of plantlets from single explants within short span of time (Bajaj, 1995). Micropropagation has been used to conserve the germplasm of many rare and endangered species of medicinal plants (Fay, 1992, Mikulik, 1999).

In vitro culture is one of the key tools of plant biotechnology that exploits the totipotence nature of plant cells, a concept proposed by Haberlndt (1902) and unequivocally demonstrated, for the first time by Stewand et al, (1958). Tissue culture is alternately called cell, tissue culture and organ culture through in vitro condition (Debergh and Read, 1991); it can be employed for large scale propagation of disease free clones and gene pool conservation.

Therefore, the objective of this study was to develop micropropagation protocol of this medicinally important herb, *Andrachne aspera* Spreng. Also to introduce the medicinal importance of these plant and to produce industrially antibiotic, anti-mosquitoes insecticide .

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2. Literature review

2.1 Description and taxonomy of *Andrachne aspera*

Euporbiaceae family comprises nearly 322 genera and 8910 species many of which have their own economic value and hence contribute to the floristic wealth of tropical and subtropical countries of the world (Bingtao Li *et al.,* 2008). The family comprises a number of endemic and endangered taxa. However the *in vitro* studies are confined only to a few genera of aesthetic, medicinal, timber yielding, rubber yielding , dye yielding, cottage industries, ornamental and food crops like *Acalypha, Baliospermum, Codiaeum, Colistanthus, Croton, Euphorbia, Emblica, Eryngium, Exoecaria, Givtia, Glochidion,Hevea, Jatrpha , Mallotus, Manihot, Phyllanthus, Putranjiva, Ricinus, Sapium and Uapaca.*

The family Euphorbiaceae is grouped into 52 tribes and five subfamilies namely, Phyllanthoideae, Oldfieldioideae, Acalyphoideae, Carotonideae, and Euphoribideae (Webster 1975).

A. aspera was grouped in Euphoribideae by Webster (1975) and Engler and Prants (1931) under subfamily Phyllanthoideae and sub tribe Porantherreae APG II and III (2003, 2009) and *A. aspera* is included in the family Phyllanthaceae (Chase, *et al.*2002, Davis and Chase,2004; Wurdack *et al.,2004*).

2.2 Origin and distribution of *A.aspera*

Andrachne aspera is mainly of arid and lowland of the subtropics, but also found in the tropic of West Africa. It is found in the wild open dry woodland or bushland or stony river beds in dry sandy, well drained soils. It is drought tolerant and grows at elevation from sea-level up to 2000 meters, occasionally to 2400 meters.

A. aspera is perennial and usually produces several stems from a woody root stock. *Aserpera* grows in Cameroon, Morocco, Egypt, Sudan, Ethiopia, Eritrea, Kenya, and Somalia, through Saudi Arabia to Pakistan.

The habitat of these plants is woodland or bushland and in dry sandy or stony and, at elevations from sea-level up to 2000 m- 2400 m. In Ethiopia it grows at an altitudinal range from 1000 to 2400m.

Importance of *A. aspera*

Several medicinal plants are traditionally used in Ethiopia for the relief of pain. Few of them have been subjected to pharmacological screening for their anti-nociceptive effects and have shown promising results (Makonnen *et al.,* 2003).

A. aspera is used in Ethiopia to prevent from snake bite unidentified gastrointestinal, to relieve pain and reduce fever. The roots of this plant are traditionally used in for various ailments (Eyob *et al.,* 2007).

It is known in Konso, south Ethiopia by the vernacular name "Koramegana" also in Boast, Eastern Shewa Zone Oromia by the vernacular name "korichaadda" and "Hakanur" "Etse- Tekeze" (Atnafu *et al,* 2018)

2.3 Plant tissue culture and its application

Plant tissue culture (PTC) is different kinds of techniques for the *in vitro* aseptic cultures of plant body structures such as cell, tissue organ and their components under controlled physical and chemical conditions and environment. PTC technology also explores conditions that promote cell division and genetic reprogramming *in vitro* conditions and it is considered an important tool both basic and applied studies as well as in traditional medicinal plants preparations (Thorpe, 1990).

Plant tissue culture is a term containing techniques used to propagate plants vegetative by ng small parts of living tissues (explants) on artificial growth mediums under sterile conditions. Explants regenerate shoots and roots, and consequently whole fertile plants under certain cultural conditions. Micropropagation is the production of whole plants through tissue culture from small parts such as shoot and root tips, leaf tissues, anther, nodes, meristems and embryos. Micropropagation is the vegetative (asexual) propagation of plants under *in vitro* conditions and is widely used for commercial purpose worldwide (Huetteman*et al.,* 1993; Pierik, 1978).

In vitro propagation offers a good alternative to multiply novel and endangered plant species. Moreover; it is also used to provide sufficient number of plantlets for planting from few stock plants which responds well to vegetative propagation. (Strivastava *et al.,*2005).

