MICROPROPAGATION AND ACCLIMATIZATION OF EUROPEAN VARIETIES OF GRAPES (*VITIS VINIFERA* L).

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ABSTRACT

An efficient, rapid and reproducible in vitro plant regeneration protocol was developed for European varieties of Vitis vinifera L. (Red Globe, Crimson Seedless, Autumn Royal and Thompson) using nodal explants on Murashige and Skoog (MS) medium which was fortified with different cytokinins and auxins with varying concentrations. The cultivars responded differently to the different PGRs used for In vitro micropropagation. The browning of the explants due to phenolics (Flavon-3-ols, tannins etc.)was reducedby washing 3-4 times with a mixture of antioxidants(Citric acid and Ascorbic acid in concentration of 150 mg/L and 100 mg/L respectively). The surface sterilization of explants was done with 0.01% $HgCl_2$ for 5-7 min. for getting aseptic cultures.The best combination for efficient shoot induction and multiplication was MS medium supplemented with BAP 2.0 mg/L for Thompson, with KN1.0 mg/L for Crimson, with BAP 4.0 mg/L for Autumn Royal and with BAP 1.0 mg/L for Red Globe, whereas the best rooting was obtained on ½ MS medium supplemented with IBA 2.0 mg/L for all varieties. The regenerated plantletswere successfully established in sand + soil (1:1) for hardening.

KEYWORDS:

Vitis vinifera, TDZ, BAP, KN, Micropropagation, Acclimitization.

1. INTRODUCTION

Fruits are valued as protective food and are considered as very rich source of minerals and vitamins. Their demand in Pakistan has been increasing due to changes in consumption pattern and population growth. Pakistan is oriental in location having agro-climatic conditions. For various horticultural crops including grapes it's conditions provide a suitable environment for better production [1].

Among the fruit crops grown worldwide grapes is one of the most important[2,3]. Its association with man is older than that of wheat and rice. Hymas (1954)traced its origin to 7000BC and stated that this crop was cultivated long before the cereals[4]. According

to the De Candolle (1886), grapes were known to be cultivated in Egypt around 4000BC.The origination of cultivation of grapes is believed to be Armenia near the Black and Caspian seas in Russia, from where it spread eastward to Iran and Afghanistan and westward to Europe[5].

Grapes belong to family *Vitaceae* and genus *Vitis.* This genus comprises of 60 species and out of them the most popular cultivated grape specie is *Vitis vinifera*[6].Windingtendrils are present on grape wines that generally arise opposite to leaf and the inflorescence is located in place of a tendril. Species of *Vitis* genera are diploid (2n=38). Most commercially important cultivars have perfect flowers[7], while unisexual male and female plants do exist[8,9].Most members of this genus can be found in either warm or temperate regions of the world.

In Pakistan, among fruits grapes rank at 10^{th} position.Although grapes is a minor fruit but it is used both in fresh and dry form. Total area under grapes cultivation in Pakistan is 15.3 thousand hectare with annual production of 66.0 thousand tones [10]. Over 70% of the grapes are grown in Baluchistan (Pishin, Quetta, Killa Abdullah, Kalat, Mastung, Loralai, Zhob District), while there is some acreage in Khyber Pakhtunkhwa (Nowshera, Swat, Hazara, Swabi, Peshawar etc.).

Tissue culture is a technique used for the production of disease-free plants of grapes. This technique has become a basic and convenient tool for providing tremendous and efficient opportunities for rapid and exponential growth of agriculture, horticulture and floriculture.*In vitro* propagation using shoot apices and axillary buds of various species and cultivars of *Vitis* has been reported since 1985 by Gray & Fischer[11]. However, micropropagation protocols for muscadine grapeshave been reported[12],our work is on cultivars of *V. vinifera* L., but studies of its cultivars have met with limited success[13].*Vitis* species, cultivars or hybrids respond differently to certain culture conditions because the degree of competence is highly depended on the particular genotype [12].

The present report describes a procedure for the micropropagation of grape varieties Red Globe, Crimson Seedless, Autumn Royal and Thompson.

