PROTOCOL OPTIMIZATION FOR *IN VITRO* PROPAGATION OF GRAPEVINE (*Vitis vinifera* L.*)* USING AXILLARY BUD CULTURE

MSc. THESIS

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OCTOBER, 2015

JIMMA, ETHIOPIA

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By

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DEDICATION

I dedicate this thesis to my family for their commitment in the success of my life.

STATEMENT OF THE AUTHOR

I, Kegna Gadisa, hereby declare that the work presented in the thesis manuscript entitled, "**Protocol Optimization for *In Vitro* Propagation of Grapevine (*Vitis vinifera* L.*)* Using Axillary Bud Culture"** for partial fulfillment of the requirements for the award of the Degree of Master of Science in Plant Biotechnology at Jimma University is an authentic record of my own work under the supervision of my major advisor Dr. Kassahun Bantte and co-advisor Beza Kinfe.

Duly acknowledging all source material I used in this thesis, I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate. It can be deposited at the University Library to be made available without special permission, provided that accurate acknowledgment of source is made. Request for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major department or the Dean of the School of Graduate Studies when in his/her judgment the proposed use of material is in the interest of scholarship.

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BIOGRAPHICAL SKETCH

The author, Kegna Gadisa, was born on January 20, 1988 in Dano Woreda, West Shoa Zone, Oromia Regional State. He attended his elementary and secondary school education at Ambo Addis ketema and Ambo Comprehensive Senior Secondary and Preparatory Schools respectively. Then he joined the then Jimma University, Ambo College in 2008 and graduated with BSc degree in crop production in 2010. After his graduation, he was employed by former Gambella Agricultural Technical, Vocational and Educational Training College as an instructor from January 2011 – 2012. In 2012, he joined the School of Graduate Studies of Jimma University to pursue his graduate studies in Plant biotechnology.

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LIST OF ABBREVIATIONS

BAP	6-Benzyl Amino Purine
DZRC	Debre Zeit Research Center
DZRCTCL	Debre Zeit Research Center Tissue Culture Laboratory
FAOSTAT	Food and Agriculture Organization of the United Nations Statistics
IBA	Indol-3-Butyric Acid
MS	Murashige and Skoog
NAA	Naphthalene Acetic Acid
PGR	Plant Growth Regulator

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PROTOCOL OPTIMIZATION FOR *IN VITRO* PROPAGATION OF GRAPEVINE (*Vitis vinifera* L.*)* USING AXILLARY BUD CULTURE

ABSTRACT

The production of grapevine through conventional propagation methods requires mature mother vines. This takes four to five years to reach maturity for woody production besides the limited amount of planting material that can be produced. In vitro propagation has a potential to quickly provide relatively large amount of planting materials to grapevine growers. This is especially very important when planting materials are required in large quantities. In vitro propagation of grapevine can be carried out by the culture of intact or fragmented shoot apical meristems, axillary bud or through adventitious bud formation. The degree of response to certain culture is highly depends on the particular genotype and part of organ used as ex-plant. Therefore, this study was initiated to optimize a protocol for in vitro propagation of grapevine using axillary bud. The material used for the study was a cultivar named Black Corinth. Experiment one was laid out in Completely Randomized Design (CRD) with three replications and five ex-plants were use per jar. Different concentrations of BAP (0.2, 0.6, 1.0, 1.5 and 2.0 mg/l) in combination with 0.1mg/l NAA were used. Experiment two was also replicated three times with factorial arrangement in Completely Randomized Design (CRD) and three shoots per jar were used. BAP (1.0, 2.0, 2.5, 3.0 and 3.5 mg/l) in combination with NAA (0.1, 0.2 and 0.3 mg/l) were used as the treatments. Similarly, experiment three was also replicated three times with factorial arrangement in Completely Randomized Design (CRD).Three shoots per jar were used. IBA (0.0, 0.8, 1.2, 1.6 and 2.0 mg/l) in combination with NAA (0.0, 0.2, 0.5, 0.8 and 1.0 mg/l) treatments were used. For shoot initiation, sterilized explants were cultured on MS basal medium supplemented with 0.6 and 1.0mg/l BAP in 0.1mg/l NAA and resulted in highest (80.00±0.00) percentage of explants showing shoot initiation and maximum number of shoot/ex-plant (1.72±0.05) was obtained on 1.5mg/l BAP. The highest shoot height (3.22±0.26) was recorded at 1.0mg/l BAP with 0.1mg/l NAA. On shoot multiplication, the maximum (2.67±0.14) number of shoots/ex-plant was obtained on 3.0 mg/l and 0.2mg/l BAP and NAA concentration respectively. The highest value (3.23±0.25) for shoot height was obtained on MS medium containing BAP and NAA at 1.mg/l and 0.2mg/l respectively. The combination of IBA and NAA at 0.8 and 0.5 mg/l, 1.2 and 1.0 mg/l, 1.6 and 1.0mg/l, 2.0 and 0.5mg/l and 2.0 and 1.0mg/l resulted in the highest value (100%) for rooting percentage. Concentration of IBA and NAA at 2.0 and 0.2mg/l respectively resulted in the highest value (12.50±0.50) for number of roots/shoot and the maximum root length (5.39±0.19) was recorded on 1.6mg/l IBA and 0.5mg/l NAA. The concentration of 1.5mg/l BAP in 0.1mg/l NAA could be the best option for shoot initiation whiles the combination of 2.5mg/l BAP + 0.1mg/l NAA was the optimal concentration for shoot multiplications. For in vitro rooting, the combination of 1.2mg/l IBA and 1.0mg/l NAA could be taken as the best alternative. About 80% of plantlets transferred to the greenhouse were successfully acclimatized. As this protocol was tested on a single cultivar, further studies using other genotypes are required so as to have a reliable protocol for in vitro propagation of grapevine.

Key words/phrases: Axillary bud, Grape vine, In vitro propagation

1. INTRODUCTION

Grapevine (*Vitis vinifera L.*) is a fruiting berry of the deciduous woody vines. It belongs to *Vitaceae* family. Its chromosome number is 2n=2x=38. Almost all cultivated varieties are hermaphroditic and self fertile. Pollination occurs mostly through wind; hence, close planting is useful for effective pollination. Generally, 80% pollination is through self fertilization in grapevines, the rest may attribute to cross pollination.

According to Food and Agriculture Organization of the United Nations Statistics (FAOSTAT, 2014), 6.9 million ha of land in the world is dedicated to grapes and the total production was 67 million tons in 2013. In Ethiopia, 2200 ha of land has been covered by vineyard and the total production was 5000 tons in 2012 (FAOSTAT, 2014). Majority of the vineyard farm is owned by foreign private companies. Among of them Awash winery Share Company which is cultivating grapevine on 60 ha has currently planned to expand to 500 ha in Merti Jeju. Castel winery has also acquired 450 ha from the government in 2008 in Batu with the aim of producing locally-grown, quality wines to help revitalize the country's winery industry. Approximately 71% of the world grape production is used for wine, 27% as fresh fruit and 2% as dried fruit (FAOASTAT, 2011).

Grapevine gives different uses to human kind such as; preventing cancer, heart disease, degenerative nerve disease, retinal disorder, constipation, etc. Grape juice is also the sources of different minerals and vitamins which makes our immunity system strong and prevent common disease (Jaladet *et al*., 2009).

Commercially cultivated grapes can usually be classified as either table or wine grapes while almost all of them belong to the same species, *Vitis vinifera*. Table and wine grapes have significant differences brought about through selective breeding. Table grape cultivars tend to have large, seedless fruit with relatively thin skin. Wine grapes are smaller, usually seeded, and have relatively thick skins which is desirable characteristic in winemaking, since much of the aroma in wine comes from the skin (Donald *et al*., 2007).

The production of grapevine through conventional propagation methods requires mature mother vines.

This takes four to five years to reach maturity for woody production (Winkler, 1976). *In vitro* propagation has a potential to quickly provide relatively large amounts of planting materials to grapevine growth. This is especially very important when planting materials are required in large quantities during expansion in acreage.

Depending on the plant species and cultural conditions, tissue culture may enable the mass production of genetically homogeneous populations and vigorous growth (Blazina *et al*., 2000). *In vitro* propagation methods allow growers to get new planting materials two years earlier than they would be available via traditional propagation methods (Sim *et al*., 2010). Thus, developing *in vitro* propagation techniques for large scale production of grapevine in order to exploit its potential is unequivocal.