In vitro propagation in many cases is more rapid than traditional methods and offer virus and disease free plants all year round. It is carried out in aseptic and favorable condition on growth media, using various plant tissue culture techniques (Zhou and Wu, 2006).

Plant micropropagation is an integrated process in which cells, tissues or organs of selected plants are isolated, surface sterilized, and incubated in a growth-promoting aseptic environment to produce clone plantlets (Altman, 2000). Micropropagation can also be described as the process of mass propagation of selected plants via *in vitro* techniques. *In vitro* propagation for mass production refers to the process of asexual reproduction by multiplication of genetically identical copies of individual plants (Omar and Aouine, 2007).

In vitro propagation techniques are preferred over the conventional asexual propagation methods because a small amount of tissue is required to regenerate millions of clones of plants in a year. Micropropagation provides a large number of potential for large scale multiplication of such useful species for the next production.

PTC involves the use of small pieces of plant tissue (explants) which are cultured in a nutrient medium under sterile conditions. Using the appropriate condition for each explant type plants can be induced to rapidly produce new shoot and node with addition of suitable hormones. These plantlets can also be divided, usually at a shoot stage, to produce a large number of new plantlets.

In vitro culture techniques are an attractive alternative to overcoming problems with the inconsistencies in secondary product formation in field-grown plants that have phyto-chemical pharmacological, and biotechnological value. (Rao and Rhvishankar,2002). *In vitro* plants have also been used as a source of biomass and to produce disease free clones that have no seasonal variation (Simons *et al*., 2006; Grzegorczyr *et al*., 2007).

Moreover, plant tissue and cell culture suspensions grown in vitro can often produce novel secondary metabolites or increase the production of molecules that may not be present at useful level in whole plant materials. (Stckigt *et al.,* 1995; Santos –Gomes *et al*., 2002)

Plant biotechnology has recently replaced most conventional method used for plant propagation and conservation, as it has overcome all obstacles facing such purpose. (Debanath and Malik 2006.Hussey, 2011)

Using tissue culture techniques for plant propagation was found to be fruitful, as it can guarantee a stable biomass availability of plants of neutraceutical interest in addition to high quality of extracts (Lucchesini and sodi, 2010). Also, *in vitro* cultivation techniques were developed over years to get high yields of uniformed agronomic compounds in a short time and to protect endangered plants from extinction (Bajaj; 1998; Shbliet *al*., 2006).

Various *in vitro* culture techniques such as, micropropagation direct regeneration, regeneration from callus, somatic embryogenesis genetic transformation, were applied to mega production of medicinal plant biomass. (Lucchesini and Sodi,2010). Using tissue culture techniques as a plant biotechnology approach was remarkably successful when applied for identification, isolation and extraction of secondary metabolites of phytochemical importance (Hussein 2011).

2.4 Composition of culture medium

Culture media contain macroelements, microelements, vitamins, other organic components (e.g. amino acids), plant growth regulators, gelling agents (agar if solid) and sucrose.

Growth and morphogenesis of plant tissue *in vitro* are largely governed by the composition of the culture medium.

The composition of the culture medium depends upon the plant species, the explants, and the aim of the experiments. In general, certain standard media are used for most plants, but some modification may be required to achieve genotype specific and stage dependent optimizations, by manipulating the concentrations of growth regulators, or by the addition of specific components to the culture medium.

Commercially available ready-made powdered medium or stock solution can be used for the preparation of culture media. A range of culture media of different formulations, and plant growth regulators are supplied by companies such as Duhefa and Sigma-Aldrich. The importance of nutrition in plant tissue culture is reported by Gautheret (1955). Murashige and Skoog medium (MS) is used most extensively (Murashige and Skoog, 1962). Since then, it is widely used for micropropagation, organ culture, callus culture

and suspension culture. The formulation is a nutrient blend of inorganic salt, vitamins, amino acid and carbohydrate.

MS provides all the essential macroelements and microelements. Potassium dihydrogen phosphate serves as a source of phosphate. Microelements like Boron, Manganese, Molybdenum, Copper, Iron, and Zinc play a vital role in metabolism. Boron plays a key role in carbohydrate metabolism. Thiamine, pyridoxine, nicotinic acid act as enzymatic cofactors in universal pathways including glycolysis and TCA cycle along with the primary and secondary metabolism in plants. Glycine serves as a source of amino acid.

According to the recommendation of the International Association for Plant Physiologists, the elements required by plants in concentration 0.5 mmol 1^{-1} are referred to as macroelements, relatively large amount of some inorganic elements also called major plant nutrients : ions of Nitrogen(N) Potassium (K), Calcium (Ca), Phosphorus (P), Magnesium (mg), and Sulphur (S); and those in concentration less than 0.5 mmol 1^{-1} are microelements, small quantities of other elements are also called trace elements : Iron (Fe), Nickel (Ni), Chlorine (Cl), Manganese (Mn), Zinc (Zn), Boron (B), Copper (Cu), and Molybdenum (Mo) (De Fossard, 1976).

