# *IN VITRO* REGENERATION OF CHENIN BLANC AND CANONANNON VARIETIES OF GRAPEVINE (*Vitis vinifera* L.) FROM LEAF EXPLANTS

**MSc THESIS**

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**HARAMAYA UNIVERSITY, HARAMAYA**

# *IN VITRO* REGENERATION OF CHENIN BLANC AND CANONANNON VARIETIES OF GRAPEVINE (*Vitis vinifera* L.) FROM LEAF EXPLANTS

**A Thesis Submitted to the College of Natural and Computational Sciences, Department of Biology, Postgraduate Program Directorate**

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**In Partial Fulfillment of the Requirements for the Degree of**

**MASTER OF SCIENCE IN APPLIED BIOLOGY**

**BERIHA GEBREWAHD**

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## HARAMAYA UNIVERSITY

## POST GRADUATE PROGRAME DIRECTORATE

As Thesis research advisors, we hereby certify that we have read and evaluated this Thesis entitled **''*In vitro* Regeneration of Chenin blanc and Canonannon Varieties of Grapevine (*Vitis vinifera* L.) From Leaf Explants''** prepared under our guidance by Beriha Gebrewahd and we recommend that it be submitted as fulfilling the thesis requirement.

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As members of the Board of Examiners of the M.Sc. Thesis Open Defense Examination, we certify that we have read and evaluated the thesis prepared by Beriha Gebrewahd and examined the candidate. We recommended that the Thesis be accepted as fulfilling the thesis requirement for the degree of **MASTER OF SCIENCE IN APPLIED BIOLOGY**.

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Final approval and acceptance of the thesis is contingent up on the submission of the final copy to the Council of Graduate Studies (CGS) through the candidates Department and Graduate Committee (DGC) of the School of Biological Sciences and Biotechnology.

# DEDICATION

I dedicate this thesis to all my family members especially to my father, relatives and to my high school teachers.

## STATEMENT OF THE AUTHOR

By my signature below, I declare and affirm that this thesis is my own work and that all sources of materials used for the thesis have been duly acknowledged. This thesis is submitted in partial fulfillment of the requirements for an M.Sc. degree at Haramaya University. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma or certificate and is deposited at the University library to be made available to borrowers under the rules of the Library. Brief quotations from this thesis are allowable without special permission provided that accurate acknowledgement of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the school of biological sciences and when in his or her judgment the proposed use of the material is in the interests of scholarship. In all other instances, however, permission must be obtained from the author.

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# BIOGRAPHICAL SKETCH

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# LIST OF ACRONYMS/ ABBREVIATIONS

| | |
|---|---|
| DZARC | Debre Zeit Agricultural Research Center |
| EU | European Union |
| MS | Murashige and Skoog |
| BAP | 6-Benzyl amino purine |
| NAA | α-Naphthalene acetic acid |
| IBA | Indole-3-butyric acid |
| IAA | Indole-3-acetic acid |
| TDZ | Thidiazuron |
| 2, 4-D | 2, 4 dichlorophenoxyacetic acid |
| CRD | Complete Randomized Design |

# TABLE OF CONTENTS

# LIST OF TABLES

**Table** **Page**

# LIST OF TABLES IN THE APPENDIX

**AppendixTable** **Page**

# LIST OF FIGURES IN THE APPENDIX

**AppendixFigure** **Page**

# In vitro Regeneration of Chenin Blanc and Canonannon Varieties of Grapevine *(Vitis vinifera* L.*)* from Leaf Explants


## *ABSTRACT*

*Grapevine (Vitis vinifera* L.*) is one of the most widely distributed fruit crops in the world. The availability of adequate amount of quality and disease free planting materials within a short time is the major limiting factor to attain large scale grapevine production using the conventional method of Propagation. Therefore, development of an optimal in vitro propagation technique is too much needed. The objective of this study was to develop an optimal in vitro regeneration protocol for two grapevine varieties (Chenin blanc and Canonannon) from leaf explants. The leaf explants have been cultured on MS basal medium prepared with (0mg/l, 1mg/l, 1.5mg/l, 2mg/l, 2.5mg/l, 3mg/l and 4mg/l) BAP and (0mg/l, 0.1mg/l, 1mg/l, 2mg/l, 2.5mg/l and 3mg/l) TDZ separately for shoot induction and multiplication. One-month sub cultured shoots of the grape varieties were rooted on the full strength 20ml MS medium supplemented with 3% (w/v) sucrose and with different concentrations of IBA (1mg/l, 2mg/l, 3mg/l and 4mg/l) and IAA (2mg/l and 4mg/l) and the plantlets were then transferred to 12cm diameter plastic bag containing sterilized red soil, sand and cow dung manure at the ratio of 1:2:1, respectively for acclimatization. From the tested seven different concentrations of BAP and six different concentrations of TDZ, the best direct shoot regeneration was obtained at 2mg/l BAP for both Chenin blanc (2.6±0.5) and Canonannon (2.3±0.2). Therefore, frequency of shoot regeneration was greatly influenced by the concentrations of growth regulators. Efficient rooting of in vitro regenerated plants and subsequent establishment was achieved on a medium supplemented with 2mg/l IBA for both varieties of grapevines. The survival rates were 75.0% and 83.3% for Chenin blanc and Canonannon, respectively. It is necessary to investigate the indirect shoot regeneration mechanism for both varieties at higher concentrations of the growth regulators that were not included in this study.*



***Key words:*** *Acclimatization, explant, growth regulators, Organogenesis, Vitis vinifera L.*

# 1. INTRODUCTION

Grapevine (*Vitis vinifera* L., Vitaceae) is one of the most widely distributed fruit crops in the world. It grows from temperate to tropical regions, but most vineyards are planted in areas with temperate climates. Wild grapevines occur primarily in the Northern Hemisphere, especially in the temperate zone in Asia, North America, Central America, and Northwest of South America in the Andes chain in Colombia and Venezuela. This distribution highly deals with historical connections with the development of human culture (Patrice *et al*., 2006).

It is believed that grape cultivation originated near Caspian Sea in Russia that spread westward to Europe and American continents and eastward towards Iran and Afghanistan (Richard *et al*., 2010). However, *V. vinifera* originated in the regions between north and south of the Caspian and Black Seas in Asia and has been distributed from region to region in all temperate climates (Krongjai, 2005). 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).

Micro-propagation of grapevine was first performed by "*in vitro*" culture of micro-cuttings (Aazami *et al*., 2010). More recently, introduction of bud proliferation has been shown to provide an alternative pathway to grapevine micropropagation (Aazami *et al*., 2010). However, the developed technique should result in rapid clonal multiplication and uniform plants, normal yield and healthy plants (Salami *et al*., 2005). In addition, the *in vitro* vegetative multiplication techniques should be designed in the form of unchanging genetic makeup, basic biological, physiological, and horticultural characteristics (Chee *et al*., 1984). Grapes are used for wine production, fresh fruit, dried fruit, and juice production. It has much health benefits through prevention of cancer, heart disease, degenerative nerve disease, Alzheimer diseases, retinal disorder, constipation and viral disease. Also it has nutritional value as it is rich in calcium, iron, vitamin C, A, E, amino acids and phosphorous. These components make the immune system strong and prevent common disease (Conde *et al*., 2007; Hulya, 2007; Jaladet *et al*., 2009).

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). 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. The prevailing average grapevine production at DZARC (100,000kg/ton/year), Merti-Jeju (350,000kg/ton/year) and Ziway (100,000kg/ha/year) with average national yield of (183,000kg/ton/year), which shows that the country is endowed with suitable environmental conditions for viticulture expansion. (Chusing, 2008).

The conventional method of grapevine propagation is time consuming and allows disease transmission. Juvenility is one of the principal naturally occurring problems hindering grapevine production (Winkler, 1974; Rossel, 1992). Grapevine 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).

Although the grapevine is the third most important fruit crop in the world after banana and citrus, today the need for grapevine fruit is increasing (Richard *et al*., 2010). Typically, this happened because of increase in the number of wine industries and more demand for fresh and dried fruits (Fayek *et al*., 2009). According to Aazami *et al*. (2010), genetic improvement of the classic cultivars in order to obtain high quality wine and table grape varieties through conventional hybridization methods does not appear to be enough.

Tissue culture and the commercial production of plantlets in different parts of the world are limited to a few outstanding regional cultivars. Even though micropropagation represents an efficient method of plant regeneration and rapid propagation of any valuable genotype, to do in vitro selection and genetic transformation need the in vitro regenerated plantlets of the varieties (Pe´ros *et al*., 1998).

Propagation of grapevine conventionally gives low yield and highly susceptible to disease. On the other hand, availability of adequate quality and disease free planting materials within a short time is the major limiting factor to attain large scale grapevine production using the conventional method of Propagation.

To overcome the shortcomings of conventional propagation method of mass production of disease free grapes within short period of time, development of optimal invitro propagation techniques was of paramount importance. Therefore, this study has been carried out to optimize the methods in vitro regeneration of the two grape varieties.

