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Multiple shoot regeneration study on three varieties of grape vine (*Vitis vinifera* L.) from shoot tip and nodal culture

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LIST OF ABBREVIATION

BAP = 6-Benzyle Aminopurine

CRD = Completely Randomized Design

DZARC = Debre Zeit Agricultural Research Center

IAA = Indol Acetic Acid

IBA = Indol-3-Butyric Acid

MS = Murashige and Skoog basal media

NAA = Naphtalene Acetic Acide

HARC = Holetta Agricultural Research Center

UAAIE = Upper Awash Agro Industry Enterprise

PGR = Plant Growth Regulators

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ABSTRACT

Grape-vine (*Vitis vinifera* L.) is a perennial deciduous woody vine which is cultivated all over the world. Conventional breeding of grape vine is impeded by long juvenile periods and inbreeding depression. The objectives of this study were to establish a protocol for *in vitro* micropropagation of selected grape vine varieties. Preliminary studies were undertaken in order to optimize the duration of sterilization of the explants and the culture used to initiate shoots. Shoot tips of variety Chenin blanc was cultured in MS medium supplemented with 0.5 mg/l after sterilizing in 1% NaOCl to determine the optimum duration of sterilization. For shoot initiation experiment, shoot-tip and nodes of the three varieties, namely, Ugni blanc, Chenin blanc and Canonannon were cultured in Murashige-Skoog medium supplemented with five different concentrations of BAP including the BAP free. Various experiments were carried out to optimize shoot multiplication using MS medium supplemented with different combination and concentrations of BAP with or without IBA. To optimize root induction different concentrations of IAA were used. Data such as number of shoots and root, shoot height, root length and node number were recorded and analyzed. Sterilization of explants using 1% of NaOCl was found to be optimum for 7 minutes duration. Cheninblanc showed high percentage of survival rate (96%) followed by Ugniblanc and Canonannon (88%) at 0.5 mg/l BAP. Among the different concentrations and combination of PGRs, a maximum mean number of shoots 7.2, 6.7, 6.1 was achieved at 1mg/l BAP combined with 0.1 mg/l IBA for Chenin blanc, Canonnanon and Ugni blanc respectively. The shoot length and node number of all varieties showed a decreasing trend when the concentration of BAP was increased, except for Canonannon, in terms of node number. Number of shoots, node and shoot height showed significant variability ($p=0.05$) at different level of BAP or BAP combined with IBA. All varieties induced root on MS medium without IAA or supplemented with different concentration of IAA. Among these rooting culture, however, MS medium with higher concentration (2 and 4 mg/l) of IAA were found to be the best for root induction in all varieties. The plantlets were acclimatized in the glasshouse and survival percentage was 92% for Chenin blanc followed by 78.6% and 73.9% for Ugni blanc and Canonannon, respectively. Thus, the achievements of this study will play a big role in the grape vine culture program.

Key words: *Vitis vinifera* L., shoot initiation, root initiation, Cytokinin, Auxin

1. INTRODUCTION

Grapevine (*Vitis vinifera* L.) is perennial deciduous woody vine. It is believed to have originated in Armenia near the Black and Caspian seas in Russia (Winkler *et al*., 1974; Weaver, 1976). Grapes have been used by humans for thousands of years. They were around during the Bronze Age. The Greek poet, Homer, who lived about 700 BC, talked of wine made from grapes. The fruit is mentioned in the Bible, and Egyptian tombs and relics have representations of grapes on them (Patrice *et al*., 2006). It has ancient historical connections with the development of human culture. The main product, wine, was considered divine, a drink of the Gods. Other Mediterranean cultures considered that 'the wine sprang from the blood of humans who had fought the Gods' (Patrice *et al.*, 2006).

Grapevine is one of the most important fruit crops grown in the world. In 2005, vineyard area and grape production was 7,488,196 ha and 66,901,419 t, respectively, ranking second in fruit production (Chusing, 2008). The most important countries mentionable are France, Italy, Germany, Spain, Greece, Portugal, Moldova, Romania, Bulgaria and Hungary (Mhatre and Bapat, 2007).

Grapes are used for wine production, fresh fruit, dried fruit, and juice production. It has many health and nutritional value such as reservation which is constituents of grape has capacity of inhibiting cancer, heart disease, degenerative nerve disease, alzheimer disease, retinal disorder, constipation and viral disease. Grape juice forms a rich source of calcium, iron, vitamin C, A, E, amino acid and phosphorous. These components make the immunity system strong and prevent common disease (Conde *et al.,* 2007; Hulya, 2007; Jaladet *et al*., 2009).

Grape is a crop that is susceptible to many diseases. The causal agents of these diseases are fungi, bacteria, viruses and nematodes (Krongjai, 2005).

Grapes are mainly cultivated in the temperate regions but some cultivars that are tolerant to high temperatures have been introduced to tropical and subtropical countries (Weaver,

1976). It is successfully grown in areas where the temperature range is from 15-40^{0}C. Even though grape vine is a temperate fruit, viticulture is possible in tropics under certain conditions such as selecting suitable cultivars, managing cultural practices such as pruning and inducing flowering by chemicals, defoliation, and withholding irrigation water.

Ethiopian wineries are importing about 300 tons of grapes per year in the form of dried raisin, grape juice concentrates, natural wine extracts, and citric acid (Alemu Geda, personal communication). The demand of Awash Winery for the 1997 Ethiopian fiscal year was 2,500 tones with annual increment of 20% and only small amount is covered by the local fresh grapes supply (Alemu Geda, personal communication).

Established grapevines can be productive for 40 years or more. Grapes are vegetatively propagated by cutting a one-year-old healthy and mature cane. In the fruit research history of Ethiopia, Debre Zeit Agricultural Research Center (DZARC) with the support of Upper Awash Agro Industry Enterprise (UAAIE) and Awash Winery has released six quality wine producing varieties in August 2004 for the first time. The prevailing average grapevine production at DZARC (100qt/ha/year), Merti-Jeju (350qt/ha/year) and Ziway (100 qt/ha/year) with average national yield of (183qt/ha/year) reveal that the country is endowed with suitable environmental conditions for viticulture expansion. At Merti 490qt/ha/year was obtained under experimental plot showing the tremendous potential of the area. In countries, where grapes are extensively grown, it has a considerable economic significance as a potential source of national revenue. Thus, expansion of vineyards in Ethiopia is essential to convert the existing potential of the country into usable form.

The conventional method of grapevine propagation (cutting) is time consuming and allows diseases transmission. A planted grapevine needs four to five years to produce propagation materials by cuttings due to its long juvenility period (Winkler, 1976). Grapevine juvenility is one of the principal natural problems hindering grapevine production (Rossel, 1992). Thus, notion of vineyard expansion can't be achieved without

the pre-establishment of techniques that make adequate amount of planting materials available within a shortest time. Thus, developing rapid propagation techniques for large scale production of grapevine in order to exploit its potential is unequivocal. Depending on the plant species and cultural conditions, tissue culture may enable the mass production of genetically homogeneous populations, vigorous growth, normal yield and healthy plants (Murashige, 1974; Blazina *et al*., 1991). It is fast and requires less space than conventional methods of propagation (cutting).

The propagation of grapes via tissue culture approach has been commercialized around the world and tissue culture eliminates viruses from the new vineyards using virus free materials (Bruce and Prett, 1996). Tissue culture of selected grape genotypes can be carried out by the culture of intact or fragmented shoot apical meristems (Gray and Fischer, 1985;, axillary-bud micro cuttings (Barlas and Skene, 1980) or through adventitious bud formation (Heloir *et al*., 1997). Thus this study aims to develop a protocol for multi-shoot regenerate from shoot tip and node using tissue culture.

