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

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The traditional way of grapevine (*Vitis vinifera* L.) propagation is time consuming and allows disease transmission from generation to generation. Moreover, it is difficult to improve this crop through conventional plant breeding methods. Therefore, the objective of this study was to develop efficient *in vitro* regeneration protocol for 'Canonannon' and 'Chenin Blanc' varieties of grapevine using leaf explants. MS medium supplemented with different concentrations of thidiazuron (TDZ) alone or in combination with α-naphthalene acetic acid (NAA), and 6-benzyl aminopurine (BAP) alone or in combination with indole-3-butyric acid (IBA) were used for regeneration of shoots from leaves. The regenerated shoots were transferred to shoot multiplication medium and subsequently to rooting medium and the plantlets were acclimatized after rooting. The rooting medium consisted of MS medium containing different concentrations of IBA or indole-3-acetic acid (IAA). The highest number of shoots per leaf explant was obtained from both 'Chenin Blanc' (2.3 ± 0.3) and 'Canonannon' (2.2 ± 0.2) on medium supplemented with 2.0 mg/L BAP. Among 16 different combinations of TDZ and NAA, the maximum number of shoots per explant (1.5 ± 0.2) was obtained from 'Canonannon' on medium containing 1.0 mg/L TDZ and 0.1 mg/L NAA. However, when these shoots were transferred to shoot multiplication medium, 10 ± 0.51 shoots per explant were obtained from 'Chenin blanc' on MS medium supplemented with 2.0 mg/L BAP. The highest number of roots per explant (8.3 ± 0.30) was obtained on medium containing 2.0 mg/L IBA. The survival rate of 'Chenin Blanc' and 'Canonannon' was 83.3 and 75 %, respectively after one month of acclimatization.

Key words: Callus induction, growth regulators, hyperhydricity, organogenesis.

INTRODUCTION

Grapevine (*Vitis vinifera* L.) is one of the most widely distributed fruit crop in the world. Although most grapevines are produced in areas with temperate climate, some cultivars have cultivation potential under high-temperature of tropical and sub-tropical conditions. According to Patrice et al. (2006), *V. vinifera* is highly distributed and constituted over 90% of the world's grapes. In case of Ethiopia, wineries are importing about 300 tons of grapes annually in the form of dried raisin, grape juice concentrates, natural wine extracts and citric

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acid (Kinfe et al., 2017). Grapevine is grown worldwide for a variety of purposes including wine, fresh fruit, juice, jams, jellies, raisins and other processed products (Ferreira et al., 2004). It is also a major horticultural crop with great applications in food and pharmaceutical industries. Regardless of its enormous uses, grapevine cultivation is affected by different biotic and abiotic stresses. Although it is the third most important fruit crop in the world after banana and citrus, the demand for grapevine fruit is increasing because of increase in the number of wine industries and more demand for fresh and dried fruits (Fayek et al., 2009). According to Aazami (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. Moreover, genetic improvement of grapevine through conventional breeding is severely limited because of its polyploidy nature and the existing cultivars are highly heterozygous (Gray and Fisher, 1985). The non-conventional methods such as *in vitro* screening and genetic engineering have enormous potential for genetic improvement of plants including grapevine. However, for this purpose, development of *in vitro* regeneration protocol is a pre-requisite (Fikadu, 2016). As response of explants to culture conditions is dependent on genotype, each cultivar of a species requires its own *in vitro* regeneration protocol. The application of these modern genetic improvement techniques in different parts of the world is limited to a few outstanding regional cultivars (Fikadu, 2016). Therefore, the objective of the present study is to develop *in vitro* regeneration protocol for 'Canonannon' and 'Chenin Blanc' varieties of grape vine that were introduced from abroad and being cultivated in Ethiopia.

MATERIALS AND METHODS

Plant material

In vitro cultured grapevine varieties, 'Chenin Blanc' and 'Canonannon', were maintained by sub-culturing of shoots and nodes at four-week intervals on MS (Murashige and Skoog, 1962) shoot multiplication medium supplemented with 1.0 mg/L BAP in combination with 0.1 mg/L IBA and 30 g/L sucrose at Addis Ababa University. The pH of the medium was adjusted to 5.8 and 7.0 g/L agar was added. The medium was then autoclaved at 121°C for 15 min and 40 ml was dispensed into each sterile Magenta GA-7 culture vessels. The cultures were maintained at temperature of 27 ± 2°C and light intensity of 40 µmol $m^{-2}s^{-1}$ at 16 h photoperiod. Unless and otherwise indicated, all cultures were maintained at these culture conditions.