**General objective:**

- To develop an optimal *in vitro* regeneration protocol for two grapevine varieties (Chenin blanc and Canonannon) from leaf explants.

**Specific objectives:**

- To determine the appropriate concentration of growth regulators (BAP and TDZ) for shoot initiation from leaf explant and multiplication.
- To determine the appropriate concentration of plant growth regulators (IBA and IAA) for efficient rooting.
- To evaluate the degree of acclimatization of plantlets (Chenin blanc and Canonannon) in the greenhouse condition.

# 2. LITERATURE REVIEW

## 2.1. Taxonomy and Morphology of Grape

Grapes belong to family Vitaceae (Cherepanov, 1995). Grapevine 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. Grapevine leaves are alternate, from full, rounded or angular to very much divided in to 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 to the petals. Each anther consists of two lobes running length-wise. Each lobe is divided into 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).

## 2.2. Structure and Growth Stage of Grapevine

Like most other plants, the grapevine has a predictable cycle of growth. The life cycle of the grapevine can be categorized under certain stages, depending upon the growth pattern of the plants. Bud break, flowering stage, the fruit stage, veraison (coloring) and harvesting stages are the complete set of life cycle of grapevine (Krongjai, 2005). There are some grape growing areas in the world, such as the subtropical climates of southern India, where grapes not shed their leaves naturally. In the spring, when the mean daily temperature reaches about 10°C, the buds begin to swell in spring, and the green shoot emerges from them. The shoots, leaves, tendrils, clusters, and new buds are rapidly grown and develop (Yingyos, 2007).

## 2.4. Grape Production and Trends in the World

Historically, grape production and consumption have been at home in Europe. The European Union 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, producing grape on about 2 million hectares and America produces about 1 million hectares, and shows a growing tendency. In Africa, grape production area 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, 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 primarily due to 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).

## 2.6. Importance of Grape

### 2.6.1. Economical uses of grapes

Grapevine is one of the most important commercial fruit crop in terms of economic value (Orhan *et al*., 2009). The grape has been used in folk medicine for its biological activities since ancient times. 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). Approximately 71% of this production is used for wine, 27% as fresh fruit, and 2% as dried fruit (Conde *et al*., 2007).

The use of Grape is based on its structure. For instance, leaves of the plant, which have stringent and haemostatic properties, are used in the treatment of diarrhea, hemorrhage, varicose veins, hemorrhoids, inflammatory disorder, pain, hepatitis, free radical related diseases, to heal wounds and as an antiseptic for eye wash (Orhan *et al*., 2009). In addition to this, the grape is used as fermented to wine and brandy, fresh fruit, and juice production

(Carimi *et al*., 2005). Based on their usage and purposes, grapes are divided into five main classes.

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

**Wine grapes:** These varieties can produce satisfactory wine in some locations. These grapes have high acidity and moderate sugar content, while grapes with high sugar content and moderately low acid are required for sweet or dessert wines. 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).

**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).

**Raisin grapes:** These include any dried grape, 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; the latter has seeds that can be removed by machine (Yingyos, 2007).

**Canning grapes:** Only seedless grapes are suitable for use in canned fruit. The Thomson Seedless variety is most commonly used, alone or in combination with other fruits as fruit salad or fruit cocktail (Yingyos, 2007). 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).

### 2.6.1 Nutritional value of grapes

Grapevine is one of the most commonly consumed fruits in the world. Grapevine 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 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, malice acids, succinic, fumaric, glyceric, p-coumaric and caffeic acids (Mattick, 1973). Lycopene, carotene, ellagic acid, resveratrol and other sulfur 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).

### 2.6.2. Health benefits 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 (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 antioxidant, 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). 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).

## 2.7. Diseases of Grape

From commercial viticulture perspective, nearly all grape varieties are propagated through stem cutting, layering and grafting in most parts of the world. However, this increases the susceptibility of cultivated varieties to disease causing agents (microbes, mites, insects, nematodes, fungi, bacteria, viruses and more importantly Phylloxera) (Alizadeh *et al*., 2010). According to Yingyos (2007) many serious diseases of fungi like powdery mildew, gray rot; viral diseases like fan leaf roll fleck, stem pitting, corkey bark and bacterial diseases like pierces and necrosis are accountable for drop in production and shortened life span of plants. According to Yingyos (2007) diseases affect production, harvesting, processing and marketing.

The incidence of diseases depend not only on the presence of the pathogen but also on the vineyard management practices and environmental factors like temperature, rainfall and humidity which have an important bearing on the epidemics of any disease (Jamadar, 2007). In most cases, disease is the result of an interaction between a susceptible host and a living pathogenic organism.

Conventional method of propagation is sometimes in a weak position by seedling heterozygosity, space and time consideration, seed and cutting dormancy and limited yield (Jaskani, 2008). Hence, one needs to focus on developing the successful commercial cultivation accessibility to suitable planting materials (Alizadeh *et al*., 2010). Thus, improvement in production and quality of grapes can be achieved by practicing genetic and sanitary clonal selection through incorporation of unconventional method like tissue culture.

## 2.8. Concept of Plant Tissue Culture

Tissue culture may be defined as the aseptic culture of cells, tissues, organs or whole plants under controlled nutritional and environmental conditions (Gonzales *et al*., 2010). The first reports regarding tissue culture date back to the beginning of the 20th century when Gottlieb Haberlandt (Haberlandt, 1902 cited in Gonzales *et al*., 2010) developed experiments to maintain mesophyll cells in culture. From this moment on, development has been constant and every year hundreds of results and reports regarding the application of

tissue culture techniques, applied to breeding programs, genetic biodiversity conservation and biopharmaceutical production have been documented. (Gonzales *et al*., 2010).

In addition, plant tissue culture plays an important role in the rapid micropropagation of many agronomically important plant species (Iktena and Reada, 2010). It represents also an efficient method of plant regeneration and rapid propagation through organogenesis and embryogenesis of any valuable genotype obtained by nonconventional methods and therefore, regeneration of whole plants by somatic embryogenesis and organogenesis has been intensively studied. Organogenesis and embryogenesis result in formation of plantlets from a determined tissue in order to form complete plants. Nevertheless, propagative potential depends on the species and on the explants source (Aazami *et al*., 2010).

The limitations of tissue culture were also studied from time to time. Accordingly, some authors have argued that plants regenerated from direct somatic embryogenesis and organogenesis ought to contain fewer mutations than those regenerated via callus phase (Gloriada *et al*., 1999). In addition, many investigators have noted that an increase in variability of plant is increased with culture age (Gloriada, *et al*., 1999).

Anyways, development of *in vitro* techniques for many plant species provide promising opportunities for rapid and reliable plant propagation based on several factors. The composition of the culture medium is an important factor in successful establishment of a tissue culture. Each tissue type may require a different formulation of growth regulators compared with genotypes of the explants relating to the objectives of the study (Torregrosa and Bouquet, 1996; Dikibo, 2008). In vitro propagation via organogenesis usually involves four stages including initiation of cultures, multiplication of shoots, rooting of shoots, and acclimatization of plants (Cardoza, 2008).

### 2.8.1. Types of plant tissue culture

George *et al.* (2008) classified plant tissue culture into two; namely: cultures of unorganized tissues and cultures of organized tissues. Cultures of unorganized tissues with their respective explants in the bracket are; callus cultures (any plant tissue or organ), cell-suspension cultures (friable callus), protoplast culture (protoplast) and microspore culture (anthers). Root cultures (tip of either primary or lateral roots), shoot culture (tips of shoots), meristem culture (meristem), nodal culture, embryo culture (immature and mature

embryos), leaf or leaf sheath cultures (leaf or leaf sheath), fruit culture and flower cultures are classified as cultures of organized tissues, with their respective explants indicated in brackets.

### 2.8.2. Micropropagation

The objective of plant propagation via tissue culture is to propagate plants true-to-type (clones), which is termed micro propagation. It is the process in which explants placed in a suitable culture media in aseptic environment results in multiple shoots available for rooting and become ready for transferring to soil after acclimatization (Singh, 2003).

According to (George *et al.,* 2008) there are five stages of micro propagation. Namely, selection of the plant tissue (explants) from a healthy vigorous 'mother plant', Establishment of the explants in a culture medium, multiplication, root formation and finally the rooted shoots are potted up and 'hardened off' by gradually decreasing the humidity by a process called acclimatization.

### 2.8.3. Culture medium of tissue culture

One of the most important factors governing the growth and morphogenesis of plant tissues in culture is the composition of the culture medium (Rai, 2007). The appropriate composition of the medium largely determines the success of cultures. Plant materials do vary in their nutritional requirements and therefore it is often necessary to modify the medium to suit a particular tissue. The basal medium employed for the culture of grape is MS (Murashige- Skoog) medium (Murashige and Skoog, 1962). A variety of growth regulators such as 6- Benzyl amino purine (BAP), alpha-Naphthalene acetic acid (NAA), 3-Indole Butyric acid (IBA) and 2,4- dichlorophenoxy acetic acid (2,4-D) will be added to the medium singly or in combinations at various concentrations and used for initiating different experiments.