2. MATERIALS AND METHODS

2.1. Plant material.

In this study, explants comprised intact nodes of *in vitro* grown grape (*Vitis vinifera* L.) varieties. Plants used in this study were four European varieties of grapes; Red Globe, Crimson Seedless, Autumn Royal and Thompson.

2.2 Surface sterilization

Plants of different varieties were collected from nurseries of Lahore and Peshawar and were grown in Botanical Garden,University of the Punjab, Lahore. Twigs bearing shoot apices were collected early in the growing season (April to May) and were washed thoroughly for 15 min under running tap water. Like other woody plants the oxidative browning was also a problem in grapes. It was resolved by washing 3-4 times with the solution of 150 mg/L Citric acid and 100mg/L Ascorbic acid in distilled water, followed by soaking for 5 min in 25 % (v/v) NaOClsolution. The explants were then washed 4-5 times with distilled water to remove every trace of the bleach.After washing the explants thoroughly they were immersed in 0.01% Mercuric chloride with 0.01% SDS for 15 min. Then under Laminar air flow cabinet explants were rinsed three times in sterile autoclaved distilled water. The excess tissues were aseptically cut off and the shoot apices (4-6 mm) were placed into MS medium[14]for the supplemented with different growth hormones of varying concentrations.

2.3. Shoot induction and Shoot multiplication

Surface sterilized nodal segments were cultured in MS medium supplemented with 1.0, 2.0, 3.0 and 4.0 mg/L concentrations of TDZ, Kinetin and BAP separately, for shoot induction and multiplication. Each experiment consisted of 10 replicates and was repeated thrice. As a control a PGR-free medium was used.The results in terms of number of shoots per explant were recorded after four to five weeks of culture.

2.4. Rooting

Sub-shoots were cut off from the main shoots and form the donor explant which was thencultured with the basal ends in solidified ½ strength MS medium supplemented with different concentrations of IBA (0.5, 1.0, 1.5, 2.0 and 2.5 mg/L). An auxin-free medium was included as a control.

2.5. Acclimatization

In vitro developed plantlets were then acclimatized in plastic cups containing a 1:1 mixture by volume of soil and sand.

2.6. Culture conditions

MS medium was solidified with 8% agar and the pH was adjusted to 5.7- 5.8 prior to autoclaving. Test tubes containing medium were sterilized in an autoclave for 15 min at

121°C. All cultures were incubated in growth room at 23± 2°C with a 16-h photoperiod under 1500 lux illumination provided by cool-white fluorescent lamps.

2.7. Statistical analysis

Ten replicates were assigned to each treatment in a factorial arrangement. The experiment was done in a completely randomized design and conducted thrice. Data were collected after four to five weeks and statistically analyzed using SPSS ver.16 (SPSS Inc., Chicago, IL., USA). The significance of differences among means was done by using Duncan's Multiple Range Test at $P = 0.05$.

3. Results and Discussion

*Vitis vinifera*is perennial woody plant which is cultivated in different parts of the world. The conventional method of its propagation is not only time consuming but the plants are susceptible to various diseases which are transmitted from one generation to another. Tissue culture techniques provide genetically similar disease free plants.

In the present study shoot tips (4-6 mm) were used for the production of *in vitro* grown plants for obtaining nodal explants to be used for micropropagation.Thies and Graves (1992) also preferred shoot tips as compared to meristem tips of *V. rotundifolia* for micropropagation[15]. Although meristem tips (0.2 – 0.4 mm) may be superior to shoot tips in terms of avoidance of systemic pathogens however they failed to give response due to oxidation. Qiu*et al.* (2004)used a mixture of ascorbic acid and citric acid for*V. aestivalis*cv. 'Norton' to prevent browning of meristem tips but he failed[12]. However in our case, this mixture was successful in preventing browning of the shoot tips from field grown plants of different varieties of grapes used.