Micro-propagation of selected *Vitis* genotypes can be carried out by the culture of intact or fragmented shoot apical meristems, axillary bud or through adventitious bud formation (Heloir *et al*., 1997). Most efficient protocols have been reported for *muscadine* other than *V. vinifera* grapes (Qiu *et al*., 2004). But the work done by Abido *et al*., (2013) on *V. vinifera*-Muscat of Alexandria genotype indicated that number of shoots formed per ex-plant (1.43) was obtained on MS medium augmented at 0.5 mg/l BAP in combination with 0.1mg/l NAA. BAP at 3.0mg/l with 0.2mg/l NAA showed the maximum number of shoots/ex-plant (3.2) and shoot height (4.5cm) for shoot multiplications. The influence of various concentrations of IBA (0-2mg/l) in combination with NAA (0-1mg/l) on root formation was determined and the combination at 1.0 and 0.5 mg/l IBA and NAA resulted in the highest value (87%) of shoots rooted and gave the highest (3.4) number of roots/shoot.

The degree of competence of a genotype in responding to *in vitro* propagation is highly depended on the particular genotype and part of organ used as ex-plant, as various *Vitis* species respond differently to certain culture conditions (Qiu *et al*., 2004).

Explant type, plant growth regulator and different combinations may have different results. Several researchers have used different plant organs as explants for *in vitro* propagation of different *Vitis vinefera* genotype. Among the work done in Ethiopia, Beza (2010) reported that BAP at 0.5mg/l was the optimum concentration that performed best on shoot initiation using nodal culture comprising active buds.

Similarly, the combination of BAP at 0.5mg/l and 1mg/l with 0.1mg/l IBA gave the best shoot number and shoot height on shoot multiplications. Finally, among used different IAA concentrations for rooting, IAA at 4mg/l resulted in the highest number of root/ex-plant.

Even though, few works had been reported on grapevine tissue culture, there is limited information on using axillary buds as ex-plant types in Ethiopia. Therefore, this study was initiated to optimize a protocol for *in vitro* propagation of a selected genotype using axillary bud.

General objective

To optimize a protocol for *in vitro* propagation of a selected genotype using axillary buds culture

Specific objectives

- To determine the optimum concentration of BAP for shoot initiation.
- To determine the optimum concentration of BAP and NAA combinations on shoot multiplications.
- To determine the optimum concentration of IBA and NAA combinations on root formations.

2. LITERATURE REVIEW

2.1. Taxonomy and Distribution of Grapevines

Grapevines are the crops that belong to the *Vitaceae* family and genus *Vitis*. The genus *Vitis* is largely distributed between 25^{o} and 50^{o}N latitude in Europe, the Middle East, North America and Eastern Asia. Additionally, a few species of *Vitis* are found in the tropics Central American countries, Caribbean and Northern South America (Fabio *et al.*, 2005). Its chromosome number is 2n=2x=38. Almost all cultivated varieties are hermaphroditic and self fertile. Pollination occurs mostly through wind; hence, close planting is useful for effective pollination. In grapevines, 80% pollination is through self fertilization. The rest may attribute to cross pollinations.

The genus *Vitis* is categorized into two subgenera:

1. ***Euvitis*** - "True grapes"; characterized by elongated clusters of fruit with berries adhering to stems at maturity, forked tendrils, diaphragms in pith at nodes also called "bunch grapes". Most of the species are in these subgenera.

2. ***Muscadinia*** - *Muscadine* grapes are characterized by small fruit clusters, thick skinned fruit, berries that detach one by one as they mature, simple tendrils and the lack of diaphragms in pith at nodes.

Based on their usage and purposes, grapevines are categorized into five main classes

Table varieties: These varieties are utilized for food and decorative purposes. They have an attractive appearance, good eating qualities and storage qualities (Yingyos, 2007).

Canning grapes: These are seedless grapes which are used in canned forms. The Thomson Seedless variety is most commonly used, alone or in combination with other fruits as fruit salad or fruit cocktail. Even though there are many wild species today, recent advances in grapevine genetic transformation offer new opportunities for genetic improvement and make it very important fruit crops (Carimi *et al.*, 2005).

Wine grapes: These are the varieties that are used for wine production. These grapes have high acidity and moderate sugar content.

It includes the varieties such as Shiraz, Carbernet, Sauvignon, Riesling and Pinot noir in which they have the outstanding bouquet and flavor essential for production of highest quality premium wines (Krongjai, 2005).

Raisin grapes: These include any dried grapes, although several standards must be met if a suitable dried raisin is to be made. The dried raisins must be soft in texture and should not stick together when storing. Few varieties can meet all of these criteria. Some of the best and most widely grown grape varieties for raisins are Thomson seedless, Black Corinth and Muscat of Alexandria (Yingyos, 2007).

Juice grapes: In the manufacture of sweet unfermented juice, the clarifying and preserving procedure should not destroy the natural flavor of the grape. In the United States, grape juice is usually produced from Concord grapes or a blend of Concord and other varieties (Krongjai, 2005).

2.2. Origin and history of grapevine cultivation

Vitis vinifera "Old world grape" is the major species of grape accounting for >90% of world production (Fabio *et al.*, 2005).

Figure 1: Top grapevine producing areas in the world (adopted from FAO, 2005)

Probably native in the area near the Caspian Sea, in Asia minor seeds have been found in excavated dwellings of the Bronze age in south central Europe (3500 - 1000 BC).

The Phoenicians carried wine varieties to Greece, Rome and southern France before 600 BC and Romans spread the grape throughout Europe. Grapes moved to the Far East via traders from Persia and India (Novello, 1999).

Grapevine (*Vitis vinifera* L.) is one of the most widely grown fruit crops in the world (Molina, 2007). Grape as an ancient food in the life and history of humankind over several millennia has increased production because the fresh fruit is nutritious for humans. They can be eaten fresh as table grapes or enjoyed in a variety of products such as juice, jelly and the ultimate processed grape product, wine produced from the controlled fermentation of grape juice (Crassweller, 2008). Therefore, it is widely used in the production of juice and wine, and an increase in industrial raw materials for developing new food products.

2.3. Ecological requirement of grape production

There are different factors that can affect grapevine production all over the world (Winkler *et al.*, 1974). The major one that interferes with grape production is a temperature. Grapevine is heat loving plants which require a hot and dry climate during its growth and fruiting periods. It well thrives under temperature ranges from 15- 40^{O}C. Temperatures above the optimal at the time of fruit growth and development highly affect the size of the fruits while temperatures below the minimum leads the crops not to bear fruits (Crassweller, 2008). There are also other factors like; rainfall, humidity, wind, soil type and pH etc. which reduce its production (Amerine *et al*., 1967). The mean annual rainfall suitable for its cultivation is 400 mm. However, at the time of flowering and fruit ripening high rainfall and humidity are favorable for disease development, particularly fungal attack. Sustained efforts to ensure high, reliable grapevine yields are usually threatened with damage to production caused by numerous diseases (Martelli, 1993). The causal agents of these diseases are fungi, bacteria, viruses and nematodes. Fungal diseases affect leaves, shoots and fruit of the grape and the common fungal grapevine diseases include powdery mildew, downy mildew and black rot (Dan *et al*., 2008).

2.4. Conventional propagation techniques of grapevines

Traditional grape propagation techniques utilize mother vines, from which dormant cuttings are taken for rooting, bench grafting or field budding.

Mother vines are generally planted from dormant rooting or potted plants and require about four to five years to produce generous amounts of cuttings (Winkler, 1974). When new clones or varieties are released from breeders or from quarantine programs, there is very little wood available for distribution and a variety of techniques have been employed to produce commercial amounts of planting stock over a short period of time. Mainly grape is a plant propagated by seed or grafting. However, seed grown plants are genetically very heterogeneous (Winkler, 1974).

Vegetative propagation is slow and only a limited number of plants can be grown from a stock plant due to the seasonal responsiveness and inability to rooting. Moreover, rooting ability is strongly influenced by plant genotype. Grafting transfer disease from their mother plants to newly growing one (Crassweller, 2008).

2.5. Tissue culture of grapevines

Many of the *in vitro* propagation techniques were developed by vine improvement associations to speed up the release of material and to avoid the spread or infection of certified material with pests and diseases (Gray and Fisher, 1986). Genetic improvement of classic cultivars in order to obtain high quality wine and table grape varieties through conventional hybridization methods does not appear to be practical and therefore unconventional approaches have been proposed (Stamp *et al.*, 1990) and when planting stock of new varieties, clones or root stocks is limited, *in vitro* propagation techniques can speed up release and offer large economic advantages (Andrew *et al.*, 1999). Many of these techniques are labor and technology intensive and their cost may not compete with that of traditional methods of propagation when source materials are not limited.

Micro-propagation is a tool used to propagate genotypes by applying *in vitro* culture methods. Depending on the plant species and culture conditions, tissue culture may enable the mass production of genetically uniform populations from elite (e.g. high yielding or disease resistant) individuals in a short period of time (Arya *et al.*,1962).

