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Paraphernalia of Growth Regulators During *In Vitro* micro-Propagation of Grapevine (*Vitis vinifera* L.) from Shoot Tips and Nodal Segments

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Abstract: As grapevine (*Vitis vinifera* L.) is rarely produced in Bangladesh because of unavailability of improved varieties, so this study was designed to solve this problem through evaluating the effects of hormonal combination for the duration of *in vitro* micro propagation of grapevine (*Vitis vinifera* L.) from shoot tips and nodal segments. Firstly, surface sterilization process was carried out by using $HgCl_2$ (mercuric chlorite) at 0.1% for 3 min and best result was found. During establishment stage, explants were cultured on MS (Murashige and Skoog) basal medium supplemented with BAP (6-benzylamino purine) 0.5, 1.0 and 2.0 mg/l and NAA (β-naphthalene acetic acid). 0.1mg/l where MS+ BAP 1.0 mg/l + NAA 0.1mg/l displayed best potential result. During shoot multiplication stage, BAP 2.0 and 3.0 mg/l and NAA 0.1, 0.2 and 0.3 mg/l and their combination were used and highest number of proliferated shoots was obtained from MS+ BAP 3.0 mg/l + NAA 0.2 mg/l. For rooting stage, NAA 0.5 and 1.0 mg/l and IBA (Indol-3-butyric acid) 0.5, 1.0 and 1.5 mg/l were used and tested. The highest rooting percentage, number of roots per shoot and root length found in MS+ 0.5 mg/l NAA + IBA 1.0 mg/l. Finally, neo-formed plantlets were transferred into pots containing peat moss and sand (1:1 v/v) and potential growth of these plantlets in environment indicates that through using the adequate amount of hormonal combination could give a better solution for the improvement and availability of grapevine (*Vitis vinifera* L.) for Bangladeshi farmers.

Keywords: Micropropagation, Surface Sterilization, Proliferated Shoots, Neo-formed plantlets

INTRODUCTION

Grape (*Vitis vinifera* L.) is a refreshing, nourishing and delicious fruit of the world. It is one of the most important fruit crops grown in the world today in terms of both total acreage and dollar value [1]. At present, its acreage of production is around 10 million hectares [2]. The world area of grape is 8.37 million hectares producing 74.30 million tons per annum [3]. It belongs to the family Vitaceae which is made up of 12 genera consisting of about 600 species and 800 varieties widely distributed all over the world [4]. Among fruits, grape occupies the first position in the world in terms of area and production [5]. As fruit, Grapes are a rich source of vitamins A, C, B_6, as well as essential minerals, such as potassium, calcium, iron, phosphorus, magnesium and selenium, which are necessary for human health [6]. In Bangladesh grape is a rare food item but throughout the world grape is a popular food. Preliminary observation trail conducted at BARI concluded that grape can be grown in Bangladesh as a fruit crop [7]. Recently farmers also want to make vineyard at commercial scale. But the problem is that non availability of virus free planting materials and good varieties of grape [8]. Some plants produce seeds remain viable only for a limited duration and some do not produce seeds and only require vegetative propagation. The vegetative propagation also faces challenge due to high rate of mortality and low rate of rooting in soil [9]. All grape varieties are propagated through grafting, layering and stem cutting in conventional method. Sometimes this method is hampered by seed and cutting dormancy, seedling heterozygosity, time consideration and space, and limited yield. The improvement in production and quality of grapes can be achieved by practicing genetic and sanitary clonal selection through incorporation of unconventional propagation method like tissue culture which is adopted an established method for the commercial propagation of herbaceous and woody plant species [10]. Propagation by axillary shooting has proved to be the most applicable and reliable method of *in vitro* propagation of *Vitis vinifera* L [11].

The main objectives of this study were to establish a reproducible cost effective protocol, to establish standardized $HgCl_2$ treatment for explants

sterilization process, to select suitable growth regulators for proper multiple shoots regeneration, elongation and root induction to produce a large number of plantlets within a short time for large scale production of *Vitis vinifera* L in Bangladesh.

MATERIALS AND METHOD

Collection of Plant Materials and Explant Preparation through $HgCl_2$Treatment

Terminal shoot segments with immature leaves from field grown plants were collected. The excess unnecessary parts like tendrils and leaves were removed from the collected materials and the remaining part of shoot segments were cut into five positions with convenient size (5-6 cm in length). The cutting positions of explant were numbered as P_1, P_2, P_3, P_4 and P_5. Shoot tips were the position number one (P_1) and nodal segments were position number P_2, P_3, P_4, P_5 respectively. Then they were collected in separated conical flask. Both the materials were washed thoroughly under running tap water for several times to reduce the dust and surface contaminants and then were taken in conical flask containing distilled water with a few drops of savlon and washed for 4-5 minutes with constant shaking. Second time washing was performed through dipping in 70% (v/v) ethanol for 30 seconds gradual change of distilled water until all traces of above chemicals were removed. The procedure of surface sterilization was carried out in side of laminar airflow cabinet. The above materials were taken into sterile flask and suspended in 0.1% $HgCl_2$ solution for different period to ensure contaminant free culture. The sterilized materials were washed 7-8 times with sterile distilled water immediately to remove all the traces of $HgCl_2$. The surface sterilized explants were sized into 2.0- 4.0 cm in length.

