ADDIS ABABA UNIVERSITY

COLLEGE OF NATURAL SCIENCES

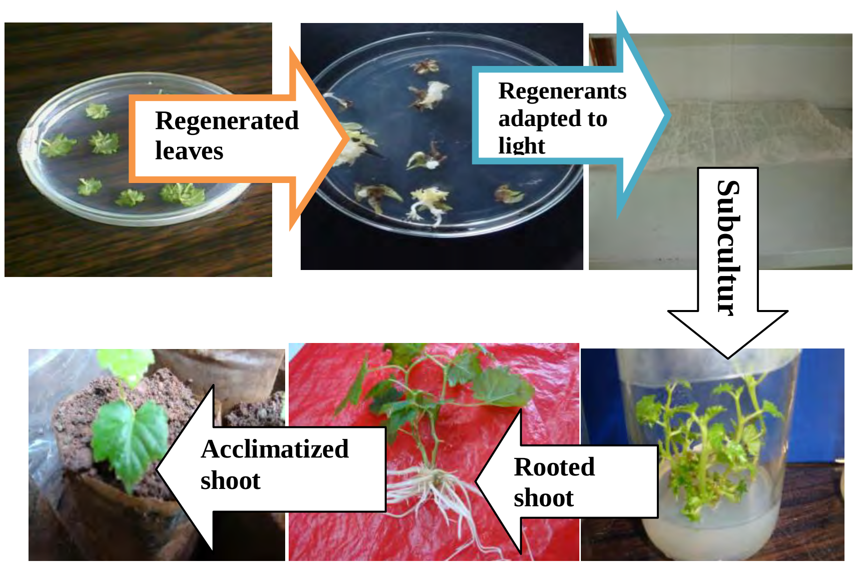
SCHOOL OF GRADUATE STUDIES

Biotechnology Program Unit

In vitro regeneration of two grapevine (*Vitis vinifera* L.) varieties from leaf explants.

By

Fikadu Kumsa Gemechu



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

Addis Ababa, Ethiopia

July, 2011

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***IN VITRO* REGENERATION OF TWO GRAPEVINE *(VITIS VINIFERA* L.*)* VARIETIES FROM LEAF EXPLANTS.**

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Contents **Pages**

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LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES AND TABLES IN THE APPENDICES

LIST OF ABBREVIATIONS

BAP	6-Benzylaminopurine
NAA	Naphthalene acetic acid
MS	Murashige and Skoog
IBA	Indole-3-butyric acid
TDZ	Thidiazuron
GRS	Growth regulators
IAA	Indole Acetic Acid

ABSTRACT

Grapevine (*Vitis vinifera* L.) is one of the most widely distributed fruit crops in the world. It belongs to the family *Vitaceae.* Conventional method of grapevine propagation is time consuming and allows disease transmission. A planted grape vine needs four to five years to be a propagation material by cutting, due to its long juvenility period. Therefore, the establishment of efficient *in vitro* regeneration to is much needed. The objective of this study was to develop protocol for the regeneration of grapevine plants from leaf explants of varieties canonannon and chenin blanc. Many factors influence the grapevine *in vitro* approach. The factor considered under this study was the effect of different hormone concentrations and combinations on two grape varieties (chenin blanc and canonannon). MS medium supplemented with 42 different combinations of auxins (NAA or IBA), and cytokinins (BAP or TDZ) was used for regeneration of shoots from leaf. From the tested six different concentrations of TDZ and eight different concentrations of BAP, the best direct shoot regeneration was obtained at 2mg/l BAP for both chenin blanc (2.3 ± 0.3) and canonannon (2.2 ± 0.2). Among sixteen different combinations of TDZ and NAA, the maximum and best shoots per explant (1.5 ± 0.2) were obtained at 1mg/l TDZ and 0.1mg/l NAA for canonannon. Both varieties did not show any regeneration of shoots at low (0.5mg/l and 1mg/l) and high (4mg/l and 5mg/l) concentrations of BAP. 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 rate of chenin blanc and canonannon cultivars were 83.3% and 75 %, respectively.

Key words/phrase: Organogenesis, Hyperhydricity, growth regulators, callus induction

1. INTRODUCTION

Grapevine (*Vitis vinifera* L.) is one of the most widely distributed fruit crop 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, *Vitis vinifera* originated in the regions between and south of the Caspian Black Seas in Asia and has been distributed from region to region in all temperate climates (Krongjai, 2005). According to Patrice *et al.* (2006), *V. vinifera* is highly distributed and produces over 90% of the world's grapes, which are either pure *V. vinifera* or hybridized with one or more American species today. About 85% of the grapes in the United States are derived from pure *vinifera* varieties (Krongjai, 2005). Thus, most important varieties of pure species in North America used for fruiting are from the varieties of *V. labrusca* (Concord, Niagara*), V. rotundifolia* (Scuppernong, Eden), and *V. rupestris* (Rupestris St. George-used mainly as phylloxera-resistant rootstocks). This old world species, *V. vinifera*, is the grape of antiquity often mentioned in the Bible.

The main product, wine, was considered divine, a drink of the gods (Patrice *et al*., 2006). Other Mediterranean cultures considered that 'the wine sprang from the blood of humans who had fought the gods' and wine has always had a major role in the way of life of Mediterranean people (Patrice *et al*., 2006).

Cultivated grapevines now exist on every continent on earth wherever the climatic conditions are favorable except for Antarctica**.** In 2001, global vineyard acreage was 7.9 million hectares. However, when compared with the period 1997-2000, the most significant vineyard acreage increased in 2001. Their distribution is different from country to country: China (57.9%), Australia (31.6%), New Zealand (28.7%), Chile (17.0%), United States (9.7%) and Iran (8.5%). In 2001, Some African countries such as Algeria, Egypt, Libya, Tunisia and South Africa have also participated in production of grape. In case of Ethiopia, wineries are importing about 300 tons of grape production per year in the form of dried raisin, grape juice concentrates, natural wine extracts and citric acid (Beza Kinfe, 2010).

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 (Rossel, 1992; Winkler (1974). Beside this, genetic improvement of grape through conventional breeding is severely limited due to a polyploidy and the highly heterozygous nature of existing cultivars (Gray and Meredith 1992; Nakano *et al*., 1994 *cited* in Das *et al*., 2002).

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. Therefore, the non-conventional methods such as micropropagation and plant regeneration systems could be used.

Micropropagation in grapevine was first performed by "*in vitro*" culture of micro-cuttings (Aazami *et al.*, 2010) to propagate the varieties. However, 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).

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 obtained by nonconventional methods, to do *in vitro* selection and genetic transformation need the *in vitro* regenerated plantlets of the varieties (Pe´ros *et al.,* 1998). The objective of this study is to develop protocol for the regeneration of grapevine plants from leaf explants using two varieties from Ethiopia.

2. OBJECTIVES OF THE STUDY

2.1. General objective

To develop protocol for the regeneration of grapevine plants from leaf explants of canonannon and chenin blanc varieties

2.2. The specific objectives

- To optimize the concentrations of growth regulators for regeneration of canonannon and chenin blanc varieties from leaf explants
- To evaluate the performance of shoots regenerated directly from leaf explants and those obtained from micropropagation using nodal and shoot culture of canonannon and chenin blanc varieties
- To root shoots obtained from *in vitro* regeneration and micropropagation
- To acclimatize the plantlets and evaluate their survival
- To identify the problem of vitrification and the technique used to reduce it

3. LITERATURE REVIEW

3.1. Taxonomy of grape

Grapes belong to the order vitals, family *Vitaceae,* and genus *Vitis* which include 14 living and 2 fossil genera and more than 1000 species. In the *Vitaceae* family, the *Vitis* genus has major agronomic importance. In total, there are about 60 species of *Vitis*, of which the most commonly grown is *Vitis vinifera*. Among them, *Vitis vinifera* is the only species extensively used in the global wine industry. It is also the only species of the genus indigenous to Eurasia and is suggested to have first appeared 65 million years ago (Patrice *et al.*, 2006).

3.2. Genus *Vitis*

The grapes are divided into two sections, *Vitis* (*Euvitis*) and *Muscadinia*. The section *Vitis* (*Euvitis*) consists of the grapevines. The chromosome number of *Euvitis* is 2n = 38, while, that of *Muscadinia* is *2n=40. Euvitis* are perennials, annually producing shoots that have tendrils. Grapevine is either deciduous or evergreen. *Vitis vinifera* is a woody climber with coiled climbing tendrils. It has small, pale and green flowers in the summer followed by bunches of berry fruits that range from green to purple-black (Pe´ros *et al.,* 1998).

3.3. Structure and growth stage of grapevine

Like most other plants, the grapevine has a predictable cycle of growth. Life cycle of the grapevine can be categorized under certain stages, depending upon the growth pattern of vitis 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 grape does 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).

3.4. Significance and use of grapevine

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. The usage is based on the structure of plant. 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).

Raisin grapes*:* These include any dried grapes, although several standards must be met if a suitable dried raisin is to be made. The dried raisins must be soft in texture and should not stick together when storing. Few varieties can me*et* 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).

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).

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).

3.5. 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).

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.

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.

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.

3.6. 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.

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 (Aazami *et al.*, 2010). 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 growth regulators compared with genotypes of the explants relating to the objectives of the study (Torregrosa and Bouquet, 1996; Dikibo, 2008).

3.6.1. 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, 1982; Reisch, 1986; Singh *et al.*, 2000; Mhatre *et al.*, 2000; Singh *et al.*, 2004, cited in Alizadeh *et al.,* 2010*).* Many tissue culture *protocols* have also been reported for *muscadine* grape (Lee and Wetzstein, 1990; Gray and Benton, 1991; Sudarsono and Goldy, 1991; Thies and Graves, 1992; Torregrosa and Bouquet, 1995; Roubelakis-Angelakis, 2001, cited in Alizadeh *et al.,* 2010).

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 & 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). Limitation of the methods that use anthers and ovules as explants are questionable due to the brief availability period of the explant. 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 *V. 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 meristems culture is also used for *in vitro* regeneration in varieties of grape (Das *et al.*, 2002). Baker & 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). However, in case of present study, an improved procedure for adventitious shoot regeneration using leaf explants of grapevine and factors affecting regeneration are described.