Carbohydrate plays an important role *in vitro* culture as an energy and carbon source; as well as an osmotic agent. In addition, carbohydrate-modulated gene expression in plant is known (Koch, 1996). Sugar (sucrose) is an important component in medium and its addition is essential for *in vitro* growth and development plants because photosynthesis is insufficient, due to the growth taking place in conditions unsuitable for photosynthesis or without photosynthesis (Pierik, 1977).

The sugar concentration depends on the type and use of growth materials; very young embryo requires a relatively high sugar concentration. Generally, growth and development increase with sugar concentration until an optimum is reached and then decreases at high concentration.

The most commonly used source of carbon is sucrose at concentration of 2-5%. Glucose and fructose are also known to support good growth of some tissue (Bhojwani and Radar, 2004). Variation in shoot response was observed in different sugars and 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 experiment (Ruby *et al.,* 2007).

Four vitamins, myo-inositol, thiamin, nicotinic acid, pyridoxine are ingredients of MS medium and have been used in varying proportion for the culture of tissue 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 Bokemann, 1975) on the other hand, obtained increased shoot formation on *Chrysanthemum* pedicels when MS vitamins were present.

2.5 Plant growth regulators

The basal medium (e.g., MS) is designed to keep plant tissue alive and thriving. Plant growth regulators or hormones are needed to manipulate the developmental program of tissues to make callus tissue proliferate, or produce roots from shoot. Growth regulators are the items most often manipulated as experimental factors to enhance tissue culture condition. The most growth regulators for tissue culture are auxins, cytokines, and gibberellins. Both natural and synthetic auxins and cytokines are used in tissue culture.

Auxins promote cell growth and root growth. The most commonly used auxins are IAA (Indole acetic acid), IBA (indole butryic acid), NAA (naphthalene acetic acid) and 2, 4-D (2, 4-dichlorophenoxy acetic acid).

Auxins are involved in the regulation of several physiological processes, as e.g. apical dominance and formation of lateral and adventitious roots. These growth regulators generally cause cell elongation and swelling of tissue, cell division, callus formation and the formation of adventitious roots as well as the inhibition of adventitious and axuillary shoot formation (Pierk,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 (Gorge,1993). IBA and IAA are widely used for root and, in interaction with a cytikinin, for shoot proliferation. Auxins are usually dissoived in either in ethanol or dilute NaOH (Bhojwani,1996)

Cytokines promote cell division and shoot growth,modification of apicaldominance ,shoot differentiation, etc. In tissue culture media cytokinins are incorporated mainly for cell division and differentiation of adventious shoots from callus and organs. An auxin like compound TDZ (thidiazuron) has increased success rate of plant regeneration in many species. The most commonly used cytokines are BAP (benzyl amino purine), zeatin and kinetin. In addition to auxin and cytokines, other hormones such as abscisic acid (Augustine and D'Souza 1997; Cardoza and D' Souza, 2002) and jasmnic acid (Blazquez*et al.,* 2004) have also been used in plant cell culture.

2.6 Culture conditions and Vessels

Cultures are grown in growth rooms or chambers. Humidity, light, and temperature have to be controlled for proper growth of cultures. A 16-h light photoperiod is optimal for tissue culture, and a temperature of 22-25°C is used in most laboratories. A light intensity of 25-50 mol m^{-2} s^{-1} is typical for tissue cultures and is supplied by cool white fluorescent lamps. A relative humidity of 50-60 % is maintained in the growth chambers. Some cultures are also incubated in the dark.

Cultures can be grown in various kinds of vessel such as Petri plates, test tubes, and Magenta boxes.

3. Objectives

3.1 General objective

To develop an efficient micropropagation protocol for *Andrachne aspera* from shoot tip and nodal explants

3.2 Specific objectives

❖ To optimize growth regulators concentration for shoot and nodal induction

❖ To optimize concentrations of growth regulators for shoot multiplication

❖ To optimize concentration of auxin for rooting

❖ To acclimatize and evaluate the survival rate of *in vitro* produced *A. aspera* plantlets in greenhouse

4. Materials and Methods

All the laboratory activities and experiments were conducted at Plant Tissue Culture and Molecular Biology Laboratory, Institute of Biotechnology, Addis Ababa University.

4.1 Plant material

The seeds of *A. aspera* were collected from a single wild type mother plant growing in the wild in Boasat Worda, East Shewa Zone, Ethiopia.

The area lies between 8°25' and 8°50'N and 39°16' and 39°50'E. Boosat fall between 1100 and 1800 m.a.s.l. (Debela, *et al.,* 2006).

4.2 MS medium stock solution preparation

Murashige and Skoog (1962) (MS) medium was used throughout the research activity. Full strength stock solution macronutrients, micronutrients, Fe-Na-EDTA and $FeSO_4$ maximum and vitamins were prepared separately. To do so, appropriate amount of each nutrient was weighed in grams per liter and dissolved in 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 or glass bottles and stored at -20°C until used.