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The potential for multiple shoot formation from nodalexplants of each variety exhibited differently on different PGR supplemented MS basal medium. As shown in Table 1, the effect of different growth regulators on number of shoots per explant was significantly affected by the concentration of TDZ and the variety. In MS medium supplemented with different concentrations of TDZ, *V. vinefera* var. Thompson produced an average number of 4 shoots per cultureat 1.0 mg/L TDZ with 50% shoot induction as compared to higher concentrations tried (Fig. D).While on the same concentration of TDZ, Red Globe gave an average of 5 shoots per explant with 30% shoot induction. Crimson seedless produced single shoot per explant while Autumn Royal showed no response to TDZ. The shoots produced on TDZ containing medium were small and stunted in appearance. Similar results were also obtained by Aazami (2010) in *V. vinifera* varieties Soltanin and Sahebi[16]and Diab*et. al.* (2011) in cv. Speryoin MS medium supplemented with 1.0 mg/L TDZ[17]. They also reported that the shoots produced on TDZ were stunted and

distorted.Mok*et al.* (1982) and Visser*et al*. (1995) have been reported both cytokinin and auxin like effects of Thiadizuron[18,19]. One of the reported disadvantages of TDZ is its often inhibitory effects on shoot elongation in many woody species [20]. Shoot inhibition occurs because the action of cytokinin oxidase is inhibited with increase in the level of endogenous cytokinins[21].

Table 1: Effect of different concentrations of TDZ on shoot induction and multiplication from nodal explants of different varieties of *Vitis vinifera* L. in MS medium.

Growth regulators	Conc. Mg/L	% Shoot induction				No. of shoots/explant			
		Red Globe	Autumn Royal	Crimson Seedless	Thompson	Red Globe	Autumn Royal	Crimson Seedless	Thompson
MS(basal)	0.0	1.0	1.0	1.0	1.0	-	-	-	-
Kinetin	1.0	67±3.33^{b}	37±6.67^{a}	67±3.33^{b}	67±3.77^{a}	4	1	2	2
	2.0	46±3.34^{a}	27±3.34^{a}	67±3.34^{b}	66±5.75^{a}	3	1	1	2
	3.0	43±5.57^{a}	10±5.77^{b}	43±5.57^{a}	53±3.33^{a}	3	1	2	1
	4.0	37±8.81^{a}	-	10±5.77^{b}	47±3.35^{b}	2	-	1	1

Data followed by different letters show significant difference at P≤0.05
Each experiment was performed in triplicate.

MS basal medium fortified with Kinetin (1.0, 2.0, 3.0 and 4.0 mg/L) showed better response in terms of number of shoots per nodal explant as compared to TDZ (Table2). Maximum number of shoots(4per explant)were produced by Red Globe followed by Thompson and Crimson Seedless (2 per explant) (Fig. 3)while Autumn Royal produceda single shoot at 1.0 mg/l concentration of KN in MS medium. At higher concentrations the average number of shoots decreased as shown in Table 2.Reisch (1986) reported negative response with increasing level of KN on shoot bio-mass in 'Concord' variety of grapes but shoot proliferation rate did not vary significantly to the treatments[22].Poudel*et al*. (2005) reported the effectiveness of KN on the establishment of *in vitro* cultures of two wild grapes[23].

Table 2: Effect of different concentrations of Kinetin on shoot induction and multiplication from nodal explants of different varieties of *Vitis vinifera* L. in MS medium.

Growth regulators	Conc. Mg/L	% Shoot induction				No. of shoots/explant			
		Red Globe	Autumn Royal	Crimson Seedless	Thompson	Red Globe	Autumn Royal	Crimson Seedless	Thompson
MS(basal)	0.0	1.0	1.0	1.0	1.0	-	-	-	-
Kinetin	1.0	67±3.33^{b}	37±6.67^{a}	67±3.33^{b}	67±3.77^{a}	4	1	2	2
	2.0	46±3.34^{a}	27±3.34^{a}	67±3.34^{b}	66±5.75^{a}	3	1	1	2
	3.0	43±5.57^{a}	10±5.77^{b}	43±5.57^{a}	53±3.33^{a}	3	1	2	1
	4.0	37±8.81^{a}	-	10±5.77^{b}	47±3.35^{b}	2	-	1	1

Data followed by different letters show significant difference at P≤0.05
Each experiment was performed in triplicate.

