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Effect of growth regulators on indirect organogenesis of two grapevines (*Vitis vinifera* L.) cultivars

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Establishment of efficient protocol for high-frequency of indirect regeneration of plantlets has a vital role in the analysis of genetic material in mass propagated plants. The optimal levels of growth regulators and light conditions were investigated on callus induction and organogenesis of cultured grapevine from *in vitro* tissues. Maximum calluses and shoots were produced by using medium supplemented with four different growth regulators as alone or in combinations. An observation of maximum calluses (51%) were recorded when 0.5 mg/l thidiazuron (TDZ), was combined with 0.5 mg/l indole-3-butyric acid (IBA) for Chenin *blanc* cv. Produced calluses were observed with different size and nearly similar colors. In this experiment, shoot initiation was observed in dark condition. The light condition did not induce the shoot on the same dark treatment. Different concentrations of 6-benzylaminopurine (BAP) and TDZ tested alone were not induced at any shoots from callus, but re-callusing the explant. Thus, shoot induction was observed when different concentration of BAP and TDZ were combined with auxins. The calluses produced from leaf did not produce high percentages of shoots. Further studies are needed to optimize the maximum percentage of somatic embryogenesis.

Key words: Callus, growth regulators, organogenesis, grapevine, dark.

INTRODUCTION

The organogenesis is a biotechnological tool used for obtaining mass production of mother plant with high quality of health (Bettoni et al., 2015). The explants can be grown into whole plant or produce callus. The produced callus can be utilized to regenerate plantlets or to extract or manipulate some primary and secondary metabolites (Pande and Gupta, 2013). 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, which is well known and it influences plant tissue culture and micro-propagation. The most common culture temperature range has been between 20 and 27°C, but optimal temperatures vary widely, depending on genotype (Kumar and Raddy, 2011).

Most times, the optimal shoot proliferations of grapevine were reported when both hormones (cytokines

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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. Similarly, the best shoot inductions were recorded at 2.0 mg/l BAP + 0.1 mg/l indole-3-acetic acid (IAA) for Canonnanon and Chenin blanc cultivars (Abido et al., 2013; Fikadu, 2011). According to Aazami (2010), different combinations of growth regulators (1.5 mgl^{-1} IBA), C (1 mgl^{-1} IBA+ 1.5 mgl^{-1} IBA) were best produced for shoots "*Soltanin*" and "*Sahebi* cultivars from meristem. 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).

Despite the 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 mass propagation should be developed (Deore and Johnson, 2008). Beside the micro-propagations, an establishment of efficient protocol for high-frequency of indirect regeneration of plantlets is so much needed. Indirect organogenesis has a vital role in the analysis of genetic material in mass propagated plant (Deore and Johnson, 2008). In this finding, a protocol of producing shoots from callus was developed.

MATERIALS AND METHODS

Source and maintenance of explant

In vitro grown verities of grapevines (Chenin blanc and Canonannon*)* were obtained from Holeta Agricultural Research Center for this study. The experiments were conducted in Addis Ababa University at plant propagation and tissue culture laboratory. Sources of plant were maintained by sub culturing shoots and nodes of *in vitro* cultivated stock plant at one-month intervals on shoot induction medium (MS medium supplemented with 1 mg/l BAP + 0.1 mg/l IBA in Magenta GA-7 box vessels and sealed with Para-film). The gelling agent was 7.5 g/l agar with adjusted pH to 5.8 prior to autoclaving at 121°C for 15 min.

Preparation of media and explants culture conditions

The MS (Murashige and Skoog, 1962) nutrient medium composed of full macro, micro and vitamin A composition was used (Appendix I). Upper most leaf explant induced from the induction medium was wounded and cultured in the medium supplemented with four different growth regulators (BAP, IBA, TDZ and NAA) as alone or in combinations, to produce calluses. The cultures were incubated in a growth room at 27.5°C under different light conditions (light and darkness), where induced calluses were identified at three to four weeks intervals. Then the induced calluses were re-culturing in combinations of different growth regulators (BAP, IBA, TDZ and NAA) to produce shoots in intervals of 30 to 45 days. The length and number of induced shoots were recorded after culture at three weeks. There were 30 replicates per treatment and the experiments were repeated three times.