Mainly 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 transfer 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.

3.6.2. *In vitro* plant regeneration systems of grapevine

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.

4. MATERIALS AND METHODS

4.1. Explant source and maintenance in the laboratory

In vitro cultivated stock plants of varieties (chenin blanc and canonannon*)* were obtained from Holeta Agricultural Research Center. The present investigations were undertaken at Addis Ababa University, College of Natural Sciences, in Plant Propagation and Tissue Culture Laboratory. Shoot cultures of *in vitro* cultivated stock plant varieties were maintained by subculturing shoot and nodes at one-month intervals on MS basal medium supplemented with 1mg/L BAP in combination with 0.1mg/L IBA and 30 g/l sucrose in Magenta GA-7 box and was sealed with Parafilm. The medium was gelled with 8g/l agar and the pH was adjusted to 5.8 prior to autoclaving at 121^{0}C for 15 min. Then the leaves from 30 days old *in vitro* regenerated plants originated from shoot and nodes were used for *in vitro* regeneration.

4.2. Stock solution and culture media preparation

4.2.1. 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 iv). The solutions were poured into plastic bottles and stored at +4^{0}c until used. The prepared stock solutions were used for a maximum one month.

4.2.2. Plant growth regulators stock solution preparation

Plant growth regulators such as BAP, TDZ, IBA and NAA 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 was added until the crystals dissolved. Volume was adjusted by double distilled water. Then, the prepared solution was stored at +4 ^{O}C.

4.2.3. Culture media preparation

Culture media were prepared by taking the appropriate volume of MS stock solutions and diluting in double distilled water. AS energy source, 30g/l sucrose was added to the solution. For leaf culture shoot regeneration, shoot multiplication and rooting; growth regulators namely 6-benzyl aminopurine (BAP), α-naphthalene acetic acid (NAA), indole-3=acetic acid (IAA), indole-3-butyric acid (IBA) and thidiazuron (TDZ) were added separately to the MS medium. The pH of the media in each case was adjusted to 5.8 with 1NNaOH or 1NHCl solution and then 0.8% agar was added to the media. Finally, the culture media was autoclaved for 15min at 121°C and 0.15kpa pressure.

For shoot induction, about 20 ml of autoclaved medium was poured into Petri dish under Laminar air flow cabinet; about 25ml into Magenta GA-7 box for further shoot growth, shoot multiplication and rooting.

4.3. Explant preparation and shoot induction

One-month old shoots of grapevine were excised aseptically from *in vitro*-grown cultures and the most uppermost expanding leaves, were cut near the junction of petiole and lamina from the shoot. The powers of plant regeneration from structure of leaf (petioles, leaf lets, and complete leaf) were tested at best growth regulators (2mg/L BAP, 2mg/l BAP in combination with 0.1mg/l IBA and 1mg/l TDZ in combination with 0.1mg/l NAA) used to regenerate shoots from complete leaf (leaf with petioles). All leaves were wounded abaxially by scalpel blade across main vein and cultured adaxial side touching the medium for all experiments. After culturing, Petri dishes were sealed with clean Parafilm. Culture were grown under appropriate photoperiod (light and dark) and maintained at 27^{0}C. Contamination was checked daily.

4.3.1. Multiplication of the regenerated shoots

The regenerated shoots were transferred to fresh medium in Petri dish after four weeks and slowly adapted to light using transparent (thin) cloth. The cloth was removed after two weeks and when the size of the shoots reach about 10mm high or more they were transferred to shoot multiplication medium and incubated at 27^0C. The shoot multiplication medium was MS medium consisted of 2mg/l BAP. Then light adapted regenerated shoots were subcultured.

In order to overcome the problem of hyperhydricity, aseptically ventilating the culture vessels, changing the agar concentration from 7% to 8%, trimming most of the leaves from plantlets during subcultures and frequent subculturing the plantlets at 21days were done.

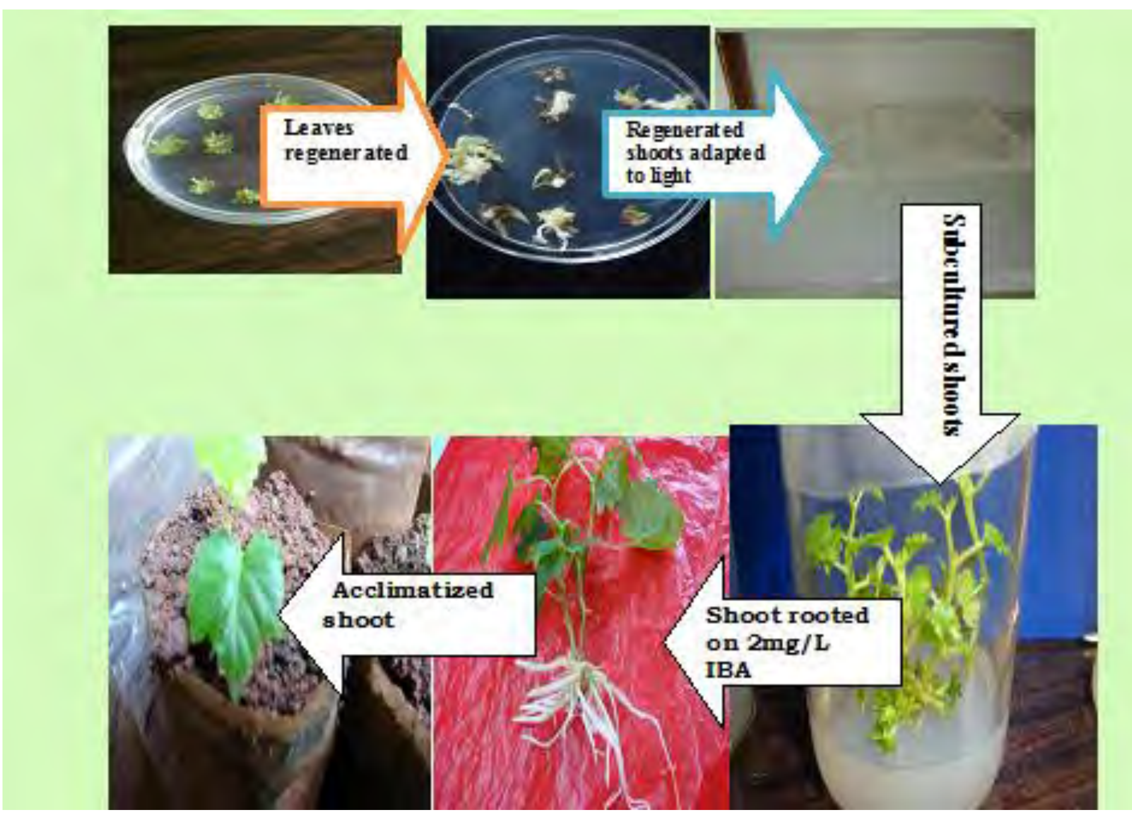

Figure 1: Regeneration methods of canonannon cultivar from leaves explant.

4.4. *In vitro* rooting of shoots

One-month subcultured shoots of canonannon and chenin blanc were rooted on 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). The length, number of main roots and length of shoots were recorded after 30 days at all IBA and IAA treatments. They were kept at 27^0C.

4.5. Acclimatization

The plantlets having sufficient root and shoot systems were taken out from the culture vessels and the roots were washed under running tap water to remove the agar and sucrose. 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. The plantlets were covered with transparent plastic bag to maintain moisture and watered within one days interval. The plastic cover was gradually removed after the plantlets successfully established in insect proof glasshouse for one month.

4.6. Experimental design, data collection and analyses

In this study, Complete Randomized Design (CRD) was used. Six explants per Petri dishes were used for the whole experiments of shoots initiations. Each experiment had five replicates of culture Petri dishes and set as experimental unit for leaf explants. The one-way analysis of variance (ANOVA) was used to compute the percentage and mean number of regenerated shoots, shoots multiplication, the number and length of rooted plantlets and their survival rate in glasshouse. All data were analyzed at p ($\alpha<0.05$) using SPSS 16 version statistical software.

5. RESULTS

5.1. Effect of different concentrations of BAP and TDZ on shoot regeneration

From the tested six different concentrations of TDZ and eight different concentrations of BAP, excluding control, the best direct shoot regeneration was obtained at 2mg/L BAP for both chenin blanc (2.3 ± 0.3) and canonannon (2.2 ± 0.2). But the capacity of leaf explants to induce direct shoot was reduced when BAP concentrations was reduced or increased for both cultivars (Table 1). Unlike interaction between auxin and cytokinin, different concentrations of BAP or TDZ alone triggered similar responses on explants of both varieties. It was shown that callus induction was significantly low at all tested levels of TDZ, and 0.5mg/l BAP, 2mg/l BAP, 4mg/l BAP, except 2.5mg/l BAP, which promoted callus in 25% and 33% of the explants of canonannon and chenin blanc, respectively. Both shoots and calli were simultaneously induced by 3mg/L BAP in both cultivars. However, TDZ alone at all tested concentrations (0.1mg/l, 0.5mg/l, 1mg/l, 2mg/l, 3mg/l and 4mg/l) and low concentrations of BAP (0.5mg/l) and high concentration of BAP (4mg/l and 5mg/l) did not induce shoots from leaves of both varieties (Table 1).