Many authors have indicated that the ideal composition of grapevine culture medium depends on the varieties in question so that the results obtained with one genotype in a given medium may differ from those obtained with other genotypes (Ibariez and Morte, 2005; Jaskani *et al.*, 2008).

The study done by Baker & Bhatia (1993) has shown that variation in the ammonium content of the medium affected somatic embryogenesis in different cultivars of grapevine. Similarly variation in pH has also been shown to affect embryogenesis and organogenesis in *Vitis vinifera* (Bornhoff and Harst, 2000).

Micro-propagation of selected *Vitis* genotypes can be carried out among others, by the culture of total or fragmented shoot apical meristems, axillary bud or through adventitious bud development (Monette, 1988). Somaclonal variation is undesirable in applications pointing to reproduce selected elite genotypes, e.g., ancient and rare clones, pathogen resistant or drought/salinity tolerant genotypes and it is caused by high levels of plant growth regulators (PGRs), mainly cytokinins, usually useful to promote shoot multiplication and thus increase yield (Gray *et al.*, 2005).

Therefore, regeneration of whole plants by tissue culture has been intensively studied. A prerequisite to develop such approaches for grapevine is the ability to establish efficient *in vitro* regeneration techniques (Molina, 2007). Hence, micro-propagation is capable of producing disease free plant material faster than conventional cloning methods all year round.

Different grapevine genotypes do not give the same response in all culture conditions that is why different genotypes of the crops are reported differently (Qiu *et al*., 2004). So that tissue culture is one of the alternative methods for the production of selected superior plants in a short period of time at high multiplication frequency.

2.5.1. Media composition

Plant tissue culture media should generally contain macronutrients, micronutrients, vitamins, amino acids or nitrogen supplements, source of carbon, growth regulators and solidifying agents. According to the International Association for Plant Physiology, the elements in concentrations greater than 0.5 mM are defined as macroelements and those required in concentrations less than 0.5 mM as microelements (Fossard, 1976).
It should be considered that the optimum concentration of each nutrient for achieving maximum growth rates varies among species.

Macronutrients

The essential elements in plant cell or tissue culture media include, besides C, H and O, macroelements: Nitrogen (N), Phosphorus (P), Potassium (K), Calcium (Ca), Magnesium (Mg) and Sulphur (S) for satisfactory growth and morphogenesis. Culture media should contain at least 25-60 mM of inorganic nitrogen for satisfactory plant cell growth. Potassium is required for cell growth of most plant species. Most media contain K in the form of nitrate chloride salts at concentrations ranging between 20 and 30 mM. The optimum concentrations of P, Mg, S and Ca range from 1-3 mM if other requirements for cell growth are provided (Torre, 1989).

Micronutrients

The essential micronutrients (minor/trace elements) for plant cell and tissue growth include Iron (Fe), Manganese (Mn), Zinc (Zn), Boron (B), Copper (Cu) and Molybdenum (Mo). Iron is usually the most critical of all the micronutrients. The element is used as either citrate or tartarate salts in culture media. However, there exist some problems with these compounds for their difficulty to dissolve and precipitate after media preparation. There has been trials to solve this problem by using ethylene diamine tetra acetic acid (EDTA) iron chelate (FeEDTA). A procedure for preparing an iron chelate solution that does not precipitate has also been developed. Cobalt (Co) and Iodine (I) may be added to certain media, but their requirements for cell growth has not been precisely established. Copper and Cobalt are added to culture media at concentrations of 0.1μM, Iron and Molybdenum at 1μM, Iodine at 5μM, Zinc at 5-30 μM, Manganese at 20-90 μM and Boron at 25-100 μM (Torres, 1989).

Carbon and energy sources

In plant cell culture media the sucrose is frequently used as carbon source at a concentration of 2-5%, other carbohydrates are also used.

These include lactose, galactose, maltose and starch, and they were reported to be less effective than either sucrose or glucose, the latter was similarly more effective than fructose considering that glucose is utilized by the cells in the beginning followed by fructose. It was frequently demonstrated that autoclaved sucrose was better for growth than filter sterilized sucrose.

Autoclaving seems to hydrolyze sucrose into more efficiently utilizable sugars such as fructose. Sucrose was reported to act as morphogenetic trigger in the formation of auxiliary buds and branching of adventitious roots (Vinterhalter *et al*., 1997).

Vitamins and Myo-inositol

Some plants are able to synthesize the essential requirements of vitamins for their growth. Some vitamins are required for normal growth and development of plants. They are required by plants as catalysts in various metabolic processes. They may act as limiting factors for cell growth and differentiation when plant cells and tissues are grown *in vitro* (Torres, 1989).The vitamins that mostly used in the cell and tissue culture media include: Thiamin (B1), Nicotinic acid, Glycine and Pyridoxine (B6). Thiamin is necessarily required by all cells for growth (Ohira *et al*., 1976). Thiamin is used at concentrations ranging from 0.1 to 10 mg/l. Nicotinic acid and pyridoxine, however; not essential for cell growth of many species. They are often added to culture media. Nicotinic acid is used at a concentration range 0.1-5 mg/l and pyridoxine is used at 0.1-10 mg/l. It was recommended that vitamins should be added to culture media only when the concentration of thiamin is below the desired level or when the cells are required to be grown at low population densities. Although it is not a vitamin but a carbohydrate, myo-inositol is added in small quantities to stimulate cell growth of most plant species (Vasil *et al*., 1998). Myo-inositol is believed to play a role in cell division because of its break down to ascorbic acid and pectin and incorporation into phospho inositides and phosphatidylinositol. It is generally used in plant cell and tissue culture media at concentrations of 50-5000 mg/l.

Gelling agents

Gelling or solidifying agents are commonly used for preparing semi solid or solid tissue culture media. In static liquid cultures the tissue or cells become submerged and die due to lack of oxygen, thus calling upon such gels that would provide a support to the growing tissues under static conditions. The most common of these include agar-agar, as well as others that are commonly known as gelatin, gelrite, alginate and phytagel etc.

Agar: A polysaccharide obtained from seaweed has several advantages over other gelling agents. Agar has several advantages over other gelling agents; mixed with water, it easily melts in a temperature range 60-100^{o}C and solidifies at approximately 45^{o}C and it forms a gel stable at all feasible incubation temperatures.

Agar gels do not react with media constituents and are not digested by plant enzymes. It is commonly used in media at concentrations ranging between 8-10g/l. They also contain phenolic substances and less pure grades may contain long chain fatty acids, inhibitory to the growth of some bacteria. As agar can be the most expensive component of plant media, there is interest in minimizing its concentration. Concentrations of agar can be considered inadequate if they do not support explants or lead to hyperhydricity. Hyperhydricity decreases as the agar concentration is raised but there may be an accompanying reduction in the rate of growth (Scherer *et al*., 1988). Other compounds successfully tested include biogel (polyacrylamide pellets) alginate, phytagel and Gelrite. The advantage of working with synthetic gelling compounds is that they form clear gels at relatively low concentrations (1.25–3.0 gm/l) and are valuable aids for detecting contamination that may develop during the span of cultures. It is also possible to have a look at roots of the plantlets and count them in the media.

Plant Growth regulators

Plant growth regulators are important in plant tissue culture since they play vital roles in cell division, elongation, growth and apical dominance. They are generally classified into auxins, cytokinins, gibberellins and abscisic acid. Moreover, proportion of auxins to cytokinins determines the type and extent of organogenesis in plant cell cultures.

Auxins: The common auxins used in plant tissue culture media include: Indole-3- acetic acid (IAA), Indole-3- butric acide (IBA) and Naphthalene- acetic acid (NAA). IAA and IBA are natural auxin occurring in plant tissues.

In tissue cultures, auxins are usually used to stimulate callus production and cell growth, to initiate shoots and rooting, to induce somatic embryogenesis, to stimulate growth from shoot apices and shoot stem culture. NAA is considered to be stable and can be stored at 4^{o}C for several months (Gamborg *et al*., 1876).

Cytokinins: Cytokinins commonly used in culture media include BAP (6-benzyloaminopurine), 2iP (6-dimethylaminopurine), kinetin (N-2-furanylmethyl-1H-purine-6-amine), Zeatin (6-4-hydroxy-3-methyl-trans-2-butenylaminopurine) and TDZ (thiazuron-N-phenyl-N-1,2,3 thiadiazol-5ylurea). Zeatin and 2iP are naturally occurring cytokinins and zeatin is more effective.

In culture media, cytokinins proved to stimulate cell division, induce shoot formation and axillary shoot proliferation and to retard root formation. Cytokinins are frequently reported to be difficult to dissolve and sometimes addition of few drops of 1N HCl or 1N NaOH facilitate their dissolution.