In vitro rooting

A 21 days sub-cultured shoots of Canonannon and Chenin blanc were rooted on full strength 20 ml MS medium supplemented with 3% (w/v) sucrose, four different concentrations of IAA (1, 2, 3 and 4 mg/l) and IBA (1, 2 and 4 mg/l). The length and number of main roots were counted and recorded after culture, at four weeks.

Acclimatization

Plantlets, having better roots and shoots systems were taken out from the culture vessels, washed under running tap water to remove the agar and sucrose. The plantlets were then transferred to 12 cm 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 day interval. Plastic cover was gradually removed after plantlets were successfully established in insect proof glasshouse for one month.

Experimental design and data analyses

The one-way analysis of variance (ANOVA) was used to compute the mean number and length of shoots and roots. Complete randomized design (CRD) was used. All data were analyzed at p ($\alpha<0.05$) using SPSS 16 version statistical software.

RESULTS

Effect of light conditions and growth regulators on induction of callus from leaf explant of grapevine

After culturing leaf explants in different concentrations of growth regulators, different amount of calluses were observed at dark. With similar treatments there was no record in light condition. Non wounded explants do not produce calluses both at dark and light conditions. An observation of maximum calluses (51%) were recorded when 0.5 mg/l TDZ was combined with 0.5 mg/l NAA for 'Chenin blanc' cultivar, while the highest calluses production in 'Canonannon' cultivars were obtained when 2 mg/l TDZ is combined with 0.1 mg/l NAA (Table 1).

The produced calluses had different size and nearly similar colors. Calluses produced by BAP alone or in combination with IBA were whitish while the TDZ alone or in combination of NAA were a little bit greenish white (Figure 1).

Effect of light conditions and growth regulators on induction of shoots from callus

In this experiment, shoot initiation was observed in the dark condition. The light condition did not induce shoot on the same treatment of dark. Different concentrations of BAP and TDZ alone were tested for shoot inductions from callus, which did not induce any shoots rather produced re-callus.

Shoot induction was observed when different concentration of BAP and TDZ were combined with auxins. Thus, 0.5 mg/l BAP +1 mg/l IBA and 1.5 mg/l

Table 1. Effect of light conditions and growth regulators on induction of callus from leaf explant of grapevine at 3 weeks after culturing.

Light conditions	Growth regulators (mg/l)				Canonannon	Chenin blanc
	BAP	IBA	TDZ	NAA	Callus formation (%)	Callus formation (%)
	0	0	0	0	0.0[d]	0.0[d]
	0.5	-	-	-	9.0[d]	3.0[d]
	1 .0	-	-	-	2.0[d]	1.0[d]
	-	-	1	-	22[b]	9.8[d]
	-	-	2	-	18[c]	14[c]
	2.5	-	-	-	26[b]	34[a]
	3 .0	-	-	-	13[c]	5.0[d]
	-	-	3	-	3.0 [d]	2.6[d]
	-	-	-	-	3.2[d]	3.1[d]
	-	-	0.5	0.01	3.4[d]	3.8[d]
Dark	-	-	0.5	0.1	8.3[d]	9.3[d]
	-	-	0.5	0.5	16[c]	51[a]
	-	-	1	0.1	19[c]	20[b]
	-	-	1	0.5	8.3[d]	9.3[d]
	-	-	1.5	0.01	10[c]	18[c]
	-	-	1.5	0.5	26[b]	9.3[d]
	-	-	2	0.01	8.3[d]	0.0[d]
	-	-	2 .0	0.1	35[a]	9.3[d]
	1.5	0.1	-	-	32[a]	17[c]
	1.5	0.5	-	-	4.1[d]	0.0[d]
	1.5	1	-	-	32[a]	41[a]
	2	0.1	-	-	0.0[d]	0.0[d]