Media Preparation with Different Hormonal Combination and Inoculation of Explants

Full strength MS [12] medium with different concentrations of BAP and NAA was used for shoot induction and for root induction MS medium with different combinations of IBA and NAA were used. For carbon source 3% sugar was used and the medium was solidified with 0.7% agar. In all tests the pH of the medium was adjusted to 5.7 ±1 before addition of agar. Finally the culture vessels containing the medium were autoclaved at 15 id/inch2 pressures and at the temperature of 121°C for 20 min to insure sterilization. Then the vessels with the medium were allowed to cool as vertically.

Prepared explants were carefully inoculated in culture vessels (test tube) containing agar gelled nutrient medium supplemented with different concentration of hormones. The cotton plugs of the culture vessels were removed inside laminar airflow cabinet in presence of spirit lamp flame. Then the inoculation procedure was applied. Inoculation of explants was made singly per culture vessels. During inoculation, special care was taken so that the explants could touch on the medium equally and did not dip into the medium. After inoculation the mouth of culture vessels were tightly plugged and marked by glass marker with inoculation date. Then the vessels were ready for incubation. InIncubation all cultures were grown in the growth chamber illuminated by 40 watts white fluorescent light intensity varied from 2000- 3000 lux. The photoperiod was maintained generally 16 hours and 8 hours dark. The culture vessels were checked daily to note the response.

Sub culture for multiple shoot and root induction

When the regenerated shoots were 2-3 cm in length, they were removed aseptically from the culture vessels and placed on a sterile petri dish and remove the old media by washing through double sterilized distil water and again transferred into test tubes containing the same of different hormones supplemented media.

The regenerated shoots were removed aseptically from the culture vessels and placed on a sterilized petri dish and they were cut 2- 4 cm in length from the basal end of the shoots. Then each of the shoot was inoculated on freshly prepared medium containing full strength of MS medium with different combination and concentration of hormonal supplements for root induction.

Acclimatization and Transplantation of Plantlets

The rooted shoots (neo-formed plantlets) were kept in their media for 14, 24, 34 days without subculture before acclimatization to enhance the efficiency of roots. Neo-formed plantlets were washed thoroughly to remove any medium residual. The neo-formed plantlets were treated with Benelet (antifungal) 1 g/l and potted into a mixture of peat moss 1:1 sand (v/v), then covered with white plastic bags, to maintain high humidity the plantlets and maintained in a growth chamber at 27+2°C under 16h illumination (45 µ mole $m^{-2} s^{-1}$) with fluorescent lamps. Bags were progressively opened weekly. After 3 weeks acclimatization had been completed, plantlets were transferred to large pots for further growth in the field.

Statistical Analysis

Variance analysis of data was done using ANOVA program for statistical analysis. Data were analyzed using SPSS software (Ver. 11.5)

RESULTS & DISCUSSION

Effects of 0.1% $HgCl_2$ with different duration on surface sterilization and different position of explants

To establish explants from field grown plants under aseptic condition surface standardization is essential. Standardization for surface sterilization was carried out by trial and error experiments. Surface sterilization was carried out by 0.1% $HgCl_2$ solution at different time duration. When the explants were treated

with 0.1% $HgCl_2$ solution for 0.5 minute, contamination was occurred as the treatments failed to kill the microorganisms attached to the explants. 30%, 65% and 85% of explants were found contamination free with healthy tissue when they were treated for 1, 2 and 3 minutes respectively. No contamination was found but partial and complete tissue killing was observed when explants were treated for 4-5 minutes and more than five minutes (Table 1).

Table-1: Effects of 0.1% $HgCl_2$ with different duration of explants surface sterilization

$HgCl_2$ Treating period (Minutes)	No. of different explants	Contamination rate after days					% of survival explants
		5	**7**	**10**	**12**	**15**	
0.5	20	6	9	14	17	19	5
1.0	20	-	1	5	11	14	30
2.0	20	-	-	-	2	7	65
3.0	**20**	**-**	**-**	**-**	**-**	**-**	**85**
4.0	20	*	*	*	*	*	50
5.0	20	**	**	**	**	**	25
>5.0	20	***	***	***	***	***	0.0

Note: **-** = No contamination,* = Partial tissue killing, ** = Moderate tissue killing and*** = Complete tissue killing.