Shoot regeneration from leaf explants

Upper most expanding young leaves from the four-week-old *in vitro* propagated two varieties of grapevine shoots were excised aseptically and cultured on shoot regeneration medium. The shoot regeneration medium is MS medium containing different concentrations of BAP alone (0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0 and 5.0 mg/L) or BAP (0.0, 0.5, 1.5, 2.0 and 3.0 mg/L) in combination with IBA (0.0, 0.1, 0.5 and 1.0 mg/L), or TDZ alone (0.0, 0.1, 0.5, 1.0, 2.0, 3.0 and 4.0 mg/L) or TDZ (0.0, 0.1, 0.5, 1.0, 1.5, 2.0 and 3.0 mg/L) in combination with NAA (0.0, 0.01, 0.1 and 0.5 mg/L). All leaves were wounded by scalpel blade across main vein and cultured on 90 mm diameter Petri dishes containing 20 ml medium with adaxial side of the leaves contacting the medium. The regenerated shoots were transferred to the same fresh medium after four weeks and all Petri dishes containing the shoots were covered with transparent (thin) cloth for two weeks. The cloth was used for light reduction. The number of leaf explants that produced shoots and induced callus, and the number of shoots per explant were recorded.

Multiplication of regenerated shoots

The shoots that were regenerated from leaf explants were excised and cultured on shoot multiplication medium in Magenta GA-7 culture vessels that contained 40 ml medium. The shoot multiplication medium was MS medium consisted of 2.0 mg/l BAP. When problem of hyperhydricity was encountered, culture vessels were ventilated aseptically under laminar air flow cabinet, the agar concentration was increased from 7 to 8%, and most of the leaves were trimmed. The number of shoot per explant were recorded and compared with the number of shoots per explant that were produced by the stock plants that were maintained on shoot multiplication medium from which leaf explants for regeneration experiment were obtained.

Rooting and acclimatization

One-month-old shoots from shoot multiplication medium were cultured on rooting medium. The rooting medium was full strength MS medium containing different concentrations of IBA (1.0, 2.0, 3.0 and 4.0 mg/L) or IAA (2.0 and 4.0 mg/L). The number of roots, length of roots and plantlets were recorded after 30 days. 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. These plantlets were then transferred to glasshouse and planted in 12 cm diameter plastic pots containing sterilized red soil, sand and cow dung manure at the ratio of 1:2:1 respectively. The plantlets were covered with transparent polythene bags and watered every other day. The polythene bags were gradually removed after two weeks and the number of survived plants was recorded after a month.

Statistical analyses

Completely Randomized Design (CRD) was used. Six explants per Petri dish were used for the whole experiments of shoot regeneration from leaf explants and each experiment had five replications. The one-way analysis of variance (ANOVA) was used to compute the percentage and mean number of regenerated shoots per-explant, the number and length of roots and their survival rate in glasshouse. All data were analyzed at p ($\alpha < 0.05$) using SPSS 16 version statistical software.