2. LITRATURE REVIEW

2.1. Taxonomy and morphology of grape

Grapes (*Vitis vinifera* L.) belong to family Vitaceae Juss., genus Vitis L. (Cherepanov, 1995). Grape-vine is a perennial plant with chromosome number 2n = 38 (Ttan and Wang, 2008). It is a deciduous woody vine 30-40 m tall with a powerful root system that penetrates the soil into a depth of 7m or more. Grape-vine's trunk is coarse and barbate. Its crust peels off in strips. Grape-vine leaves are alternate, from full, rounded or angular to very much divide into lobes (Trimble and Tryon, 1979; Fabio and Andrea, 2002). The flowers are very fragrant and polygamous. They are functionally female (with short, distant and bent sterile stamens) or bisexual. Staminate flowers and carpellary flowers are only typical of some grape-vine varieties. The corolla is made up of five greenish petals formally united at the tip. The five stamens are present opposite the petals. Each anther consists of two lobes running length-wise. Each lobe is divided in to two pollen sacks. The ovary consists of two halves each with two ovules. Each ovule has one embryo sack containing the egg. Immediately after a flower opens (cap fall) the stigma is coated with a sweet and sticky solution secreted to hold the pollen grains (Strausbaugh and Core, 1952; Fabio and Andrea, 2002).

Grape-vine fruits are berries with very juicy, dense or viscous pulp inside. In some varieties, their taste may be quite different, ranging from very sweet to sour. Grape-vine berries are of different sizes, shapes and colors (from yellow to almost black) and form bunches or clusters (Shutts, 1968). Grape-vine is a cross-pollinating plant (wind- and insect-pollinated). It may be also self-pollinating, sometimes cleistogamous (Fabio and Andrea, 2002).

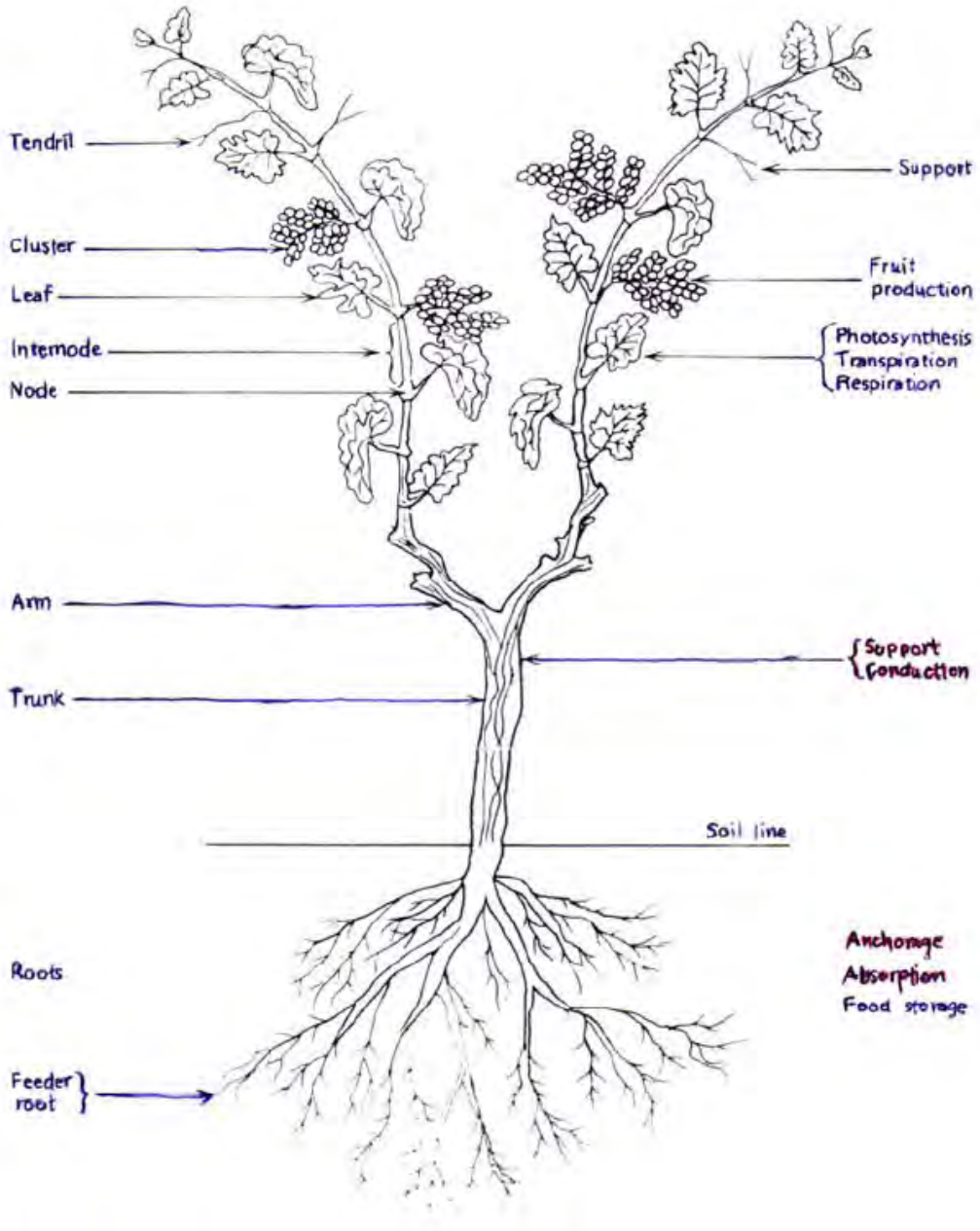

Figure 1. Important structures and functions of a grapevine (Pearson and Goheen, 1988).

2.2. Origin and geographical distribution

Grape is believed to have originated in Armenia near the Black and Caspian seas in Russia (Winkler *et al*., 1974; Weaver, 1976). Grapes have been used by humans for thousands of years. Archaeological evidence shows that the grape has been a food of man from the earliest of times. Seeds have been found in excavated dwellings of the Bronze-age in south-central Europe (3500 - 1000 BC). Egyptian hieroglyphics detail the culture of grapes in 2440 BC. The Phoenicians carried wine varieties to Greece, Rome, and southern France before 600 BC, and Romans spread the grape throughout Europe (Jackson and Danny, 1987; Deng *et al*., 2010).

During the ancient Greek and Roman civilizations, grapes were revered for their use in winemaking. The Greek poet, Homer, who lived about 700 BC, talked of wine made from grapes. The fruit is mentioned in the Bible, and Egyptian tombs and relics have representations of grapes on them. It has ancient historical connections with the development of human culture (Weaver, 1976; Patrice *et al*., 2006).

Currently, there are three main species of grapes: European grapes (*Vitis vinifera*), North American grapes (*Vitis labrusca* and *Vitis rotundifolia*) and French hybrids (Deng *et al*., 2010). The genus *Vitis* is broadly distributed, largely between 25° and 50° N latitude in eastern Asia, Europe, the Middle East, and North America. Additionally, a few species of *Vitis* are found in the tropics-Mexico, Guatemala, the Caribbean, and northern South America (Ghulam *et al*., 2006).

Nowadays, grapevines are cultivated all over the world, between latitudes 4° and 51° in the Northern Hemisphere (NH) and between 6° and 45° in the Southern Hemisphere (SH) across a large diversity of climates (oceanic, warm oceanic, transition temperate, continental, cold continental, Mediterranean, subtropical, attenuated tropical, arid and hyper arid climates) (Schultz and Stoll, 2010). In tropical and subtropical areas, grapevines may grow continuously and produce more than once per year (Tonietto and Carbonneau, 2004).

2.3. Grape production and trends in the world

Grapevine (*Vitis vinifera* L.) is one of the most important fruit crops grown in the world today in terms of both total acreage and dollar value (Galletta and Himerlic, 1989). Grape berries are consumed as table fruit, wine, juice, and raisins. Grapevines and their products, particularly wine, have been important elements in human life, foods and religions (Lavee, 2000).

In 2005, vineyard area and grape production was of 7,488,196 ha and 66,901,419 t, respectively, ranking second in fruit production next to banana (Conde *et al*., 2007; Chusing, 2008). Approximately 71% of this production is used for wine, 27% as fresh fruit, and 2% as dried fruit (Conde *et al*., 2007). Historically, grape production and consumption have been at home in Europe. The most important countries mentionable are France, Italy, Germany, Spain, Greece, Portugal, Moldova, Romania, Bulgaria and Hungary. The European Union (EU 27) occupies a leading position on the world wine market. Globally, it accounts for 49.9% of growing areas and 39.1% of grape production (Mhatre and Bapat, 2007). Asia is ranked second in wine harvested area, production grape on about 2 million hectares and America produces about 1 million hectares, and shows a growing tendency. In Africa, grape production was 550,244 hectares in 1961, and this figure had reduced to 339,655 hectares by 2006.

