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Full Length Research Paper

IN VITRO explants regeneration of the grape 'Wink' (*VITIS VINIFERA* L. 'Wink')

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The effects of different hormones and their concentration combinations, different explant types, dark periods, on the regeneration of the grape 'Wink' was investigated using *IN VITRO* leaves, petioles, internodes and radicles. The results showed that of all the media prepared, the explants on following media produced the highest regeneration rate: 78.74±1.60% of the leave explants on the medium prepared with MS+18.20 µM, TDZ+0.49 µM IBA regenerated with a mean of 6.75±0.75 shoots per explant; 39.33±1.47% of the petioles on the medium prepared with MS+9.10 µM TDZ+0.49 µM IBA regenerated with a mean of 3.55±0.50 shoots per explant and 41.37±1.13% of internodes on the same medium regenerated with a mean of 4.74±0.64. However, radicles did not generate on any of the media. TDZ enhanced regeneration rate of leaves better than BA. From 0 week to 4 weeks in dark, leave explants displayed a higher rate of regeneration for 2 or 3 weeks. Adventitious shoots were rooted on 3/4 MS+1.73 µM IBA medium, and the rooted plantlets survived after acclimatization and they were transplanted to the greenhouse.

Key words: Grape, explants, regeneration, hormones, adventitious shoots.

INTRODUCTION

Vitis vinifera L. 'Wink', one of the Eurasian species, originated in Japan and is a popular late-maturing variety widely cultivated in China. However, its vine grows too prosperously and requires too much pruning. Therefore, growth inhibition of the plant is necessary. To improving plant characteristics, such as plant growth, genetic engineering techniques are often applied, in which achieving a high rate of regeneration is key step.

Reports have been published on grape regeneration using organ regeneration (Stamp et al., 1990; Tao et al., 2005; Li et al., 2007) or somatic embryogenesis regeneration (Li et al., 2000; Pinto-Sintra 2007; Yuan et al., 2007; Araya et al., 2008; Zhi et al., 2010). Of the two popular methods, somatic embryogenesis regeneration is too tedious. It requires different mediums, takes more time (over half a year) to induce somatic embryo and uses immature pollens which are limited by phenophase (Tsvetkov et al., 2000; Mulwa et al., 2007; López-Pérez et al., 2008; Dhekney et al., 2008; Vidal et al., 2009). Organ regeneration is also limited because plants regenerated from the explants transferred by exogenous gene may alter heredity, but the variation is very low. Plants regenerated from leaf explants display even no variation (Yang et al., 2006; Jin et al., 2009). Therefore, organ regeneration is relatively simpler and more feasible.

In this study, we used explants from the grape 'Wink' to develop a protocol of regeneration with a purpose of laying a foundation for the application of genetic engineering techniques to research on the growth of *V. vinifera* L. 'Wink'.

MATERIALS AND METHODS

Plant material

Micropropagation of *V. vinifera* L. 'Wink' was established *in vitro* from nodal sections (1 to 2 cm in length) of mature plants. A ¾ MS medium was prepared and supplemented with 1.73 µM IBA, 3% (w/v) sucrose and 0.55% agar (PH 5.8). The medium samples of 30 to 40 ml each were put in jars and autoclaved at 121°C for 20 min. One nodal section with one *in vitro* micro-shoot was proliferated in each jar. Then, the *in vitro* micro-shoots on the medium were

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Abbreviations: MS, Murashige and Skoog medium (1962)**; TDZ,** thidiazuron (N-phenyl N'1, 2, 3-thidiazol-5-ylurea); **BA,** 6-benzyladenine; **IBA,** indole-3-butyric acid.

incubated at 25±2°C under a 16-h photoperiod provided by cool-white fluorescent tubes at an intensity of 32 to 40 µmol $m^{-2}s^{-1}$. The shoots grew into plantlets 4 weeks later and were cut to obtain new nodal sections which were then transferred to fresh media of the same preparation as described above and were multiplied there.