RESULTS

Shoot regeneration from leaf explants

Shoots were regenerated directly from leaf explants after

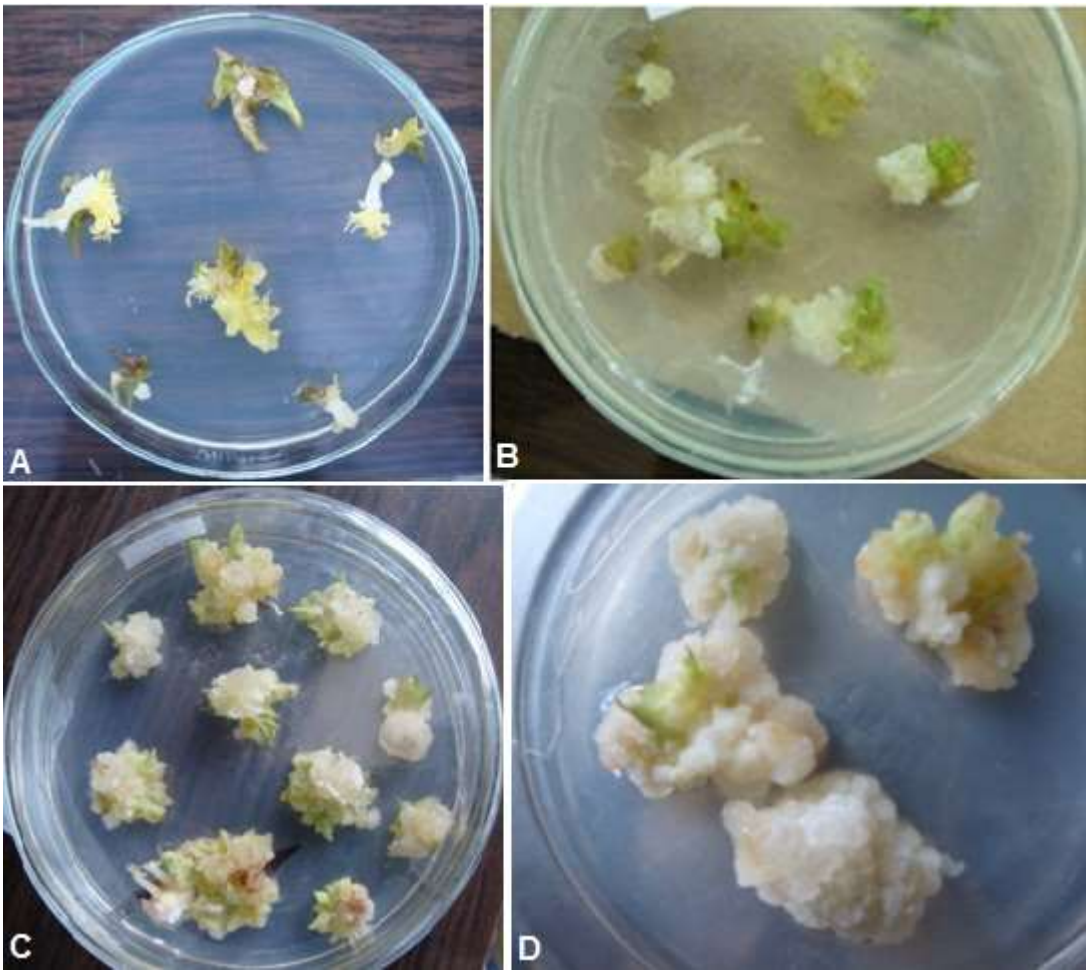

Figure 1. Direct shoot regeneration and callus induction from leaf explants after 30 days of dark incubation: Direct shoot regeneration from 'Canonannon' (A) and 'Chenin Blanc' (B) on MS medium containing 2.0 mg/l BAP and 0.1 mg/L IBA. Callus induction of 'Canonannon' on MS medium containing 1.5 mg/L BAP and 1.0 mg/L IBA (C) and 2.0 mg/L BAP and 0.1 mg/L IBA (D).

four weeks of culture (Figure 1A and B) and there was significant difference in percentage of shoot regeneration and number of shoots per explant among different concentrations of BAP and TDZ. The highest number of shoots per leaf explant were obtained on the medium containing 2.0 mg/L BAP from both 'Chenin Blanc' (2.3 ± 0.3) and 'Canonannon' (2.2 ± 0.2) varieties. However, the number of shoots regenerated from leaf explants of both cultivars was reduced when the concentration of BAP was reduced or increased from 2.0 mg/L (Table 1). Different concentrations of BAP or TDZ alone triggered similar responses on explants of both varieties. Callus induction was significantly low at all concentrations of TDZ used in this experiment, but explants of both cultivars that were cultured on medium containing 0.5, 2.0, and 4.0 mg/L BAP produced callus though the size and percentage was low. Shoots were regenerated from calli on medium containing 3.0 mg/L BAP. However, shoots were not regenerated from leaf explants that were cultured on medium containing TDZ alone at all tested concentrations, and on medium containing 0.5, 4.0 and 5.0 mg/L BAP.

Effect of TDZ and NAA on shoot regeneration

Among sixteen different combinations of TDZ and NAA, 45% of 'Canonannon' and 29.8% of 'Chenin Blanc' leaf explants exhibited direct regeneration on medium containing 1.0 mg/L TDZ in combination with 0.1 mg/L NAA (Table 2). There was significant difference in percentage of shoot regeneration, number of shoots per explant and percentage of callus induction among different concentrations of TDZ in combination with NAA. The maximum mean number of shoots per explant was also obtained on this medium. Leaf explants cultured on medium containing 0.5 mg/L TDZ combination with 0.01

Table 1. Effect of different concentrations of BAP and TDZ on *in vitro* shoot regeneration of 'Canonannon' and 'Chenin Blanc' cultivars of grapevine from leaf explants.