Generally, worldwide grape production shows decrement. For instance, vineyards accounted for a total area of 9,336,513 ha under plantation in 1961, and decreased to 7,399,546 ha by 2006. The highest amount of reduction was observed in Europe, where the harvested area for wine was reduced from 6,435,356 ha in 1961 to 4,081,412 ha by 2006 (Hajar, 2006; Lazanyi, 2008). The decrease was due to primarily to a vine yard grubbing program in the former Soviet Union followed by grubbing and other supply control program initiated in the EU (World vineyard grape and wine report, 2005).

2.4. Ecology of grape production

Climate is often the most influential factor in producing grapes for wine (Winkler *et al*., 1974). The primary climatological factor is temperature. Grape-vine is a heat-loving plant, but it is also quite cold-resistant (able to survive frosts of -18°C). Grapes, generally, require a hot and dry climate during its growth and fruiting periods. It is successfully grown in areas where the temperature range is from 15- 40^0C. High temperatures above 40^0C during the fruit growth and development reduce fruit set and consequently the berry size. Low temperatures below 15^0C followed by forward pruning impair the bud break leading to crop failure (Perry, 1974; Crassweller, 2008).

Secondary factors are rainfall, humidity, wind, soil type and pH, and combinations of these (Amerine *et al*., 1967). Grape requires 300-500 mm of annual rainfall for optimal growth (Negrul, 1959; Cherepanov, 1995). However, humidity associated with rains during flowering and fruit ripening is not favorable and invites the attack of fungal diseases (John, 2008).

Grapes are adapted to a wide variety of soil types, ranging from gravelly sands to heavy clays, from shallow to very deep, and from low to high fertility (Winkler *et al*., 1974). The soil should be well drained, having good water holding capacity and devoid of any hard pan or impervious layer in the top 90-cm, with water table at least 6.5 m below. Grapes can also be grown successfully over a wide range of soil pH (4.0-9.5). However, soils having pH range of 6.5-8.0 are considered ideal (Cherepanov, 1995).

2.5. Importance of grape

2.5.1. Nutritional value of grapes

Grape is one of the most commonly consumed fruits in the world. Grape-vine is a valuable source of food. Its berries contain various nutrient elements 10-33% sugars, 0.5-

1.4% organic acids, 0.3-0.5% mineral substances, 0.3-1.0% pectic substances, vitamins C, vitamins B and β-carotene. Grape juice contains 16.1-20.8% sugars (6.2-8.8% glucose; 5.7-9.3% fructose) (Conde *et al.,* 2007).

Grapes have a lot of important vitamins such as vitamin A, B1, B2, B6 and C6 (Ameine, 1967). Grapes also contain acids such as tartaric acids, malic acids, succinic, fumaric, glyceric, p-coumaric and caffeic acids (Mattick, 1973). Lycopene, β-carotene, ellagic acid, resveratrol and other sulphur compounds are found in grape skins. Grapes have important anti-oxidants such as anthocyanins, flavones, geraniol, linalol, nerol and tannins. Moreover, grapes contain all the necessary minerals such as calcium, chlorine, copper, fluorine, iron, magnesium, manganese, phosphorus, potassium, silicon and sulfur (Ameine, 1967; Alonso, 2002).

2.5.2. Health benefit of grape

The grape is a significant source of antioxidants as well as biologically active dietary components. Polyphenols are the most important phytochemicals found in grape that possess many biological activities and health-promoting benefits (Silva *et al*., 1991; Shrikhande, 2000). The phenolic compounds mainly include anthocyanins, flavanols, stilbenes (resveratrol) and phenolic acids (Novaka *et al*., 2008; Jaladet *et al*., 2009). Anthocyanins are pigments, responsible for a wide range of colours in grape and red wines and mainly exist in grape skins. Flavonoids are widely distributed in grapes, especially in seeds and stems (Cantos *et al*., 2002; Spacil *et al*., 2008). It has been reported to produce a variety of pharmacological effects. These effects contain anti-oxidant, anti-cancer, anti-obesity effect, and anti-inflammatory properties. Grapes may also promote heart health, support immunity and strengthen bones (Jaladet *et al*., 2009). Grapes contain ellagic acid, substance that blocks the production of enzyme that cancer cell needs to grow (Hulya, 2007; Lee *et al.*, 2009). Resveratrol has also been shown to modulate lipoprotein metabolism, reduce the synthesis of lipids, inhibit aggregation of platelets and suppress cellular processes associated with tumorigenesis (Zhang *et al*., 2006).

2.6. Diseases of grape

Grape is a crop that is susceptible to many diseases. However, the degree of susceptibility varies depending on the variety. The causal agents of these diseases are fungi, bacteria, viruses and nematodes (Krongjai, 2005). Fungal diseases affect leaves, shoots, and fruit of the grape and the important fungal grapevine diseases include powdery mildew, downy mildew and black rot (Soytong *et al*., 2005; Dan *et al.,* 2008). Fungal diseases on the leave can drastically reduce photosynthesis, and, in extreme cases, can cause defoliation. Fungal diseases can render fruit unusable and can very easily cause severe losses in yield (Soytong *et al.,* 2005; Uyovbisere, 2007).

Grapevine is also a host of large number of viruses responsible for severe yield losses (Hartman and Bachi, 2009). The most important viruses are Grapevine fanleaf, Arabis mosaic, Tobacco ring spot and Tomato ring spot. Other viruses that are less prevalent include Raspberry ring spot, Peach rosette, Tomato black ring and many others (Lazer *et al*., 2002; Infante and Fiore, 2009).

Grapevines are affected by bacterial diseases that have local or worldwide importance. Crown gall caused by *Agrobacterium vitis* occasionally by *Agrobacterium tumefaciens* causes serious economic loss in viticulture worldwide (Szegedi *et al*., 2005). This bacterium, like others, is also systemic in the host plant and may occur in grapes without symptom development. Besides the above mentioned microbial pathogens and several pests of grapevine, e. g. phylloxera, nematodes and mites are also transmitted by propagating material (Murrell and Lo, 2000; Hartman and Bachi, 2009)

The effects of diseases on grape production are found throughout the record of viticulture. Diseases affect the production, harvesting, processing, marketing, and the consumer. The lower quality will be reduced yield, and increase the costs of producing and harvesting (Krongjai, 2005; Uyovbisere *et al*., 2007).

2.7. Tissue culture in grape

There are two basic methods of propagation of grape vines; these are sexual propagation by using seed and asexual (vegetative) propagation (Chanana and Gill, 2008). Sexual methods of propagation do not produce progeny exactly like the parent. These are disappointing when they mature and produce fruit. Asexual or vegetative propagation of grape vine includes cutting, layering, budding and grafting (Jean *et al*., 1998). These 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 three years to produce generous amounts of cuttings. However, such propagation methods are restricted due to the slow and limited number of plants production, transmition of systemic diseases and unwanted mutation, requirement of large propagation space and an extended period to produce plants (Skiada *et al*., 2009). These factors contribute to a high production cost (Laimer *et al*., 2009) and often prevent an efficient and rapid production of planting materials to meet the market demand. For this reason, tissue culture has been proposed (Jean *et al*., 1998).

Micropropagation is the culture of different somatic cells, tissues or organs of plants under controlled *in vitro* conditions with the aim of producing a large number of progeny plants. This implies *in vitro* cloning based on the fact that different plant parts, buds, meristems, tissues and cells are capable of regeneration into whole plants under adequate *in vitro* conditions (Kumar *et al*., 2006 and George, 2008). The first attempts at initiating cultures of plant cells were made by the German botanist G. Haberlandt at the turn of this century. However, it has only been during the last three decades that rapid developments in plant cell, tissue, and organ culture have occurred (Mather and Roberts, 1998). The first true plant tissue cultures were obtained by Gautheret in 1934 from cambial tissue of *Acer pseudoplatanus.*

The main advantages of micropropagation are a) enormous capacity to multiply target plant material compared to conventional cloning methods, b) the ability to produce progeny all-year round, c) the production of disease-free plant material.