When lower concentrations of $HgCl_2$ were used in short duration they fail to kill the microorganisms attached to the surface of the explants. When higher concentrations of $HgCl_2$ were used in short duration, it showed some efficiency but when those higher concentrations were applied in long duration tissue killing occurred.

In case of positional treatment of explants with 0.1% $HgCl_2$ solution at different time duration were survive 35% of explants on P_1, 45% of explants on P_2 and 50% of explants on P_3, 60% of explants on P_4 and 65% of explants on P_5 (Table 2).

Table-2: Effects of 0.1% $HgCl_2$ with different position of explants surface sterilization

Different position of explants	No. of different explants	Treating period in Minutes $HgCl_2$					% of survival explants
		1.0	2.0	**3.0**	4.0	5.0	
P_1	20	4	2	**1**	***	***	35
P_2	20	2	3	**4**	***	***	45
P_3	20	#	4	**4**	2	***	50
P_4	20	#	2	**4**	4	2	60
P_5	**20**	#	**2**	**4**	**4**	**3**	**65**

Note: # = Contamination, *** = Complete tissue killing

Effect of Hormones for Establishment of Grapevine culture from Shoot Tip and Nodal Segment

In vitro establishment of grapevine (*Vitis vinifera* L.) shoot tips and stem segments were cultured on MS medium supplemented with different concentration and combinations of plant growth regulators (BAP and NAA). Results of this study have been presented in Table 3. In shoot tip culture the highest percentage of shoot induction (90%) was noticed in MS+ 0.5mg/l BAP and 0.1mg/l NAA. The highest mean number of shoots formed per explants was obtained 1.35 in MS+ 1.0mg/l BAP and 0.1mg/l NAA within 10-18 days. The highest length of shoot was recorded 2.84 cm in MS+ 2.0 mg/l BAP and 0.1mg/l NAA. Lowest percentage of shoot multiplication was 75% and length of shoot was 2.67 cm MS+ 0.5mg/l BAP+ 0.1mg/l NAA. However, this result could be ascribed to the mode of action of BAP as cytokinins at 1.00 mg/l that stimulate both cell division and promote growth of axillary shoots in plant tissue culture as reported by Trigiano and Gray [13] and George *et al.* [14].These results are compatible with the previous studies which disclosed that BA is the most effective among other cytokinins for inducing shoot development in Vitis [15, 16]. Further, BAP at 1.00 mg/l concentration was the best for shoot proliferation in both 'Soltanin' and 'Sahebi' cultivars that was reported by Aazami [17].

In stem segment culture the highest percentage of shoot induction (95%) and the highest mean number of shoots formed per explants was obtained 1.30 in MS+0.5mg/l BAP and 0.1 mg/l within 8-15 days. The highest length of shoot was recorded 2.15 cm was obtained in MS+ 1.0 mg/l BAP+ 0.1 mg/l NAA. Lowest percentage of shoot induction was 75% and length of shoot was 0.56 cm was obtained in MS+ 1.0 mg/l BAP+ 0.1 mg/l NAA and MS+ 2.0 mg/l BAP+ 0.1 mg/l NAA respectively. This result could be ascribed to the mode of action of BAP a scytokinin that stimulate both cell division and enhance growth of axillary shoots in plant tissue culture as reported previously by Trigiano and Gray [13] and George *et al.* [14]. The shoot proliferation confided upon the balance of cytokinins

and auxins and using lowest concentration of NAA affected well the initiation of grapevine *in vitro* reported by Tapia and Read [18]. Torrey and Reinert [19] reported that auxin increased the activating enzymes that break down starch and had the ability to increased proliferation of organogenesis.

Table- 3: *In vitro* establishment of grapevine (*Vitis vinifera* L.) shoot tips and stem segment cultured on MS medium supplemented with different concentration and combinations of plant growth regulators (BAP and NAA). Data were recorded after four weeks

Explants	Hormones supplement used in MS medium mg/l	No. of Explants inoculated	%of explants responded	Days to shoot formation	Mean number of shoots formed / explants	Length of Shoots in cm (M±S.E.)
Shoot tip culture	BAP +NAA					
	0.5 + 0.1	20	90	12-20	1.35	2.67±0.084
	1.0 + 0.1	**20**	**85**	**10-18**	**1.35**	**2.71±0.14**
	2.0 + 0.1	20	75	15-20	1.00	2.84±0.42
Stem segment culture	BAP +NAA					
	0.5 + 0.1	**20**	**95**	**8-15**	**1.30**	**1.69±0.089**
	1.0 + 0.1	20	75	6-13	0.95	2.15±0.26
	2.0 + 0.1	20	80	10-18	0.80	0.56±0.17