The concentrated stock solutions of the major salts, minor salts and vitamins are prepared to be used in the preparation of the media and stored under refrigeration. Auxins are dissolved in 1N KOH and cytokinins in 1N HCL before making up the final volume with distilled water. Iron EDTA stock solution is stored in amber colored bottle.

The medium is prepared by adding appropriate quantities of the stock solutions and correct volume is made up with the distilled water. The pH will be adjusted in all cases to 5.8 by using 1 N KOH and 1 N HCL and agar 0.8%(w/v) is used for semi-solid medium for culture initiation/establishment only and liquid medium were used for multiplication and rooting of micro shoots. Before autoclaving, the media is poured into washed and dried test tubes (up to 20ml) and culture bottles (15-20 ml for liquid culture) which are then, capped and labeled properly. These are then autoclaved at 121 $^{o}$C for 15 minutes at 15-psi pressure and transferred to the inoculation room where they are stored under aseptic conditions till their use. (Rai, 2007).

Plant tissue culture media should generally contain some or all of the following components: macronutrients, micronutrients, vitamins, amino acids or nitrogen supplements, source(s) of carbon, undefined organic supplements, growth regulators and solidifying agents (Saad and Elshahed, 2012). It should be considered that the optimum concentration of each nutrient for achieving maximum growth rates varies among species (Defossard R, 1976).

**Carbon and energy sources:** In plant cell culture media, besides the sucrose, frequently used as carbon source at a concentration of 2-5%, other carbohydrates are also used. Other mono or disaccharide and sugar alcohols like glucose, maltose or sorbitol may be used depending upon plant species (Rahman et al., 2010). 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 is 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 axillary buds and branching of adventitious roots. (Vinterhalter and Vinterhalter, 1997).

**Plant growth regulators:** Hormones are organic compounds naturally synthesized in higher plant, which affect the growth and development of plants by exerting a profound influence on physiological processes (Hopkins and Hüner, 2009). They are usually active at a different site in the plant from where they are produced and are only present and active in very small quantities (George et al., 2008). Apart from natural hormones, synthetic hormones have been developed which act-like the natural ones. These synthetically

produced hormones are commonly called plant growth regulators and have biological activity which equals or exceeds that of the equivalent endogenous hormones (Gaspar et al., 1996).

The synthetic plant growth regulators are used in commercial applications largely than natural ones because they are more effective, low cost, and have greater chemical stability (Hopkins and Hüner, 2009). In addition to the nutrients, it is generally necessary to add one or more growth substances, such as auxins, cytokinins, and gibberellins, to support good in vitro growth of tissues and organs.

The requirement for growth substance varies depending on the type and source of the explants and on their endogenous level (Bhojwani and Razdan, 1996). The balance composition of the medium, particularly the plant hormones and the nitrogen source (nitrate versus ammonium salts or amino acids) have profound effects on the morphology of the tissues that grow from the initial explants. In tissue culture plant growth regulators act alone and in interaction to produce the final effect (George *et al*., 2008).

Cytokinin is similar to adenine and was first discovered in 1941 as the active component in coconut milk that promoted growth of plant cells in tissue culture (Cardoza, 2008), and it is added to culture medium to promote cell division, stimulation of DNA synthesis to yield shoot formation and auxiliary shoot proliferation, can delay senesce and sometimes to inhibit root formation (George *et al*., 2008).

The irreversible degradation of cytokinins, catalyzed by cytokinin oxidase, is an important mechanism by which plants modulate their cytokinin levels (Brugie`re *et al*., 2003). Cytokinins like BAP, furfuryl amino purine (kinetin), thidiazuron (TDZ) and zeatin have been commonly employed alone and/or in combination with other cytokinins or/and auxins for proliferation and multiplication of different plant species (Gaspar *et al*., 1996). Of these BAP is the most active, cheapest and easily autoclaved without exhaust. Therefore, it is the one most frequently used, particularly in commercial micro propagation establishments where cost and ease of handling are major considerations (George *et al*., 2008).

Auxins induce cell division, cell elongation, apical dominance, adventitious root formation, and somatic embryogenesis (Rai, 2007). Auxins like NAA, 2, 4 dichlorophenoxyacetic acid (2, 4-D), indole-3 acetic acid (IAA) and IBA are the most frequently used in plant

tissue culture alone and in combination. IAA (indole-3-acetic acid) is the most important naturally occurring auxin but its use in plant tissue culture media is limited because it is unstable in the medium (Doods and Roberts, 1985).

Generally, the type of morphogenesis that occurs in a plant tissue culture largely depends on the ratio and concentration of auxins and cytokinins in the medium. With this regard, adventitious and auxiliary shoot proliferation occurs when the ratio of auxin to cytokinin is low, whereas root induction of plantlets, embryogenesis and callus initiation occur when the ratio of auxin to cytokinin is high (George *et al*., 2008).

**Amino acids:** The required amino acids for optimal growth are usually synthesized by most plants, however, the addition of certain amino acids or amino acid mixtures is particularly important for establishing cultures of cells and protoplasts. Amino acids provide plant cells with a source of nitrogen that is easily assimilated by tissues and cells faster than inorganic nitrogen sources. Amino acid mixtures such as casein hydrolysate, L-glutamine, Lasparagine and adenine are frequently used as sources of organic nitrogen in culture media. (Torres K.C, 1989).

**pH and solidifying agents:** The hydrogen ion concentration of the tissue culture medium is important since it influences the uptake of various components of the medium as well as regulating a wide range of biochemical reactions occurring in plant tissue cultures by altering pH of the cells (Owen *et al*., 1991; Singh *et al*., 2013). Most media are adjusted to a pH of 5.2–5.8. Values of pH lower than 4.5 or higher than 7.0 greatly inhibit growth and development in vitro (Rai, 2007).

Cultures can be grown in either a liquid or solid medium. Solidifying agents are used for preparing solid and semisolid tissue culture media to enable explants to be placed in right contact with nutrient media (not submerged but on surface or slightly embedded) to provide aeration (Puchooa *et al*., 1999).

The most frequently used solidifying agent is Agar. Agar is high molecular weight polysaccharide obtained from sea weeds and can bind water. It is added to the medium in concentration ranging from 0.5% to 1 %( w/v). Agar is preferred over other gelling agents because it is inert, neither does it react with media constituents nor digested by plant enzymes (Cameron, 2006). Hardness of the culture medium greatly influences the growth

of cultured tissues. There are a number of gelling agents such as agar, agarose and gellan gum. (Prakash *et al.*, 2002).

**Light and temperature:** The average constant growth room temperature employed in a large sample of experimental reports was found to be 25°C ranging from 17 to 32°C. In many plants, in vitro root induction on the shoot micro-cuttings produced requires a slightly lower temperature than is necessary for shoot multiplication and growth. Fluorescent tubes are used almost universally to provide light for cultures (George et al., 2008).

## 2.2. Organogenesis of Grapevine

The organogenesis is a biotechnological tool used for obtaining mass production of mother plant with high quality of health (Betton *et al.*, 2015). The explants can be grown into whole plant or produce callus. Plant mass production can be affected by several factors such as light, temperature, plant varieties, and type of explant, components of media, sources and orientation of explants (Kumar and Raddy, 2011).

Temperature influences the various physiological processes, such as respiration and photosynthesis, It is not surprising that it profoundly influences plant tissue culture and micro-propagation. The most common culture temperature range is been between 20°C and 27°C, but optimal temperatures vary widely, depending on genotype (Kumar and Raddy, 2011). Most of the time, the optimal shoot proliferations of grapevine were reported when, both hormones (Cytokines and auxins) were combined. For instance for *Muscat of Alexandria cv* maximum number of proliferated shoots was obtained on MS medium containing 3.0 mg/l BAP + 0.2 mg/l NAA.

According to (Aazami, 2010) different combinations of growth regulators (1.5 mg/l BA), C (1 mg/l IBA+ 1.5 mg/l BA) were produced the best shoot for"*Soltanin*"and "*Sahebi* cultivars from meristem. So developing *in vitro* propagation of grapes was not only for the wine industry, but also due to the demand for fresh and dried fruit (Abido, 2013). Beside the micro-propagations, an establishment of efficient protocol for high-frequency of indirect regeneration of plantlets is so much needed. Even though the indirect regeneration of grapevine were not success with different cultivars of grapevine, it was reported that

shoots were initiated from callus of ‘Canonannon’ cultivar at 0.5mg/l BAP+1mg/l IBA at 35 days, after culture (Fikadu Kumsa, 2016).

## 2.9. Invitro Plant Regeneration Systems of Grapevine

The micropropagation of grape has been reported previously by many authors. Thus, use of *in vitro* techniques for propagation of various *Vitis vinifera* cultivars has been well-documented (Alizadeh *et al.,* 2010*).*

Despite years of investigation, the application of tissue culture techniques in the grape-growing industry is still limited (Pe´ros *et al*., 1998). Hence, different cost effective protocols for organogenesis should be developed (Deore and Johnson, 2008). Thus, an establishment of such efficient protocol for high-frequency direct regeneration of plantlets from leaf explants of *Vitis vinifera* has a vital role in the analysis of genetic material and mass propagation of plants in short period of time. Accordingly, effective mass propagation of grapevine from shoot tips of some grapevine cultivars was successful on different concentrations of growth regulators (Beza Kinfe, 2010).