Currently *in vitro* cell cultures are applied for cell behavior study, large-scale propagation of selected materials, plant modification and improvement, pathogen-free plants and germplasm storage (Loyala-Vergas and Vazquez-Flota, 2005). Nowadays micropropagation becomes essential techniques in the improvement and genetic manipulation of plants particularly vegitatively propagated crops.

Micropropagation in grapevine was first performed using entire plantlets obtained from microcuttings (Jean *et al*., 1998). According to previous reports Barlas and Skene, 1980, Gray and Fischer, 1985, numerous explant parts such as meristem, shoot tip and node culture, were successfully used. In addition, somatic embryogenesis of *Vitis vinifera* L. has been studied (Martinelli and Gribaudo, 2001). Previously several types of tissues such as unfertilized ovules, anthers (Mauro et al. 1986), immature zygotic embryos and immature leaves (Stamp and Meredith,1988) tendrils (Salunkhe et al.,1997) immature ovaries (Nakaro et al., 2000), leaf discs (Das et al., 2002) and filaments (Nakajma and Matsuta, 2003) have been successfully used. Among them anthers are considered the most suitable explant for the regeneration of somatic embryos (Bouquet and Torregrosa, 2003).

Micropropagation was applied for selected *Vitis genotypes* using the culture of intact or fragmented shoot apical meristems, axillary-bud microcuttings or through adventitious bud formation (Heloir *et al*., 1997). Raymond *et al*., (1984), developed protocol for micropropagation of grapevine from shoot tip culture, nodal segments, shoot tips and leaf disk. Optimal cultural condition for regeneration of *V. vinifera* somatic embryos from anthers and floral tissues (stigmas and styles) were also developed by Morgana *et al*., (2004). However, most efficient protocols have been reported for muscadine grapes (Qiu *et al*., 2004).

Tissue culture techniques, in combination with molecular techniques, have been successfully used to incorporate specific traits through gene transfer. *In vitro* techniques for the culture of protoplasts, anthers, microspores, ovules and embryos have been used

to create new genetic variation in the breeding lines, often via haploid production. Cell culture has also produced somaclonal and gametoclonal variants with crop-improvement potential.

Markers linked to disease-resistance genes are currently utilized for discarding susceptible seedlings of grapes at the initial stage of development in large-scale breeding programs conducted in Germany, Italy and the USA (DiGaspero *et al*., 2007). Different methods of DNA delivery such as agrobacterium, biolistics and silcon carbide whiskers were used for the transformation of grape (soloki *et al*., 2005).

There are many institutes and research centers worldwide on gene and genomic research of grape vine. The Institute for Wine Biotechnology (IWBT) is the one trying to develop genetic markers, primers and probes for the genetic fingerprinting of *Vitis vinifera* varieties. Another objective of IWBT is to establish and optimize efficient transformation and regeneration techniques and procedures. The Grapevine Biotechnology programme includes fundamental studies of gene regulation and expression in grapevine. The overall aim is to develop disease-resistant cultivars so that less pesticide are required in spraying of vineyards (Evans, 2007).

Although tissue-culture protocols were available for grapes the degree of response is highly dependent on the particular genotype, as various *Vitis* species, cultivars or hybrids respond differently to certain culture conditions (Qiu *et al*., 2004). Hence, it is vital to develop new protocol for rapid multiplication of the available grapes varieties found in Ethiopia.

3. OBJECTIVES

3.1 General objective

The general objective of this study is to develop a protocol for multiple shoot regeneration of grape vine from shoot and nodal culture.

3.2 Specific objectives

- To observe the optimum duration for explant sterilization.
- To determine the appropriate growth regulators concentration for shoot initiation and multiplication.
- To determine effect of combination of different concentration of BAP and IBA on shooting
- To determine the optimum concentration of IAA for root induction
- To acclimatize plantlets and determine the survival rate of plantlets.

4. MATERIALS AND METHODS

4.1 Donor plant preparation and growth condition

Three varieties of grape, which are released by Debre Zeit Agricultural Research Center (DZARC), have been used as plant material for this study. The names of the varieties are Chenin blanc, Ugni blanc and Canonannon. Chenin Blanc originated in Anjou, south of the Loire Valley in North-Western France and was recognized there as early as the ninth century. Ugni Blanc may have originated in the Levant and has been in Italy since Roman times. Stem cuttings of these varieties were obtained from Debre Zeit Agricultural Research Center, Fruit Crops Research Division. The stems were planted in a pot containing soil, compost and sand at the ratio of 3:2:1, respectively and allowed to sprout in glasshouse, at Holetta Agricultural Research Center (HARC), at average temperature of 25± 2^0C under natural sun light condition.

4.2 Stock solution and medium preparation

4.2.1 MS stock preparation

The MS (Murashige and Skoog 1962) nutrient medium with its full macro, micro and vitamin compositions were used (Appendix I). Stock solution was prepared by weighing the powder and dissolving in double distilled water and stored in a refrigerator at +4^0C temperature.

4.2.2 Growth regulators preparation

Growth regulators such as benzyl aminopurin (BAP) for shoot induction and shoot multiplication and indol acetic acid (IAA) for rooting were used in this study. The growth regulators were prepared by weighing the powder in a 1: 1 ratio (1mg/1ml) and dissolved

by 1N NaOH and/or ethanol according to their instruction and the total volume was adjusted by distilled water. Finally the stock was kept in a refrigerator at + 4^0C.

4.2.3 Culture medium preparation

Culture medium was prepared by taking 100 ml/l macro, 10 ml/l micro and 10 ml/l vitamin stock solution of MS medium. Then 3% sucrose was dissolved and different concentrations of BAP for initiation and multiplication or IAA for rooting was added. The pH was adjusted to 5.8 using 1N NaOH and/or 1 N HCl. Then 7.5 g agar was added and 40 ml medium was dispensed into magenta culture vessel. Finally the medium was autoclaved at a temperature of 121^0C with a pressure of 0.15 kpa for 15 min.

4.3 Explants collection and surface sterilization

Shoot tips and nods having 2-3 cm length were collected. The expanded leaves were removed and the remaining tissues thoroughly washed with running tap water and a drop of 'Tween 20'. The explants were soaked in 70% ethanol for 30 second and bleach in 1% active sodium hypochlorite (NaOCl) for 7 min, followed by five rinses with sterile distilled water.

4.4 Culture condition

All cultures were incubated in a growth room under 16-h light at 2700 lux light intensity and 8 h dark cycle at 25^0C + 2^0C until new plantlets were formed on the surface of the explants.

4.5 Experiments

4.5.1 Preliminary observation trial for the optimum sterilization duration

To identify the optimum sterilization time for sodium hypochlorite (NaOCl) ten shoot-tip samples of variety Chenin blanc was sterilized under different time duration (5, 7 and 9 minute) and cultured on MS medium supplemented with 0.5 mg/l of BAP.

4.5.2 Determination of the optimum BAP concentration for shoot initiation and survival

Explants taken from the three varieties were sterilized for 7 minute in 1% NaOCl and cultured on MS media supplemented with five different concentration of BAP (0.5, 1, 2, 3, 4 mg/l) including the control (without BAP). A Magenta jar with five explants is a unit of replication and there were five replications for each treatment.

4.5.3 Determination of the optimum BAP concentration for shoot multiplication

The plantlets induced from the induction medium were placed in the MS medium supplemented with six different concentration of BAP (0.25, 0.5, 1, 2, 3, 4, mg/l) Table 2. 25 explants were used for each treatment. Then the cultures were incubated in a growth room at 27^0C for 4 weeks.

4.5.4 Determination of the optimum combinations of BAP and IBA concentration for shoot multiplication

Four different concentrations of BAP (0.5, 1, 2, and 4mg/l) including growth regulator free medium in combination with 0.1mg/l IBA (Table 6). For each treatment 25 explants were used. Then the cultures were incubated in a growth room at 27^0C for 4 weeks.