Growth regulators Concentrations (mg/L)		'Canonannon'			'Chenin Blanc'		
		Callus induction (%)	Regeneration (%)	No. of shoots per explant	Callus induction (%)	Regeneration (%)	No. of shoots per explant
Control	0.0	0.0^{d}	0.0^{c}	0 ± 0.0^{c}	0.0^{d}	0.0^{c}	0 ± 0.0^{c}
BAP	0.5	8.3^{bd}	0.0^{c}	0 ± 0.0^{c}	0.0^{d}	0.0^{c}	0 ± 0.0^{c}
	1 .0	0.0^{d}	0.0^{c}	0 ± 0.0^{c}	21.0^{bc}	3.0^{b}	1 ± 0.1^{b}
	1.5	0.0^{d}	4.3^{b}	1 ± 0.0^{b}	4.2^{cd}	8.0^{b}	1.2 ± 0.2^{ab}
	2 .0	0.0^{d}	88.4^{a}	2.2 ± 0.2^{a}	0.0^{d}	86.0^{a}	2.3 ± 0.3^{a}
	2.5	25.0^{a}	5.8^{b}	1 ± 0.1^{b}	33.0^{a}	0.0^{c}	1.3 ± 0.1^{ab}
	3 .0	12.5^{b}	1.4^{b}	1 ± 0.1^{b}	4.2^{cd}	3.0^{b}	1 ± 0.0^{b}
	4 .0	0.0^{d}	0.0^{c}	0 ±0. 0^{c}	0.0^{d}	0.0^{c}	0 ± 0.0^{c}
	5 .0	0.0^{d}	0.0^{c}	0 ±0. 0^{c}	0.0^{d}	0.0^{c}	0 ± 0.0^{c}
$T^{D}Z$	0.1	0.0^{d}	0.0^{c}	0 ± 0.0^{c}	0.0^{d}	0.0^{c}	0 ± 0.0^{c}
	0.5	0.0^{d}	0.0^{c}	0 ±0. 0^{c}	0.0^{d}	0.0^{c}	0 ± 0.0^{c}
	1.0	21.0^{ab}	0.0^{c}	0 ± 0.0^{c}	8.3^{cd}	0.0^{c}	0 ± 0.0^{c}
	2.0	17.0^{ab}	0.0^{c}	0 ± 0.0^{c}	13.0^{cd}	0.0^{c}	0 ± 0.0^{c}
	3.0	17.0^{ab}	0.0^{c}	0 ± 0.0^{c}	17.0^{c}	0.0^{c}	0 ± 0.0^{c}
	4.0	0.0^{d}	0.0^{c}	0 ± 0.0^{c}	0.0^{d}	0.0^{c}	0 ± 0.0^{c}

Means followed by the same letters in the same column are not significantly different at 5 % level of probability. Data are represented as mean ± SD.

and 0.1 mg/L NAA exhibited callus induction and shoot regeneration in 'Canonannon' cultivar. However, leaf explants of both cultivars cultured on medium containing 0.5 mg/L TDZ in combination with 0.5 mg/L NAA induced calli (14.6% of 'Canonannon' and 50% of 'Chenin Blanc') and no shoots were regenerated from these calli.

Effect of BAP and IBA on shoot regeneration

Among different concentrations of BAP in combination with IBA, 90% of leaf explants of 'Chenin Blanc' and 71.7% of 'Canonannon' exhibited direct regeneration on medium supplemented with 2.0 mg/L BAP in combination with 0.1 mg/L IBA (Table 3). Similarly, the highest number of shoots per explant was obtained on this medium for both cultivars. At lower concentrations of BAP (0.5 and 1.5 mg/L), 'Chenin Blanc' did not show any response of regeneration while 'Canonannon' exhibited 1.0 ±0.0 shoots per explant on medium containing 0.5 mg/L BAP combined with 0.1 mg/L IBA and 1.5 mg/L BAP combined with 0.5 mg/L IBA. The highest percentage of callus induction, 40% of 'Chenin Blanc' and 31.3% of 'Canonannon' was exhibited by the leaf explants cultured on 1.5 mg/L BAP in combination with 1.0 mg/L IBA (Table 3 and Figure 1C and D). However, shoots were not regenerated from these calli of 'both cultivars.

Shoot multiplication

When shoots obtained from regeneration experiment were cultured on shoot multiplication medium (Figure 2A and B), the highest number of shoots per explant (10 ± 0.51) from 'Chenin Blanc' and 4.7 ± 0.3 for 'Canonannon' were obtained on medium containing 2.0 mg/L BAP. However, when shoots from *in vitro* maintained stock plants were cultured on the above same medium, the highest number of shoots per explant obtained from 'Chenin Blanc' and 'Canonannon' were only 3.3 ± 0.3) and 4.3 ± 0.3 respectively (Figure 3).