Table 1: The effect of different concentrations of BAP and TDZ on shoot induction per leaf explants of grapevine

Growth regulators concentrations (mg/l)	Canonannon			Chenin blanc		
	% regeneration	No. of shoots/ Explant	% of induced callus	% regeneration	No. of shoots/ explant	% of induced callus
BAP						
0	0.0^{c}	0 ± 0.0^{c}	0.0^{d}	0.0^{c}	0 ± 0.0^{c}	0.0^{d}
0.5	0.0^{c}	0 ± 0.0^{c}	8.3^{bd}	0.0^{c}	0 ± 0.0^{c}	0.0^{d}
1	0.0^{c}	0 ± 0.0^{c}	0.0^{d}	3.0^{b}	1 ± 0.1^{b}	21.0^{bc}
1.5	4.3^{b}	1 ± 0.0^{b}	0.0^{d}	8.0^{b}	1.2 ± 0.2^{ab}	4.2^{cd}
2	88.4^{a}	2.2 ± 0.2^{a}	0.0^{d}	86.0^{a}	2.3 ± 0.3^{a}	0.0^{d}
2.5	5.8^{b}	1 ± 0.1^{b}	25.0^{a}	0.0^{c}	1.3 ± 0.1^{ab}	33.0^{a}
3	1.4^{b}	1 ± 0.1^{b}	12.5^{b}	3.0^{b}	1 ± 0.0^{b}	4.2^{cd}
4	0.0^{c}	$0 \pm 0. 0^{c}$	0.0^{d}	0.0^{c}	0 ± 0.0^{c}	0.0^{d}
5	0.0^{c}	$0 \pm 0. 0^{c}$	0.0^{d}	0.0^{c}	0 ± 0.0^{c}	0.0^{d}
TDZ						
0.1	0.0^{c}	0 ± 0.0^{c}	0.0^{d}	0.0^{c}	0 ± 0.0^{c}	0.0^{d}
0.5	0.0^{c}	$0 \pm 0. 0^{c}$	0.0^{d}	0.0^{c}	0 ± 0.0^{c}	0.0^{d}
1	0.0^{c}	0 ± 0.0^{c}	21.0^{ab}	0.0^{c}	0 ± 0.0^{c}	8.3^{cd}
2	0.0^{c}	0 ± 0.0^{c}	17.0^{ab}	0.0^{c}	0 ± 0.0^{c}	13.0^{cd}
3	0.0^{c}	0 ± 0.0^{c}	17.0^{ab}	0.0^{c}	0 ± 0.0^{c}	17.0^{c}
4	0.0^{c}	0 ± 0.0^{c}	0.0^{d}	0.0^{c}	0 ± 0.0^{c}	0.0^{d}

The data present the regeneration rate and mean number of shoots per explant ± standard error of the experiments. Means having the same superscript letters in a column were not significantly different at 5% probability level.

During the first 14 days, explants became yellowish and produced bud like structure up to day 20. First shoots appeared on the 25th days on induction medium; sometimes at the wounded edges and mostly from swollen petiole tip. On the 30th day, their number increased and easily identified (Figure 2). Almost all regenerated shoots in the darks were shown fresh white color (Appendix II).

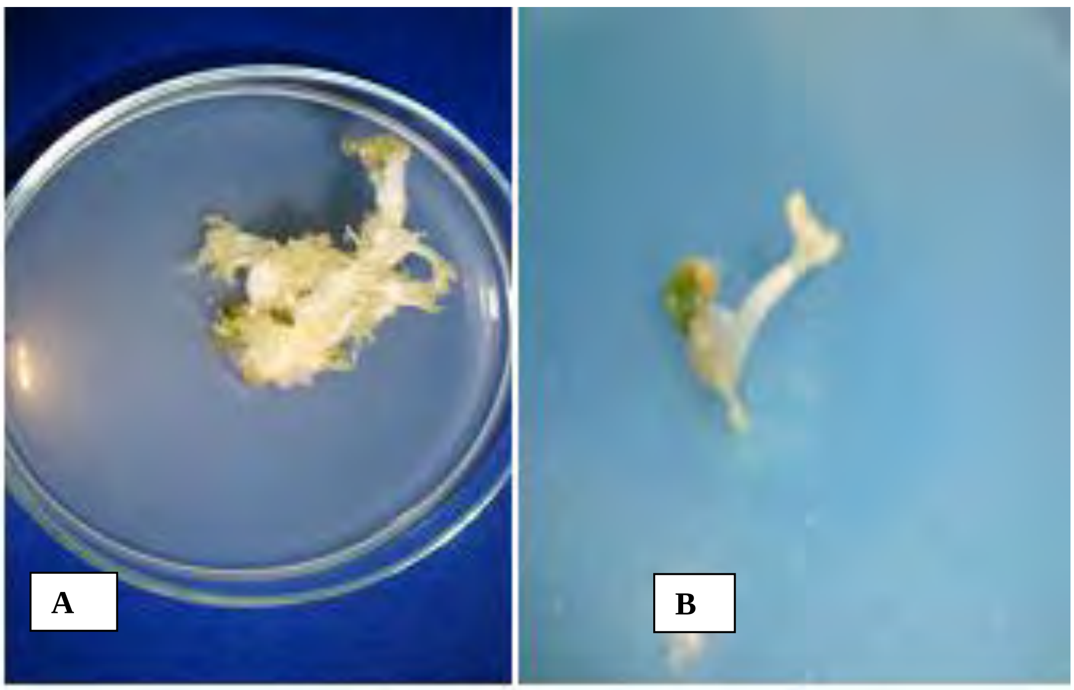

Figure 2: Direct regenerants of grapevine (*Vitis vinifera*) from leaf explants on 2mg/L BAP after 30days (**A**=Canonannon and **B**=Chenin blanc).

5.2. Effect of TDZ and NAA combinations on shoot induction of grapevine (*Vitis vinifera*)

Among sixteen different combinations of TDZ and NAA excluding control, the maximum and best shoots per explants were obtained when 1mg/l TDZ was combined with 0.1mg/l NAA for both cultivars. Thus, about 45% and 29.8% of shoots were directly regenerated from chenin blanc and canonannon leaves, res pectively (Table 2). Lower concentrations of TDZ in combination with different c oncentrations of NAA gave no response except some shoots obtained at 0.5mg/l and 0.1mg/l TDZ in combination with 0.01mg/L NAA for canonannon cultivar. Combination of 0.5mg/l TDZ with 0.01mg/l and 0.1mg/l NAA have shown significant shoots and callus induction in canonannon cultivar. Similarly, combinations of 0.5mg/l TDZ and 0.5 mg/l NAA, have no response in shoot inductions for both cultivars (Chenin blanc and canonannon) rather it promoted highly significant(about 50%) callus in chenin blanc cultivar. But, there were no response of both shoots and callus induction at 0.1mg/l and 3mg/l TDZ in combination with 0.1mg/l NAA for both varieties (Table 2). These results suggest that TDZ in combination with NAA had a synergistic effect in adventitious shoot production from grapevine leaf explants.

Table 2: Effect of TDZ and NAA combinations on shoot initiation from leaf explants

Growth regulators concentrations (mg/l)		**Canonannon**			**Chenin blanc**		
TDZ	NAA	%regeneration	Mean No. of shoots/explant	% of induced callus	% of regeneration	Mean No. of shoots/ Explant	% of induced callus
0	0	0.0^{c}	0 ± 0.0^{c}	0.0^{c}	0.0^{c}	0 ± 0.0^{c}	0.0^{c}
0.1	0.01	8.5^{b}	1.3 ± 0.3^{ab}	0.0^{c}	0.0^{c}	0 ± 0.0^{c}	0.0^{c}
0.1	0.1	0.0^{c}	0 ± 0.0^{c}	0.0^{c}	0.0^{c}	$0 \pm 0. 0^{c}$	0.0^{c}
0.5	0.01	6.4^{b}	1 ± 0.0^{b}	2.4^{b}	0.0^{c}	0 ± 0.0^{c}	2.8^{b}
0.5	0.1	6.4^{b}	1 ± 0.0^{b}	7.3^{b}	0.0^{c}	0 ± 0.0^{c}	8.3^{b}
0.5	0.5	0.0^{c}	0 ± 0.0^{c}	14.6^{b}	0.0^{c}	0 ± 0.0^{c}	50.0^{a}
1	0.01	0.0^{c}	0 ± 0.0^{c}	0.0^{c}	0.0^{c}	0 ± 0.0^{c}	0.0^{c}
1	0.1	29.8^{a}	1.5 ± 0.2^{a}	18.2^{b}	45.0^{a}	1.4 ± 0.2^{a}	18.4^{b}
1	0.5	0.0^{b}	0 ± 0.0^{c}	7.3^{b}	0.0^{c}	0 ± 0.0^{c}	8.3^{b}
1.5	0.01	19.0^{a}	1 ± 0.0^{b}	9.8^{b}	0.0^{c}	0 ± 0.0^{c}	16.7^{b}
1.5	0.1	7.0^{b}	1 ± 0.0^{b}	0.0^{c}	0.0^{c}	0 ± 0.0^{c}	0.0^{c}
1.5	0.5	6.4^{b}	1.3 ± 0.3^{a}	24.4^{a}	25.0^{a}	1.3 ± 0.3^{a}	8.3^{b}
2	0.01	6.0^{b}	1 ± 0.0^{b}	7.3^{b}	15.0^{b}	1 ± 0.0^{b}	0.0^{c}
2	0.1	6.0^{b}	1 ± 0.0^{b}	34.1^{a}	15.0^{b}	1 ± 0.0^{b}	8.3^{b}
2	0.5	0.0^{c}	$0 \pm 0. 0^{c}$	0.0^{c}	0.0^{c}	0 ± 0.0^{c}	0.0^{c}
3	0.01	0.0^{c}	$0 \pm 0. 0^{c}$	0.0^{c}	0.0^{c}	0 ± 0.0^{c}	0.0^{c}
3	0.1	0.0^{c}	$0 \pm 0. 0^{c}$	0.0^{c}	0.0^{c}	0 ± 0.0^{c}	0.0^{c}

The data present the regeneration rate and mean number of shoots per explant ± standard error of the experiments. Means having the same superscript letters in a column were not significantly different at 5% probability level.