4.5.5 Determination of the optimum IAA concentration for rooting

Shoots having one cm and more length were cut 2 mm below their basal node and planted into the medium containing Indole-3-acetic acid (IAA) at different concentration (0, 0.5, 1, 2, 4 mg/l). For each treatment 25 explants were cultured and the cultures were incubated in a growth room at 27^0C for 4-6 weeks.

4.5.6 Acclimatization

The plantlets having length of 3 cm or more were planted into small pots containing sterile soil, compost and sand in the ratio of 2:1:1, respectively and then covered by plastic bags (in order to keep humidity) for one week in glasshouse. The number of survived plantlets was recorded after four weeks.

4.6 Experimental design and data analysis

All experiments were replicated and laid out in completely randomized design (CRD). Analysis of variance was conducted using JMP SAS computer software version 8.0. Means were compared using the least squares means procedure. Number of explants survived, number of shoots, nodes, roots per explants, shoots height and root length were recorded in a month interval.

5. RESULTS

5.1 preliminary observation of the duration of sterilization of explants

According to the preliminary experiment to determine the optimum duration for sterilization of explants using sodium hypochlorite (1%), 100 % clean cultures were obtained when treating the explants at 7 and 9 minutes (Table1). Although shoot-tip cultures treated for 9 minutes did not show contamination, most of them died due to prolonged sterilization time.

Table1. Percentage of contaminated and clean culture for shoot-tip cultures of the variety Chenin blanc sterilized with 1% of NaOCl at different times.

Treatment durations	No.of explants	No. of explants contaminated	Percentage of clean culture
5 minutes	10	8	20
7 minutes	10	0	100
9 minutes	10	0	100

5.2 Effect of different concentration of BAP for shoot initiation and survival rate

Results on the effect of BAP on shoot initiation for the three varieties of grapes are presented in the Appendix I (Table 2) and (Fig 2). The result revealed that all varieties respond to all treatments including the hormone free. Morphologically best shoots were induced on the medium that contains 0.5 mg/l BAP (Fig 3). Highest percentage of survival rate of cultured explants was obtained at 0.5 mg/l of BAP. This holds true for all the three varieties in which the maximum percentage (96 %) for Chenin blanc. Ungni blanc and Canonannon scored the same percentage of survival rate (88%). Based on this result, 0.5 mg/l BAP concentration was used as an optimum PGRs for shoot initiation survival.

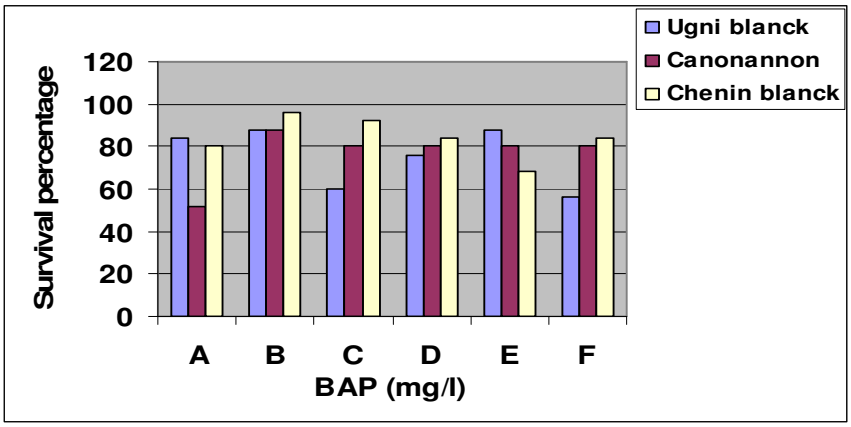

Figure 2. Survival percentage of shoots of the three varieties cultured on MS medium supplemented with five different concentrations of BAP.

Note: A= control, B= 0.5 mg/l BAP, C=1mg/l BAP, D= 2 mg/l BAP, E= 3 mg/l BAP and F= 4 mg/l BAP

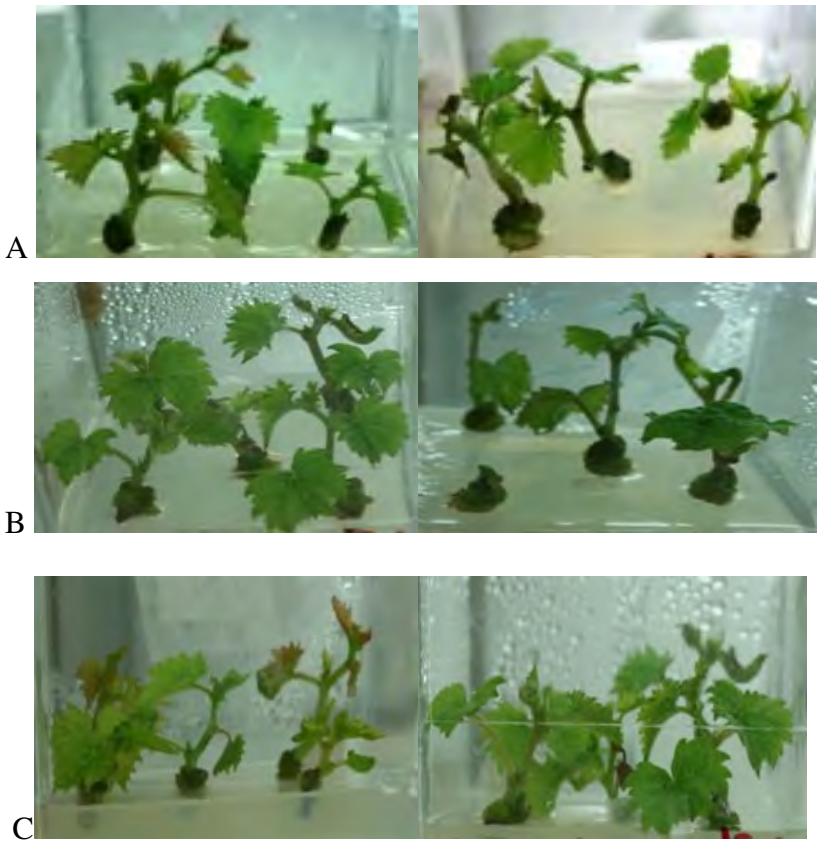

Figure 2. Shoot initiation from nodal culture in MS culture media supplemented with 0.5 mg/l BAP. Shoots initiated from nodes of the varieties Ugni blanc (A), Chenin blanc (B) and Canonannon (C).

5.3 Effect of different concentrations of BAP on number of shoot, node and height

Among the seven BAP concentration 0.5 mg/l of BAP produced significantly maximum mean number of shoots for the three varieties (Table 2). Canonannon gave maximum mean number of shoots (5.6) followed by Ugnin blanc (5.3) and Chenin blanc (5.0). The best mean height of shoot was attained at 1 mg/l of BAP for Ugni blanc and Chenin blanc moreover Ugni blanc score maximum height on hormone free medium as Canonannon (Table 3). Chenin blanc scored the maximum mean number of node (3.6) at 0.25 mg/l BAP as compared to Ugni blanc and Canonannon (3.1) (Table 4). Shoots that were proliferating at different concentration of BAP are presented in Fig 4.

According to the analysis of variance (Table 5), there was significant difference for the response of BAP concentration in number of shoot among the three varieties. The effect of PGRs on shoot number was not significant between the three varieties (Table 5). Shoot height and number of nodes showed significant variability at various levels of BAP and among the three varieties (Table 5). The effect of BAP on shoot height and number of node dependent on the type of the variety since the interaction of PGRs (BAP) with varieties was significant.