Gibberellins: Gibberellins comprise more than twenty compounds, of which GA3 is the most frequently used gibberellins. These compounds enhance growth of callus and help elongation of dwarf plantlets. Other growth regulators are sometimes added to plant tissue culture media as abscisic acid, a compound that is usually supplemented to inhibit or stimulate callus growth depending upon the species. It enhances shoot proliferation and inhibits later stages of embryo development (Anagnostakis *et al*., 1974). Although growth regulators are the most expensive medium ingredients, they have little effect on the medium cost because they are required in very small concentrations.

2.5.2. Selection of mother plants

Plant tissue culture technique, which involves propagation of plants in a laboratory, allows for rapid production of clean, disease free, and vigorous plant material in a shorter time period compared to conventional propagation techniques. In order to get suitable explants at a time, appropriate establishment of mother plant is the priority consideration for every successful mass propagation program.

The genotypes may have been selected deliberately from a grapevine that has demonstrated desirable traits (good yields, disease resistance, desirable berry sizes, etc.) and propagated as explants taken from that mother vine (Robinson, 2006).

2.5.3. *In vitro* initiation and multiplication of grapevines

The procedures of plant tissue culture have developed to such a level that any plant species can be regenerated *in vitro* through several methodologies. Plant cells retain the ability to change to a meristematic state and differentiate into a whole plant if it has retained an intact membrane system and viable nucleus.

In Plant tissue culture more often we use explants to initiate their growth in culture. The non-dividing, differentiated, quiescent cells of the explants first undergo changes to achieve meristematic state when grown on a nutrient medium.

Skoog and Miller (1957) suggested that organ formation is controlled by quantitative interaction of different substances in their proportion rather than absolute concentration of substances. Cytokinin in the medium leads to the promotion of bud differentiation and development. Effect of different concentrations of cytokinin with auxin studied on establishment stage for shoot tip explants cultured *in vitro* had attributed to the mode of action of BAP as cytokinins at1.0mg/l on stimulation both cell division and promotes growth of axillary shoots in plant tissue as reported by Tamas (1987).

Auxins are capable to control various distinctive processes such as promotion of stem elongation and growth but, they are not effective against shoot proliferation (Goussard, 1981). On the other hand, the shoot proliferation depends upon the balance of cytokinins and auxins. Addition of low level of auxins had positively affected the initiation of grapevines *in vitro* (Tapia *et al*., 1998). Torrey and Reinert (1961) had reported that auxin increases the activation enzymes that could break down starch and has the ability to move the active leading to increase proliferation of organogenesis. Aazami (2010) had reported that the study of the effects of the four hormonal treatments on the shoot production in the two cultivars showed that BA at 1.5mg/l concentration was the best for shoot proliferation in both '*Soltanin*' and '*Sahebi*' cultivars. The presence of BA, even at relatively low levels (i.e., 2.5 and 5.0 μM) enhanced bud multiplication. At relatively high BA concentrations (>10.0 μM) axillary bud proliferation was apparent while at the same time the growth of the main shoot was suppressed (Banilas *et al*., 2007). Beza (2010) report indicated that among different BAP concentrations used on shoot initiation, 0.5 mg/l BAP produced maximum number of shoots (5.6) for Canonannon then followed by Ugnin blanc (5.3) and Chenin blanc (5.0). The maximum shoot height was attained at 1.0mg/l BAP for Chenin blanc while Ugni blanc and Canonannon varieties gave the maximum height on hormone free medium. Similarly, the combination of at 1.0mg/l BAP with 0.1mg/l IBA gave the maximum shoot number (7.2, 6.7 and 5.2) for Chenin blanc, Canonannon and Ugnin blanc respectively on shoot multiplications.

In vitro rooting and acclimatization

When grapevines are cultured on growth regulators free MS media, they developed rooting but root mass showed a decline as the media contained higher concentration of cytokinins (Ghulam *et al*., 2006).

Thus, cytokinin was found to inhibit the root induction. However, IAA and NAA in the media containing BAP (0.2 mg/l) promoted root induction (Ghulam *et al*., 2006). NAA proved to be better in root induction as compared to IAA in different grape vines *in vitro* propagation.

The combination of different auxin levels on rooting had showed significant effects. The combination of IBA and NAA at 1.0 and 0.5 mg/l resulted in the highest mean value 87% for rooting percentages (Abido *et al.*, 2013). The obtained results showed that the used auxin (NAA and IBA) produced the best results in almost all studied traits. These results could be explained on the bases that auxin induced number of responses which involved cell division, cell enlargement, protein and nucleic acids synthesis which are concomitants of auxin induced growth and changes in wall plasticity of plant cell and increase the apical dominance as there are essential and rapid processes involved in growth and elongation (Abido *et al*., 2013). The work done by Beza (2010) using different IAA concentration on root formations gave maximum number of roots (4) and (5.2) for Ugni blanc and Canonannon on 4.0mg/l IAA. For Chenin blanc maximum number of root (3.7) was obtained at 2.0mg/l IAA. Concerning the length of the roots, Chenin blanc and Canonannon induced a maximum length of 9.7 and 8.6 cm at 2.0 and 4.0mg/l IAA, respectively while the maximum root length (6.2 cm) was obtained on 2.0 mg/l IAA for Ugni blanc variety.

A successful tissue culture method of propagation must result in re establishment in soil of high frequency of the tissue culture derived plant. The work done by Thomas (1998) illustrated that survival percentage of plantlets was 50-70% after kept plantlets in rooting medium for 14 days before acclimatization, but; as rooted plantlets were kept in the rooting media for long time (35 days) the survival percentage was increased to 80-90%.

3. MATERIALS AND METHODS

3.1. Plant material

Black Corinth is the cultivar that was released as raisin variety. Its yield production at Debre Zeit Research Centre was 8.5tons/ha/year and mostly used as dried berries of the small, black seeded sweet seedless cultivar. It can be eaten raw, especially when ripe when it is sweet to the taste. It may also be referred as table grapes for this purpose.

3.2. Ex-plant source and preparation

The cultivar was obtained from Debre Zeit Research Center, Ethiopian Institute of Agricultural Research. The explants were taken from field grown plants. Initial explants comprised intact nodes; bearing dormant buds were harvested from the middle part of the shoots.

The explants were washed under tap water with 3 drops of Tween-20 for 15 minutes. Subsequently, they were immersed in 70% ethanol for one minute and rinsed using sterilized double distilled water. Finally, the explants were surface sterilized with 1.0% of sodium hypochlorite (NaOCl) solution containing two drops of Tween-20 for 15 minutes. Then they were rinsed three times for 5 minutes each using sterilized double distilled water (Banilas *et al*., 2007). Axillary buds having a length of 10-12 mm were used for culturing.

3.3. Media preparation

MS (Murashige and Skoog 1962) nutrient medium with its full macro, micro, vitamins and iron source compositions was used to prepare stock solution. The stock solutions were prepared by weighing the powder of each elements/nutrient and dissolving them in double distilled water and stored in a refrigerator at $+4^{o}C$ temperatures (Appendix 4).

The growth regulators (BAP, NAA & IBA) were prepared by dissolving first in 1N NaOH and finally adjusting the volume by sterilized double distilled water in a 1: 1 ratio (1mg/1ml) and then kept in a refrigerator at $4^{o}C$.

Culture medium was prepared by taking appropriate amount of each stock solution (ml) in one liter (1L) of medium (Appendix 4). Sucrose at 3% was added.

The pH was adjusted to 5.8 using 1N NaOH and/or 1 N HCl. Then 3g gelrite was added in 1littre of media for culture establishment and shoot multiplications, while 1.5g gelrite was used for root formation. The media was put on hot heat stirrer plate until the gelrite dissolved and becomes transparent. Then after 40 ml medium was dispensed into plastic culture jars using dispenser, finally the medium was autoclaved at a temperature of 121^{o}C with a pressure of 105 kpa for 15 minutes and all media cultures were kept in dark room.

3.4. Experiment one: Effect of different concentrations of BAP on shoot initiation

The sterilized explants were cultured on basal MS medium augmented with various concentrations of BAP (0.2, 0.6, 1.0, 1.5 and 2.0 mg/l) in 0.1mg/l NAA. The experiment was replicated three times in Completely Randomized Design (CRD). Five explants were used per jar. Subculture was done at 15th and 30th days. Data on percentages of explants showing shoot initiation, number of shoots/ex-plant and shoot height (cm)/ex-plant were collected after 4 weeks of culture.

3.5. Experiment two: Effect of different concentrations of BAP and NAA combinations on shoot multiplications

Uniform explants from initiation stage of about 3-4 cm length were sub cultured on fresh MS medium containing BAP (1.0, 2.0, 2.5, 3.0 and 3.5 mg/l) in combination with NAA (0.1, 0.2 and 0.3 mg/l). The experiment was replicated three times with factorial arrangement in Completely Randomized Design (CRD). Three shoots were used per jar. Data were collected for number of shoots formed and shoot height (cm) per propagules after 30 days of culture.