## 2.10. Tissue Culture of Grape

The micropropagation of grape has been reported previously by many authors. Thus, use of in vitro techniques for propagation of various *Vitis vinifera* cultivars has been well-documented (Chee and Pool, 1984; Mhatre *et al*., 2000; Singh *et al*., 2004, cited in Alizadeh *et al*., 2010*).* 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).

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). However, the study done by Baker and 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).

In plant tissue culture, different explants are used to regenerate the whole plant (Sebastiani *et al*., 2001). Most of micropropagation in grapevine was performed by "in vitro" culture of micro-cuttings (Aazami, 2010). Limitations of the methods that use anthers and ovules as explants are questionable due to the brief availability period of the explants. Hence, there is high potential to use leaves as explant material in somatic organogenesis and embryogenesis at high frequency. Leaves are readily available throughout the year, and useful for the genetic improvement of *Vitis vinifera* through the transformation of important agronomic traits (Das *et al.*, 2002).

In addition, introducing micropropagated shoot cultures provide a constant and reliable source of sterile leaf explant material for generating embryogenic and organogenic cultures are important (Meyerson, 1994). Thus, embryogenic and organogenic cultures are useful in grape transformation studies and to provide an alternative pathway to grapevine micropropagation (Meyerson, 1994). Shoot apical meristem culture is also used for in vitro regeneration in varieties of grape (Das *et al.*, 2002). Baker and Bhatia (1993) reported adventitious shoot regeneration using leaf explants of crops such as apple, pear, *Rubus* and *Vitis.* But somatic organogenesis has been achieved from immature ovules of muscadine (Xu *et al*., 2005).

Grape is a plant propagated by seed or grafting. However, seed grown plants are genetically very heterogeneous (Winkler, 1974). Vegetative multiplication is slow and only a limited number of plants can be grown from a stock plant. Propagation by grafting includes failure to root and seasonal responsiveness to rooting. Moreover, rooting ability is strongly influenced by plant genotype. Grafting transfers disease from their mother plants to newly growing one. It is also one of the traditional ways of harvesting system. Micropropagation and plant regeneration are one of the alternative methods for the production of selected superior plants in a short period of time and at high frequency (Pe´ros *et al*., 1998).

According to Rossel (1992), the expansion of vineyard cannot be achieved without the pre-establishment of techniques that make adequate amount of planting materials available within short time. Hence, it is better to develop the technique for large-scale production. This might need to develop an effective technique based on the plant species and cultural conditions within the small size place and then to field system.

# 3. MATERIALS AND METHODS

## 3.1. Description of the Study Area

The study was conducted at Tissue Culture Research Laboratory of Plant Biotechnology in Tigray, specifically at Ellala near to Mekelle Agricultural Research Center, Northern Ethiopia, that is located at a latitude of 13°29′N, longitude of 39°28′E and altitude of 2076 meters above sea level (MARC, 2012).

## 3.2. Explant and its Surface Sterilization

*In vitro* cultivated stock plants of grape varieties (Chenin blanc and Canonannon) were obtained from Debre Zeit Agricultural Research Center (DZARC) and used as source of explants. Shoots of grapevine have been excised from stock plants and the most uppermost expanding leaves were cut near the junction of petiole and lamina from the shoots. Thereafter, leaves were washed thoroughly twice under running tap water containing liquid soap solution and three drops of Tween-20. Then, explants were taken to laminar airflow chamber and rinsed in 70% alcohol for one minute and washed with sterile distilled water three times each for five minutes. Finally, the explants were treated with 10% (v/v) sodium hypochlorite solution (4% active chlorine) for 20 minutes. After discarding the sodium hypochlorite solution, the explants were washed with sterile distilled water three times each for five minutes and the surface sterilized explants were aseptically excised and sized to 1 cm long and 0.5 cm diameter for culturing. Following to (Fikadu Kumsa, 2011).

## 3.3. Stock Solution and Culture Media Preparation

### 3.3.1. Murashige and Skog (MS) stock solution preparation

In the present study, the media stock solutions were prepared separately by weighing the recommended amount of macronutrients, micronutrients and vitamins (Appendix II). For example macro nutrients stock solution was prepared first with 2 liter capacity beaker having about 500 ml of distilled water. Then amount of the required macronutrients was taken and added to the beaker. Then after, it was stirred using magnetic stirrer, dispensed and a homogenous solution was obtained. Finally, some distilled water was added and obtained a final 100 ml solution. The same procedures were followed for preparing stock

solutions of micronutrients and other organic additives. The stock solutions were kept in a refrigerator until use and the prepared stock solutions were used for a maximum of one month. Following to (Beza Kinfe, 2010).

### 3.3.2. Plant growth regulators stock solution preparation

Plant growth regulators such as BAP and TDZ were weighed in such a way that every ml of a solution contains 1mg of a given growth regulator and three to four drops of 1M NaOH and 1N HCl for auxins and cytokinins, respectively until the crystals were dissolved. Thereafter, the volume was adjusted by adding double distilled water to obtain a final stock solution. The stock solutions were labeled and stored in refrigerator until use with a specified time period. Following to (Beza Kinfe, 2010).

### 3.3.3. Culture media preparation

Full strength Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962) was used as a culture medium. MS basal medium consisted of 30g/l of sucrose for leaf culture shoot regeneration, shoot multiplication and rooting. Plant growth regulators namely 6-benzyl aminopurine (BAP) and thidiazuron (TDZ) were added separately to the MS medium. The pH of the medium was adjusted to 5.8 using 1 N KOH and 1 N HCl before being gelled with 8g/l agar and autoclaved at 121$^{o}$C, 15 psi for 20 minutes. While molten the medium (40 ml) was dispensed into Petri dishes for culturing and stored under aseptic condition until use for culturing. Following to (Beza Kinfe, 2010).

Similarly, the MS basal medium consisted of 60g/l of sucrose was used for efficient rooting of the leaf culture. Plant growth regulators namely Indole-3-butyric acid (IBA) and Indole-3-acetic acid (IAA) were added separately to the MS medium.

## 3.4. Shoot Induction and Multiplication

For shoot initiation, leaves were wounded abaxially by sterilized scalpel blade across main vein. Thereafter, the wounded leaves were cultured on MS basal medium prepared as indicated above and supplemented with (0, 1, 1.5, 2, 2.5, 3 and 4mg/l) of BAP and (0, 0.1, 1, 2, 2.5 and 3mg/l) of TDZ separately. The culture was maintained in a growth room at a temperature of 25 ± 2$^{o}$C under 16/8-hours photoperiod adjusted with fluorescent light having 2500 μmol $m^{-2}S^{-1}$ light intensity with 75-80% relative humidity of the incubation

chamber. The experiment was laid out in a completely randomized design (CRD) with three replicates. Following to (Fikadu Kumsa, 2011).

The regenerated shoots were transferred to a fresh medium in Petri dishes after four weeks and slowly adapted to light using transparent cloth. The cloth was removed after two weeks and when the size of the shoots reached above 12mm they were transferred to shoot multiplication medium and incubated at 27$^{o}$C. The shoot multiplication medium was MS medium consisted of 2mg/l BAP. Then, the light adapted regenerated shoots were sub-cultured on the MS medium. Following to (Fikadu Kumsa, 2011).

## 3.5. *In Vitro* Rooting of Shoots

One-month sub-cultured shoots of the grape varieties were rooted on 20ml of full strength MS medium supplemented with 3% (w/v) sucrose supplemented with different concentrations of IBA (1, 2, 3 and 4mg/l) and IAA (2 and 4mg/l) alone. All the cultures have been incubated at 25±2$^{o}$C under 16/8 hours of photoperiod adjusted with fluorescent light having 2500 μmol $m^{-2}S^{-1}$ light intensity with 75-80% relative humidity of the incubation chamber. The experiment was laid out in a completely randomized design (CRD) in three replicates. Following to (Fikadu Kumsa, 2011).

## 3.6. Acclimatization of the Plantlets

The plantlets having sufficient root and shoot systems were taken out from the culture vessels and their roots were washed under running tap water to remove the agar and sucrose. Finally, plantlets were soaked in hot water about 40$^{0}$C for about 5 minutes to kill contaminants. The plantlets were then transferred to 12cm diameter plastic bag filled with sterilized red soil, sand and cow dung manure at the ratio of 1:2:1. The plantlets were then covered with transparent plastic bag to maintain moisture and watered every other day. The plastic cover was gradually removed and the plantlets were grown in insect proof glasshouse for one month. While hardening of the plantlets, the relative humidity, temperature and light intensity were adjusted as indicated in table1. Following to (Fikadu Kumsa, 2011).