4.3 Plant growth regulators stock solution preparation

Plant growth regulators such as 6-Benzyl Amino-Purine (BAP), kinetin (KIN) for shoot induction and Indole-Butyric Acid (IBA), Naphthalene Acetic Acid (NAA), 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 1:1 (1 mg/ml) and dissolved by 2-3 drops of 1NNaOH and /or 1N HCl based on the requirement of the plant growth regulators. Then, the volume was adjusted by double distilled water and magnetic stirrer. Finally, growth regulator stock solutions were stored in a refrigerator at $+4^0$ C for short term use.

4.4 Culture medium preparation

Culture medium was prepared by taking 100 ml/l macronutrient and 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, kinetin, and NAA for shoot induction

and IAA and IBA for rooting was 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 and 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. Immediately after autoclaving, the medium was taken and kept in laminar air flow cabinet bench or kept in refrigerator for one or two days until used.

4.5 *In vitro* seed germination

Seeds of *A. aspera* were released from the fruit cover and washed with detergent under running tap water to reduce the contamination. Seeds were surface sterilized in 70% ethanol for 2-3 minute and rinsed 4 to 5 times by using sterile double distilled water, to lower the toxic effect of ethanol, followed by 20% Clorox solution (NaOCl) for 10 min and rinsed 5 times using distilled water under a laminar flow hood. The sterilized seeds were aseptically germinated on 50 ml MS (Murashige and Skoog, 1962) medium without hormone containing 7 g/l agar and 30 g/l sucrose (w/v) were dispensed in culture vessel (10x6 cm, height, width, respectively). Before addition of agar, pH of the medium was adjusted to 5.6-5.8 with 0.1 N HCl or 0.1N NaOH and autoclaved at 121°C for 15 min under 105 KPa pressure. Five seeds per Magenta culture vessel were cultured in 6 replicates per treatment. Cultures were maintained at 22-25°C under 16 h photoperiod and light intensity of 22μmol $m^{-2}s^{-1}$ under white fluorescent lamp.

.Seeds were removed from pod and surface sterilized by washing with OMO (detergent soap) thoroughly under running tap water for 30 min, then washed by distilled water. Antiseptic solution of 20% Clorox (sodium hypochlorite) was used under the Laminar air –flow cabinet and seeds were immersed in the solution for 10 min with continuous shaking. Seeds were then rinsed with sterilized distilled water for 8-10 times. Finally, 70% ethanol alcohol was added to the seeds for 3 min then rinsed with sterile distilled water for 8-10 times.

Shoot initiation

Shoot tips were excised from seedlings and cultured on full strength MS medium containing 30 g/l sucrose (w/v), BAP (0.0, 0.5, 1.00, 1.5, 2.00, 2.5 mg/l) and kinetin (0.0, 0.5, 1.00, 1.5, 2.00, 2.50 mg/l) and 7 g/l agar was added after adjusting the pH to 5.8before autoclaving.

Cultures were incubated under light intensity of 22 μmol m^{-2} s^{-1} and 16 h photoperiod provided by white light fluorescent lamps at 22-25°C. All experiments (total of 30 treatments) were performed by using five plants per culture vessel in 6 replications.

Number of initiated explants was recorded after four weeks.

4.6 Shoot multiplication

Shoots from initiation culture were used as explants in shoot multiplication, Shoots were transferred to MS medium containing different concentrations of BAP (0.0, 0.5, 1.00, 1.50, 2.00, 2.50 mg/l) in combination with NAA (0.0, 0.25, 0.5, 1.00, 1.50, 2.00 mg/l) and kinetin (0.0, 0.5, 1.00, 1.50, 2.00, 2.50 mg/l). All experiments were performed by five explants per culture vessel in 6 replications. The cultures were kept at 22-25° C under 16 h photoperiod and light intensity of 22 μmol m^{-2} s^{-1} for 30 days.

4.7 Rooting

For rooting, half strength MS medium containing IAA (0.0, 0.25, 0.5, 1.00, 1.50, and 2.00) or IBA (0.0, 0.25, 0.50, 1.00, 1.50, 2.00 mg/l), 30 g/l sucrose was used. The pH was adjusted to 5.8 followed by addition of 7 g/l agar. Well-developed micro shoots were excised and transferred to rooting media. Five explants were transferred to each culture vessel (1cm). The cultures were maintained at 22-25°C with 16 h photoperiod at light intensity of 22 μmol m^{-2} s^{-1}.

Data were recorded after 1 month for rooting percentage, number of roots, root length and callus percentage.

4.8 Acclimatization

Well-developed rooted plantlets were removed from the medium and washed gently under running tap water to remove the MS medium that helps to prevent the new plant

from attacking of microbes. The *in vitro* developed rooted shoots were transplanted in to the pots that containing sterilized mixture of soil, sand and peat in the ratio of 2:1:1v/v respectively. The pots were covered with transparent poly-ethylene bags and kept in the culture room for 15 days and watered at an interval of 2 to 3 days. After two weeks the poly-ethylene bags were removed and transferred to greenhouse. Finally, the plants were fully exposed to the normal growth condition for a month.