In the present study, treatment with different concentrations of BAP showed the highest shoot inductionand proliferation rate (100%)for Thompsonat 2.0mg/L of BAP (Fig. E)followed by Red Globe (77%) on MS supplemented with 1.0mg/L BAP (Table 3) (Fig. A).However, MS medium supplemented with 1.0 mg/L BAP showed average number of 4 shoots per explant after four weeks of inoculation by Thompsonwhile Red Globe showed an average of 2 shoots on MS supplemented with 1.0mg/L BAP.

In case of Autumn Royal and Crimson seedless, average number of shoots was 2 on MS medium supplemented with 3.0 mg/l BAP.Although, 4.0mg/L BAP providedthe best shoot induction(47%) and proliferation with well expanded leaves in variety Autumn Royal (Fig. B).

Table 3: Effect of different concentrations of BAPon **s**hoot induction and multiplication from nodal explants of different varieties of *Vitis vinifera* L. in MS medium.

Growth regulators	Conc. Mg/L	% Shoot induction				No. of shoots/explant			
		Red Globe	Autumn Royal	Crimson Seedless	Thompson	Red Globe	Autumn Royal	Crimson Seedless	Thompson
MS(basal)	0.0	1.0	1.0	1.0	1.0	-	-	-	-
BAP	1.0	77±3.33 ^{a}	10±5.73 ^{c}	53±3.33 ^{a}	96±3.33 ^{a}	2	1	1	4
	2.0	53±3.33 ^{b}	27±3.34 ^{a}	43±3.34 ^{b}	100±0.00 ^{a}	2	1	1	2
	3.0	40±5.77 ^{bc}	36±8.13 ^{a}	40±0.00 ^{b}	90±5.77 ^{a}	1	2	2	2
	4.0	33±6.67 ^{c}	47±3.33 ^{a}	26±5.57 ^{c}	93±5.75 ^{a}	1	1	2	2

Data followed by different letters show significant difference at P≤0.05
Each experiment was performed in triplicate.

Although all the concentrations of BAP exhibited shooting inall the varietiesused (Table 1), but withincrease in the concentration of BAP the number of shoots decreased. Lee and Wetzstein (1990) worked on 'Summit' variety of grapes and observed strong inhibition of shoot elongation with almost no elongated shoots on 10 µM BA concentrations and higher than that[24].

BAP has been reported to be the most commonly used cytokinin for shoot induction and multiplication in grape tissue culture [13,15,25]. The effect of BAP has also been reported beneficial for multiple shoots induction in grape by Alizadeh*et al.* (2009)[26]. BAP has been known for breaking apical dominance and enhancement of axillary branching, but higher levels may sometimes suppress growth and is even toxic to the tissues [27].

In our study, BAP showed significant results for three varieties (Red Globe, Autumn Royal and Thompson) among the four tried for both the shoot induction and shoot proliferation. Bigger (2010) reported micropropagation of *Vitis vinifera* cvNortons in MS medium supplemented with 4.0 µM BA and 0.5mg/l Thiamine[28].

Best proliferated *In Vitro*shoot cultures were inoculated on half-strength MS medium exhibited with different concentrations of IBA for root induction. Among all varieties of grapes, Thompson showed highest rooting (92%) with early root initiation after15 days in half-strength MS medium fortified with IBA (2.0 mg/L)followed by Red globe and Crimson seedless(68%). While Autumn Royal exhibited 49% shoot induction on the same concentration of IBA. However other concentrations of IBA also showed rooting but the response to rooting recorded on other concentration was low (Table 4) (Fig. A-E). The rooted plantlets of Thompson were successfully hardened inside the culture room in autoclaved mixture of soil and sand (1:1) while other plantlets dried off after a couple of days.

Table 4: Effect of different concentrations of IBA on root induction from *in vitro* grown shoots of different varieties of *Vitis vinifera* L. in MS medium

Growth regulator	Concentrations Mg/L	Varieties of *Vitis vinifera* L.			
		Red Globe	Autumn Royal	Crimson Seedless	Thompson
IBA	0.5	60 ± 5.12^{b}	22 ± 2.82^{a}	26 ± 5.85^{bc}	63 ± 5.22^{b}
	1.0	63 ± 5.50^{b}	21 ± 3.53^{a}	37 ± 6.17^{cd}	72 ± 5.85^{b}
	1.5	68 ± 5.50^{bc}	47 ± 4.59^{a}	43 ± 6.61^{c}	86 ± 6.85^{bc}
	2.0	68 ± 5.85^{cd}	49 ± 5.53^{b}	68 ± 5.94^{bc}	92 ± 7.45^{a}