Hyperhydricity (vitrification) was a serious problem observed during this work. As a result of hyperhydricity, some regenerated shoots of both cultivars that were cultured on shoot multiplication medium started to lose leaves after three weeks of culture. This problem was observed more frequently on 'Chenin Balnc' cultivar than 'Canonnanon'. However, the percentage of hyperhydric shoots was reduced when the concentration of agar was increased from 7 to 8%, when the cultures were ventilated under laminar airflow cabinet twice a week and when the shoots were subcultured every three weeks instead of four weeks.

Rooting and acclimatization

Shoots started to produce roots in the first 10 days after

Table 2. Effect of TDZ and NAA combinations on *in vitro* shoot regeneration of 'Canonannon' and 'Chenin Blanc' cultivars of grapevine from leaf explants.

Growth regulators concentrations (mg/L)		'Canonannon'			'Chenin Blanc'		
TDZ	NAA	Callus induction (%)	Regeneration (%)	No. of shoots per explant	Callus induction (%)	Regeneration (%)	No. of shoots per explant
0.0	0.0	0.0^{c}	0.0^{c}	0.0 ± 0.0^{c}	0.0^{c}	0.0^{c}	0 .0 ± 0.0^{c}
0.1	0.01	0.0^{c}	8.5^{b}	1.3 ± 0.3^{ab}	0.0^{c}	0.0^{c}	0.0 ± 0.0^{c}
0.1	0.1	0.0^{c}	0.0^{c}	0.0 ± 0.0^{c}	0.0^{c}	0.0^{c}	0.0 ±0. 0^{c}
0.5	0.01	2.4^{b}	6.4^{b}	1.0 ± 0.0^{b}	2.8^{b}	0.0^{c}	0.0 ± 0.0^{c}
0.5	0.1	7.3^{b}	6.4^{b}	1.0 ± 0.0^{b}	8.3^{b}	0.0^{c}	0.0 ± 0.0^{c}
0.5	0.5	14.6^{b}	0.0^{c}	0.0 ± 0.0^{c}	50.0^{a}	0.0^{c}	0.0 ± 0.0^{c}
1.0	0.01	0.0^{c}	0.0^{c}	0.0 ± 0.0^{c}	0.0^{c}	0.0^{c}	0.0 ± 0.0^{c}
1.0	0.1	18.2^{b}	29.8^{a}	1.5 ± 0.2^{a}	18.4^{b}	45.0^{a}	1.4 ± 0.2^{a}
1.0	0.5	7.3^{b}	0.0^{b}	0.0 ± 0.0^{c}	8.3^{b}	0.0^{c}	0.0 ± 0.0^{c}
1.5	0.01	9.8^{b}	19.0^{a}	1.0 ± 0.0^{b}	16.7^{b}	0.0^{c}	0.0 ± 0.0^{c}
1.5	0.1	0.0^{c}	7.0^{b}	1.0 ± 0.0^{b}	0.0^{c}	0.0^{c}	0.0 ± 0.0^{c}
1.5	0.5	24.4^{a}	6.4^{b}	1.3 ± 0.3^{a}	8.3^{b}	25.0^{a}	1.3 ± 0.3^{a}
2.0	0.01	7.3^{b}	6.0^{b}	1.0 ± 0.0^{b}	0.0^{c}	15.0^{b}	1.0 ± 0.0^{b}
2 .0	0.1	34.1^{a}	6.0^{b}	1.0 ± 0.0^{b}	8.3^{b}	15.0^{b}	1.0 ± 0.0^{b}
2.0	0.5	0.0^{c}	0.0^{c}	0.0 ±0. 0^{c}	0.0^{c}	0.0^{c}	0.0 ± 0.0^{c}
3.0	0.01	0.0^{c}	0.0^{c}	0.0 ±0. 0^{c}	0.0^{c}	0.0^{c}	0.0 ± 0.0^{c}
3.0	0.1	0.0c	0.0^{c}	0.0 ±0. 0^{c}	0.0^{c}	0.0c	0.0 ± 0.0^{c}

Means followed by the same letters in the same column are not significantly different at 5 % level of probability

Table 3. Effect of BAP and IBA combinations on *in vitro* shoot regeneration of 'Canonannon' and 'Chenin Blanc' cultivars of grapevine from leaf explants.