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

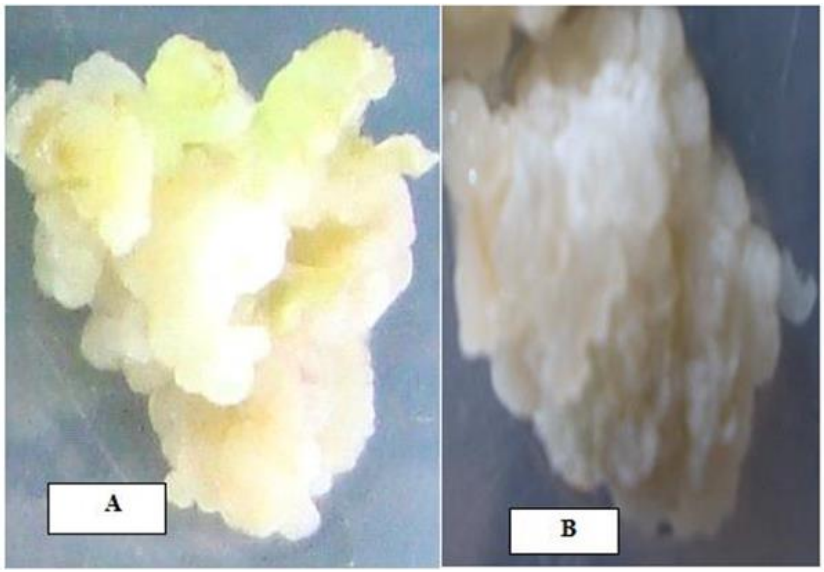

Figure 1. Induced callus from leaf explants of 'chenin blanc' cultivar (A= 0.5 mg/l TDZ +0.5 mg/l NAA; B=1.5 mg/l BAP+1 mg/lIBA) at 30 days after culture.

TZD + 0.5 mg/l NAA induced shoots for both cultivars (Canonannon and Chenin blanc), while 1.5 mg/l BAP + 1 mg/l IBA produced shoots for Chenin blanc cultivar in the dark incubation (Table 2). The induced shoots were white

Table 2. Effect of light conditions and growth regulators on induction of shoots from callus after culture at 4 weeks.

Light conditions	Growth regulators (mg/l)				Canonannon	Chenin blanc
	BAP	IBA	TDZ	NAA	Shoots induction/treatments	Shoots induction/treatments
Dark	0	0	0	0	-	-
	0.5	1			+	+
	1	1			-	-
	1.5	1			-	+
	2	0.1			+	-
	2.5	0.1			-	-
	3	0.1			-	-
			0.5	0.5	-	-
			1	0.5	-	-
			1.5	0.5	+	+
			2	0.5	-	-
			3	0.5	-	-
Light	0	0	0	0	-	-
	0.5	1			-	-
	1	1			-	-
	1.5	1			-	-
	2	0.1			-	-
	2.5	0.1			-	-
	3	0.1			-	-

+ =there was an induction of shoots; -=shows there was no shoot induction.

in color. Detail clear structures of the leaf were not observed.

Clearly visible shoots were observed at 35 days, after culture. It was gradually adapted to light incubation for further sub-culturing (Figure 2). Once the induced shoots are adapted to light, it changed to green. The general steps to obtain the green whole plantlets were summarized as the following steps (Figure 3).

Figure 2. Induced shoots of ‘canonannon’ cultivar from cultured callus on medium supplemented by 0.5 mg/l BAP+1 mg/l IBA at 35 days, after culture.

Effect of BAP on number and length of shoots derived from callus

The induced shoots were sub-cultured with different concentrations of BAP. Accordingly, the best mean numbers 5.5 ± 0.2 and 5.0 ± 0.1 were recorded for 0.5 mg/l BAP for canonannon and chenin blanc cultivars, respectively. Maximum length of shoots were recorded for 0.5 and 2 mg/l BAP for both cultivars (Table 3).

Effect of IBA and IAA on number and length of roots derived from callus shoot

Maximum mean numbers of roots were recorded for IAA and IBA in both cultivars. Thus, better root numbers (7.1 ± 0.5 and 6.1 ± 0.2) were produced at 4 and 2 mg/l IAA, respectively for ‘canonannon’ cultivar. Similarly, 6.1 ± 0.3 mean number of ‘Chenin blanc roots were observed at 4 mg/l IAA. Mean length of roots were recorded at 4 mg/l IAA and 2 mg/l IBA for both cultivars (Table 4).