Data followed by different letters show significant difference at $P\leq0.05$

Micro-shoots of the plants of cv. Norton were inoculated on ½ strength MS medium fortified with different concentrations of IBA[28].According to his reportsrooting was increased with increasing concentrations of IBA for all varieties of the grapes. Similarly addition of low level of auxins has been reported for *in vitro* rooting in grapes. Auxin IBA is the most effective in most of the cases apparently because it is not destroyed by IAA oxidase or other enzymes [13,29].

The rooted plantlets of all the varieties were initially hardened inside the culture room in sterile sand: soil (1:1). The plantlets of only Cv. Thompson grown on 4.0mg/L BAP responded to acclimatization (Fig. F).The reason of poor response of other varieties for acclimatization was that the leaves of *in vitro* raised plants are generally not competent photosynthetically because they are growing in sucrose rich medium but are active in transpiration and respiration, which may be the reason for low establishment of plantlets during hardening[30].

3. CONCLUSION

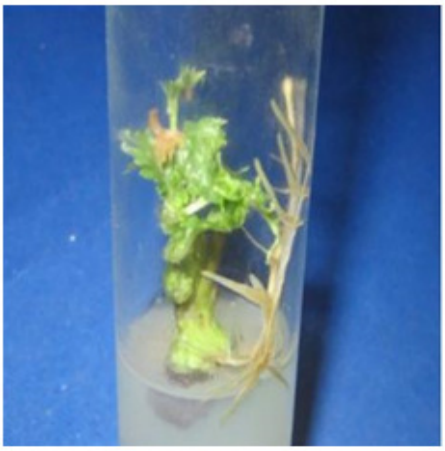
The present protocol is for the development of multiple shoots from nodal explants of European varieties being cultivated in Pakistan. The protocol described here is efficient,

reproducible and provide a rapid technique for mass multiplication of these potential grape varieties. To our knowledge, using these European varieties collectively this is the first report the micropropagation.

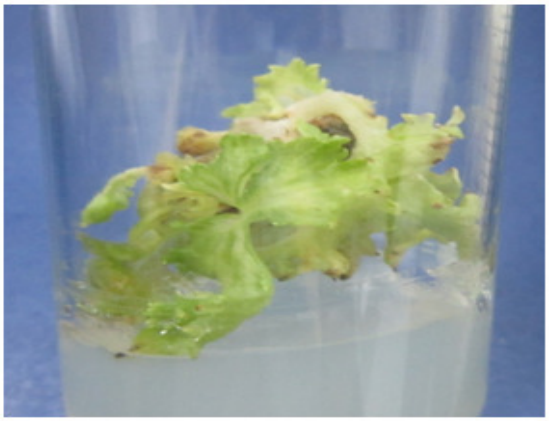
ACKNOWLEDGEMENTS

The authors are grateful to the Higher Education Commission, Pakistan and Department of Botany, University of the Punjab, Lahore Pakistan for providing financial support for this study.

A



B



C

D E

F

Fig:*In vitro* plant regeneration in *Vitis vinifera* L.
(A) Shooting on MS + TDZ 1.0 mg/L and *In vitro* rooting on ½ MS +IBA 2.0 mg/L cv. Red Globe. (B) Shooting on MS + BAP 4.0 mg/L and *In vitro* rooting on ½ MS +IBA 2.0 mg/L cv. Autumn Royal. (C) Shooting on MS + KN 1.0 mg/L and *In vitro* rooting on ½ MS +IBA 2.0 mg/L cv. Crimson Seedless. (D) Shooting on MS + TDZ1.0 mg/L and *In vitro* rooting on ½ MS +IBA 2.0 mg/L cv. Thompson. (E) Shooting on MS + BAP 2.0

mg/L and *In vitro* rooting on ½ MS +IBA 2.0 mg/L cv. Thompson. (F) Acclimatized plant of Thompson in sand soil (1:1).

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