4.9 Data analysis

Data were statistically analyzed using statistical package for the social sciences (SPSS). Standard error was calculated for all the experiments. Analysis of variance (ANOVA) was used to analyze the obtained results for shoot number, number of node, shoot height, root number and root length per explants. The least significant difference (LSD) was tested using statistical analysis software SPSS 20.0 version at 0.05 levels. Means were separated with probability of 0.05 according to the Tukeys HSD.

5. Results

5.1 *In vitro* seed germination

The study revealed that sterilization of *A. aspera* seed with 70% ethanol for 3 to 5 minute was found to be effective to control contamination. Below 70% concentration resulted in 100% contamination while no germination was observed at the mentioned concentration (Above 80%).

The second surface sterilization was carried out by 20% Clorox (sodium hypochlorite) for 10 minute provided 100% contamination free seeds. Cultures were contaminated when the concentration of Clorox was below 20% and exposure time was below 10 minute. However, when the concentrations of Clorox were above 20%, it affected seed germination.

Figure 1Seed germination on MS medium

5.2 Shoot initiation

Shoot tips cultured on the MS medium supplemented with BAP and kinetin alone and the control showed significant variation ($p \leq 0.05$) in terms of percentage of shoots induced. All shoot tips produced proliferated shoots on MS medium supplemented with 1.5 mg/l BAP and 0.5 mg/l kinetin (Tables 1 and 2).

On the other hand, the lowest shoot induction percentage was observed on medium containing 2.5 mg/l BAP only. And 2.5 mg/l KIN shoots exhibited less new growth of shoots.

Table 1 Effects of different concentrations of BAP on shoot induction of *A. aspera* Data expressed as Mean ± SE

BAP (mg/l)	No. Shoots	Number of node	Shoot length
0.0	0.27±0.09[d]	0.23±0.10[c]	0.25±0.10[d]
0.5	1.80±0.15[c]	3.27±0.27[b]	4.40±0.34[ab]
1.0	2.93±0.34[b]	3.50±0.32[b]	2.83±0.26[c]
1.5	5.03±0.29[a]	4.93±0.28[a]	4.05±0.24[ab]
2.0	2.20±0.21[bc]	4.10±0.32[ab]	4.93±0.37[a]
2.5	1.93±0.23[c]	3.30±0.31[b]	3.33±0.35[bc]

Different letters within the same column represent significant differences according to Tukeys multiple range test at 5% level.

Figure 2 Shoot initiations on different concentrations of BAP

Table 2 Effect of kinetin on shoot initiation

Kinetin (mg/l)	No. of shoot	No. of node	Shoot length(cm)
00	0.17±0.07[c]	0.17±0.07[b]	0.25±0.11[b]
0.5	2.50±0.27[a]	3.00±0.28[a]	2.97±0.35[a]
1.0	2.73±0.29[a]	3.17±0.24[a]	2.93±0.24[a]
1.5	1.60±0.13[b]	2.43±0.28[a]	3.10±0.43[a]
2.0	1.40±0.10[b]	2.50±0.23[a]	2.88±0.32[a]
2.5	1.47±0.10[b]	2.33±0.16[a]	2.43±0.27[a]

Different letters within the same column represent significant differences according to Tukeys multiple range test at 5% level.

Figure 3 Shoot initiations on different concentrations of kinetin

5.3 Shoot multiplication

Effects of BAP and NAA on the shoot multiplication of *A. aspera*

The effect of NAA in combination with BAP on shoot multiplication after four weeks of culture was highly significant ($p \leq 0.05$). The results of this study indicated that using BAP in combination with NAA for shoot multiplication was better than kinetin in combination with NAA.

In all treatments, there were differences in the rate of shoot multiplication. The mean number of shoots per explants ranged from 2.23±0.14 to 5.17±0.29 (Table 3 and Fig. 4). Among all the treatments, 0.5 mg/l BAP in combination 0.25 mg/l NAA resulted in the highest mean number of shoots per explants (5.17±0.29) (Table3).

MS medium supplemented with 0.5 mg/l kinetin combined with 0.25 mg/l NAA, and 0.5 mg/l NAA resulted in the first (2.61±0.70) and the second (2.00 ±0.48) highest mean shoot number respectively. Shoot explants cultured on MS medium supplemented with 2.00 mg/l BAP in combination with 1.5 mg/l NAA and 2.0 mg/l kinetin in combination with 1.5 mg/l NAA produced the lowest mean number of shoot per explants (2.23 ±0.14) and (1.08±0.09) respectively. Shoot length was significantly the highest (1.05±0.59 shoot number and 1.43±0.13shoot length on growth regulators free medium(00) but it produced the lowest mean number of shoots per explants (1.08± 0.098). The highest mean shoot number per explant (5.17±0.29), number of node per explants (5.23±0.21)

and shoot length(4.30±0.22 cm) were recorded on MS medium supplemented with 0.5 mg/l BAP combined with 0.25 mg/l NAA and 0.5 mg/l kinetin combined with 0.5 mg/l NAA respectively. With increasing the concentrations of kinetin in combination with NAA, the number of shoot per explants decreased.