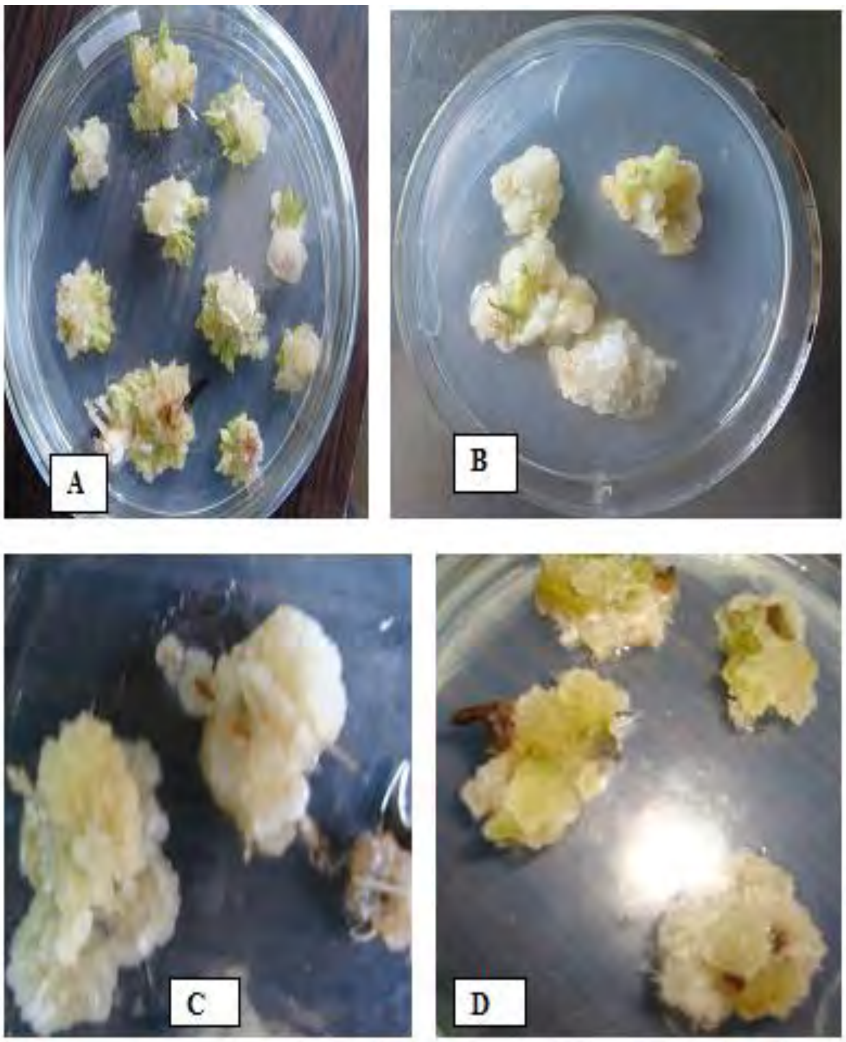

Figure 3: Induced calli from canonannon (A & B) and chenin blanc (C & D) on different concentrations of growth regulators from leaf explants at dark incubation after 30 days. (**A**=1.5mg/l BAP and 1mg/l IBA, **B**= 2mg/l BAP and 0.1mg/l NAA, **C**= 0.5mg/l TDZ and 0.5mg/l NAA, **D**=2mg/l TDZ and 0.1mg/l NAA)

5.3. Effect of BAP and IBA combinations on direct shoot inductions

Using different BAP and IBA concentrations, the best and significant direct shoot induction was obtained at 2mg/l BAP in combination with 0.1mg/l IBA for both varieties. Accordingly, the best responses in terms of multiple shoot regenerations were 71.7% and 90% for canonannon and chenin blanc, respectively (Table 3). Unlike canonannon cultivar, chenin blanc had no shoot induction response at lower concentrations of BAP (0.5mg/l and 1.5mg/l) while canonannon cultivars had shown significant mean number (1.0±0.0) of shoots per explants when 0.5mg/l BAP combined with 0.1mg/l IBA (Table 3). However, shoot inductions from chenin blanc cultivar was observed when 2mg/l BAP is combined with 0.1mg/l and 0.5mg/l IBA. Shoot induction for canonannon was observed when 2mg/L BAP is combined with all tested IBA concentrations (0.1mg/l, 0.5mg/l and 1mg/l). Higher calli 31.3% and 40% was obtained when 1.5mg/l BAP was combined with 1mg/l IBA for canonannon and chenin blanc, respectively (Table 3). In addition, there were no response of both shoot and callus induction at 0.5mg/l and 3mg/l BAP in combination with 0.5mg and 1mg/l NAA for both varieties. The data for percent of plant regeneration from leaf explants was recorded after 30 days of *in vitro* culture on different media (Table 3).

Table 3: Effect of BAP and IBA combinations on shoot initiations from leaf of two grape varieties

Growth regulators concentrations (mg/L)		Canonannon			Chenin blanc		
BAP	IBA	% regeneration	Mean No. of shoots/ explant	% of induced callus	%regenerati on	Mean No. of shoots/ explant	% of induced callus
0	0	0.0^{c}	0 ± 0.0^{c}	0.0^{d}	0.0^{c}	0 ± 0.0^{c}	0.0^{d}
0.5	0.1	6.5^{b}	1 ± 0.0^{b}	12.5^{c}	0.0^{c}	0 ± 0.0^{c}	0.0^{d}
0.5	0.5	0.0^{c}	0 ± 0.0^{c}	0.0^{d}	0.0^{c}	0 ± 0.0^{c}	0.0^{d}
0.5	1	0.0^{c}	0 ± 0.0^{c}	0.0^{d}	0.0^{c}	0 ± 0.0^{c}	0.0^{d}
1.5	0.1	0.0^{c}	0 ± 0.0^{c}	31.3^{a}	0.0^{c}	0 ± 0.0^{c}	16^{b}
1.5	0.5	8.7^{b}	1 ± 0.0^{b}	3.1^{cd}	0.0^{c}	0 ± 0.0^{c}	0.0^{d}
1.5	1	0.0^{c}	0 ± 0.0^{c}	31.3^{a}	10^{b}	1 ± 0.0^{b}	40^{a}
2	0.1	71.7^{a}	1.4 ± 0.1^{a}	0.0^{d}	90^{a}	1.3 ± 0.1^{a}	0.0^{d}
2	0.5	8.7^{b}	1.3 ± 0.0^{a}	0.0^{d}	10^{b}	1 ± 0.1^{b}	0.0^{d}
2	1	4.3^{b}	1 ± 0.0^{b}	21.9^{b}	0.0^{c}	0 ± 0.0^{c}	24^{b}
3	0.1	13.5^{b}	1 ± 0.0^{b}	16.9^{c}	7.9^{b}	1 ± 0.0^{b}	0.0^{d}
3	0.5	0.0^{c}	0 ± 0.0^{c}	0.0^{d}	0.0^{c}	0 ± 0.0^{c}	0.0^{d}
3	1	0.0^{c}	0 ± 0.0^{c}	0.0^{d}	0.0^{c}	0 ± 0.0^{c}	0.0^{d}

The data present the regeneration rate and mean number of shoots per explant ± standard error of the experiments. Means having the same superscript letters in a column were not significantly different at 5% probability level.

There were no any plant regenerations from leaflets (leaf without petiole) and petiole. However, complete leaf (leaf with petiole) placed on induction medium enlarged and exhibited varied responses. The cut petiole tip parts exposed to the medium increased in size, and adventitious shoots proliferated (Figure 3).

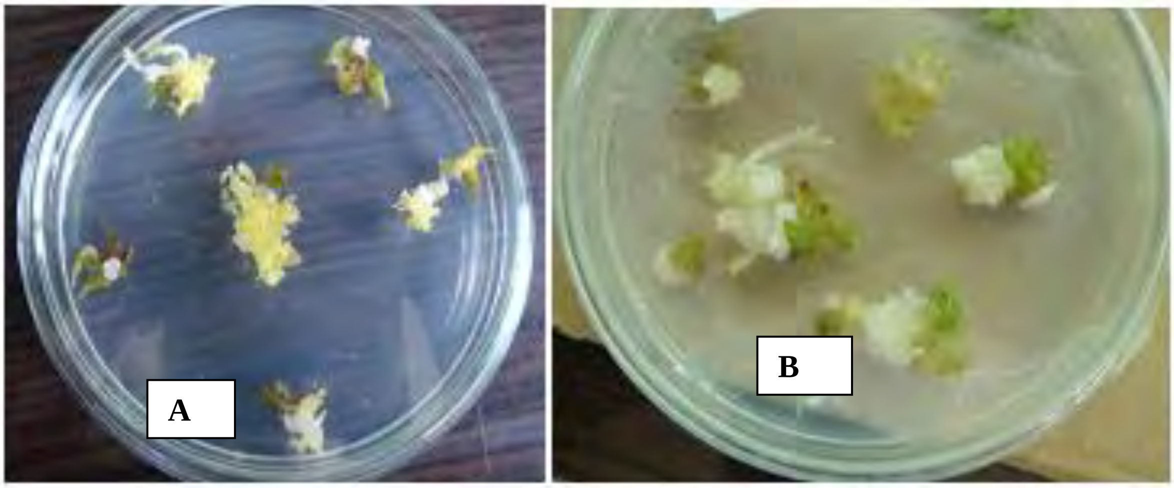

Figure 4. Shoots of grapevine regenerated from leaf explants on MS medium supplemented with 2mg/L BAP+0.1mg/L IBA after 30 days (**A**=canonannon, and **B**= Chenin blanc).

5.4. Shoot multiplication

Chenin blanc regenerated from leaves had shown best number of shoots (10 ± 0.51) at 2mg/L BAP. Similarly, canonannon gave 4.7 ± 0.3 shoots as compared to the shoots multiplied from regenerated plantlets and *in vitro* cultivated stalk plants on the same medium composition (Table 4).

Table 4: Comparisons of mean number of shoots multiplied from regenerated plantlets and *in vitro* cultivated stock plants on similar medium composition.

Growth regulators concentrations (mg/l)	**Canonannon**		**Chenin blanc**	
	Mean No of shoots /explant from leaf	Mean No of shoots/explant from stock plant	Mean No of shoots /explant from leaf	Mean No of shoots/explant from stock plant
1BAP+0.1IBA	2.7 ± 0.12^{b}	3.0 ± 0.1^{b}	3.3 ± 0.2^{b}	2.8 ± 0.2^{a}
2BAP	4.7 ± 0.3^{a}	4.3 ± 0.3^{a}	10 ± 0.5^{a}	3.3 ± 0.3^{a}
Total	3.7 ± 21	3.7 ± 0.2	6.7 ± 0. 5	3.1 ± 0.2

The data represent the regeneration rate and mean number of shoots per explant ± standard error of the experiments. Means having the same superscript letters in a column were not significantly different at 5% probability level.