Growth regulators concentrations (mg/L)		'Canonannon'			'Chenin Blanc'		
BAP	IBA	Callus induction (%)	Regeneration (%)	No. of shoots per explant	Callus induction (%)	Regeneration (%)	No. of shoots per explant
0.0	0.0	0.0^{d}	0.0^{c}	0.0 ± 0.0^{c}	0.0^{d}	0.0^{c}	0.0 ± 0.0^{c}
0.5	0.1	12.5^{c}	6.5^{b}	1.0 ± 0.0^{b}	0.0^{d}	0.0^{c}	0.0 ± 0.0^{c}
0.5	0.5	0.0^{d}	0.0^{c}	0.0 ± 0.0^{c}	0.0^{d}	0.0^{c}	0.0 ± 0.0^{c}
0.5	1.0	0.0^{d}	0.0^{c}	0.0 ± 0.0^{c}	0.0^{d}	0.0^{c}	0.0 ± 0.0^{c}
1.5	0.1	31.3^{a}	0.0^{c}	0.0 ± 0.0^{c}	16.0^{b}	0.0^{c}	0.0 ± 0.0^{c}
1.5	0.5	3.1^{cd}	8.7^{b}	1.0 ± 0.0^{b}	0.0^{d}	0.0^{c}	0.0 ± 0.0^{c}
1.5	1.0	31.3^{a}	0.0^{c}	0.0 ± 0.0^{c}	40.0^{a}	10^{b}	1.0 ± 0.0^{b}
2.0	0.1	0.0^{d}	71.7^{a}	1.4 ± 0.1^{a}	0.0^{d}	90.0^{a}	1.3 ± 0.1^{a}
2.0	0.5	0.0^{d}	8.7^{b}	1.3 ± 0.0^{a}	0.0^{d}	10.0^{b}	1.0 ± 0.1^{b}
2.0	1.0	21.9^{b}	4.3^{b}	1.0 ± 0.0^{b}	24.0^{b}	0.0^{c}	0.0 ± 0.0^{c}
3.0	0.1	16.9^{c}	13.5^{b}	1.0 ± 0.0^{b}	0.0^{d}	7.9^{b}	1.0 ± 0.0^{b}
3.0	0.5	0.0^{d}	0.0^{c}	0.0 ± 0.0^{c}	0.0^{d}	0.0^{c}	0.0 ± 0.0^{c}
3.0	1.0	0.0^{d}	0.0^{c}	0.0 ± 0.0^{c}	0.0^{d}	0.0^{c}	0.0 ± 0.0^{c}

Means followed by the same letters in the same column are not significantly different at 5 % level of probability.

Figure 2. Shoot multiplication, rooting and acclimatization: Multiplication of shoots of leaf explant origin of 'Chenin Blanc' (A) and 'Canonannon' (B) on MS medium containing 2.0 mg/L BAP after 30 days. Rooted shoots of 'Canonannon' (C and D) and 'Chenin Blanc' (E and F) on MS basal salt medium supplemented with 2.0 mg/l IBA after 30 days of culture. Acclimatized plantlets of 'Chenin Blanc' (G) and 'Canonannon' (H) after 30 days.

culture on rooting medium. The highest mean number of roots per plantlet produced by 'Canonnannon' was 7.0 ± 0.92 whereas that of 'Chenin Blanc' was 6.7 ± 0.73 on medium containing 2.0 mg/L IBA (Table 4). The best mean root length produced by 'Canonnanon' and 'Chenin Blanc' were 5.5 ± 0.63 and 5.4 ± 0.50 on the above same medium, respectively.

There was no significant difference in mean shoot length per plantlet among the control and medium containing 1.0 and 2.0 mg/L IBA in both cultivars. When the performances of IBA and IAA were compared in the number and length of roots as well as length of shoot per plantlet, IBA performed much better than IAA. After one month acclimatization of the plantlets in glasshouse, 83.3% of 'Chenin Blanc' and 75% of 'Canonannon' survived and no aberrant plants were observed (Figure 2C to H).