3.6. Experiment three: Effect of different concentrations of IBA and NAA combinations on root formations

To achieve root formation, shoots resulted from multiplication stage were excised into 3-4 cm length and re-cultured on hormone free MS medium for a week. Then, the shoots were transferred onto MS media with combinations of IBA (0.0, 0.8, 1.2, 1.6, and 2.0mg/l) and NAA (0.0, 0.2, 0.5, 0.8 and 1.0mg/l). The experiment was replicated three times with factorial arrangement in Completely Randomized Design (CRD).

Three shoots were used per jar. Data were collected for percentage of rooted shoots, number of roots/shoot and length of root (cm) and data were recorded after 35 days.

Acclimatization: The rooted shoots (plantlets) were kept in their media for 35 days without subculture before acclimatization to enhance rooting efficiency. Newly formed plantlets were washed thoroughly to remove any medium residual. Then the plantlets were treated with 1gm/l koside (anti fungi) and potted into sterilized mixture of peatmass1:1sand (v/v) then covered with a white plastic bag to maintain high humidity. The plantlets were transferred to greenhouse for gradual acclimatization and after 20 days the transparent plastic bags were removed. Finally, the data of survival percentage was taken 15 days after removal of plastic covering bags.

Data analysis: Data was analyzed using SAS 9.2 version software. Least Significance Difference (LSD) was used for mean separation at $p< 0.05$ probability level.

4. RESULTS AND DISCUSSION

4.1. Effect of Different Concentrations of BAP in Combination with 0.1mg/l NAA on Shoot Initiation

Results on effect of different concentrations of BAP in combination with 0.1mg/l NAA are presented in Table1 below. The analysis of variance indicated that the effects of BAP concentrations were significantly different for all characters (Appendix 1). The mean values for percentage of explants showing shoot initiation were significantly different ($P<0.0002$). The mean values for number of shoots/ex-plant were also significantly different ($P<0.0006$). Similarly, the mean values for shoot height differed significantly ($P<0.0001$).

The highest (80.00±0.00) percentages of explants showing shoot initiation were obtained at 0.6mg/l and1.0mg/l BAP whereas maximum (1.72±0.05) number of shoots /ex-plant was recorded at 1.5mg/l BAP (Table1, Figure 2: B,C and D). In the same way, the maximum height (3.22±0.26 cm) of the shoots was recorded at1.0mg/l BAP.

Table 1: Effect of different concentrations of BAP in combination with 0.1mg/l NAA for shoot initiation of Black Corinth cultivar

BAP concentration (mg/l)	Percentage of explants Showing initiation (Mean±SD)	No. of shoots per ex-plant (Mean±SD)	Shoot heights (cm) (Mean±SD)
0.2	40.00^{b}±0.00	1.50^{ab}±0.00	2.45^{c}±0.05
0.6	80.00^{a}±0.00	1.25^{bc}±0.00	2.81^{b}±0.25
1.0	80.00^{a}±0.00	1.00^{c}±0.00	3.22^{a}±0.26
1.5	73.33^{a}±11.54	1.72^{a}±0.05	2.85^{b}±0.13
2.0	53.33^{b} ±5.77	1.27^{bc}±0.25	2.08^{d}±0.16
CV	**9.68**	**8.62**	**5.33**

Means with the same superscript letter in the same column are not significantly different at $p< 0.05$ probability level, CV= Coefficient of Variation

Regarding the percentages of explants showing initiation, the BAP concentrations at 0.6mg/l and 1.0mg/l and 1.5mg/l gave similar and better results. Even though there was no statistical difference among 0.6mg/l, 1.0mg/l and 1.5mg/l, BAP at 0.6mg/l could be taken as the best option economically as it reduces the cost of the growth regulator.

The minimum (40.00±0.00) percent of shoots showing initiation was observed at 0.2mg/l BAP (lowest concentration). In this study, it was found that as the concentration of BAP increased from 0.2mg/l to 1mg/l, the percentages of explants showing shoot initiation also increased from 40.00±0.00 to 80.00±0.00 and then starts declining as the concentrations kept increasing to 2mg/l BAP.

With respect to the number of shoots/ex-plant, treatments were significantly different in which the highest value (1.72±0.05) was obtained at 1.5mg/l BAP while the minimum (1.00±0.00) was recorded at 1.0mg/l BAP.

For shoot height, the treatments differed significantly, and the maximum (3.22±0.26) and minimum (2.08±0.16) shoot heights were obtained at 1.0mg/l and 2.0mg/l concentrations respectively. For this trait, as the BAP concentrations increased, the shoot height was also increased till it reached to the optimum (1.0mg/l BAP) and reduced as the BAP concentration kept increasing. This result is consistent with previous study on *in vitro* propagation of grapevine (*Vitis vinifera* L.) 'Muscat of Alexandria' genotype by Abido *et al*. (2013), *who* reported that BAP at 1.0mg/l concentration was the best for shoot initiation. The current results are also in agreement with those obtained by Mhatre *et al.* (2000) and Singh *et al.* (2004). This finding could be attributed to the mode of action of BAP as cytokinins promote growth of axillary buds/shoot at low level of NAA concentration in combination with BAP as reported by Tamas (1987).

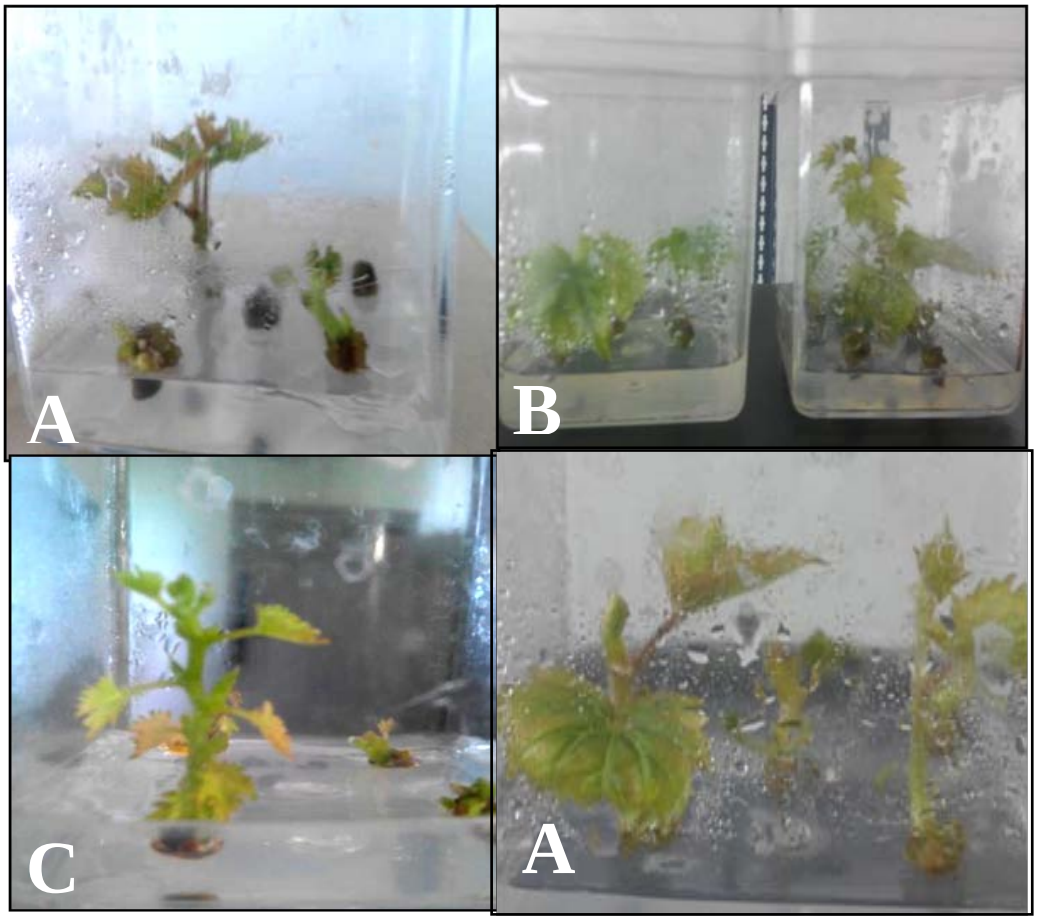

Figure 2: Shoot initiation from axillary buds in different BAP concentrations; **A** = 0.2mg/l, **B** = 0.6mg/l, **C** = 1.0mg/l, **D** = 1.5mg/l BAP in 0.1mg/l NAA

4.2. Effect of Different Concentrations of BAP and NAA Combinations on Shoot Multiplication

The analysis of variance shows that BAP and NAA combinations were significantly different for number of shoots/ex-plant and shoot height ($P<0.0001$) (Appendix 2).