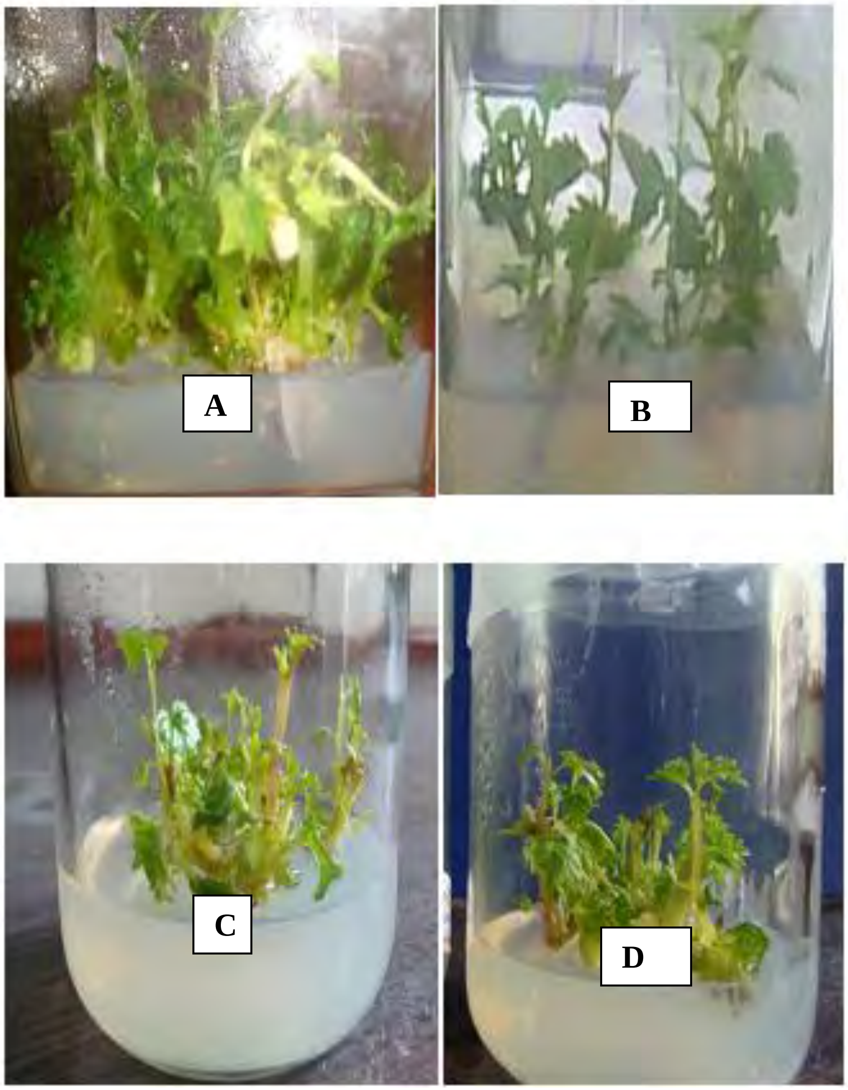

Figure 5: Shoot proliferation of two varieties of grapevine at 2mg/L BAP after 30 days. (**A**= chenin blanc originated from leaf, **B**= chenin blanc originated from stock plant, **C**= canonannon originated from leaf, **D**=canonannon originated from stock plant).

5.5. Hyperhydricity and mechanism to reduce it

Hyperhydricity (vitrification) was a serious problem observed during this work. During the conduction of this research, the regenerated plantlets of both grapevine varieties were losing leaves from shoot tips. But such condition was highly observed on chenin blanc cultivar. As the time of culture increased, the shoots of both varieties reduced to stem (Figure 5). This result, suggested that the time interval for subculture should be 21 days for both varieties.

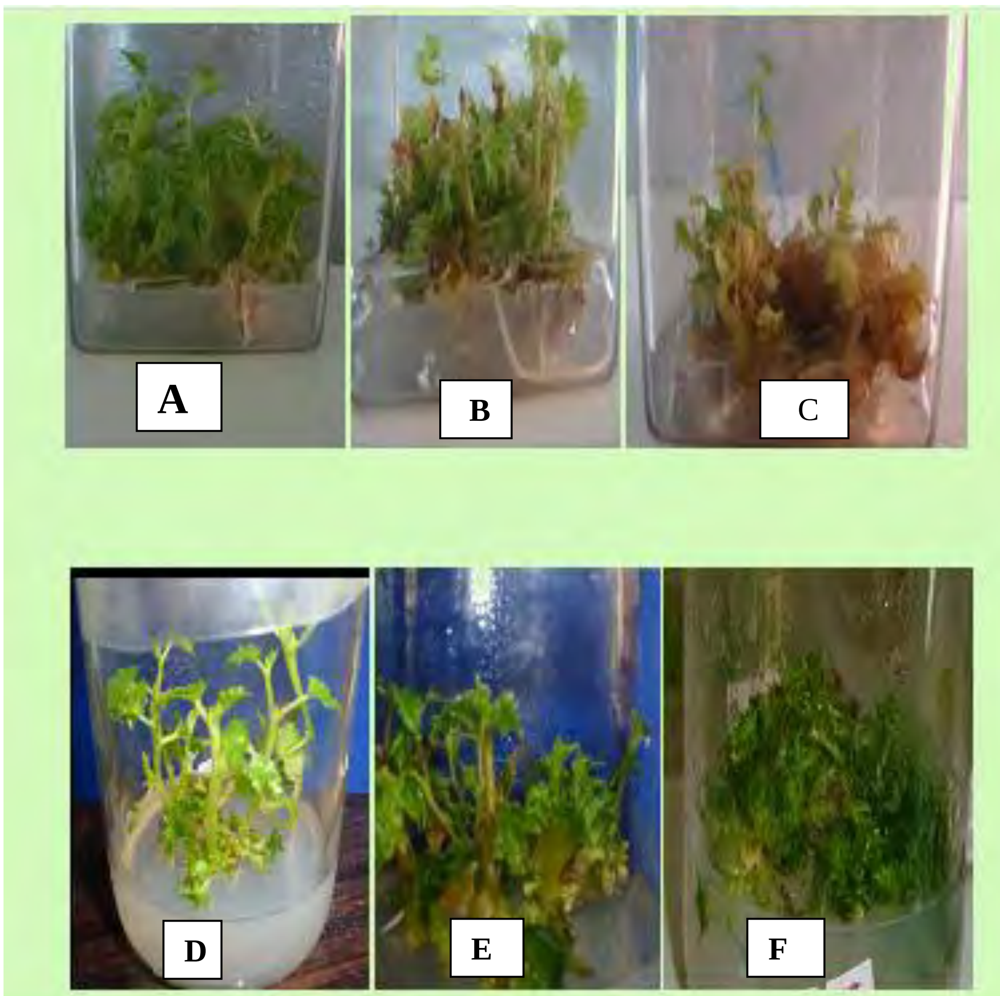

Figure 6: Effect of vitrification on two grape varieties at different intervals of time and MS medium supplemented with 1mg/L BAP + 0.1mg/L IBA. **(A**= chenin blanc at 21 days, **B**= chenin blanc after 30 days, **C**= chenin blanc after 5 weeks, **D**= Canonannon at 21 days, **E**= Canonannon after 30 days, **F**= Canonannon after 5 weeks).

5.6. Rooting

Well established plantlets were transferred to MS basal medium supplemented with IBA (0, 1, 2, 3 and 4mg/l) and IAA (2mg/l and 4mg/l) for rooting (Table 5). The root induction of shoots was obtained in the first 10 days after IBA treatment, which is important requirement for acclimatization of *in vitro* plantlets. Highest mean number and length of main roots were obtained at MS medium of 2mg/L IBA for both varieties of grapevine (Table 5). Thus about 7.0 ± 0.92 and 6.7 ± 0.73 mean number of main roots were obtained for canonannon and chenin blanc, respectively. Among the tested IBA and IAA, the best length of plantlets were obtained at 0, 1 and 2mg/l IBA when compared to 3mg/l IBA , 4mg/L IBA , 2mg/l IAA and 4mg/l IAA concentrations. Thus, the growth regulators of root have a synergistic effect on both root and shoot part.

Table 5: Effect of different IBA and IAA concentrations on rooting

	Canonannon			Chenin blanc		
IBA(mg/l)	Number of roots	Length of roots	Length of shoots	Number of roots	Length of roots	Length of shoots
0	3.9 ± 0.23^{b}	4.7 ± 0.42^{ba}	$7.5 \pm 0\ .23^{a}$	3.6 ± 0.16^{b}	3.6 ± 0.22^{b}	7.2 ± 0.47^{a}
1	4.3 ± 0.54^{b}	4.3 ± 0.63^{ba}	7.4 ± 0.37^{a}	4.5 ± 0.42^{bc}	4.9 ± 0.60^{a}	7.3 ± 0.30^{a}
2	7.0 ± 0.92^{a}	5.5 ± 0.63^{a}	8.3 ± 0.30^{a}	6.7 ± 0.73^{a}	5.4 ± 0.50^{a}	7.9 ± 0.40^{a}
3	3.2 ± 0.29^{b}	2.9 ± 0.53^{b}	6.8 ± 0.34^{a}	3.7 ± 0.6^{b}	2.8 ± 0.40^{cb}	5.2 ± 0.55^{c}
4	2.3 ± 0.31^{bc}	2.9 ± 0.23^{b}	3.9 ± 0.34^{b}	2.4 ± 0.26^{b}	2.1 ± 0.40^{c}	3.1 ± 0.58^{d}
IAA(mg/l)						
2	2.4 ± 0.51^{bc}	3.4 ± 0.51^{ba}	3.8 ± 0.49^{b}	2.2 ± 0.37^{b}	3.6 ± 0.60^{bc}	2.8 ± 0.29^{d}
4	3.14 ± 0.55^{b}	2.3 ± 0.47^{b}	3.43 ± 0.48^{b}	5.4 ± 0.92^{ac}	2.4 ± 0.20^{bc}	5.6 ± 0.29^{c}

Levels not connected by the same superscript letter in the same column are significantly different at 5% probability level.