Note: **M**= mean and **S.E.** = standard error

Hormonal Effects Observation during Shoot multiplication and Root Induction

Shoot multiplication of grapevine on MS medium supplemented with different concentration and combinations of plant growth regulators (BAP and NAA) have been presented in Table 4. The highest mean number of shoots formed per explants was obtained 3.1 and the highest length of shoot was recorded 4.2 cm in MS+ 3.0mg/l BAP and 0.2mg/l NAA. Lowest mean number of shoots formed per explants was obtained 2.3 and length of shoot was 2.5cm in MS+ 3.0mg/l BAP+ 0.1mg/l NAA and MS+ 3.0mg/l BAP+ 0.3mg/l NAA respectively. For multiplication rate, cytokinins were effective in this respect when used in combination with an auxin. Butiuc-keul *et al*. [20] reported that supplementations of culture media with cytokinins improved the multiplication rate of grapevine for instance number of shoots/explants increased with the increase of BAP concentration until 2.0 mg/l with all NAA treatments.

Table-4: Effect of different concentrations and combinations of BAP, NAA for multiple shoots proliferation of grapevine (*Vitis vinifera* L.). Data were recorded after four weeks

Sub culture	Hormones supplement used in MS medium mg/l	Mean number of shoots formed / explants	Length of shoots in cm (M±S.E.)
Sub culture for multiple shoots proliferation	BAP +NAA		
	2.0+0.1	2.5	2.9±0.23
	2.0+0.2	2.4	3.3±0.12
	2.0+0.3	2.7	2.7±0.20
	3.0+0.1	2.3	3.1±0.15
	3.0+0.2	**3.1**	**4.2±0.15**
	3.0+0.3	2.6	2.5±0.18

Results of root induction of regenerated shoot on MS medium supplemented with different concentration and combinations of plant growth regulators (NAA and IBA) have been presented in Table 5. The highest percentage of root induction per shoot was 85%, the highest mean number of roots formed per explants was obtained 3.3 and highest length of root was recorded 4.3 cm was obtained in MS+ 0.5 mg/l NAA + 1.0 mg/l IBA. Lowest percentage of root induction per shoot was 65% and length of root was 2.5 cm was obtained in MS+ 1.0 mg/l NAA + 1.0 mg/l IBA and MS+ 0.5 mg/l NAA + 0.5 mg/l IBA respectively. These results were close to those of Barreto and Nookaraju =[21] and Butiuc- keul *et al.* [20] who reported that in V. vinifera cv. "Perlette" up to 95% rooting of micro-cuttings were obtained on MS medium supplemented with IBA and NAA.

Acclimatization

Acclimatization of plantlets is one of the most important steps in tissue culture to free living conditions. Data in Table 6 represented that mean survival percentage of plantlets was 58% after keeping plantlets in rooting medium for 14days before acclimatization. The survival percentages were increased to 83% after 34 days on rooting medium before acclimatization. These results were similar to that obtained by Thomas [22] who found that large survival percent of neo-formed plantlets were successfully acclimatized and cultivated in greenhouses.

Gok *et al*. [23] clarified that the survival percent of neo-formed plantlets (80%) were exposed to open filed environmental conditions. It is clear that leaving theneo-formed plantlets that kept in the rooting medium for longer period of time for increased the efficiency of roots which led to the increase the survival percentages of acclimatized plantlets [24].

Table-5: Effect of different concentrations and combinations of NAA, IBA for roots formation of grapevine (*Vitis vinifera* L.)

Sub culture	Hormones supplement used in MS medium mg/l	% of rooted / shoot	Mean number of roots formed / explants	Length of roots in cm (M±S.E.)
Sub culture for root induction	NAA+ IBA			
	0.5+0.5	75	1.9	2.5±0.36
	0.5+1.0	**85**	**3.3**	**4.3±0.14**
	0.5+1.5	80	3.0	3.8±0.23
	1.0+0.5	75	2.5	4.0±0.13
	1.0+1.0	65	2.7	4.2±0.17
	1.0+1.5	70	3.1	4.1±0.15

Table 6: According to time (14, 24, 34 days)effect of deferent concentrations of auxin (NAA & IBA) in rooting media on survival percentage of grapevine (*Vitis vinifera* L.) in acclimatization stage

Days formation for roots	Survival % of plantlets in different concentration of hormones supplement used in MS medium mg/l NAA+ IBA						Mean % of Survival plantlets
	0.5+0.5	0.5+1.0	0.5+1.5	1.0+0.5	1.0+1.0	1.0+1.5	
14 days	40	65	70	45	60	65	58
24 days	65	90	85	65	75	75	76
34 days	**75**	**90**	**90**	**75**	**80**	**85**	**83**

CONCLUSION

This study provides an *in vitro* rapid micro propagation of grapevine (*Vitis vinifera* L.) through observing $HgCl_2$ and hormonal effects. We obtain best and adequate concentration of $HgCl_2$ for surface sterilization as well as appropriate combination of hormones for the achievement of better-quality grapevines (*Vitis vinifera* L.).

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