Table3.Effects of BAP and NAA on the shoot multiplication of *A. aspera*. Data expressed as Mean ± SE

BAP NAA	**No. Shoot**	No. Node	**Shoot length (cm)**
00+00	1.03±.076[d]	1.20±0.11[c]	1.32±.13[c]
0.5+0.25	5.17±0.29[a]	5.23±0.22[a]	4.30±0.22[a]
1.0+0.5	4.13±0.30[b]	4.97±0.31978[a]	4.03±0.26[a]
1.5+1.0	2.30±0.15[c]	3.1000±0.23[b]	2.73±0.21[b]
2.0+1.5	2.23±0.14[c]	2.97±0.23[b]	2.70±0.22[b]

Different letters within the same column represent significant differences according to Tukey's multiple range test at 5% level.

Figure 4 Shoot multiplications on MS medium containing 0.5 mg/l BAP in combination with 0.25mg/l NAA

Table 4 Effects of different KIN and NAA on shoot multiplication. Data expressed as Mean ± SE

Kinetin + NAA	No. of Shoot	No. of Node	Shoot length
00+00	1.50±0.59[d]	1.39±0.42[c]	1.43±0.12[c]
0.5+0.25	2.61±0.70[c]	2.9000±0.17[b]	2.37±0.10[b]
1.0+0.5	2.00±0.48[a]	2.10±0.15[a]	1.83±0.12[a]
1.5+1.0	1.43±0.13[b]	1.83±0.19[b]	1.57±0.18[b]
2.0+1.5	1.08±0.08[c]	1.80±0.090[b]	1.53±0.73[b]

Different letters within the same column represent significant differences according to Tukey's multiple range tests at 5% level.

5.4 *In vitro* rooting

The highest mean number of root per shoot (6.00±0.71) and root length (6.00±0.55 cm) were obtained on half strength MS medium containing 0.25 mg/l IAA. The lowest mean number of roots per shoot (1.00±0.00) and root length (1.00±0.00 cm) were obtained on half strength MS medium containing 2.00 mg/l IAA.

Table 5. Effect of half strength MS medium containing different concentrations of IAA on in vitro rooting of *A. aspera*. Data presented as Mean ± SE

IAA(mg/L)	No. Root	Root Length (cm)
00	0.00±0.00[e]	0.00±0.00[e]
0.25	6.00±0.717[a]	6.00±0.55[a]
0.50	4.00±0.32[b]	4.00±0.45[b]
1.00	3.20±0.37[c]	2.80±0.49[c]
1.50	1.80±0.20[d]	1.40±0.24[d]
2.00	1.00±0.00[d]	1.00±0.00[d]

Different letters within the same column represent significant differences according to Tukey's multiple range test at 5% level.

The highest mean number of roots per shoot (2.00±0.32) and root length (2.80±0.37) were obtained on medium containing 0.25 mg/l IBA. The lowest mean number of roots per explant (0.80±0.20) and mean root length (0.00±0.00 cm) were obtained on growth regulators free medium which was used as a control (Fig. 5).

Table 6 Effects of half strength MS medium containing different concentrations of IBA on in vitro rooting of *A. aspera*. Data expressed as Mean ± SE

IBA(mg/l)	No. roots	Root length
0.0	0.00±0.00^{d}	0.00±0.00^{d}
0.25	2.00±0.32^{a}	2.80±0.37^{a}
0.50	2.00±0.00^{a}	1.30±0.20 ^{b}
1.00	1.00±0.00^{b}	1.00±0.00^{bc}
1.50	1.00±0.00^{b}	0.30±0.20 ^{c}
2.00	0.800±0.20^{bc}	0.00±0.00 ^{d}

Different letters within the same column represent significant differences according to Tukey's multiple range test at 5% level.

Figure 5 Rooting on half strength MS medium containing 0.25 mg/l IAA

5.5 Acclimatization

After one month, all the plantlets transferred to the greenhouse grew healthy, and survived (Fig.6). They had normal leaf development and did not show any detectable differences in morphology.

These plants were observed on 15th and 30th day almost 90% of *in vitro* regenerated plants were successfully established in soil.

A B C

Figure 6 Acclimatization of *in vitro* rooted shoots of *A.aspera* in green house. A) Plant covered with plastic bag B) Plant transferred to greenhouse C) After 30 days of acclimatization

6. Discussion

6.2 Sterilization of the seed

This study used various sterilization agents to avoid the contamination of microorganisms from *A .aspera.* The small size seeds were washed with detergent Omo thoroughly in tap water followed double distilled water for effective reduction of contamination.

The seeds surface sterilized with 70% ethanol alcohol for 2-3minute and 20% of Clorox for 10 minute showed higher percentage of clean seed. The reduction below 70% alcohol and 20% Clorox concentration obtained contamination of seed by microorganisms.