DISCUSSION

Different types and concentrations of growth regulators significantly affected frequency of shoot regeneration, number of shoots per explant and percentage of callus induction of the two cultivars, 'Chenin Blanc' and 'Canonannon' in our study. In many woody plant species,

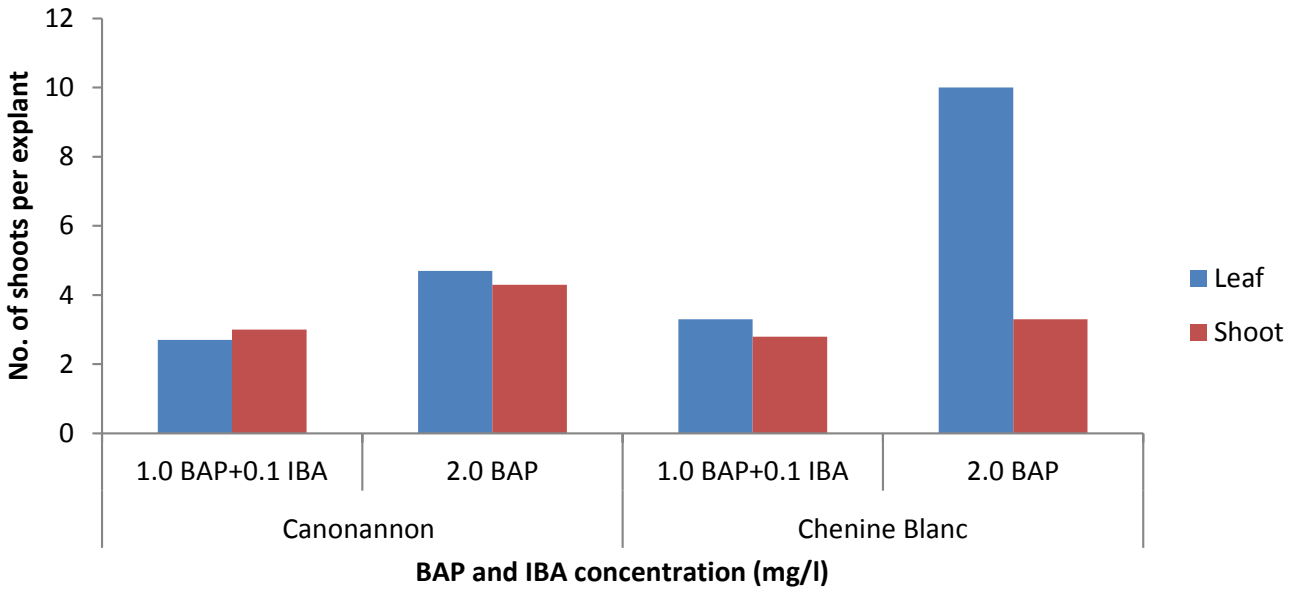

Figure 3. Number of shoots per explant that were produced by shoots of leaf explant origin and shoots that were maintained *in vitro* as stock on MS medium containing 2.0 mg/L BAP or 1.0 mg/ BAP in combination with 0.1 mg/L IBA.

Table 4. Effect of different concentrations of IBA and IAA on rooting of 'Canonannon' and 'Chenin Blanc' cultivars of grapevine.

Type of *GRs	GRs conc. (mg/L)	'Canonannon'			'Chenin Blanc'		
		Number of roots	Length of roots	Length of shoots	Number of roots	Length of roots	Length of shoots
Control	0.0	3.9 ± 0.23^{b}	4.7 ± 0.42^{ba}	7.5 ± 0 .23^{a}	3.6 ± 0.16^{b}	3.6 ± 0.22^{b}	7.2 ± 0.47^{a}
IBA	1.0	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}
	2.0	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}
	3.0	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}
	4.0	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	2.0	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}
	4.0	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}

*GRs = Growth regulators.
Means followed by the same letters in the same column are not significantly different at 5 % level of probability.

callus induction and plant regeneration have been achieved using TDZ (Huetteman and Preece, 1993). In addition, this cytokinin promotes efficient micro-propagation of many recalcitrant woody species at relatively low concentration (< 1.0 µM). However, in our study, although significant shoot induction was obtained on medium supplemented with TDZ combined with different concentrations of NAA, the highest percentage of shoot regeneration was exhibited by a medium supplemented with BAP in combination with IBA. Aazami (2010) also reported that BAP was the most effective among other cytokinins in promoting plant regeneration of *V. vinifera* cultivars 'Soltanin' and 'Sahebi' from shoot apical meristem. During *in vitro* culture, presence of cytokinin in the medium promotes shoot regeneration. However, in the present study, it also promoted callus induction, which is in agreement with the work of Baker and Bhatia (1993) who worked on shoot regeneration from leaf explants of quince (*Cydonia oblonga*).