Table 2 Mean no of shoots per explants in different BAP concentration

No. of explant	BAP (mg/l)	Mean no. of shoots/explant		
		Ugni blanc	Chenin blanc	Canonannon
25	0	1.0 ± 0.0^{c}	1.0 ± 0.0^{c}	1.0 ± 0.0^{c}
25	0.25	2.5 ± 1.4^{bc}	2.4 ± 0.8^{b}	2.6 ± 1.4^{b}
25	0.5	5.3 ± 1.9^{a}	5.0 ± 0.9^{a}	5.6 ± 1.7^{a}
25	1	2.8 ± 0.1^{bc}	2.9 ± 0.5^{b}	2.8 ± 0.5^{b}
25	2	3.4 ± 0.3^{ab}	3.4 ± 0.3^{b}	3.4 ± 0.2^{b}
25	3	2.8 ± 0.7^{bc}	3.2 ± 0.3^{b}	3.4 ± 0.4^{b}
25	4	2.8 ± 0.6^{bc}	3.3 ± 0.3^{b}	3.0 ± 0.3^{b}

Numbers connected by the same superscript letters in the same column are not significantly different at 5% probability level

Table 3 Mean height of shoot per explants in different BAP concentration medium

	Mean height of shoot /explants (cm)		
BAP (mg/l)	Ugni blanc	Chenin blanc	Canonannon
0	2.49 ± 0.3^{a}	1.73 ± 0.1^{c}	2.39 ± 0.2^{a}
0.25	2.02 ± 0.5^{bc}	2.34 ± 0.4^{ab}	2.02 ± 0.5^{abc}
0.5	1.68 ± 0.2^{c}	1.60 ± 0.1^{c}	1.68 ± 0.2^{bcd}
1	2.82 ± 0.3^{a}	2.71 ± 0.2^{a}	2.05 ± 0.2^{ab}
2	2.18 ± 0.1^{bc}	2.03 ± 0.3^{bc}	2.08 ± 0.1^{ab}
3	1.86 ± 0.1^{bc}	1.91 ± 0.1^{bc}	1.47 ± 0.1^{cd}
4	1.73 ± 0.1^{c}	1.56 ± 0.1^{c}	1.36 ± 0.0^{d}

Numbers connected by the same superscript letters in the same column are not significantly different at 5% probability level

Table 4 Mean no of nodes per explants in different BAP concentration medium

	Mean no. of Nodes/explants		
BAP (mg/l)	Ugni blanc	Chenin blanc	Canonannon
0	2.4 ± 0.4^{abc}	1.4 ± 0.1^{c}	2.9 ± 0.9^{a}
0.25	3.1 ± 1.1^{a}	3.6 ± 0.9^{a}	3.1 ± 1.1^{a}
0.5	3.1 ± 0.8^{a}	3.2 ± 0.8^{a}	3.1 ± 0.8^{a}
1	2.9 ± 0.3^{ab}	2.8 ± 0.7^{ab}	2.1 ± 0.4^{ab}
2	2.0 ± 0.4^{abc}	3.3 ± 0.8^{a}	2.4 ± 0.5^{ab}
3	1.2 ± 0.5^{abc}	1.8 ± 0.4^{bc}	1.2 ± 0.4^{b}
4	1.6 ± 0.5^{bc}	1.7 ± 0.2^{bc}	1.0 ± 0.3^{b}

Numbers connected by the same superscript letters in the same column are not significantly different at 5% probability level

Table 5. Analysis of variance for the effect of plant growth regulators (BAP) and varieties on number of shoots, shoot height and number of nodes during shoot multiplication.

Source of variation	**DF**	**Parameters**	**SS**	P-value
PGRs	6	***No. of shoots***	899.31	< 0.0001*
Variety	2		0.01	0.98
PGRs × Variety	12		14.93	0.95
		Shoot height		
PGRs	6		52.37	<0.0001*
Variety	2		13.36	<0.0001*
PGRs × Variety	12		21.63	<0.0001*
		No. of nodes		
PGRs	6		246.77	<0.0001*
Variety	2		35.84	<0.0001*
PGRs × Variety	12		78.43	<0.0001*

Figure 4. Shoot multiplication of the three varieties on MS media. A= control B= 0.25 mg/l BAP C= 0.5 mg/l BAP D= 1mg/l BAP E= 2MG/L BAP

F= 4 mg/l BAP. The number represents the variety Ugni blanck (1), Chenin blanck (2) and Canonannon (3) respectively.

5.4 Effect of different concentrations and combinations of BAP and IBA on shoot multiplication, shoot height and node number

According to the results presented in Table 6, 1 mg/l BAP combined with 0.1 mg/l of IBA gave highly significant maximum mean number of shoots (7.2) for the variety Chenin blanc. In this combination, Canonannon had also given 6.7 mean number of shoot which is significantly different from the other combination except 2 mg/l BAP combined with 0.1 mg/l IBA. Among the two hormone combinations, the combination having 0.5 and 1 mg/l BAP with 0.1 mg/l IBA produced the best height for the variety Chenin blanc, Canonannon and Ugni blanc, respectively (Table 7). The variety Ugni blanc gave more number of nodes as compared to Chenin blanc and Canonannon (Table 8).

Analysis of variance (Table 9) indicated that the different combination of BAP and IBA showed variability in number of shoot, shoot height and number of nodes. All parameters, except number of shoot exhibited significant variability among the three varieties. The ANOVA also revealed that there was significant interaction between PGRs and varieties.

Table 6. Mean no of shoots per explants in different concentration of BAP and IBA combinations of MS medium.

		Mean no. of shoots/explants		
BAP (mg/l)	IBA (mg/l)	Ugni blanc	Chenin blanc	Canonannon
0.5	0.1	2.2 ± 0.6^{b}	3.4 ± 1.0^{bc}	2.9 ± 0.1^{b}
1	0.1	5.2 ± 1.5^{a}	7.2 ± 1.5^{a}	6.7 ± 1.2^{a}
2	0.1	6.1 ± 1.6^{a}	4.7 ± 0.7^{b}	4.8 ± 1.3^{ab}
4	0.1	1.2 ± 0.3^{b}	2.2 ± 0.5^{c}	3.8 ± 0.9^{b}

Numbers connected by the same superscript letters in the same column are not significantly different at 5% probability level

Table 7. Mean height of shoots per explant in different combinations of BAP and IBA on MS medium.

		Mean height of Shoots/explants (cm)		
BAP (mg/l)	IBA (mg/l)	Ugni blanc	Chenin blanc	Canonannon
0.5	0.1	1.8 ± 0.4^{a}	2.2 ± 0.2^{a}	1.9 ± 0.3^{a}
1	0.1	2.0 ± 0.2^{a}	1.9 ± 0.2^{a}	1.8 ± 0.1^{ab}
2	0.1	1.8 ± 0.1^{a}	1.3 ± 0.4^{b}	1.5 ± 0.1^{bc}
4	0.1	1.0 ± 0.2^{b}	0.7 ± 0.2^{c}	1.3 ± 0.1^{c}

Numbers connected by the same superscript letters in the same column are not significantly different at 5% probability level

Table 8. Mean no of nodes per explant in MS medium containing different combinations of BAP and IBA.

		Mean no. of Nodes/explants		
BAP (mg/l)	IBA (mg/l)	Ugni blanc	Chenin blanc	Canonannon
0.5	0.1	2.6 ± 0.5^{a}	3.2 ± 0.8^{a}	2.4 ± 0.6^{b}
1	0.1	3.5 ± 0.5^{a}	3.2 ± 0.6^{a}	3.1 ± 0.2^{a}
2	0.1	3.0 ± 0.5^{a}	2.0 ± 0.5^{b}	2.3 ± 0.3^{b}
4	0.1	1.6 ± 0.6^{b}	0.8 ± 0.1^{c}	2.0 ± 0.2^{b}

Numbers connected by the same superscript letters in the same column are not significantly different at 5% probability level

Table 9. Analysis of variance for the effect of plant growth regulators and varieties on number of shoots, shoot height and number of nodes during shoot multiplication.