Even though the maximum mean number and length of

In vitro regenerated upper leaves were wounded and cultured

↓

Initiation of callus in 4 weeks (in dark)

↓

Culturing callus on shoot initiation media

↓

Initiated shoots excised from callus and cultured on multiplication media (in light)

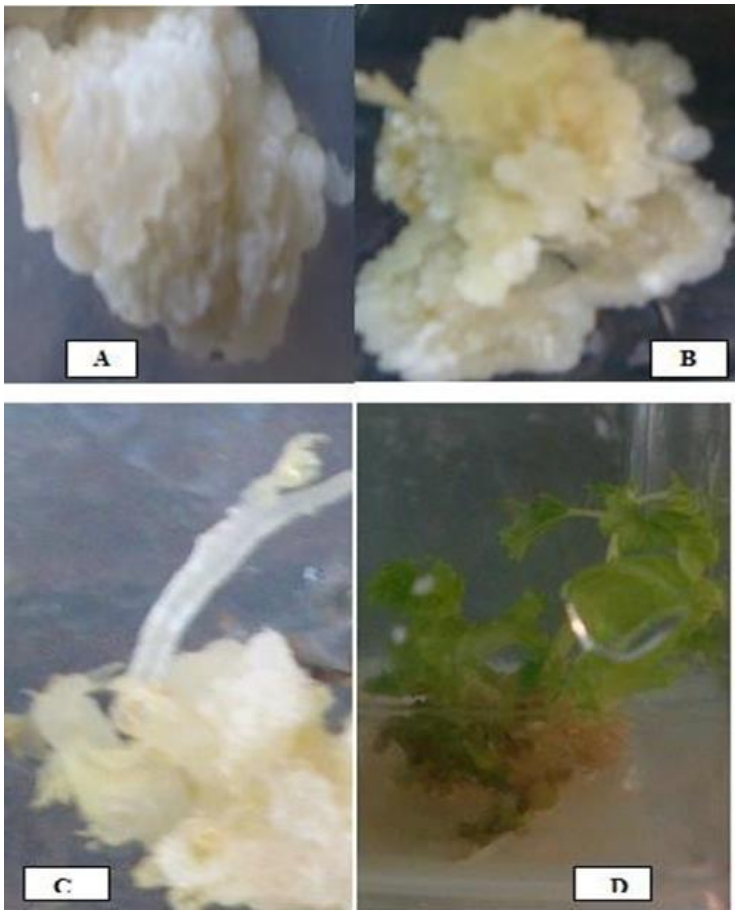

Figure 3. Shoot induction from callus (A= callus, B= Globular structure of callus on initiation, C= Induced Shoot, D= Whole plantlets).

Table 3. Effect of BAP on number and length of shoots derived from callus at 3 weeks after culture.

BAP (mg/l)	'Canonannon'		'Chenin blanc'	
	Mean number of shoots/explant	Mean length shoots/explant	Mean number of shoots/explant	Mean length shoots/explant
0	0.0 ± 0.0^{d}	0.0 ± 0.0^{d}	0.0± 0.0^{d}	0.0± 0.0^{d}
0.5	5.5± 0.2^{a}	7.5 ± 0.1^{a}	5.0± 0.1^{a}	7.3 ± 0.2^{a}
1	2.6±0.1^{c}	4.4 ± 0.3^{ab}	2.1± 0.4^{c}	4.9 ± 0.4^{ab}
1.5	3.1 ± 0.2^{b}	5.5 ± 0.2a	2.2± 0.3^{c}	5.2 ± 0.3^{a}
2	5.5 ± 0.1^{a}	6.8 ± 0.3^{a}	4.8± 0.1^{ab}	6.1 ± 0.3^{a}
2.5	2.6± 0.2^{c}	3.2 ± 0.3^{b}	2.2± 0.2^{c}	3.2 ± 0.4^{b}

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

Table 4. Effect of IBA and IAA on number and length of roots derived from callus shoot at 3 weeks after culture.