The interaction effect of BAP and NAA on shoot multiplication showed that the best performance (2.67±0.14) for number of shoot/ex-plant was obtained on 3.0 mg/l and 0.2mg/l BAP and NAA concentration respectively (Table 2). Whereas the minimum (1.38±0.03) number of shoots/ex-plant was recorded at the highest concentrations (3.5mg/l BAP and 0.3mg/l NAA).

Table 2: Effect of different concentrations of BAP and NAA combinations for shoot multiplication of Black Corinth cultivar

Conc. of PGR (mg/l)		Characters	
		No. Shoot/ex-plant	Shoot height (cm)
BAP	NAA	Mean±SD	Mean±SD
1	0.1	1.50^{f}±0.00	1.72^{f}±0.05
1	0.2	2.07^{bc}±0.11	3.23^{a}±0.25
1	0.3	2.25^{b}±0.00	2.61^{b}±0.16
2	0.1	2.00^{c}±0.00	2.04^{de}±0.07
2	0.2	2.03^{c}±0.06	2.17^{cd}±0.06
2	0.3	2.23^{b}±0.03	2.13^{cd}±0.11
2.5	0.1	2.58^{a}±0.15	2.67^{b}±0.07
2.5	0.2	1.95^{cd}±0.09	1.77^{f}±0.07
2.5	0.3	1.70^{e}±0.00	1.57^{f}±0.08
3	0.1	1.81^{de}±0.03	2.33^{c}±0.14
3	0.2	2.67^{a}±0.14	1.61^{f}±0.10
3	0.3	2.08^{bc}±0.03	1.60^{f}±0.01
3.5	0.1	2.00^{c}±0.00	1.71^{f}±0.07
3.5	0.2	2.21^{b}±0.03	1.85^{ef}±0.05
3.5	0.3	1.38^{f}±0.03	1.60^{f}±0.00
CV.		**3.02**	**5.36**

Means with the same superscript letter in the same column are not significantly different at 0.05 probability level, CV= Coefficient of variation

This result is in agreement with Abido (2013) who reported optimum concentrations at 3.0 and 0.2mg/l BAP and NAA respectively.

Augmenting the culture medium with BAP at 1.0mg/l and 0.2mg/l NAA showed highest value (3.23±0.25) shoot height which means the optimal concentration for this particular growth regulator, whereas any concentration above or lower than this concentration resulted in poor performance for this character (Table 2, Figure 3: A and C). This is in agreement with the works of Banilas and Korkas (2004) who reported similar results on micro-propagation of grapevine through lateral bud development. As the levels of BAP increased (>2.5mg/l) the shoots showed stunted and distorted growth. This is due to plant cells response to the hormones induced synthesis of few minor RNAs and some proteins that are inhibitors of respiration, transcription and translation which inhibit cytokinin mediated cell enlargement as reported by Gottieb and Skoog, (1954).

This is attributed to the mode of action of cytokinins on stimulation both cell division and promotion of growth of axillary buds in plant tissue culture as reported by Gray *et al*., (1986). It could be inferred from the above results that NAA and BAP combinations at the appropriate balance were rewarding in many fruit tree species as reported by Zimmerman and Swartz (1994).

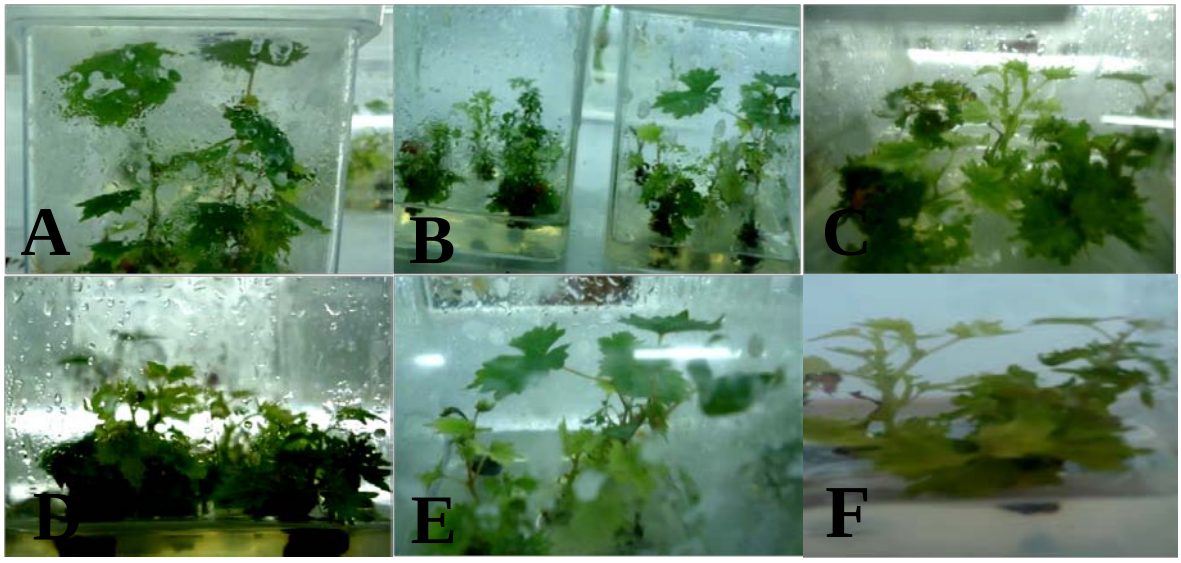

Figure 3: Different concentrations of BAP in combination with NAA performed best on shoot multiplications

Key; **A**=1.0mg/l BAP with 0.2mg/l NAA, **B**=1.0mg/l BAP with 0.3mg/l NAA, **C**=3.0mg/l BAP with 0.2mg/l NAA, **D**=2.0mg/l BAP with 0.3mg/l NAA, **E**=3.0mg/l BAP with 0.1mg/l NAA, **F**=1.0mg/l BAP with 0.1mg/l NAA

4.3. Effect of Different Concentrations of IBA and NAA Combinations on Root Formation

The analysis of variance indicated that the effects of IBA and NAA combinations were significantly different on all traits under the study (Appendix Table 3).

Data presented in Table 3 showed the mean values for percentage of rooted shoots, number of roots per shoot and root length. The mean values for percentage of rooted shoots were significantly different ($P<0.0001$). The means values for numbers of roots per shoot were also significantly different ($P<0.0001$). Similarly, the means values for root length differed significantly ($P<0.0001$).

The interaction effect of growth regulators showed highly significant differences on percent of rooted shoots. The combination of IBA and NAA at 0.8 and 0.5 mg/l, 1.2 and1.0 mg/l, 1.6 and 1.0mg/l, 2.0 and 0.5mg/l, and 2.0 and 1.0mg/l resulted in the highest value (100.00±0.00) for percentage of rooted shoots while 1.6mg/l IBA with 0.2mg/l NAA showed the least mean value (55.55±0.00) for this character (Table 3, Figure 4). Similarly, the main effect of IBA and NAA had also showed that significant differences on percent of rooted shoots. IBA at 0.8, 1.6 and 2.0 mg/l, and NAA at 0.5 and 0.8 mg/l gave the maximum (100%) for rooting percentages. Increasing NAA from 0.2mg/l to 1.0mg/l keeping IBA at 1.2mg/l and 1.6 mg/l showed a significant increase in the percent of rooted shoots from 69.44±9.62 to 100.00±0.00 and from 55.55±0.00 to 100.00± 0.00 respectively. These results are similar to that of Lewandowski (1991), Barreto *et al*. (2007) and Butiuc-keul *et al*. (2009b) who reported up to 95% *in vitro* rooting in grapevine, "Perlette" cultivar on MS medium supplemented with IBA and NAA.