The highest percentage of shoot regeneration was obtained on medium supplemented with 2.0 mg/L BAP in combination with 0.1 mg/L IBA in both varieties. Such high number of shoot formation per explant can be used for a variety of purposes, including plant improvement through *in vitro* selection and as a prerequisite for genetic engineering if the regeneration of shoots is from callus. Similarly, if regeneration is directly from the explants without passing through callus phase, that can be used for mass propagation of true-to- type clones. Although *in vitro* regeneration experiments were done on grapevine

varieties of ‘Cabernet Sauvignon’, ‘French Colombard’, ‘Grenache’, ‘Thompson Seedless’, ‘White Riesling’, *V. vinifera x rupestris* and *V. rupestris* using leaf explants (Stamp et al., 1990), this is the first report of regenerating shoots from leaves of ‘Canonannon’ and ‘Chenin Blanc’ on *in vitro* regeneration using leaf explants as each cultivar requires its own regeneration protocol.

The effect of light and type of leaf explant on *in vitro* regeneration was also studied and the explants cultured in light and dark conditions responded differently. In our study, leaves without petiole and petioles were cultured on different concentrations of TDZ, BAP or TDZ in combination with NAA, and BAP in combination with IBA. However, these explants did not regenerate shoots. First regenerated shoots were observed on the 25th day, sometimes at the wounded edges and mostly from swollen petiole tip. On the 30th day, the number of regenerated shoots increased and could be easily identified. Such response of leaf explants was observed in the previous work on other grapevine cultivars (Pe´ros, 1998). This time of regeneration is shorter when compared to the work of Aazami (2010) and Stamp et al., (1990). Thus, our results indicated that culture age of four weeks in the dark is necessary for shoot regeneration of grapevine varieties of ‘Canonannon’ and ‘Chenin Blanc’ using leaf explants.

The number of shoots produced per explant on shoot multiplication medium showed significant difference between shoots used from *in vitro* stock plants and the shoots used from regenerated leaf explants. There was also significant difference in the number of shoots per explant between the two cultivars. The shoots that were obtained from leaf explants through regeneration and cultured on shoot multiplication medium produced 10 ± 51 mean number of shoots per explant in ‘Chenin Blanc’ cultivar whereas the same explants produced 4.7 ± 0.29 shoots per explant in ‘Canonannon’ cultivar. Contrary to this, the highest mean number of shoots produced per explant from *in vitro* maintained stock shoots was 4.3 ± 0.3 for ‘Canonannon’ and 3.3 ± 0.3 for ‘Chenin Blanc’ cultivar. This could be probably due to the shoots obtained through regeneration from leaf explants are more juvenile than the shoots that were maintained on shoot multiplication medium. Generally, higher mean root number, root length and shoot length per plantlet were exhibited by shoots cultured on medium containing different concentrations of IBA than shoots cultured on different concentrations of IAA. The highest mean number of roots per plantlet produced by ‘Canonannon’ was 7.0 ± 0.92 whereas that of ‘Chenin Blanc’ was 6.7 ± 0.73 on medium containing 2.0 mg/L IBA. Kinfe et al., 2017) reported that among different concentrations of IAA used for rooting, 5.2 ± 1.0^{a} and 3.5 ± 0.6^{a} roots per plantlet were obtained from ‘Canonannon’ and ‘Chenin Blanc’ respectively on MS medium containing 4.0 mg/L IAA. However, she did not use IBA for rooting experiments. In our study, the shoots that exhibited highest number of shoots per explant showed better survival percentage during acclimatization.

Conclusion

Different types and concentrations of growth regulators significantly affected frequency of shoot regeneration, number of shoots per explant and percentage of callus induction of the two cultivars, ‘Chenin Blanc’ and ‘Canonannon’ in our study. Even though significant shoot induction was obtained on medium supplemented with TDZ combined with different concentrations of NAA, the highest percentage of shoot regeneration was exhibited by a medium supplemented with BAP in combination with IBA. The highest percentage of shoot regeneration was obtained on medium supplemented with 2.0 mg/L BAP in combination with 0.1 mg/L IBA in both varieties. The number of shoots produced per explant on shoot multiplication medium showed significant difference between shoots used from *in vitro* stock plants and the shoots used from regenerated leaf explants. There was also significant difference in the number of shoots per explant between the two cultivars. The shoots that were obtained from leaf explants through regeneration and cultured on shoot multiplication medium produced 10 ± 51 mean number of shoots per explant in ‘Chenin Blanc’ cultivar whereas the same explants produced 4.7 ± 0.29 shoots per explant in ‘Canonannon’ cultivar. Generally, higher mean root number, root length and shoot length per plantlet were exhibited by shoots cultured on medium containing different concentrations of IBA than shoots cultured on different concentrations of IAA.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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