Table 1. Adjustment of the greenhouse microclimate during acclimatization of the plantlets

| Week | Relative humidity | Temperature | Light intensity (Lux) |
|---|---|---|---|
| 1 | 80-90 | 25 ± 2$^0$C | 12000 |
| 2 | 70-80 | 26 ± 2$^0$C | 25000 |
| 3 | 60-70 | 27 ± 2$^0$C | 50000 |
| 4 | 50-60 | 27 ± 2$^0$C | 70000 |

## 3.7. Data Collected

The following *in vitro* plant growth variables were recorded to serve as quantitative data.

**Percent of explants shoot regeneration:** Percent of shoot formed from the cultured explants of the two varieties after four weeks.

**Average number of days to shoo emergence:** number of days needed by the explants to induce shoots from the first day of culturing in the shooting media.

**Average number of days to root emergence:** number of days needed by the explants to induce roots from the first day of culturing in the rooting media.

**Mean number of shoots/explant:** is the average number of dissectible shoots regenerated from each cultured explant in each treatment.

**Mean number of roots/explant:** is the average number of dissectible roots regenerated from each cultured explant in each experiment.

**Mean length of shoot/root**: is the average length of shoots and roots developed from each cultured explant.

**Survival capacity:** is the competence or the ability of the *in vitro* derived plantlets to endure in the *in vivo* condition for acclimatization. Data on this parameter were taken after one month plantelets were transferred to poly-house. Accordingly, the survival rate was

calculated after three weeks as the ratio of plantlets survived to the total number of plantlets transferred to the poly-house and expressed in percentage.

## 3.8. Data Analysis

Data were subjected to analysis of variance (one-way ANOVA) using SAS version 9.2 software and means were compared using the Least Significant Difference (LSD) test at $p<0.05$. Following to (Beza Kinfe, 2010).

# 4. RESULTS AND DISCUSSION

## 4.1. Shoot Induction and Regeneration

Leaf explants were cultured for shoot initiation and regeneration on different concentrations of BAP and TDZ separately. Analysis of variance showed that hormone type and concentrations and varieties showed significant ($p< 0.05$) effect on number of days for shooting, percent shoot regeneration, number of shoots/explant and length of shoot/explant (Tables 2). Compared to the control, BAP at all concentration levels showed significant positive effect on shoot initiation days of the two varieties of grape. The highest regeneration about 87.5% and 86.6% on 2mg/L BAP were obtained for canonannon and chenin blanc, respectively (Table 2). Similar responses were obtained during the development of micropropagation protocol of the two varieties (Fikadu Kumsa, 2011).

However, in the present study it also promoted callus, which is in consisted with the work of Baker & Bhatia (1993). The data for percent of plant regeneration from leaf explants was collected after 30 days of *in vitro* culture on different media. And also the best mean length of shoot was attained at 2 mg/l of BAP for Canonannon (2.1±0.3) and Chenin blanc (2.5±0.5) (Table 2). This result related to (Beza Kinfe, 2010).

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. Percent shoot regeneration, number of shoots/explant, and length of shoot/explant were significantly ($P<0.05$) higher at 2mg/L of BAP than the rest of concentrations in both grape varieties. These parameters, however, were found to reduce with deviation of BAP concentration form 2mg/L, suggesting 2mg/L of BAP is an optimal for shooting of both grape varieties.

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. This result is in agreement with that of Beza Kinfe (2010). Similarly, the best shoot inductions were recorded at 2.0 mg/l BAP + 0.1mg/l IAA for Canonannon and Cheninblanc cultivars (Abido *et al*., 2013; Fikadu Kumsa, 2011).The two varieties showed

no significant difference in their shooting response to the tested concentrations of BAP (Table 2).

It was shown that callus induction was significantly low at (0mg/l, 1mg/l, 1.5mg/l 2mg/l 3mg/l and 4mg/l) BAP except 2.5mg/l BAP, which promoted callus in 24.6% and 28.3% of the explants of canonannon and chenin blanc, respectively. Both shoots and callus were simultaneously induced at 3mg/L BAP in both varieties. However at low concentrations of BAP (1mg/l) and high concentration of BAP (4mg/l) shoot induction from leaves of both varieties was at low level (Table 2). This result was similar to (Fikadu Kumsa, 2011).

Table 2. The effect of different concentrations of BAP on shoot induction from leaf explants of the two grapevine varieties (Values are mean ±SE, n=3)

| Hormones | | | Canonannon | | |
|---|---|---|---|---|---|
| BAP (mg/l) | %of regeneration | No of days to shooting | No of shoots/ explant | Length of shoots/explant | %of induced callus |
| 0 | $0.0^{d}$ | | $0.0\pm0.0^{c}$ | $0.0\pm0.0^{cd}$ | $0.0^{d}$ |
| 1 | $0.6^{d}$ | $14.0^{c}$ | $0.3\pm0.0^{c}$ | $0.6\pm0.2^{c}$ | $1.1^{cd}$ |
| 1.5 | $4.0^{c}$ | $18.3^{ab}$ | $1.3\pm0.5^{b}$ | $1.5\pm0.5^{ab}$ | $0.0^{d}$ |
| 2 | $87.5^{a}$ | $16.3^{bc}$ | $2.6\pm0.5^{a}$ | $2.1\pm0.3^{a}$ | $0.0^{d}$ |
| 2.5 | $6.3^{b}$ | $19.6^{a}$ | $1.3\pm0.5^{b}$ | $1.0\pm0.5^{bc}$ | $24.6^{a}$ |
| 3 | $1.1^{d}$ | $18.6^{ab}$ | $1.3\pm0.5^{b}$ | $1.0\pm0.5^{bc}$ | $12.0^{b}$ |
| 4 | $0.6^{d}$ | $17.0^{abc}$ | $0.3\pm0.0^{c}$ | $0.6\pm0.2^{cd}$ | $2.0^{c}$ |
| BAP(mg/l) | | | Cheninblanc | | |
| 0 | $0.0^{d}$ | | $0.0\pm0.0^{c}$ | $0.0\pm0.0^{d}$ | $0.0^{f}$ |
| 1 | $3.0^{c}$ | $14.0^{c}$ | $1.0\pm0.1^{ab}$ | $0.8\pm0.2^{cd}$ | $20.5^{b}$ |
| 1.5 | $7.3^{b}$ | $18.3^{ab}$ | $1.0\pm0.1^{ab}$ | $1.8\pm0.2^{b}$ | $7.3^{c}$ |
| 2 | $86.6^{a}$ | $16.3^{bc}$ | $2.3\pm0.2^{a}$ | $2.5\pm0.5^{a}$ | $2.3^{e}$ |
| 2.5 | $8.3^{b}$ | $19.6^{a}$ | $2.0\pm0.2^{a}$ | $1.3\pm0.5^{bc}$ | $28.3^{a}$ |
| 3 | $2.5^{c}$ | $18.6^{ab}$ | $1.3\pm0.1^{ab}$ | $1.1\pm0.2^{cd}$ | $4.6^{d}$ |
| 4 | $3.0^{c}$ | $17.0^{abc}$ | $0.3\pm0.1^{b}$ | $0.6\pm0.2^{d}$ | $0.6^{ef}$ |

Means having the same superscript letters in a column are not significantly different at 5% probability level.

Contrary to BAP, TDZ at all concentrations showed no positive shooting response on both varieties (Data was not presented as there was no any shooting response). This is in accordance with previous studies that have clearly put that BAP is the most effective hormone, among other cytokinins, for promoting regeneration of *Vitis vinifera* cultivars belonging to "Soltanin" and "Sahebi" from shoot apical meristem (Aazami, 2011). And also it was shown that callus induction was significantly low at all tested levels of TDZ on the explants of Canonannon (21.1%) and chenin blanc (24.6%) with regard to the induction of callus at 2.5mg/l concentration of BAP.

## 4.2. *In Vitro* Rooting of Shoots

Well established shoots were transferred to MS basal medium supplemented with IBA (0, 1, 2, 3 and 4mg/l) and IAA (2 and 4mg/l) separately for rooting. Rooting of shoots was usually attained at low concentration of auxins in many *in vitro* cultures (Nitzche and Wenzel, 1977).

The best rooting performances i.e., number of days for rooting, number of roots /shoot and root length was observed under IBA treatment than IAA in both varieties (Table 3). Though root emergence were observed on the 14th day of culturing, number of roots /shoot and root length was obtained at MS medium supplemented with 2mg/L of IBA (Table 3). Among the tested IBA and IAA concentrations, the best length of plantlets were obtained at 1mg/1 and 2mg/l IBA when compared to 3mg/l IBA , 4mg/L IBA , 2mg/l IAA and 4mg/l IAA concentrations. Two mg/l IBA and 1mg/l IBA have been reported to induce optimum rooting for both varieties of Canonannon and Chenin blanc, respectively (Fikadu Kumsa, 2011).