Table 3: Effect of different concentrations of IBA and NAA combinations on root formation of Black Corinth cultivar

PGRs Concentrations		Characters		
		Rooting percentage	NRPS	Root length(cm)
IBA (mg/l)	**NAA (mg/l)**	Mean±SD	Mean±SD	Mean±SD
0.0	0.0	64.46^{bc}±3.86	4.44^{m}±0.10	3.93^{f}±0.11
0.0	0.2	88.88^{ab}±11.12	5.55^{l}±0.95	3.18^{hij}±0.09
0.0	0.5	100.00^{a}±0.00	$7.44f^{ghi}$±0.10	3.66^{fg}±0.02
0.0	0.8	100.00^{a}±0.00	$6.88g^{hijk}$±0.38	2.22^{mn}±0.08
0.0	1.0	66.64^{bc}±0.03	7.00^{ghij}±0.00	2.45^{lm}±0.04
0.8	0.0	100.00^{a}±0.00	7.3^{ghij}±0.05	2.02^{n}±0.01
0.8	0.2	88.88^{ab}±11.12	11.55^{ab}±0.50	3.18^{hij}±0.04
0.8	0.5	100.00^{a}±0.00	5.77^{kl}±0.38	4.48^{e}±0.02
0.8	0.8	66.66^{bc}±0.00	8.77^{def}±0.38	5.12^{abc}±0.09
0.8	1.0	58.33^{c}±0.00	6.16^{jkl}±0.28	3.26^{hi}±0.20
1.2	0.0	66.67^{bc}±0.02	5.83^{kl}±0.3	4.68^{de}±0.04
1.2	0.2	69.44^{bc}±9.62	6.33^{ijkl}±0.33	2.98^{ijk}±0.03
1.2	0.5	77.77^{abc}±19.24	7.72^{fgh}±0.25	2.42^{lm}±0.16
1.2	0.8	96.00^{a}±3.92	11.11^{b}±0.84	3.52^{gh}±0.02
1.2	1.0	100.00^{a}±0.00	11.66^{ab}±0.33	2.87^{jk}±0.22
1.6	0.0	100.00^{a}±0.00	9.33^{cd}±0.33	5.30^{ab}±0.17
1.6	0.2	55.55^{c}±0.00	8.11^{efg}±0.19	4.98^{bcd}±0.05
1.6	0.5	77.77^{abc}±19.24	9.16^{cde}±0.28	5.39^{a}±0.19
1.6	0.8	88.88^{ab}±11.12	5.61^{lk}±0.34	2.47^{lm}±0.15
1.6	1.0	100.00^{a}±0.00	7.22^{ghij}±0.19	2.28^{mn}±0.04
2.0	0.0	100.00^{a}±0.00	10.00^{c}±0.00	4.73^{de}±0.20
2.0	0.2	66.64^{bc}±0.03	12.50^{a}±0.50	2.75^{kl}±0.11
2.0	0.5	100.00^{a}±0.00	9.19^{cde}±0.50	5.21^{abc}±0.20
2.0	0.8	69.45^{bc}±0.00	6.78^{hijk}±0.40	4.89^{cd}±0.17
2.0	1.0	100.00^{a}±0.00	9.21^{cde}±0.50	3.41^{gh}±0.26
	CV	**5.10**	**4.96**	**3.66**

Means with the same superscript letter in the same column are not significantly different at $p<0.05$ probability level, PGRs= Plant growth regulators, NRPS=Number of roots per shoot, CV= Coefficient of variation

Regarding number of roots/shoot, the interaction effect showed highly significant effect on the character. Increasing NAA from 0.2mg/l to 1.0mg/l keeping IBA at 1.2mg/l showed a significant increase in number of roots per shoot from 6.33±0.33 to 11.66±0.33.

Concentration of IBA and NAA at 2.0mg/l and 0.2mg/l respectively resulted in the highest (12.50±0.50) number of roots/shoot. The least response (4.44±0.10) was recorded with the PGR free medium (control). This result is contrary to that of Abido *et al*., (2013) who reported (3.4) roots/shoot for 'Muscat of Alexandria' genotype at 1.0mg/l IBA with 0.5mg/l NAA.

The interaction effect of growth regulator had exerted significant differences on root length (cm). The highest (5.39±0.19) root length was recorded at 1.6mg/l IBA and 0.5mg/l NAA. The results of the present study showed that the auxins (IBA and NAA) used, gave the best result for the character under the study. These results could be explained on the bases that auxin induced number of responses which involved cell division, cell enlargement, protein and nucleic acids synthesis which are naturally accompanying of auxin induced growth and changes in plant cell and increase the apical dominance as there are essential and rapid processes involved in growth and elongation (Wilkins,1989).

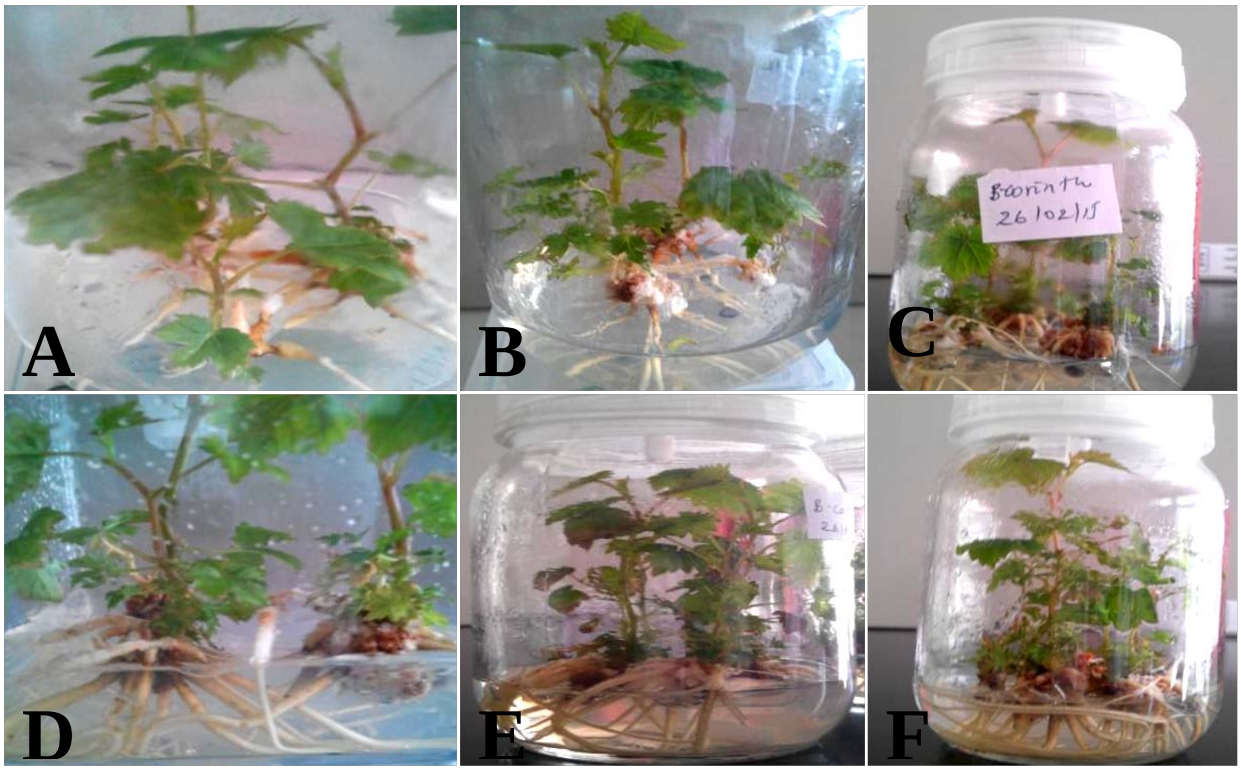

Figure 4: Different IBA and NAA concentrations performed best in root formations

Key; A=0.0mg/l IBA in 0.5mg/l NAA, **B**=0.0mg/l IBA in 0.8mg/l NAA, **C**=0.8mg/l IBA in 0.2mg/l NAA, **D**=1.2mg/l IBA in 1.0mg/l NAA, **E**=1.6mg/l IBA in 0.5mg/l NAA, **F**=2.0mg/l IBA in 0.2mg/l NAA

Acclimatization

Among transferred newly formed plantlets with satisfactory shoot and roots, 80% were successfully acclimatized and cultivated in greenhouse (Figure 5). In this study the newly formed plantlets remained on the rooting media for 35 days to enhance the rooting efficiency of the plantlets. This result is similar to that of Thomas (1998) who found that 80-90% successfully acclimatized plantlets transferred to sterilized soil after 34 days on the rooting media. It is clear that leaving rooted plantlets on the rooting medium for longer period of time (optimum period) increased the efficiency of roots which led to the increase of the survival percentages of acclimatized plantlets.

Figure 5: Acclimatization in greenhouse

A= first week of acclimatization (as covered)
B= at 20th day of acclimatization
C= 15 days after covering plastic bag has been removed
D= 30 days after covering plastic bag has been removed

5. SUMMARY AND CONCLUSION

The conventional propagation techniques of grapevine utilize mother vines which are generally planted from dormant rooting or potted plants and require about four to five years to produce enough amounts of cuttings in terms of their woody production. When new clones or varieties are released from breeders or from quarantine programs, there is very little planting materials available for distribution and variety of techniques have been employed to produce commercial amounts of planting stock over a short period of time. The most important aspect of *in vitro* propagation techniques is its ability to quickly provide relatively large amounts of planting stock to grape growers and especially, it is very important when plant materials are limited due to the scarcity of a clone or variety. *In vitro* propagation of grapevine can be carried out by the culture of intact or fragmented shoot apical meristems, axillary buds or through adventitious bud formation. The degree of response to certain culture is highly depends on the particular genotype and part of organ used as ex-plant. Several researchers have used different plant organs as explants for *in vitro* propagation of different *Vitis vinefera* genotype. But there is limited information on using axillary buds as explants for such work in Ethiopia. Therefore, this study was initiated with the objective of optimizing a protocol for *in vitro* propagation of a selected genotype using axillary bud.