Source of variation	**DF**	**Parameters**	**SS**	**P-value**
PGRs	3	***No. of shoots***	711.23	<.0001*
Variety	2		16.88	0.1402
PGRs ×Variety	6		134.87	<.0001*
		Shoot height		
PGRs	3		43.72	<.0001*
Variety	2		1.45	0.0040*
PGRs × Variety	6		8.099	<.0001*
		No. of nodes		
PGRs	3		121.20000	<.0001*
Variety	2		7.54667	0.0212*
PGRs × Variety	6		31.86000	<.0001*

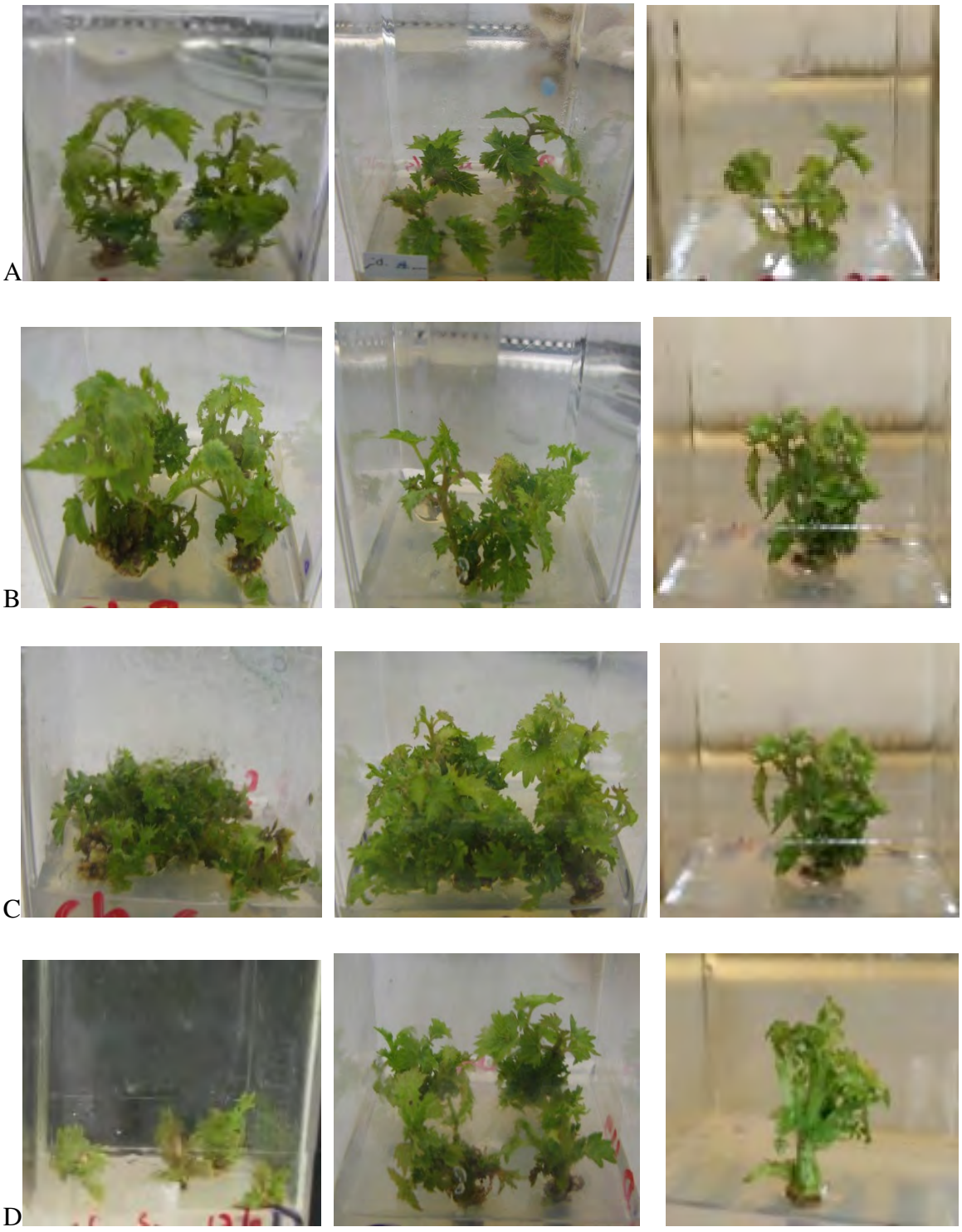

Figure 5. Shoot multiplication of the three varieties at different concentration of BAP combined with 0.1 mg/l IBA. (A) 0.5 mg/l BAP and 0.1 mg/l IBA, (B) 1 mg/l BAP and 0.1mg/l IBA, (C) 2 mg/l BAP and 0.1 mg/l IBA and (D) 4 mg/l BAP and 0.1 mg/l IBA.

5.5 Effect of IAA on root induction

The rooting result revealed that the plantlets cultured on rooting media were induced roots in all media supplemented with IAA including hormone free media. A maximum mean number of roots (4), (5.2) were counted for Ugni blanc and Canonannon, respectively in a medium supplemented with 4 mg/l IAA. For Chenin blanc maximum mean no of root (3.7) was obtained in a medium that contains 2 mg/l IAA (fig. 6) (Table 10). Concerning the length of the roots, Chenin blanc and Canonannon induced a maximum mean length of 9.7 cm and 8.6 cm at 2 and 4 mg/l IAA, respectively. Ugni blanc produced maximum mean length of root (6.2 cm) at 2 mg/l IAA. Significant variation in mean number of root among treatments was observed for the varieties Canonannon and Chenin blanc and variety Chenin blanc showed significant variation on the length of roots (Table 11).

Table 10. Mean number of roots per explants in different concentration of IAA on MS medium.

IAA(mg/l)	Mean no. of roots per explants		
	Ugni blanc	Chenin blanc	Canonannon
0	1.7 ± 0.5^{b}	1.7 ± 0.6^{c}	2.0 ± 0.4^{c}
0.5	2.5 ± 0.2^{ab}	2.4 ± 0.3^{bc}	2.2 ± 0.5^{bc}
1	3.4 ± 0.7^{a}	3.3 ± 0.4^{ab}	2.9 ± 0.6^{bc}
2	3.8 ± 1.8^{a}	3.7 ± 0.5^{a}	3.6 ± 0.8^{b}
4	4.0 ± 0.4^{a}	3.5 ± 0.6^{a}	5.2 ± 1.0^{a}

Numbers connected by the same superscript letters in the same column are not significantly different at 5% probability level

Table11. Mean length of roots per explants in different concentration of IAA medium

	Mean length of roots per explants (cm)		
IAA(mg/l)	Ugni blanc	Chenin blanc	Canonannon
0	4.1 ± 0.8^{a}	4.1 ± 0.5^{c}	5.1 ± 1.5^{a}
0.5	3.8 ± 1.0^{a}	4.8 ± 0.6^{bc}	5.1 ± 4.2^{a}
1	5.5 ± 1.7^{a}	7.5 ± 1.3^{ab}	6.8 ± 1.5^{a}
2	6.2 ± 2.1^{a}	9.7 ± 0.7^{a}	7.9 ± 4.2^{a}
4	5.4 ± 1.3^{a}	6.7 ± 2.8^{bc}	8.6 ± 1.7^{a}

Numbers connected by the same superscript letters in the same column are not significantly different at 5% probability level

A

B

C

Figuer 6. Root induction in MS medium supplemented with different IAA concentrations. Maximum number of roots was induced at 4 mg/l IAA for variety Ugni blanc (A), at 2 mg/l IAA for variety Chenin blanc (B) and at 4 mg/l IAA for variety Canonannon (C)

5.6 Acclimatization

The number of plantlets transferred to the pot and the survival percentage in the glasshouse are presented in Table 12. Survival rate of the acclimatized plantlets was 78.6%, 92% and 73.9% for Ugni blanc, Chenin blanc and Canonanoon respectively. Pictures showing the growth condition of grape plantlets in the glass house at different time are presented in (Fig.7).

Table 12. The number of plantlets transferred in to the soil and survival rate in the glass house.

Variety	Plantlets transferred	No. of survived plantlets	Survival percentages
Ugni blanc	28	22	78.6%
Chenin blanc	25	23	92%
Canonannon	23	17	73.9%

A 

B 

C 

Figure 7. Acclimatization of the *in vitro* plantlets of three varieties of grape vines in the greenhouse under natural sun light condition. (A) Plantlets of grape vine in the glasshouse after one week of removing the plastic bag, (B) Plantlets grown after four weeks of removing the plastic bag and (C) Well –developed plantlets after three month of acclimatization.