Effects of different hormones

In vitro expanded and immature leaves along with their petioles (0.1 cm in length) were transversely cut along the midrib (0.5 cm in length and 0.5 cm in width). These leaf explants were placed with adaxial or abaxial side in contact with the media and were cultured in MS media supplemented with TDZ in different concentrations of 4.55, 9.10, 13.65 or 18.20 µM and IBA in the concentration of 0.49 µM, or in MS media supplemented with BA in the concentrations of 4.44, 8.88, 13.32 or 17.76 µM and IBA in the concentration of 0.49 µM. Each of the foregoing prepared media was added with 3% (w/v) sucrose and 0.55% agar. All the explants on the media were kept in dark for two weeks to induce callus and/or adventitious shoots. The other culturing conditions were the same as described earlier.

Effects of different explants

Leave explants obtained in the same way as described earlier, were placed with the adaxial sides in contact with MS media supplemented with TDZ in the concentrations of 13.65, 18.20, 22.75 or 27.30 µM and IBA in the concentrations of 0.05, 0.49, 0.99, 1.48 or 2.47 µM. A 4×5 complete factorial design of the four concentrations of TDZ and five concentrations of IBA produced 20 treatments.

Young petioles, young internodes and radicles from 3 to 4 week-old plants were obtained, cut and used as explants. Each of the explants was cultured in MS media supplemented with TDZ in the concentrations of 4.55, 9.10, 13.65 or 18.20 µM and IBA in the concentrations of 0.05, 0.49, 0.99, 1.48 or 2.47 µM. A 4×5 complete factorial design of the four concentrations of TDZ and five concentrations of IBA produced 20 treatments.

The other preparation and culturing conditions for the foregoing explants were the same as described earlier.

Effects of dark periods

Leaf explants were placed with the adaxial side in contact with the MS medium supplemented with 18.20 µM TDZ, 0.49 µM IBA, 3% (w/v) sucrose and 0.55% agar. These explants were dark treated for 0, 1, 2, 3 or 4 weeks, respectively. They were then cultivated under a 16-h photoperiod (32 to 40 µmol $m^{-2}s^{-1}$) at 25±2°C for 7 weeks (including dark periods). The other culturing conditions were the same as described in the section of "Effects of different hormones".

The experiment was repeated three times. The percentage of callus was recorded after 1 or 2 weeks. The percentage of callus was categorized on a scale from 0 to 4: 0 = 0%, 1 = 25%, 2 = 50%, 3 = 75%, and 4 = 100%. The regeneration percentage and the number of adventitious shoots of each explant were recorded 7 weeks after the beginning of the experiment. The adventitious shoots were cut and transferred to 3/4MS media supplemented with 1.73 µM IBA. These shoots would be rooted in this medium.

Transplant of plantlets

The resulting plantlets were acclimatized in natural light in room for one week. Then the lip of the jar was removed to let the plantlets to adapt to the natural environment for 3 to 4 days. Thirdly, the plantlets were taken out of the jar and cleaned of the medium. Finally, they were transplanted to the medium in pots in the greenhouse. The medium was made of peat, vermiculite and perlite (3:1:1). Proper temperature (25±1°C) and relative humidity (60% RH-80% RH) were provided for them to grow.

Data analysis

Regeneration rate was defined as the average percentage of each treatment with the number of the explants that developed shoots divided by the number of total explants. The mean of shoots was presented as the total number of shoots divided by the number of the explants which regenerated the shoots. The regeneration percentage and the mean of shoots were presented as the mean ± standard error. Data was analyzed by the Duncan's test using DPS data program (version 3.01). The related formulas were as follows:

Regeneration percentage = the number of developed shoots / the number of total explants × 100%

Mean of shoots = the total number of developed shoots / the number of explants regenerated shoots × 100%

RESULTS

Effects of different hormones

The way the leaves were placed on the medium had a great influence on regeneration rate (Table 1). The regeneration rate was higher, and the mean of shoots was greater when the adaxial side of the leaf explant was in contact with the MS medium (Figure 1 A to C) than when the abaxial side was in contact with the same medium. This was true of the cases with media of different preparations (Table 1).