Table 3. Effect of different IBA and IAA concentrations on rooting of both grape vine (*Vitis vinifera* L) varieties. (Values are mean±SE, n=3)

| Hormones | Canonannon | | | Cheninblanc | | |
|---|---|---|---|---|---|---|
| IBA(mg/l) | No of days to rooting | Number of roots/explant | Length of roots/explant | No of days to rooting | Number of roots/explant | Length of roots/explant |
| 1 | $17.3^{b}$ | $4.5\pm0.5^{b}$ | $3.6\pm0.2^{b}$ | $17.3^{b}$ | $4.5\pm0.5^{b}$ | $5.1\pm0.2^{b}$ |
| 2 | $29.6^{a}$ | $7.6\pm0.2^{a}$ | $5.1\pm0.7^{a}$ | $29.6^{a}$ | $7.1\pm0.7^{a}$ | $6.0\pm0.5^{a}$ |
| 3 | $14.3^{b}$ | $3.1\pm0.2^{c}$ | $2.6\pm0.2^{cd}$ | $14.3^{b}$ | $3.5\pm0.5^{c}$ | $3.1\pm0.2^{cd}$ |
| 4 | $17.6^{b}$ | $2.5\pm0.5^{c}$ | $2.8\pm0.2^{cd}$ | $17.6^{b}$ | $2.5\pm0.5^{d}$ | $2.1\pm0.2^{e}$ |
| IAA(mg/l) | | | | | | |
| 2 | 18.33 | $2.5\pm0.5^{d}$ | $3.1\pm0.2^{bc}$ | 18.33 | $2.3\pm0.2^{d}$ | $3.6\pm0.2^{c}$ |
| 4 | $20.6^{b}$ | $3.1\pm0.2^{c}$ | $2.3\pm0.2^{d}$ | $20.6^{b}$ | $5.3\pm0.2^{b}$ | $2.8\pm0.2^{d}$ |

Means having different superscript letters in a column are significantly different at 5% probability level.

## 4.3. Acclimatization of the plantlets

The acclimatized plantlets of *in vitro* regenerated plants were achieved under a controlled environment and humidity. *In vitro* plantlets of grapevine were very sensitive to *ex vitro* conditions. Micropropagated plantlets can not tolerate and survive the environmental conditions when directly taken to the glasshouse. Successful acclimatization depends on the reduction of light intensity, temperature and gradual reduction of humidity during the first five days of acclimatization.

After 30 days they look healthy plants (Appendix Fig. 6). Additionally, the rooted plantlets using 2mg/l IBA also exhibited the best survival rate for both cultivars. The survival rates were 75% and 83.3% for Chenin blanc and Canonannon, respectively (Table 4). This result was in contrast with (Fikadu Kumsa, 2011). That means the higher survival rate was with that of Canonannon rather than Chenin blanc. 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.

Table 4. The survival percentage of the in vitro derived plantlets for acclimatization

| Hormones | Percentage | |
|---|---|---|
| IBA(mg/l) | Canonannon | Chenin blanc |
| 1 | 41.6[b] | 31.9[b] |
| 2 | 83.3[a] | 75.0[a] |
| 3 | 16.6[c] | 16.6[bcd] |
| 4 | 13.8[c] | 4.1[d] |
| IAA(mg/l) | | |
| 2 | 19.4[c] | 25.0[bc] |
| 4 | 27.7[bc] | 12.5[cd] |

# 5. SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

## 5.1 Summary and Conclusion

From the obtained results, it can be concluded that, the frequency of leaves showing adventitious shoot induction varied with the presence of both BAP and TDZ alone. Among the tested different concentrations for shoot regeneration, BAP gave the maximum number of shoots for both varieties when compared to TDZ. On the other hand, from the different concentrations of IBA and IAA tested for root induction, IBA gave the maximum number of roots for both varieties when compared to IAA. Based on this study:-

- BAP was the best cytokinin which gave the maximum shoot induction and regeneration for both varieties of grapevine (*Vitis vinifera* L.) from leaf explants.
- The shoots, obtained from leaf explants of Canonannon have shown the maximum mean number (2.6±0.5) on 2mg/l BAP. This can be enabled us to get the propagules of this particular cultivar within short period of time.
- IBA was the best auxin which gave the maximum root induction and acclimatization for both varieties of grapevine (*Vitis vinifera* L.) from leaf explants with short period of time.

## 5.2. Recommendations

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

- The indirect shoot regeneration from the obtained callus was not tested at higher levels of the growth regulators. Hence, it is necessary to investigate the indirect shoot regeneration mechanism for both varieties at higher concentrations of the growth regulators that were not included in this study, such as Kinetin, 2-Ip or BA which are potential cytokinins for culture establishment and shoot proliferation.
- The effect of sub-culturing on the multiplication of shoots needs a further study, to determine whether the sub-culturing increases or decreases shoot multiplication to a significant extent.

- In the present study IBA was the best auxin for maximum root induction from the tested ones. Hence, it is necessary to investigate other auxins which are significant for root induction and acclimatization.

# 6. REFERENCES


Aazami, M.A. 2010. Effect of some growth regulators on "*in vitro*" culture of two *Vitis vinifera L.* cultivars. Romans Biotechnology Letters 15:55181- 83111.

Abido M.A.M., Hassanen A.S.A. and Rayan G.A. 2013. *In vitro* Propagation of Grapevine (*Vitis vinifera* L.) Muscat of Alexandria cv. For Conservation of Endangerment *Middle-East Journal of Scientific Research,* 13:328-337.

Alizadeh, M., Singh, S.K. and Patel, V.B. 2010. Comparative performance of *in vitro* multiplication in four grape (*Vitis* spp.) root stock genotypes. *International. Journal of Physiology. Production* 4: 50.

Alonso, A.M., Dominguez, C., Guillen D.A., and Barroso, C.G. 2002. Determination of antioxidant power of red wine and white wine by a new electrochemical method and its correlation with polyphenolic content. *Journal of Agriculture. Food Chemistry.* 50(11):3112-5.

Amerine, M.A., Berg, H.W. and Cruess, W.V. 1967. The Technology of Wine Making, Westport, Connecting culture: A vital Publishing Commody.

Baker, B.S. and Bhatia, S.K. 1993. Factors effecting adventitious shoot regeneration from leaf explants of quince (Cydonia oblonga). Plant Cell, Tissue. Organ. Culture 35: 273-27.

Bettoni J.C., Costa M.D., Gardin P.P.J., Kretzchmar A.A., and Souza J.A., (2015). *In vitro* propagation of grapevine cultivars with potential for south of Brazil, *American journal of Plant Science,* 6: 1806-1815.

Beza Kinfe 2010. Multiples shoot Regeneration of Grape Vine *(Vitis vinifera L.)"* LAP Lambert Academic, Germany, Publishing, pp. 1-64.

Bhojwani, S.S. and Razdan, M.K. 1996. *Plant Tissue Culture: Theory and Practice*. Amsterdam, Netherland, Elsevier Science B.V, pp.767.

Bornhoff and Harst. 2000. Establishment of embryo suspension cultures of grapevines (*Vitisvinifera L.*). *Vitis* 39: 27-29.

Brugie`re, N., Jiao S., Hantke, S. Zinselmeier, C. Roessler, J.A. Niu, X. Jones, R.J. and Habben, J.E. 2003. Cytokinin oxidase gene expression in maize is localized to the vasculature, and is induced by cytokinins, abscisic acid, and abiotic stress. *Plant Physiology* 132: 1228-1240.

Cameron, S.I. 2006. Tissue culture gel firmness: measurement and effects on growth. pp. 329–337 *In*: S.D. Gupta and Y. Ibaraki (eds.), *Plant Tissue Culture Engineering*, Dordrecht, Netherland, Published by Springer.

Cantos, E., Espin, J.C. and Tomas-Barberan, F.A. 2002. Varietal differences among the polyphenol profiles of seven table grape cultivars studied by LC-DAD-MSMS. *Journal of Agriculture. Food Chemistry,* 50: 5691–5696.

Cardoza, V. 2008. Tissue Culture: The Manipulation of Plant Development. pp. 113-133 *In*: C.N. Stewart (Ed.) *Plant Biotechnology and Genetics: Principles, Techniques, and Applications*. Hoboken, New Jersey John, USA, Wiley & Sons, Inc.

Chee, R., Pool, R.M. and Bucher, D. 1984. A method for Large-scale *In vitro* Propagation of *Vitis*. New York State College of Agriculture and Life Sciences.

Cherepanov, S.K. 1995. Vascular Plants of Russia and Neighboring Countries. Student. Petersburg. p. 958.

Conde, C., Paulo, S., Fontes, N., Alberto, C.P., Tavares, R.M. and Joao, S.M. 2007. Biochemical Changes throughout Grape Berry Development and Fruit and Wine Quality. *Food Global Science Books* 1(1): 1-22.

Das, D.K., Reddy, M.K., Upadhyaya, K.C. and Sopory, S.K. 2002. An efficient leaf-disc Culture method for the regeneration via somatic embryogenesis and transformation of grape (*Vitis vinifera L.*). *Plant Cell Reproduction*. 20:999–1005.

DeFossard R. Tissue culture for Plant Propagation. Armidale: University of New England; 1976.

Deore, A.C. and Johnson, E.T. 2008. High-frequency plant regeneration from leaf-disc cultures of *Jatropha curcas L.*: an important biodiesel plant. Plant Biotechnology. Reproduction. 2: 7–11.