Growth regulators (mg/l)		Canonannon		Chenin blanc	
IBA	IAA	Mean number of roots/explant	Mean length of roots /explant	Mean number of roots/explant	Mean length of roots /explant
00	0.0	0.0± 0.0^{d}	0.0 ± 0.0^{d}	0.0± 0.0^{d}	0.0± 0.0^{d}
-	1.0	3.9 ± 0.4^{c}	5.6 ± 0.5^{ab}	2.0 ± 0.1^{d}	5.1 ± 0.5^{ab}
-	2.0	4.2 ± 0.2^{b}	6.0 ± 0.2^{a}	3.2 ± 0.2^{c}	6.3 ± 0.4^{a}
-	3.0	5.5 ± 0.1^{ab}	6.5 ± 0.2^{a}	4.8 ± 0.1^{b}	6.2 ± 0.1^{a}
-	4.0	7.1 ± 0.5^{a}	6.9 ± 1.1^{a}	6.1 ± 0.3^{a}	6.9 ± 0.8^{a}
1.0	-	4.8 ± 0.6^{b}	3.9 ± 0.3^{c}	4.5 ± 0.5^{b}	3.9 ± 0.5^{c}
2.0	-	6.1 ± 0.2a	7. 5± 0.5^{a}	5.0 ± 0.6^{ab}	7.2 ± 0.7^{a}
3.0	-	3.4 ± 0.1^{c}	2.6 ± 0.4^{d}	3.3 ± 0.5^{c}	2.5 ± 0.3^{d}
4.0	-	3.3 ± 0.7^{c}	3.1 ± 0.2^{c}	2.5 ± 0.3^{d}	1.5 ± 0.2^{d}

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

Figure 4. Induced roots of 'Canonannon' cultivar. A=Induced roots at 4 mg/l IAA. B= induced roots at 2 mg/l IBA.

roots were observed at medium supplemented with 4 mg/l IAA and 2 mg/l IBA, roots obtained from 2 mg/l IBA was thicker and longer than roots obtained from 4 mg/l IAA (Figure 4).

Acclimatization

The shoots with better mean length and number were survived when brought to *ex-vitro* conditions. The survival percentages were 90 and 71% for chenin blanc and canon annon cultivars, respectively.

DISCUSSION

Different concentrations of growth regulators, showed different capability of growing calluses from leaf explants. The best light condition to produce calluses were dark incubation at 27.5°C which, is confirmed with previous reports used to produce calluses from different explants of different genera (Salunkhe et al., 1997). But the same treatments incubated at light conditions did not produce callus.

For the studied varieties, non wounded leaf explants did not produce callus at both light conditions (dark and

light). An observation of maximum calluses (51%) were recorded when 0.5 mg/l TDZ is combined with 0.5 mg/l NAA for 'Chenin blanc' cultivar.

Meanwhile, the highest calluses production in 'Canonannon' cultivars, were obtained when 2 mg/l TDZ was combined with 0.1 mg/l NAA. Thus, a combinations or alone treatment of different concentrations of growth regulators are important to produce better calluses. Similar techniques were developed by Muhammad et al. (2008), while investigating effect of growth hormones on micropropagation of V*. vinifera L*. of different varieties were carried out.

The produced calluses were observed with different size and nearly similar colors but, calluses produced by BAP alone or in combination with IBA were whitish while the TDZ alone or in combination of NAA were a little bit greenish white (Figure 1). This condition indicated different concentrations of growth regulators which can produce different size of calluses while, a type of used growth regulators also contribute for the production of different color of calluses of same varieties.

Initiating shoots from leaf callus was contradicted with initiation shoots from grape tendril callus which was not successful (Salunkhe et al., 1997). Shoot initiation was found good at combinations of TDZ and BAP with auxins. Corresponding findings were reported on embryogenesis of grape from tendrils (Salunkhe et al., 1997). The induced shoots were white in color during the early development with an effect of dark when compared to light incubation, which produced green plantlets.

CONCLUSION AND RECOMMENDATION

Possible shoot inductions from callus of two cultivars of grapevine were identified in this study. Leaf callus did induce a medium rate of shoot induction but, further studies are needed to optimize the maximum percentage of somatic embryogenesis from other explants.

Conflicts of Interests

The authors have not declared any conflict of interests.

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Appendix I. 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
$MgS0_4.7H_2O$	1.8
KH_2P0_4	1.7
Micronutrients	
Fe-Na-EDTA	4
$ZnSo_4.7H_2O$	0.86
H_3BO_3	0.62
$MnS0_4.4H_2O$*	2.23
$MnS0_4.H_2O$*	1.69
$Cuso_4.5H_2O$	0.0025
KI	0.083
$Na_2Mo0_4.2H_2O$	0.025
$CoCl_{2.6}H2O$	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