Under the condition of the adaxial side of the leaf explant in contact with the MS medium, when the medium was supplemented with 8.88 µM BA and 0.49 µM IBA, the regeneration rate and the mean of shoots were the highest (Table 2). However, when the concentration of BA was 13.32 µM or more, both the regeneration rate and the mean of shoots began to fall, even to zero, with callus becoming loose and flocculent.

Comparing the results shown in Tables 1 and 2, we can see that the regeneration rate and the mean of shoots were higher when the medium was supplemented with TDZ than when the medium was supplemented with BA, while the concentration of IBA was fixed.

Effects of different explants

Table 3 showed that, after one week of culturing in dark, 13.65 µM TDZ induced 75 to 100% of callus, but the percentages of callus decreased when the concentrations of TDZ increased with the same concentration of IBA. When the duration of culturing in dark lasted for two weeks, all the callus reached 100% with the MS medium

Table 1. Adventitious shoot regeneration response of the grape 'Wink' via *in vitro* leaf explants with different placements as affected by thidiazuron (TDZ) and indole-3-butyric acid (IBA).

TDZ (µM)	IBA (µM)	Adaxial surface in contact with medium		Abaxial surface in contact with medium	
		Regeneration (%) (±SE)	Mean no. shoots (±SE)	Regeneration (%) (±SE)	Mean no. shoots (±SE)
4.55	0.49	16.5±1.38^{d}	2.49±0.30^{c}	15.48±0.86^{d}	1.3±0.16^{d}
9.10	0.49	28.65±2.19^{c}	3.36±0.18^{b}	21.67±2.08^{c}	1.8±0.14^{c}
13.65	0.49	43.47±1.69^{b}	4.16±0.21^{b}	31.36±0.88^{b}	2.6±0.15^{b}
18.20	0.49	78.41±1.97^{a}	6.79±0.83^{a}	68.55±0.90^{a}	3.3±0.15^{a}

Different letters in the same column denote significant differences at P<0.05 by Duncan's test.

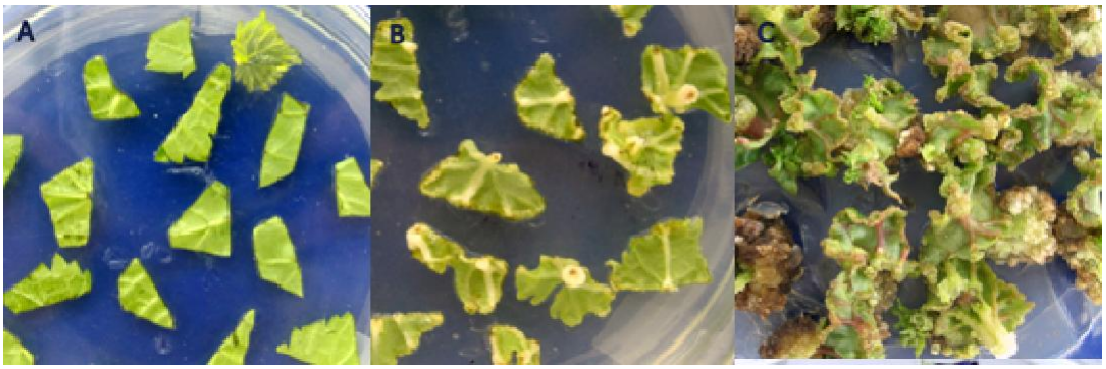

Figure 1 A to C. Adventitious shoot regeneration from *in vitro* explants of the grape 'Wink'. A to C: The process of regeneration of the leaf explant with the adaxial side in contact with the MS medium supplemented with 18.20 µM TDZ and 0.49 µM IBA, and in dark for two weeks.

Table 2. Adventitious shoot regeneration response of the grape 'Wink' via *in vitro* leaf explants as affected by 6-benzyladenine (BA) and indole-3-butyric acid (IBA).

BA (µM)	IBA (µM)	Adaxial surface in contact with medium	
		Regeneration (%) (±SE)	Mean no. shoots (±SE)
4.44	0.49	8.57±1.24^{b}	1.38±0.16^{b}
8.88	0.49	12.56±2.14^{a}	2.39±0.13^{a}
13.32	0.49	6.07±0.42^{c}	1.22±0.18^{b}
17.76	0.49	0^{u}	0^{c}

Different letters in the same column denote significant differences at P<0.05 by Duncan's test.

supplemented with all the selected concentration combinations of TDZ and IBA.