Dikibo, F.I. 2008. A Seminar Presentation on Application of Tissue Culture for Propagating Glycine max (*L.*) Merr. Via Half Seed Explant Culture. University of Port Harcourt.

Doods, J.H. and Roberts, L.W. 1985. *Experiments in Plant Tissue Culture 2nd Edition*. Cambridge 32 East 57th Street, New York, USA: Cambridge University Press, pp.232.

Evans, P. 2007. Library of Fruit Science Missouri State University –Mountain Grove Campus. This page maintained by: webmaster@library.missouristate.edu.

Fabio, M. and Andrea, B. 2002. "Grape Post harvest Operations" with emphasis in developing Countries. Department of Food Science and Technology, University of Viterbo, Italy.

Fayek, M.A., Jomaa, A.H., Shalaby, A.B. and Al-Dhaher, A.M. 2009. Meristem tip culture for in vitro eradication of grapevine leaf roll associated virus-1 (GLRaV-1) and grapevine fan leaf virus (GFLV) from infected flame seedless grapevine plantlets. Ini. Inv. 4: 1-11.

Fikadu Kumsa. 2011. *In vitro* regeneration of two grapevine (*Vitis vinifera* L.) varieties from leaf explants. MSc. Thesis, Addis Ababa University, Addis Ababa, Ethiopia.

Fikadu Kumsa and Tileye Feyissa. 2016. Hyperhydricity (Vitrification) of Micropropagated Shoots of Two Grapevine (Vitis vinifera L.) Cultivars, *International. Journal. Current. Trend. Pharmacological biology. Medicine. Science.* 1:71-76.

Galletta, G.J. and Himerlic, D.G. 1989. Small Fruit Crop Management. Prentice Hall, New Jersey. p. 383.

Gaspar, T., Kevers, C. Penel, C. Greppin, H. Reid, D.M. and Thorpe, T.A. 1996. Plant hormones and plant growth regulators in plant tissue culture: Review. *In Vitro Cellular and Developmental Biology Plant* 32: 272-28.

George, E.F., Michael, A. H. and De Klerk, G. J., 2008. Plant Propagation by Tissue Culture 3rd Edition, 403–422. Published by Springer, P.O. Box 17, 3300 AA Dordrecht, The Netherland.

Ghulam, M. S., Kashif, M. I. and Rashid, A. (2006). Effect of diverse hormonal regimes on *In vitro* growth of grape germplasm. *Pakistan. Journal. Botany*., 38(2): 385-391.

Gloriada, A.B., Vieira, C.L.M. and Marcelo Carnier Dornelas, C.M. 1999. Anatomical studies of *in vitro* organogenesis induced in leaf-derived explants of passion fruit. Pesq. Agropec. Brasil. Brasília 34:.2007-2013.

Gonzales, G.R., Quiroz, K., Carrasco, B. and Caligari 2010. Literature review Plant tissue culture: Status, opportunities and challenges. Cien. Inv. Agr. 37:5-30.

Hajar, B. 2006. Table Grape Trade in Syria National Agricultural Policy Center (NAPC). Syrian Commodity Brief No 5.

Heloir, M. C., Fournioux, J. C., Oziol, L. and Bessis, R. (1997). An improved procedure for the propagation *in vitro* of grapevine (*Vitis vinifera* cv. Pinot noir) using axillary bud microcuttings. *Plant Cell Tissue*. *Organ*. *Culture*. 49: 223-225.

Hopkins, W.G. and Hüner, P.A. 2009. *Introduction to Plant Physiology Fourth Edition*. 111 River Street, Hoboken, London, John Wiley & Sons, Inc., pp. 503.

Hulya, H.O. 2007. Total antioxidant activities, phenolics, anthocyanins, polyphenoloxidase activities of selected red grape cultivars and their correlations. Scientia Horticulturae 111: 235–241.

Ibariez, A., Valero, M. and Morte, A. 2005. Establishment and in vitro clonal propagation of the Spanish autochthonous table grapevine cultivar Napoleon: An improved system where proliferating cultures alternate with rooting ones. Anales de Biologíans 27:211-220.

Iktena, H. and Reada, P.E. 2010. The effects of growth regulators on micropropagation of grapevine (*Vitis* Spp.) 'Marechal Foch' and 'Lacrosse'. *International Journal of Fruit Science* 10: 367- 378.

Ithaca Chusing, B. 2008. Some biological effects of Shiraz grape products, grown at Suranaree University of Technology farm on myeloma cells. Ph D Thesis, Suranaree University of Technology.

Jaladet, M.S., Jubrael. and Shymaa, H. A. 2009. Genetic diversity analysis of a number of grape (*Vitis vinifera L.*) varieties in Kurdistan region-Iraq using rapid markers. *Journal of Duhok University.*12 (1):17-22.

Jamadar, M.M. 2007. Etiology, epidemiology and management of anthracnose of grapevine. Thesis submitted to the University of Agricultural Sciences, Dharwad in partial fulfillment of the requirements for the Degree of Doctor of philosophy in Plant pathology.

Jaskani, M.J., Abbas, H., Sultana, R., Khan, M.M., Qasim, M. and Khan, A.I. 2008. Effect of growth hormones on micropropagation of *Vitis vinifera L. cV. Perlette Pakistan Journal of Botany.* 40: 105-109.

Krongjai, T. 2005. The transformation of grape callus with chitinase. A thesis submitted in partial fulfillment of the requirement for the Degree of Master of science in Biotechnology. Suranaree University of Technology.

Kumar N. and Raddy M.P. 2011. In vitro plant propagation: A review, *Journal of Forest Science,* 27: 61-72.

Lavee, S. 2000. Grapevine (*Vitis vinifera*) growth and performance in warm climates. In: Temperate Zone Fruit Crops in Warm Climates (Erez A. ed.). pp. 343-366. Netherland: Kluwer Academic Publishers.

Lazanyi, J. 2008. Trends in Wine Production and Trade: Applied Studies in Agribusiness and Commerce – APSTRACT issue 1-2, Agro inform Publishing House, Budapest. Mattick, L.R. and Moyer, J.C. 1973. Acidity in Concord grape juice. New York Food and Life Science.6:8.

Meyerson, M.E., Benton, C.M. and Gray, D.J. 1994. A Comparison of shoot micropropagation among bunch and Muscatine grape species and cultivars. Proc. Fla. State Horticulture. Sociology. 107: 311-312.

Mhatre, M. and Bapat, V.A. 2007. Protocols for Micro-propagation of Woody Trees and Fruits. Bhabha Atomic Research Centre, Trombay, Mumbai, pp. 249–258.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco cultures. Physiology Plant arum, 15: 473-497.

Orhan, D.D., Orhan, N., Ozcelik, B. and Ergun, F. 2009. Biological activities of *Vitis Vinifera L*. leaves. *Turky. Journal of Biology*. 33:341-348.

Nitzche, M.W. and Wenzel, G. (1977). *Haploid in Plant Breeding*. Berlin, Hamburg, Parey, pp. 85-90.

Novaka, I., Janeiroa, P., Serugab, M. and Oliveira-Brett, A. M. 2008. Ultrasound extracted flavonoids from four varieties of Portuguese red grape skins determined by reverse-phase high-performance liquid chromatography with electrochemical detection. Anal. Chim. Acta. 630: 107–115.

Owen, H.R., Wengaerd, D. and Miller, A.R. 1991. Culture medium pH is influenced by basal medium, carbohydrate source, gelling agent, activated charcoal, and medium storage method. *Plant Cell Report* 10: 583–586.

Patrice, T., Thierry, L. and Mark, R. T. 2006. Historical origins and genetic diversity of wine grapes. Trends in Genetics 22(9): 511-513.

Pe´ros, J.P., Torregrosa, L. and Berger, G. 1998. Variability among Vitis vinifera cultivars in micropropagation, organogenesis and antibiotic sensitivity. *Journal. Experiment of Botany*. 49:171–179.

Prakash S, Hoque MI, Brinks T. Culture media and containers. In: International Atomic Energy Agency (ed.): Low cost options for tissue culture technology in developing countries. Proceedings of a technical meeting, 26-30 August 2002, Vienna, Austria.

Puchooa, D., Purseramen, P.N. and Rujbally, B.R. 1999. Effects of medium support and gelling agent in the tissue culture of tobacco (*Nicotiana tabacum*). *Science and Technology Research Journal* 3: 129-144.

Rahman, M.H., Islam, R. Hossain, M. and Islam, M.S. 2010. Role of sucrose, glucose and maltose on conventional potato micro propagation. *Journal of Agricultural Technology* 6(4): 733-739.

Rai, R. 2007. *Genetics and Plant Breeding: Introduction to Plant Biotechnology*. National Research Centre on Plant Biotechnology, Lal Bhadur Shastri Building Pusa Campus, New Delhi, India.

Richard, M.S., Margaret, M.A. and Robert, M.S. 2010. Plant regeneration via somatic embryogenesis from leaf and floral explants of "Chancellor" Wine Grape. Plant Tiss. Culture and Biotechnology. 20: 157-170.