Efficient rooting of *in vitro* regenerated plants and subsequent establishment was succeeded on a medium supplemented with 2mg/L IBA for both varieties of grapevine (Figure 6).

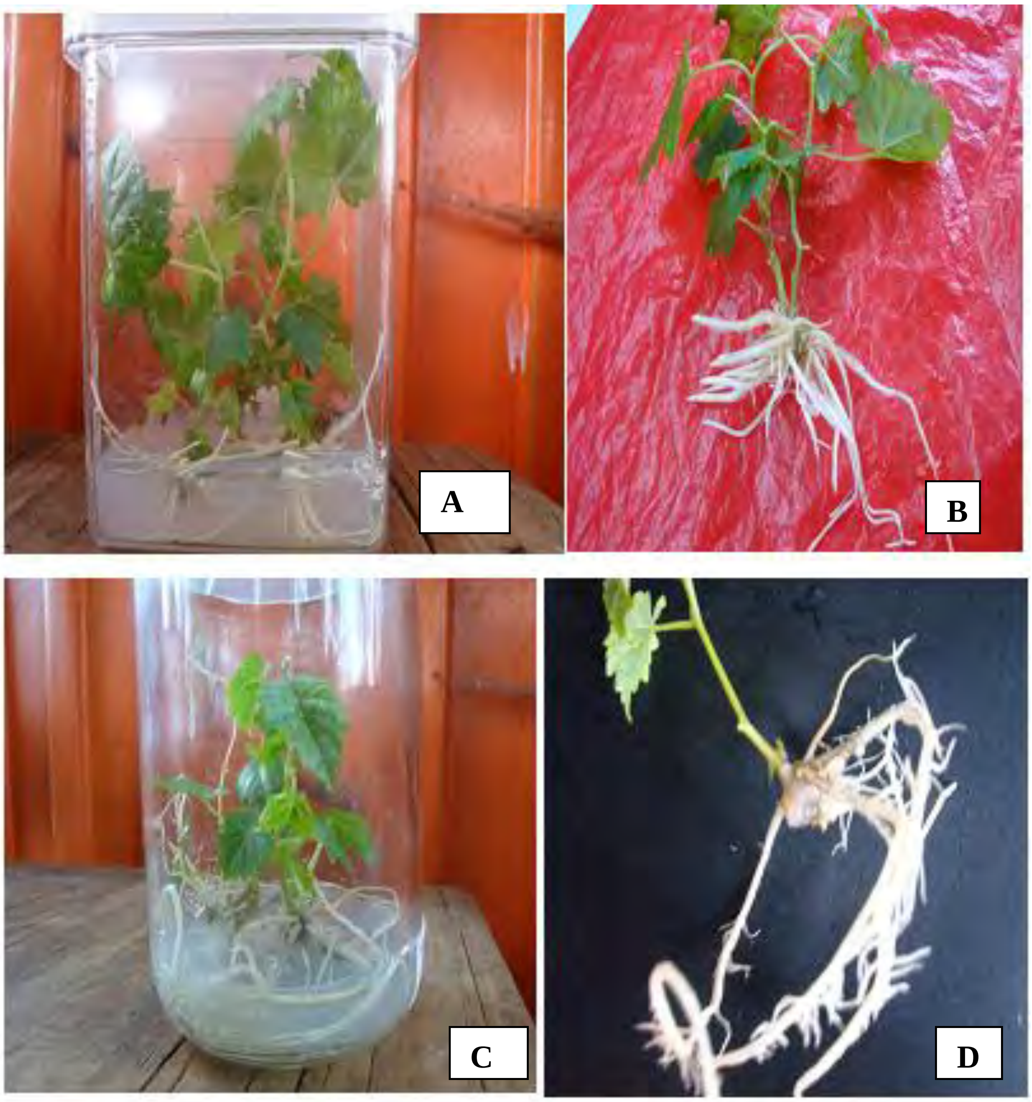

Figure 7: Rooted shoots of leaf derived *in vitro* plantlets on MS basal salt medium supplemented with 2mg/L IBA after 30 days.(**A**&**B**= canonannon and **C**&**D**= chenin blanc).

5.7. Acclimatization

The acclimatized plantlets of *in vitro* regenerated plants were achieved under controlled atmosphere and humidity. After 30 days they look healthy plants (Fig. 8). The survival rates were 83.3% and 75% for chenin blanc and canonannon, respectively.

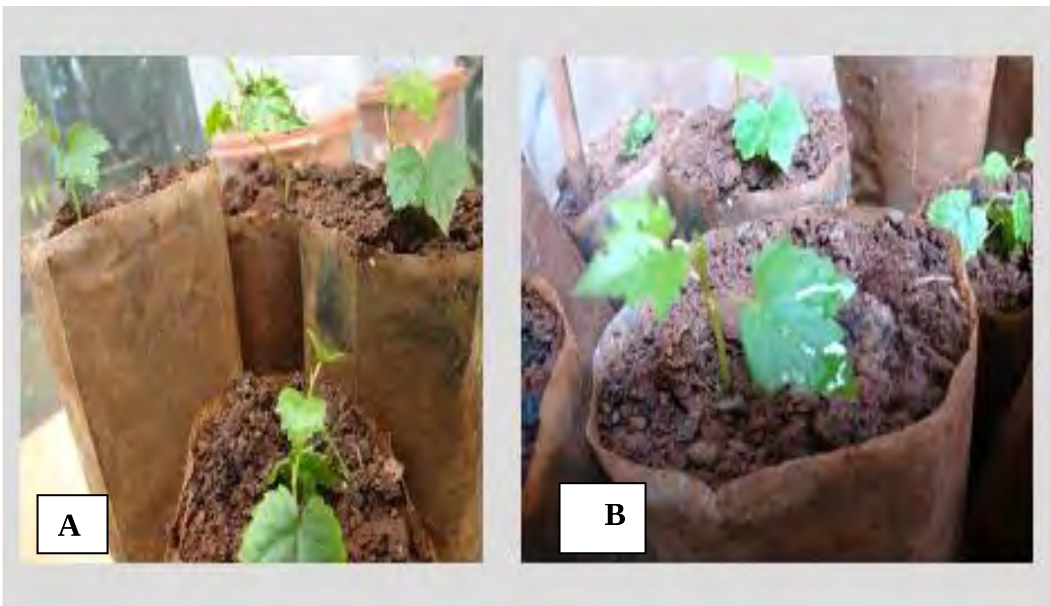

Figure 8: Acclimatized plantlets of grapevine regenerated from leaf explants after 30 days. (**A**= Chenin blanc, and **B**= Canonannon)

6. DISCUSSION

6.1. Regeneration

In this study, *in vitro* regeneration of grapevine from leaf explants was conducted for the establishment of culture conditions under strong hormonal control. The highest regeneration about 88.4% and 86% on 2mg/L BAP were obtained for canonannon and chenin blanc, respectively. The same responses were obtained during the development of micropropagation protocol of the cultivars (Beza Kinfe, 2010).

Many factors influence grapevine *in vitro* regeneration. The factors considered in this study were the effect of cytokinin and auxin concentrations on regeneration from leaf explants and *in vitro* multiplication of grapevines. With regard to plant growth regulators, in micropropagation, the general principle is that during *in vitro* culture, presence of cytokinin in the medium promotes the induction of shoot morphogenesis. 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. Even though significant shoot induction was obtained on medium supplemented with TDZ combined with different concentrations of NAA, the best mean number of shoot regeneration was obtained from medium supplemented with BAP combined with IBA. This is in accordance with previous studies that have clearly put that BAP is most effective among other cytokinins for promoting plant regeneration of *V.vinifera* cultivars belonging to "Soltanin" and "Sahebi" from shoot apical meristem (Aazami, 2010). Thus, from twelve different combinations of BAP and IBA, shoots were regenerated at 0.5mg/l BAP +0.1 IBA, 1.5mg/l BAP+0.5mg/l IBA, 2mg/l BAP+0.1mg/l IBA, 2mg/l BAP+0.5mg/l IBA and 2mg/l BAP+1mg/l IBA for canonannon cultivar.

However, BAP in combination with IBA regenerated shoots at 2mg/L BAP+0.1mg/l IBA and 2mg/l BAP+0.5mg/L IBA for chenin blanc. On the other hand, maximum plant regenerations were obtained on MS medium supplemented with 2 mg/L BAP+0.1mg/L IBA for both varieties. Such high number of shoot formation per explant is very advantageous for a variety of purposes, including both classical and molecular breeding of selected plants (Ahroni, 1997). This implies that such technique is a pre-requisite for successful application of other tissue culture techniques. Eventhough leaves of shoot cultures have been used as an explant in Cabernet Sauvignon, French Colombard, Grenache, Thompson Seedless, White Riesling, *V. vinifera x rupestris* and *V. rupestris (*stamp *et al.,* 1990) to induce shoots *in vitro,* this is the first report of regenerating shoots from leaves of canonannon and chenin blanc.

Another factor focused in this study was, the orientation of leaves and photoperiod. The leaves culture incubated in light and dark responded differently. Thus, most cultures incubated in light produced callus. However, the same cultures on the same medium compositions gave shoots in the dark. This is in consistence with the findings of Billings *et al.* (1988); Chevreau *et al.* (1988); Fasolo *et al.* (1989) *cited* in Baker & Bhatia (1993) for tissue culture of quince (*Cydonia oblonga).* For this study, leaflets (leaf without petiole) and petioles were not giving any regeneration except complete leaf (leaf with petiole) which has regenerated yellowish colour shoots. Thus, first shoots appeared on the 25th days on induction medium; sometimes at the wounded edges and mostly from swollen petiole tip. On the 30th day, their number increased and easily identified. Almost all induced shoots have shown fresh white color (Appendix II). Such response of leaf explants was observed in the previous work on other varieties of plant (Pe´ros, 1998). Induction and development of leaf to shoot on MS medium had taken 30 days after incubation.