6. DISCUSSION

6.1 Preliminary observation of the duration of sterilizing explants

Determination of the duration of sterilization of explants was very essential to avoid the problem of contamination during *in vitro* culture (Loyala and Vazques, 2006). In fact, the sterilization treatment may vary from season to season as the microbial populations are dependent on seasons (George and Sherrington, 1984). The most commonly used sterilizing agents for obtaining aseptic tissues are sodium hypochlorite and calcium hypochlorite. In the present study, sterilizing the explants with 1% NaOCl for 7 minutes was found to be optimum. This sterilization should be preceded by 70 % alcohol for 30 seconds.

Previously, on the study of the effect of growth hormones on micropropagation of *vitis vinifera* L. Cv. Perlette Muhammad *et al*. (2008), the problem of contamination was solved with 10% chlorox treatment for 5, 10 and 15 minutes. Both higher treatment for 10 and 15 minutes showed non-significant differences and successfully controlled contamination.

6.2 Effect of different concentration of BAP on shoot Initiation and survival

Shoot initiation experiment was conducted for the establishment of optimum culture condition for subsequent shoot multiplication process. Shoots were induced in all treatments including the control. This is in contrast with the works of Mon *et al*., (2008) in the study of production of plantlets through direct and indirect embryogenesis, shoot were not induced even at low level of BAP (2 µM/l). Even though shoots were induced at hormone free medium morphologically best shoots were induced on the medium that contains 0.5 mg/l BAP. Shoots induced on hormone free medium were thin and weak.

The survival percentage of shoots showed significant variation at different concentration of BAP. The maximum percentage of survival was 96% for the variety Chenin blanc and 88 % for the verities Ugni blanc and Canonnanon. This study is in consistence with the

result of Chee *et al*. (1984) where the highest percentage of shoot initiation were obtained on MS medium supplemented with 0.5 mg/l BAP.

6.3 Effect of different concentration and combination of BAP and IBA on shoot multiplication

In this study, shoot multiplication potential of three grape varieties was tested in different concentrations of BAP with or without IBA. According to Heloir *et al*., 1997, in the study of in vitro propagation of grape vine using axilary bud micro cuttings, BAP was the most effective among other cytokinins for inducing shoots. Besides development of multi shoots did not occurred in hormone free medium but only the main shoot developed without shoot multiplication. Ghulam *et al*., (2006) also reported similar findings when they grew two different varieties of grapes on the media containing BAP and NAA. The presence of BAP, even at relatively low levels (i.e., 0.25 mg/l), enhanced shoot multiplication (Table 2 and 6). As the concentration increases abnormal growth was observed in all varieties. The shoots become short and bushy; the leaves were thick and fragile. This situation is in agreement with the works of Banilas and Korkas, (2004) in rapid micropropagation of grapevine through lateral bud development.

According to this experiment, increased concentration of BAP has a negative effect for lengthen the induced shoots and increase the number of nodes. Ghulam *et al*., (2006) reported similar results for different varieties of grapes on the media containing BAP and NAA. But Poudel *et al*. (2005) in the study of the effect of growth regulators on *in vitro* propagation of *vitis vicifolia*, reported that the shoot length increased as BAP concentration increased. In this study, however, when BAP was combined with IBA the length of shoots and number of nodes was improved. This implies that auxin has a positive effect on shoot elongation when it was combined with lower concentration of BAP.

6.4 Effect of IAA on root induction

In the rooting experiment, the primary roots were visible after two weeks of culturing on MS medium supplemented by different concentration of IAA. However, more developed and mature roots were obtained after a month. Rooting of shoots was usually attained at low concentration of auxins in many *in vitro* cultures (Nitzche and Wenzel, 1977). In the present study of rooting, however, maximum number of roots was obtained at maximum concentration of IAA. A maximum mean number of roots (3.7) was counted for Chenin blanc in a medium supplemented with 2 mg/l IAA. Ugni blanc and Canonannon gave a maximum mean number of root in a medium that contains 4 mg/l IAA (Table 10). This result is in consistence with rooting result obtained for *Vitis ficifolia* variety ganebu (Poudel *et al.,* 2005).

6.5 Acclimatization

In this experiment, plantlets about 4 cm tall were transferred into the soil because plantlets less than 3 cm were not survived. Moreover those plantlets uncovered with plastic bags started to weaken immediately after transferred to the soil. This implies the plastic cover protects the plantlets from the external stress for some times until they adapt to the outside environment. After one week, the plastic cover should be removed to avoid fungal development because among the five plantlets remains with the plastic cover all of them were contaminated by fungi. Survival rate of the acclimatized plantlets was 78.6%, 92% and 73.9% for Ugni blanc, Chenin blanc and Canonanoon respectively. Chee *et al*. (1984), reported 64-94 % survival in the study of *in vitro* micropropagation of *vitis*. Only survival percentage of the variety chenin blanc were maximum but the rest two varieties has relatively low survival percentages.

7. CONCLUSION

Conventional propagation methods require large amount of materials and space for propagation, and an extended period to produce plants. These limitations prevent an efficient and rapid production of planting materials to meet the market demand. Thus micropropagation is recommended to assist the conventional method. The present *in vitro* propagation study of grapes found in Ethiopia is the first of its kind. This preliminary study was under taken to optimize the duration of the sterilization time and plant growth regulators for shoot and root initiation. Based on this studies,

- Seven minutes of sterilization in 1% NaOCl followed by culturing on basal Ms medium supplemented with 0.5 mg/l BAP was found to be effective for sterilization and shoot initiation, respectively.

- The best multiplication of shoots were obtained at 1mg/l BAP with 0.1 mg/l IBA for the variety Chenin blanc and Canonannon, while 2 mg/l BAP with 0.1 mg/l IBA for Ugni blanc.

- High levels of IAA, 2 mg/l for Chenin blanc and 4 mg/l for Ugni blanc and Canonannon were found to be effective in enhancing root number and length.

- In the acclimatization process the survival rate of Chenin blanc was better than the other two varieties.

- In this *in vitro* micro propagation of grape vine, it is possible to demonstrate culture establishment, shoot proliferation and rooting by using the different concentrations of BAP, IBA and IAA.

8. RECOMMENDATION

Based on the results of the present study the following recommendations are made

- Further studies may be needed using other hormones such as Kinetin, 2-Ip or BA which are potential cytokinins for culture establishment and shoot proliferation.

- Micropropagation techniques such as shown in the present study should be applied to solve the problem associated with the propagation of the crop.

- Since various diseases, especially virus affect grapes, virus indexing should be included in the study of the micropropagation of grape vines.

- The effect of sub-culturing on the multiplication of shoots should be further studied, to determine whether the sub-culturing increases or decreases shoot multiplication to a significant extent.

- The protocol could be used for mass propagation of grape after some minor refinement.

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10. APPENDIX

Table 1. Compositions of MS basal medium.

Components	Concentration
Macronutrient	
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
Micronutrient	
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	0.2
Nicotinic acid	0.05
Pyridoxine B6)	0.05
Thiamin B1)	0.01

Table 2. Survival percentage of shoots of the three varieties cultured on MS medium supplemented with five different concentration of BAP at N=25 per treatment.

Variety	Replication	0	0.5	1	2	3	4 (mg/l BAP)
Ugni	1	3	5	2	4	5	5
	2	5	4	0	3	3	4
	3	4	5	5	3	5	0
	4	4	4	4	4	5	2
	5	5	4	4	5	4	3
	Total	21	22	15	19	22	14
	Percentage	84%	88%	60%	76%	88%	56%
Chenin	1	5	5	4	4	0	5
	2	4	5	5	4	3	5
	3	4	5	5	3	5	3
	4	3	5	4	5	5	3
	5	4	4	5	5	4	5
	Total	20	24	23	24	17	21
	Percentage	80%	96%	92%	96%	68%	84%
Canonanon	1	0	4	5	5	4	4
	2	2	5	5	4	4	3
	3	0	5	2	3	3	4
	4	4	4	4	4	5	4
	5	4	4	4	4	4	5
	Total	10	22	20	20	20	20
	Percentage	40%	88%	80%	80%	80%	80%

Declaration

I the undersigned, declare that this thesis is my original work and has not been presented for any academic degree in any other university and that all sources of materials used for the thesis have been correctly acknowledged.

Name: Beza Kinfe Signature_____Date_____

This Thesis has been submitted for examination with my approval as an advisor:

Dr. Tileye Feyssa Signature _____Date_____