Rossel, W. 1992. Tissue Culture in Disease Elimination and Micropropagation. In: Biotechnology: Enhancing Research on Tropical Crops in Africa.CTA/IITA Co. publication. IITA, Abdan.

Saad, A.I. and Elshahed, A.M. 2012. Plant Tissue Culture Media. pp. 29-40 *in*: A. Leva and L.M.R. Rinaldi (eds.). *Recent Advances in Plant in vitro Culture*. Http://www.4shared.com/gate.

Salami, A., Ebadi, A., Zamani, Z. and Ghasemi, M. 2005. Improvement in apex culture in an Iranian grapevine (Vitis vinifera L. 'Bidaneh Sefid') through fragmented shoot apices. *International Journal of Agriculture Biology.* 7: 333-336.

Sebastiani, L., Minnocci, A., Vitagliano, C., Gribaudo, L. and Novello, V. 2001. Morphological and anatomical studies on in vitro grapevine (*Vitis vinifera L*. cV. Nebbiolo) plants by low temperature scanning electron microscopy. *Advanced Horticulture Science.* 15: 103-111.

Shrikhande, A.J. 2000. Wine by-products with health benefits. *Food Res. Internat*. 33:469–474.

Singh, R., 2003. Tissue Culture Studies of Sugarcane. MSc**.** Thesis submitted to the Department of Biotechnology and Environmental Sciences Thapar Institute of Engineering and Technology Patiala –147004, India. 60p.

Spacil, Z., Novakova, L. and Solich, P. 2008. Analysis of phenolic compounds by high performance liquid chromatography and ultra performance liquid chromatography. *Talanta*, 76: 189–199.

Strausbaugh, P.D. and Core, Earl. L. 1952. Flora of West Virginia (Part I). W.Va. Univ. Bull. Ser. 52: 1-274 West Virginia University.

Torregrosa, L. and Bouquet, A. 1996. Adventitious bud formation and shoot development from in vitro leaves of Vitis Muscadinia hybrids. Plant Cell, Tissue. and Organ. Culture.45:245-252.

Trimble, G. R., and Tryon, E. H. 1979. Silvicultural control of wild grapevines. W.V. Univ. Agric. For. Exp. Stn. Bull. 667. West Virginia University.

Ttan, L. and Wang, Y. 2008. Seedless grape breeding for disease resistance by using embryo rescue. *Vitis*, 47 (1), 15–19.

Vinterhalter D, Vinterhalter BS. Micro propagation of Dracaena sp. In: Bajaj YPS (Ed.). Biotechnology in agriculture and forestry 40, High-tech. and Micro propagation VI. Berlin, Heidelberg: Springer; 1997. p131- 146.

Weaver, R.J. 1976. Grape Growing. New York: John Wiley& Sons Inclination. Winkler, A.J.J.A., Cook, W.M., Kliewer. and Lider, L.A. 1974. *General Viticulture*. Barkley, Central A: University of California Press.

World Vineyard Grape and Wine Report 2005, vine yard grubbing program in the former Soviet Union followed by grubbing and other supply control program initiated in the EU.

Xu, X.Lu.J, Ren, Z., Wang, H. and Leong, S. 2005. Callus induction and somatic embryogenesis in muscadine and seedless bunch grapes (*vitis*) from immature ovule culture. Proc. Fla. State Hort. Soc. 118:260-262.

Yingyos, J. 2007. Genetic engineering and in vitro selection for grape cultivar improvement. A thesis submitted in Partial fulfillment of the requirements for the Degree of Doctor of Philosophy. Suranaree University of Technology.

Zhang, Y., Liu, Y., Wang, T., Li, B., Li, H., Wang, Z. and Yang, B. 2006. Resveratrol, a natural ingredient of grape skin: Anti arrhythmic efficacy and ionic mechanisms. *Biochemistry Biophysiology Resourse. Community* 340:1192-119.

# 7. APPENDICES

## Appendix I

Table 1. The number of plantlets acclimatized in the glass house from the two varieties (Values are mean ±SE, n=3)

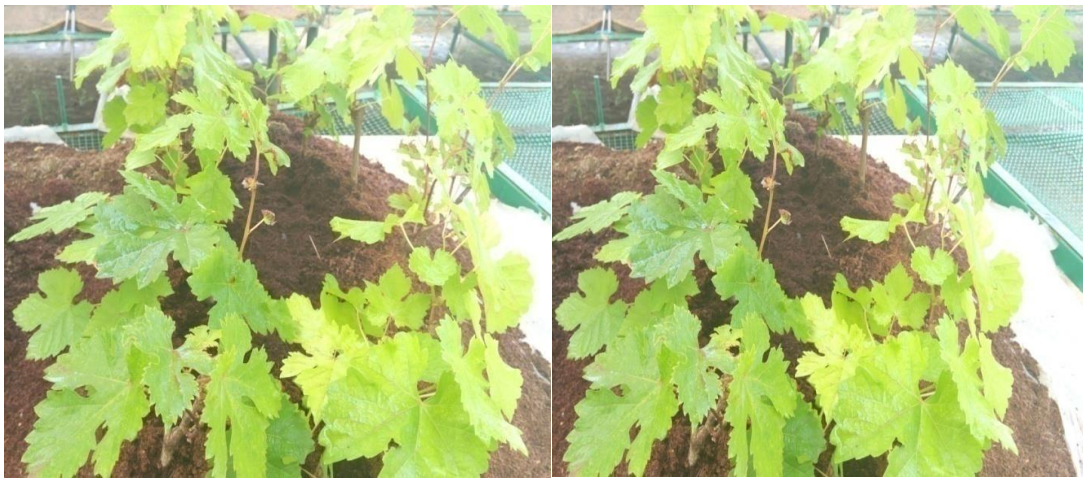
| Hormones | No of acclimatized plantlates | |
|---|---|---|
| IBA(mg/l) | Canonannon | Chenin blank |
| 1 | $5.0\pm1.0^{b}$ | $3.0\pm1.0^{b}$ |
| 2 | $10.0\pm1.0^{a}$ | $6.0\pm1.0^{a}$ |
| 3 | $2.0\pm1.0^{c}$ | $1.3\pm0.5^{cd}$ |
| 4 | $1.6\pm1.5^{c}$ | $0.3\pm0.5^{d}$ |
| IAA(mg/l) | | |
| 2 | $2.3\pm2.0^{c}$ | $2.0\pm1.0^{bc}$ |
| 4 | $3.3\pm1.5^{bc}$ | $1.0\pm1.0^{cd}$ |

## Appendix II

Table 2. Nutrient composition and concentration of MS basal medium

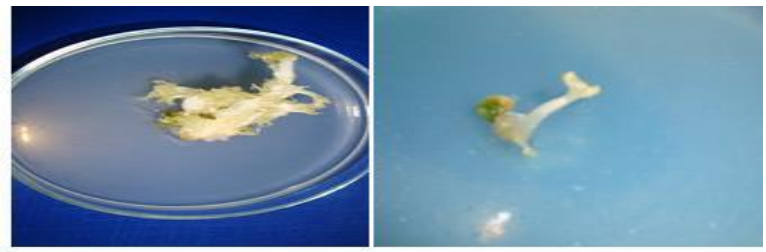
| Components | |
|---|---|
| Macronutrients | Concentration (g/L) |
| NH4NO3 | 16.5 |
| KNO3 | 19.0 |
| CaCl2.H2O | 4.4 |
| MgS04.7H20 | 1.8 |
| KH2P04 | 1.7 |
| Micronutrients | |
| Fe-Na-EDTA | 4 |
| ZnSo4.7H20 | 0.86 |
| H3BO3 | 0.62 |
| MnS04.4H20* | 2.23 |
| MnS04.H20* | 1.69 |
| Cuso4.5H20 | 0.0025 |
| KI | 0.083 |
| Na2Mo04.2H20 | 0.025 |
| CoCl2.6H20 | 0.0025 |
| Vitamins | |
| Myo-inositol | 0.1 |
| Glycin (glycol) | 0.2 |
| Nicotinic acid | 0.05 |
| Pyridoxin (B6) | 0.05 |
| Thiamin (B1) | 0.01 |

## Appendix III



**a** **b**

Figure 1. The explant establishment of Canonannon (a) and Chenin blanc (b) respectively



**a** **b**

Figure 2. Direct regenerants of canonannon(a) and chenin blanc(b) from leaf explants on 2mg/L BAP after 30 days

**a** **b**

Figure 3. Shoot proliferation of Canonannon (a) and Chenin blanc (b) respectively at 2mg/L BAP after 30 days

Figure 4. Single shoot of Canonannon on MS medium supplemented by 2mg/L BAP in the dark incubation after 30 days

**a** **b**

Figure 5. Rooted shoots of leaf derived *in vitro* plantlets of Canonannon (a) and Chenin blanc (b) respectively on MS basal medium supplemented with 2mg/L IBA after 30 days

**a** **b**

Figure 6. The acclimatized plantlets of Canonannon (a) and Chenin blanc (b) regenerated from leaf explants respectively after 30 days