This is shorter incubation period when compared to the work of Azami (2010) and Stamp *et al.* (1990). Thus, results indicated that an incubation period of 4 weeks in the dark is necessary for shoot regeneration of grapevine (*Vitis vinifera* L.) from leaf explants.

The youngest leaves when used as explants, gave the highest regeneration response. This is in agreement with the work of Sankhla *et al.* (1993) as cited in Baker & Bhati (1993). Other structures of leaf (petioles and leaf without petioles) did not show any shoot regeneration at best hormone that was used to generate shoots from whole leaf (leaf with petiole).

6.2. Shoot multiplication

In this study, shoot proliferation potential of two grape varieties was tested by using 1mg/l BAP+ 0.1mg/L IBA and 2mg/L BAP. This is similar with Fayek *et al.* (2009) who indicated considerable shoot proliferation after cytokinin application in subsequent subculture stages. Accordingly, chenin blanc regenerated from leaves had shown the best mean number of shoots (10±51) at 2mg/L BAP. Similarly, canonannon resulted in best mean number of shoots (4.7 ± 0.29) as compared to the shoots propagated from stock plantlet nodal culture and shoot. Hence, the result of this study revealed that the shoots obtained via *in vitro* regeneration from leaves of grapevine are suitable for mass propagation in a short period of time.

6.3. Vitrification

Vitrification was a serious problem during the conduction of this rsearch. This is in agreement with what has been repeatedly reported in grapevine tissue culture (Morini *et al.*, 1985; Heloir *et al.*, 1997 cited in Banilas and Korkas, 2005).

In the system presented here, most of the plantlets were hyperhydric but they developed into normal, healthy plants by using different techniques. Thus, level of hyperhydricity was reduced by well aeration of culture vessels under aseptic condition. This is in agreement with the previous works (Dillen and Buysens, 1989 *cited* in Ahroni, 1997). Recovery of non-hyperhydric plants from *Vitis vinifera* was obtained by modifying agar concentration from the normally used 7% to 8%. Similar work was done by previous worker (Gonzales *et al.*, 2010). According to Banilas and Korkas (2005) and Gonzales *et al.* (2010) hyperhydricity is also linked to shoot-tip necrosis, a physiological disorder shown by *in vitro* plants because of high relative humidity and destabilized composition of medium. The vitrified plantlets of chenin blanc and canonannon immediately lost their shoot tips, which is in agreement with the previous work of Banilas and Korkas (2005) on grapevine c*V.*agiorgitiko.

6.4. *In vitro* rooting and acclimatization

In vitro plantlets of grapevine were very sensitive to *ex vitro* conditions. Micropropagated plantlets 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. Four mg/l IAA and 2mg/l IAA have been reported to induce optimum rooting for both varieties of canonannon and chenin blanc, respectively (Beza Kinfe, 2010). However, these concentrations of IAA suppressed shoot growth and promoted only root parts. Two mg/l IBA was found to be the best. Additionally, the rooted plantlets using 2mg/l IBA also exhibited the best survival rate for both cultivars. IBA was not tested in the previous reports for chenin blanc and canonannon.

7. CONCLUSION

From the obtained result it can be concluded that, the frequency of leaves showing adventitious shoot induction varied with the presence of both TDZ or BAP alone or in combination with NAA and IBA. Among different combinations of BAP and auxins tested for shoot regeneration, IBA gave maximum number of shoots for both varieties when compared to NAA.

- ✓ The obtained results indicated that an incubation period of four weeks in the dark is necessary for regeneration of two varieties of grapevine (*Vitis vinifera* L.) from leaf explants.

- ✓ Well aeration method, changing the concentrations of gelling agent (agar) and trimming most of the leaf from shoot used for subculturing and using shoot rather than the nodal culture have shown reduced vitrification.

- ✓ The shoots, obtained from chenin blanc leaf explants have shown the maximum mean number (10 ± 0.51) on 2mg/l BAP. This enabled us to get the propagules of this particular cultivar within short period of time.

- ✓ The plant regeneration from leaf explants was obtained from only adaxially cultured leaves. There was no response of the abaxially cultured leaves.

- ✓ The best time interval for subsequent subculture for the studied varieties is 21 days.

8. RECOMMENDATIONS

- The indirect shoot regeneration from obtained callus was not tested at higher levels of growth regulators. Hence, it is necessary to investigate the indirect shoot regeneration mechanism for both cultivars at higher concentrations of growth regulators that were not included in this study.

- Even though vitrification was serious problem during multiplications of grapevines, techniques such as ventilating the culture vessels, using 8% agar concentration and using shoots instead of nodal are recommended to get normal *in vitro* growth.

- The presence of appropriate tissue culture protocol is important in the elimination or minimization of disease load in plants as it facilitates micropropagation and *de novo* plant regeneration. Another option to combat plant disease is to produce genetically modified plants using the established regeneration protocol.

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10. APPENDICES

Appendix I

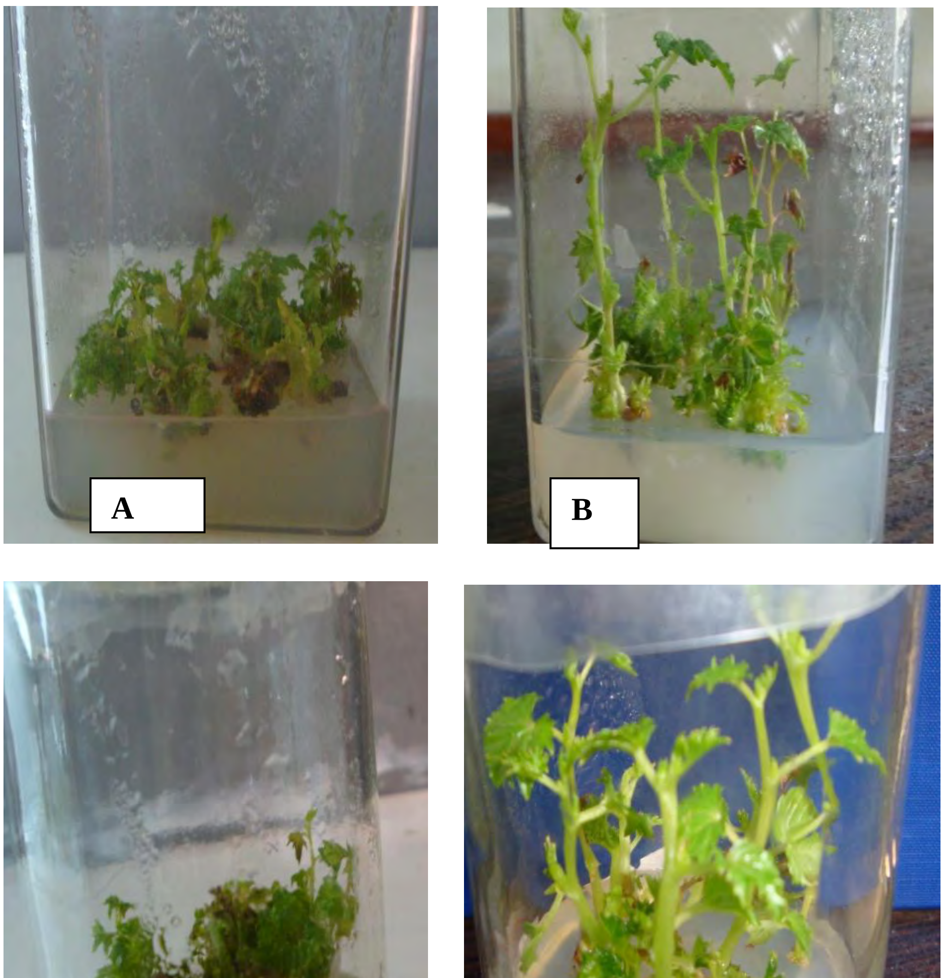
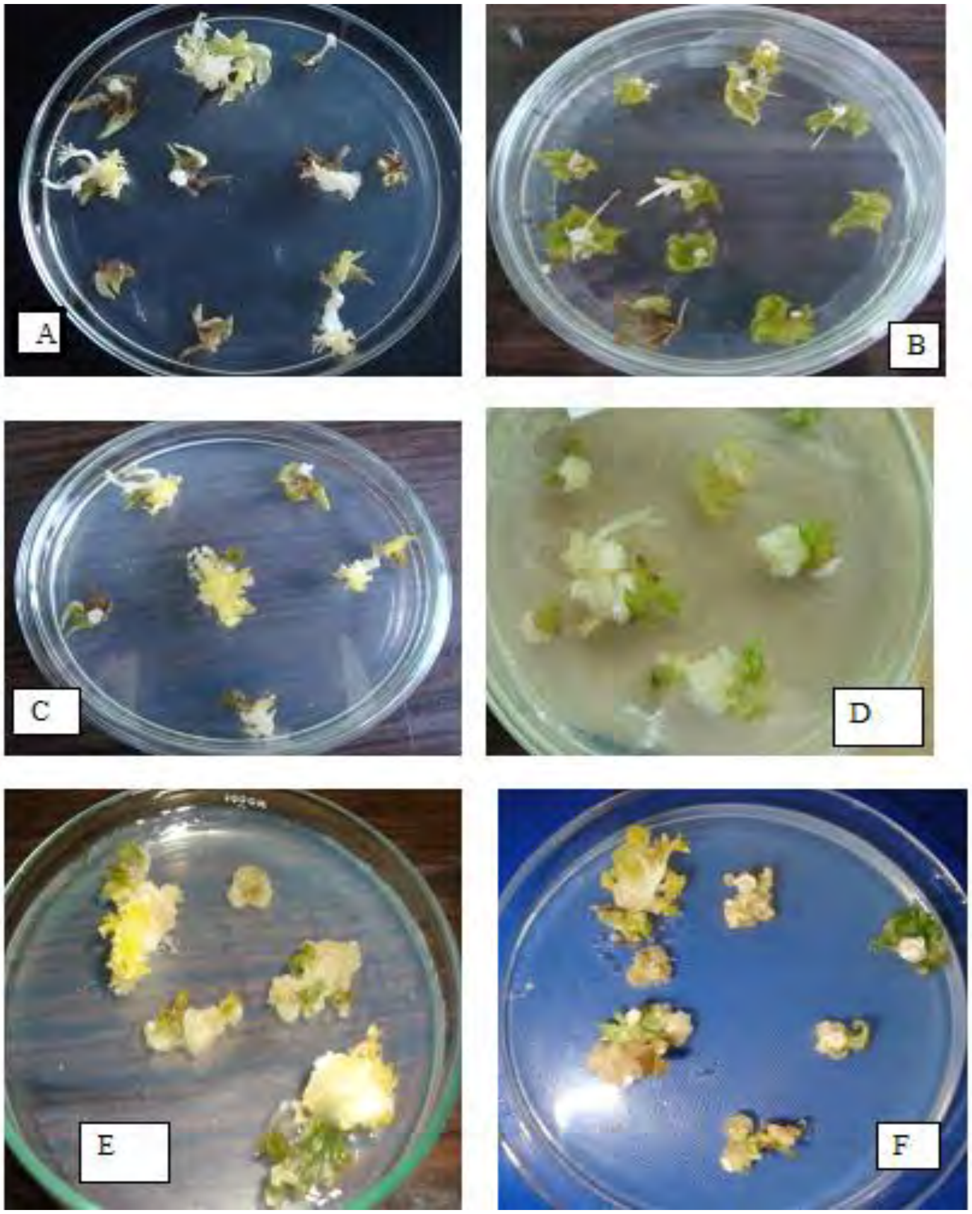