Black Corinth is a cultivar that was used for this study as a mother plant from which the explants were harvested. The explants comprised intact nodes; bearing dormant buds were taken from field grown plant. The explants were washed under tap water for 15 minutes. Subsequently, they were immersed in 70% ethanol for one minute and surface sterilized with 1.0% of sodium hypochlorite (NaOCl) solution containing two drops of Tween-20 for 15 minutes. Axillary buds having a length of 10-12 mm were used for culture. The stock solutions were prepared from its full components by weighing the powder of each elements/nutrient and dissolving them in double distilled water and stored in a refrigerator at $+4^{o}$C temperatures.

Culture medium was prepared by taking appropriate amount of each stock solution (ml) in one liter (1L) of medium. Sucrose at 3% was added and pH was adjusted to 5.8 using 1N NaOH and/or 1N HCl.

Then 3gm of gelrite was added in one liter of media for shoot initiation and shoot multiplications, while 1.5gm of gelrite was used for root formation.

Different concentrations of BAP (0.2, 0.6, 1.0 1.5 and 2.0 mg/l) in combination with 0.1mg/l NAA were used for shoot initiation. Sub-culturing was done at 15^{th} and 30^{th} dates. Data on percentages of explants showing shoot initiation, number of shoots/ex-plant and shoot height (cm)/ex-plant were collected after 4 weeks of culturing. BAP (1.0, 2.0, 2.5, 3.0 and 3.5 mg/l) in combination with NAA (0.1, 0.2 and 0.3 mg/l) was used for shoot multiplication. Data were collected for number of shoots formed and shoot height (cm) per propagules after 30 days of culture. For rooting, shoots from shoot multiplication were excised into 3-4 cm length and transferred onto MS media containing IBA (0.0, 0.8, 1.2, 1.6, and 2.0 mg/l) in combination with NAA (0.0, 0.2, 0.5, 0.8 and 1.0 mg/l). Data were collected for rooting percentage, number of roots/shoot and root length (cm), and data were recorded after 35 days.

Among BAP concentrations in combination with 0.1mg/l NAA used for shoot initiation, 0.6mg/l, 1,0mg/l and 1.5mg/l were found to be better for percentage of explants showing shoot initiation, shoot height and number of shoots/ex-plant respectively. The highest (80%) for percentage of explants showing shoot initiation was obtained on 0.6mg/l and 1,0mg/l BAP while maximum number of shoot/ex-plant (1.72) recorded at 1.5mg/l BAP and shoot height (3.22 cm) was the best performance that obtained on 1.0mg/l BAP.

The effect of BAP and NAA combination for shoot multiplications were also significantly different on number of shoots/ex-plant and shoot heights. The maximum number of shoots/ex-plant (2.67) and shoot height (3.23cm) were obtained at 3.0 mg/l and 0.2 mg/l BAP and NAA, and 1.0 mg/l BAP and 0.2 mg/l NAA respectively. It could be inferred from the above results that, NAA and BAP combinations at the appropriate concentration are essential in grapevine tissue culture and shoot multiplication instead of BAP alone.

Addition of auxins (IBA and NAA) to MS medium combining at 0.8 and 0.5 mg/l, 1.2 and1.0 mg/l, 1.6 and 1.0 mg/l, 2.0 and 0.5 mg/l, and 2.0 and 1.0 mg/l IBA and NAA concentrations resulted in the highest value (100%) for percentages of rooting respectively.

The interaction between IBA and NAA at 2.0 and 0.2 mg/l could be taken as the best performance (12.50) for number of roots/shoot. The highest value (5.39) for root length was also recorded on 1.6mg/l and 0.5mg/l IBA and NAA concentrations.

In conclusion,

- The concentration of 1.5mg/l BAP in 0.1mg/l NAA could be the best option for shoot initiation, since the primary aim of mass propagation is to produce high amount of plantlets rather than their height.
- The combination of 2.5mg/l BAP + 0.1mg/l NAA was the optimal concentration for shoot multiplications.
- For *in vitro* rooting, the combination of 1.2mg/l IBA and 1.0mg/l NAA was the optimum concentration that could be taken as the best alternative.

Rooted plantlets were taken out of *in vitro* rooting media after 35 days and maintained under greenhouse conditions where plantlets were kept in pots filled with soil and covered with white plastic bag to maintain humidity. About 80% of the *in vitro* rooted plantlets were acclimatized successfully. In general, grape vines tissue culture could be the best option for mass propagation of the crop to meet the current high demand for planting materials. It could also save high amount of money being spent to import the cuttings from abroad. Therefore, micro-propagation can help the future commercial plantations of the country.

For the future:-

- The seasonal effects of axillary bud contaminations at initiation stages of tissue culture need to be further studied under Ethiopian conditions.
- Since at the initial stage of culture, surface contaminations from explants are the major problem in grapevines, it would be good if meristem organ is used for mother plant preparation.
- 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.
- As this protocol was tested on a single cultivar, further studies using other genotypes is required so as to have a reliable protocol for *in vitro* propagation of grapevine.

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7. APPENDICES

Appendix Table 1: Analysis of variance for effect of different concentration of BAP in combination with 0.1mg/l NAA on percent of explants showing shoot initiation, number of shoot/ex-plant and shoot height

		Percent of explants showing initiation		Number of Shoot/ex-plant		Shoot height	
Source of variation	**DF**	**Mean Square**	**Pr >F**	**Mean Square**	**Pr >F**	**Mean Square**	**Pr >F**
Rep	2	6.66	0.84	0.0125	0.4364	0.100	0.0402
BAP	4	960.0	0.0002***	0.2248	0.0006***	0.5632	<.0001***

DF=Degree of Freedom, ***= very highly significant

Appendix Table 2: Analysis of variance for the effect of different concentrations of BAP in combination with NAA on number of shoot/ex-plant and shoot height for shoot multiplication

		Number of shoot/ex-plant		Shoot height	
Source of variation	**DF**	**Mean Square**	**Pr >F**	**Mean Square**	**Pr >F**
Rep	2	0.007	0.209	0.0019	0.8511
BAP	4	0.148	<.0001***	0.8426	<.0001***
NAA	2	0.277	<.0001***	0.2198	<.0001***
BAP*NAA	8	0.494	<.0001***	0.7859	<.0001***

DF=Degree of Freedom, ***= very highly significant

Appendix Table 3: Analysis of variance for the effect of different concentrations of IBA and NAA combinations on percent of rooted shoot, number of roots/shoot and root length

		Percent of rooted shoots		Number of roots per shoot		Root length(cm)	
Source of variation	**DF**	**Mean Square**	**Pr >F**	**Mean Square**	**Pr >F**	**Mean Square**	**Pr >F**
Rep	2	0.1891	0.4247	0.3036	0.1592	0.024	0.2608
IBA	4	0.2160	0.4191	21.27	<.0001***	3.50	<.0001***
NAA	4	1.8523	<.0001***	4.29	<.0001***	4.70	<.0001***
IBA*NAA	16	3.1670	<.0001***	14.60	<.0001***	3.60	<.0001***

DF=Degree of Freedom, ***= very highly significant

Appendix Table 4: Murashige and Skoog MS (1962) media components

Code	Nutrients	Stock solution(gm)		Volume of stock for 1L full MS media
MS1	Ammonium nitrate(NH4NO3)	33.0	In 1000 ml	50 ml
	Potassium nitrate(KNO3)	38		
MS2	Magnesium sulphate($MgSO_4.7H_2O$)	18.07	In 500 ml	5 ml
	Manganese sulphate($MnSO_4.H_2O$)	1.69		
	Zinc Sulphate($ZnSO_4.7H_2O$)	0.86		
	Copper Sulphate($CuSO_4.5H_2O$)	0.0025		
MS3	Calsium Chloride($CaCl_2.2H_2O$)	33.22	In 500 ml	5 ml
	Potassium Iodide(KI)	0.083		
	Cobalt Chloride($CoCl.6H_2O$)	0.0025		
MS4	Potassium dibasic phosphate(KH_2PO_4)	17	In 500 ml	5 ml
	Boric acid(H_3BO_3)	0.62		
	Sodium molbdate($Na_2MoO_4.2H_2O$)	0.025		
MS5	NaEDTA	3.726	In 500 ml	5 ml
	Iron sulphate($FeSO_4.7H_2O$)	2.78		
MS6	Myo-inositol	10.0	In 500 ml	5 ml
	Glycine	0.2		
	ThiamineHCl	0.01		
	Pyridoxine HCl	0.05		
	Nicotine acid	0.05		