The surface sterilized 80% of ethanol alcohol and 20% of Clorox inhibits the germination of seed. This result indicates the concentration of 70% alcohol and 20% of and exposure time were used for disinfectant were interrelated factors to obtain contamination free explants.

This is similar to the finding of Silva *et al.,* (2013) who reported the best sterilization for the seed of *Carton floribudus* were 70% alcohol for 3minutes of exposure time.

6.3 Soot induction from shoot tip and node explants

In culture initiation experiment, shoot tip and node of 1cm of explants cultured on control and MS medium supplemented with 0.5, 1.0, 1.5, 2.0, 2.5 mg/l BAP and kinetin separately, showed a significant variation ($p \leq 0.05$). Cytokinin type and concentration were always described as the key leaders that control the performance of plant multiplication in the tissue culture (Schuelter *et al.* 2009).

The result thus conclusively showed that cytokinis were essential for shoot induction and proliferation of tip and node explants of *A. aspera*. BAP at 1.5 mg/l and kinetin at 1.00 mg/l resulted in high shoot induction. This is similar to the finding of Matin *et al*. (2009) that reported the influence of in direct *in vitro* morphogenesis of mesophyll cells of *Euphorbia nuvlia*, Kinetin reduces the rate of morphogenesis, where as BAP induced somatic embryo genesis.

The numbers of initiated shoots were found to vary in the different concentration of cytokinins and the control treatment. The maximum shoot initiation was recorded from medium containing 1.5 mg/l BAP and 1.00 mg/l kinetin.

The effects of BAP on shoot induction, highest shoot number was attained at 1.5 mg/l treatment and the number of shoot obtained (5.03±0.29) (fig.1), the number of node obtained (4.93±0.29)and average length more seen in the treatment 2mg//l that obtained (4.93±0.37cm). This finding was similar with the work of Catapan *et al* .2002) who got on *Phyllantus fraternus* used 1.0 mg/l-2mg/l BAP to gate 6.9±0.21and 3.1±0.06number of shoots respectively. According to the work of Catapan the increasing of BAP reduced the number of shoot and shoot length. This finding in agreement the present study of A. aspera.

The effects of kinetin on shoot induction, highest shoot number attained at 1.0 mg/l Kinetin and the number of shoot reached its level (2.73±0.29), the number of nodes (3.17±0.24) but the highest shoot length (3.10±0.43) was on 1.5mg/Kinetin. This is similar to the Upadhya *et al.,* (2015) who reported the maximum shoot number (3.2±0.22) and shoot length (2.27±0.2 cm) was attained when the explants were cultured on the MS medium supplemented with 1mg/l Kinetin. The shoot number reduces 1.88 to 0 when the kinetin increases from 2-10 mg/l.

On the other hand, increasing BAP and kinetin above 1.5 mg/l caused the reduction of shoot induction which is in agreement with the report of Hu and Wang (1983) who reported that the higher concentration of cytiokinin reduced the number of multiplied shoots. The control medium also showed multiple shoot development but they were smaller in size.

The present study did n't agree with the work of Catapan *et al*.,(2001) who obtained 100% shoots on *phyllnthus stipulantus* on MS supplemented with 0.31-5.00μM BAP or Kinetin

The current result is in agreement with the finding of other workers who have noted the effectiveness of BAP than kinetin for shoot induction of *A. aspera* shoot and nodal explant cultured on MS medium supplemented with different concentrations of BAP and kinetin (Ghanti *et al., 2009*).

6.4 Shoot multiplication

There was a significant difference ($p \leq 0.05$) among the treatment effects both in terms of shoot number, number of node and shoot length per explant. In this study, four different concentrations of BAP combined with NAA and kinetin combined with NAA were used for shoot multiplication.

BAP alone or in combination with NAA were found to be the most suitable plant growth regulator for micropropagation of *A. aspera* rather than a combination of kinetin and NAA.

In present study, the highest mean number of shoot per explants was (5.17±0.29) with (4.30±0.22 cm) mean shoot length and (5.23±0.22) number of nods per explants were records on MS medium supplemented with 0.5mg/l BAP in combined with 0.25mg/l NAA.

The results of this study was similar with the finding of Ghabbia *et al* (2016) who obtained the maximum shoot number (5.20) of *Euphorbia pulcherrima* when the hypocotyls were cultured on the MS medium supplemented with BAP+NAA. Lower shoot length (1.03 cm) was obtained on medium containing 1.5 mg/l BAP+0.5 mg/l NAA for shoot multiplication. On the other hand, Patel *et al.*,(2018) obtained the highest mean shoot number and shoot length per explants (5,31± 0.1) and (6.5± 0.5 cm) respectively were obtained by these authors. From *Phyllantus niruri* on MS medium containing 0.5 mg/l BAP+0.5 mg/l NAA and 1.0 mg/l BAP+0.5 NAA respectively which was in agreement with the present study.