Figure 1: Growth of grapevine at two different temperatures (27 ^{0}C and 30^{0}C); **A**&**C** were kept at 30^{0}C while **B**&**D** were kept at 27^{0}C for 30 days. (A&B= Canonannon, C&D= Chenin blanc)

Appendix II



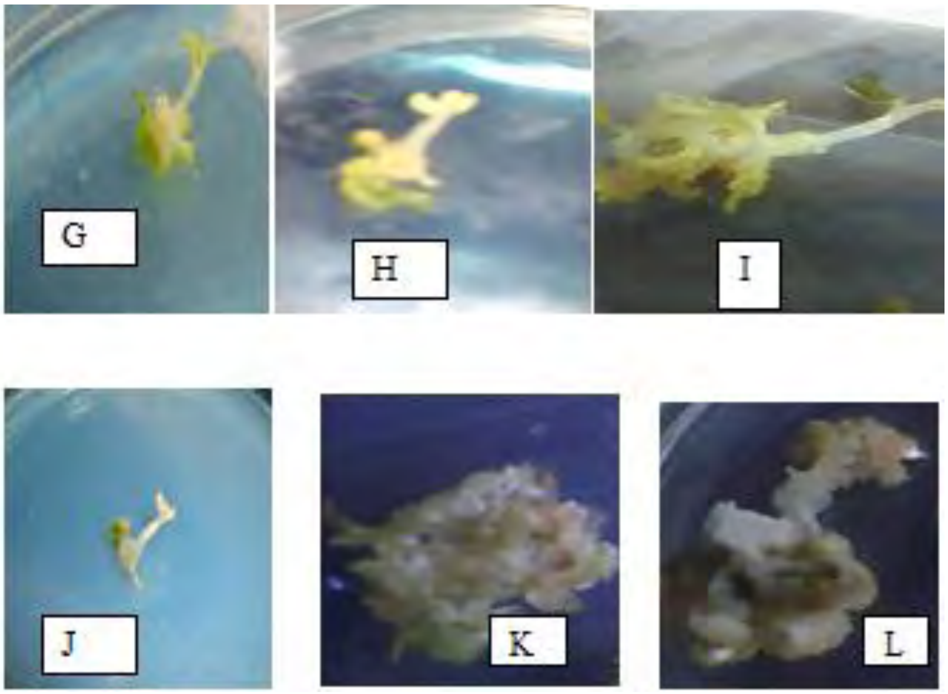


Figure 2: Different generated shoots per different concentrations of growth regulators from leaf explants after 30 days.

Keys:

A= Canonannone on MS medium with 2mg/l BAP

B= Chenin blanc on MS medium with 2mg/l BAP

C= Canonannon on MS medium supplemented with 2mg/l BAP+0.1mg/l IBA

D= Chenin blanc on MS medium supplemented with 2mg/l BAP+0.1mg/l IBA

E= Canonannon on MS medium supplemented with 1mg/l TDZ+0.1mg/l NAA

F= Chenin blanc on MS medium supplied with 1mg/l TDZ+0.01mg/l NAA

G=Single shoot of Chenin Blanc on MS medium supplemented by 2mg/L BAP+0.1mg/l IBA

H= Single shoot of Canonannon on MS medium supplemented by 2mg/L BAP

I= Single shoot of Canonannon on MS medium supplemented by 2mg/L BAP+0.1mg/l IBA

J= Single shoot of Chenin Blanc on MS medium supplemented by 2mg/L BAP

K= Single shoot of Canonannon on MS medium supplemented by 1.5 TDZ+0.5mg/l NAA

L= Single shoot of Chenin blanc on MS medium supplemented by 1.5TDZ+0.5mg/l NAA

Appendix III

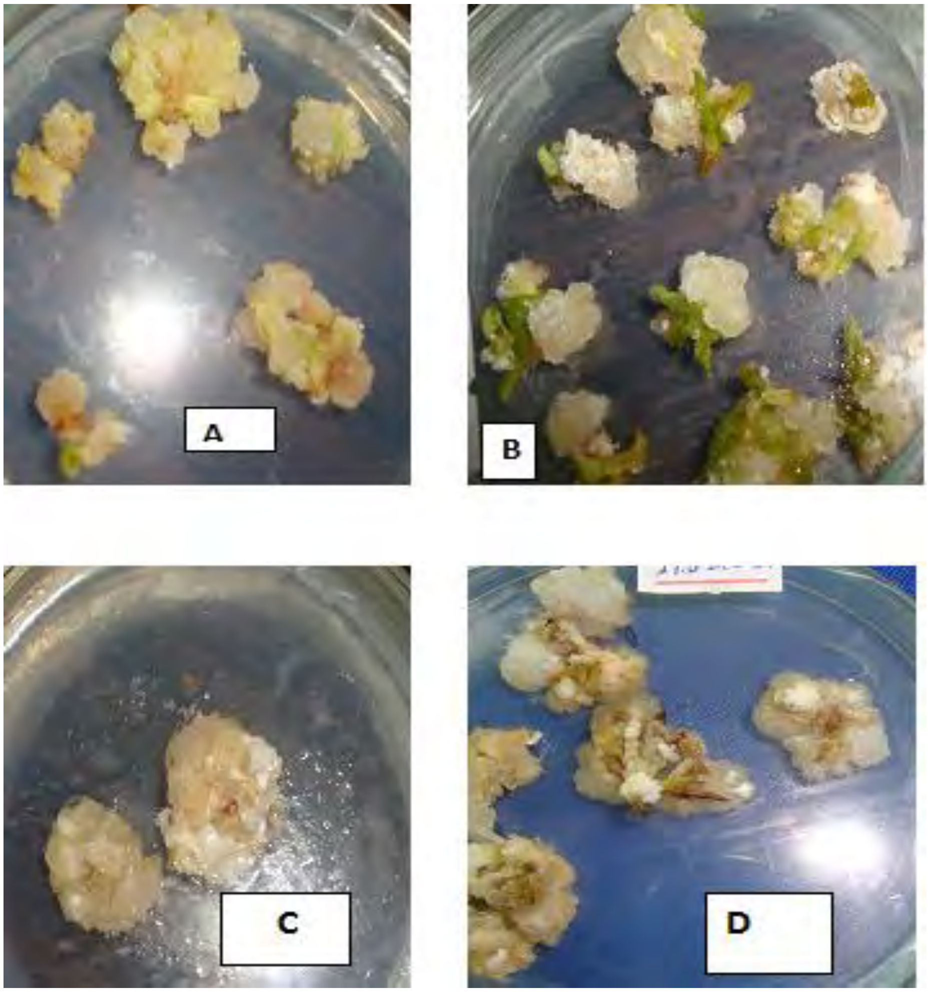


Figure 3: Promoted callus from two varieties of grapevine on different concentrate of growth regulators from leaf explants in the **light incubation** after 30 days.

A=2mg/l BAP +0.1mg/l IBA on canonannon leaf explant
B= 2mg/l BAP on canonannon leaf explant
C= 0.5mg/L TDZ and 0.5mg/L NAA on leaf of chenin blanc cultivar
D=1mg/L TDZ and 0.1mg/L NAA on chenin blanc leaf explant

Appendix IV

Table 1: Nutrient composition and concentration of MS basal medium

Components	Concentration (g/L)
Macronutrients	
NH_4NO_3	16.5
KNO_3	19.0
$CaCl_2.H_2O$	4.4
$MgS0_4.7H_20$	1.8
KH_2P0_4	1.7
Micronutrients	
Fe-Na-EDTA	4
$ZnSo_4.7H_20$	0.86
H_3BO_3	0.62
$MnS0_4.4H_20$*	2.23
$MnS0_4.H_20$*	1.69
$Cuso_4.5H_20$	0.0025
KI	0.083
$Na_2Mo0_4.2H_20$	0.025
$CoCl_{2.6}H20$	0.0025
Vitamins	
Myo-inositol	0.1
Glycin (glycol)	0.2
Nicotinic acid	0.05
Pyridoxin (B6)	0.05
Thiamin (B1)	0.01

*are alternative

DECLARATION

I, the undersigned, declare that this thesis is my original work in the Department of Biotechnology of Addis Ababa University and has not been presented for any academic degree in any other university. All assistance received from other individuals and organizations has been acknowledged and full reference is made to all published and unpublished sources used.

Name: Fikadu Kumsa *Signature*_______________ Date 14/07/2011