BAP in combination with NAA found to be high-quality, attractive culture maximum number of shoots per explant for shoot regeneration in many plants (Rosaool *et al.,* 2009; Islam and Bari, 2013) which is in agreement with the present study.

The finding of Kafane (2018) on *Pterolobium stellatum* showed that MS medium supplemented at 0.5 mg/l KIN +0.25mg/l NAA resulted in maximum mean shoot

number on nodal explants (1.56 mg/l±0.17) and shoot length (3.25±0.33 cm), which is similar result with present study.

The present study showed when the concentration of BAP and NAA raised lower number of shoot per explants. In contrast with Ali *et al.,*(2014) who reported MS medium 0.5mg/l BAP+0.25mg/l NAA produced the highest mean number(20.4±0.920) of shoots per explants. This study reported that the increasing BAP and NAA in combination in the MS medium provided more shoot multiplication

Generally, the present study showed that the explants of *A. aspera* produced multiple shoots when BAP combined with NAA than kinetin combined with NAA.

6.5 . Rooting and Acclimatization

Auxins are mainly used in root induction and their effect varies with type and concentrations used in different plant species (Singh, 2002). IAA was reported as potential auxin for rooting in several plants including *Arahisstenosperma* and *A. villosa*(Vijayalakshmi and Giri, 2003). In this study, different concentrations of IAA and IBA alone on half MS medium was used for root formation of *A. aspera* plant. The highest number of root per shoot (6.00±0.71) was obtained and mean root length (6.00± 0.55 cm) on medium containing 0.25 mg/l IAA. This is similar to finding of Reham*et a., (*2017) who reported 90% rooting in half strength MS supplemented with 0.5 mg/l IAA while the decline of root indicated increasing of IAA above 1.0 mg/l which is similar with this study. In contrast, with the study of Gharbia H *et al.* (2016) who reported 66% pre explant or 4.86 plant of *Euphorbia pulcerriama* also indicated that increasing IAA above 1.0 mg/l provided effective *in vitro* root formation which is different from this study. IBA was not effective in this study when compared to IAA. According to Catapan *et al.,* (2002) rooting was activated with 93-100% of the microshoots on MS medium without growth regulators, although 1.25-5.0 μM NAA significantly increase the number of root per explant.

After development of extensive root system, these plantlets were gradually acclimatized to natural environment and finally planted in specially prepared plastic pot. The soil was

prepared in the ratio of 1:1 (sand soil, loom soil) and dried. Then this soil was sterilized in autoclave to prevent contamination. Finally, plantlets were planted into the pot soil. These plants were observed on 15th and 30th day. *A. aspra* potted plantlets showed 90% survival. This could be attributed to the weedy nature of plant as it supports its survival at harsh environmental condition (Pandhure *et al.,* 2010).

7. Conclusions

Successful propagation of *Andrachne aspra* was obtained via tissue culture techniques. Using BAP (1.5 mg/l BAP) and BAP X NAA(0.5 mg/l BAP+0.25 mg/l NAA) was found to be best for shoot induction and shoot multiplication, respectively, while best rooting was achieved in micro shoots treated with IAA at 0.25 mg/l. Moreover, 90% of plantlets survived after being transplanted to greenhouse conditions. The plant has wide important to treat bacterial disease, anti-malaria prevention and from snake bite, it should be plant in to produce traditional and modern medicines. The propagated plant must cultivate in the natural area to reduce endangered condition.

8. Recommendations

- ✓ Explants other than shoot tips culture for in vitro propagation of *A.aspera* should be considered to investigate if they can offer better and efficient responses.
- ✓ Further studies on the performance of the tested cytokinins in combination with other type of hormone (cytokinins and /or auxins) should be carried out for better result on shoot induction and multiplication.
- ✓ The effect of different concentrations of auxins on induction of roots should be further studied.
- ✓ It is also recommended to practice other techniques like culturing of explants in liquid medium to reduce the cost of agar for mass propagation of *A.aspera*

However, more research is still needed to investigate the effect of *in vitro* propagation of this plant on the quality and quantity of its active ingredients and to prevent it from extinction it is endangered next species.

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Appendix 1.

Table 4. Nutrient composition and concentration of full strength MS basal medium.

Components Macronutrients	Concentration (gm/L)
NH_4NO_3	16.5
KNO_3	19.0
$CaCl_2.2H_2O$	4.4
$MgSO_4.7H_2O$	3.7
KH_2PO_4	1.7
Micronutrients	
Fe-Na-EDTA	4.0
$ZnSO_4.7H_2O$	0.86
H_3BO_3	0.62
$MnSO_4.4H_2O$	2.23
$CuSO_4.5H_2O$	0.0025
KI	0.083
$Na_2MoO_4.2H_2O$	0.025
$COCl_2.6H_2O$	0.0025
Organic supplements	
Myo-inositol	1.0
Glycin (Glycocoll)	0.2
Nicotinic acid	0.05
Pyridoxin (B6)	0.05
Thiamin (B1)	0.01