More importantly, 18.20 µM TDZ induced higher regeneration rate and mean shoots than the other concentrations of TDZ with the same concentration of IBA. At 18.20 µM TDZ, 0.49 µM IBA induced the highest regeneration rate (78.74±1.60) and mean shoots (6.75±0.75).

After one week of culturing in dark, 4.55 µM TDZ TDZ induced 100% of callus in all concentrations of IBA, but the percentage of callus was a slightly decreased when the concentrations of TDZ increased (Table 4). Our data showed that the callus reached 100% with the MS medium supplemented with all the selected concentration combinations of TDZ and IBA when the culturing period in dark lasted for two weeks.

9.10 µM TDZ induced higher regeneration rate and mean shoots than the other concentrations of TDZ no matter in which concentration of IBA. At 9.10 µM TDZ, 0.49 µM IBA induced the highest regeneration rate (39.33±1.47) and mean shoots (3.55±0.50) (Figure 1 D to F).

In Table 5, the percentage of callus and the rate of regeneration of internode explant were of the same trend as that of the petiole explant (Figure 1 G to I). However, the highest mean of shoots was induced at a different

Table 3. Callus induction and adventitious shoot regeneration response of the grape 'Wink' via *in vitro* leaf explants as affected by thidiazuron (TDZ) and indole-3-butyric acid (IBA).

TDZ (µM)	IBA (µM)	Callus coverage (0-4)	Adaxial surface in contact with medium	
			Regeneration (%) (±SE)	Mean no. shoots (±SE)
13.65	0.05	3-4	37.26±2.72^{f}	3.38±0.44^{e}
13.65	0.49	3-4	43.51±1.42^{e}	4.22±0.28^{cd}
13.65	0.99	3-4	42.79±1.37^{e}	3.78±0.55^{de}
13.65	1.48	2-3	35.35±1.53^{f}	3.45±0.30^{e}
13.65	2.47	2-3	33.06±1.58^{g}	2.58±0.67^{f}
18.20	0.05	3-4	71.53±2.46^{c}	4.76±0.51^{c}
18.20	0.49	3-4	78.74±1.60^{a}	6.75±0.75^{a}
18.20	0.99	2-3	76.32±2.02^{b}	5.76±0.52^{b}
18.20	1.48	2-3	69.62±1.89^{c}	4.73±0.49^{c}
18.20	2.47	1-2	61.68±2.07^{d}	3.25±0.38^{e}
22.75	0.05	2-3	20.06±1.05^{i}	1.22±0.20^{h}
22.75	0.49	2-3	24.78±0.01^{h}	2.35±0.41^{fg}
22.75	0.99	1-2	19.56±0.53^{i}	1.87±0.49^{g}
22.75	1.48	1-2	0^{j}	0^{i}
22.75	2.47	1-2	0^{j}	0^{i}
27.30	0.05	1-2	0^{j}	0^{i}
27.30	0.49	1-2	0^{j}	0^{i}
27.30	0.99	1-2	0^{j}	0^{i}
27.30	1.48	1-2	0^{j}	0^{i}
27.30	2.47	0-1	0^{j}	0^{i}

Classification of callus coverage on leaf explants: 0 = 0%, 1 = 25%, 2 = 50%, 3 = 75%, and 4 = 100%. Callus coverage observed on leaf explants for each plant growth regulator combination of concentrations. Different letters in the same column denote significant differences at P<0.05 by Duncan's test.

preparation with the MS medium supplemented with 9.10 µM TDZ and 0.99 µM IBA (Table 5).

Only a few radicle explants developed callus on the MS medium with different concentrations of TDZ (13.65, 18.20, 22.75 or 27.30 µM) and IBA (0.05, 0.49, 0.99, 1.48 or 2.47 µM). And none of the callus could induce adventitious shoots.

Effects of dark periods on shoot organogenesis

Table 6 showed that when the best medium preparation was selected and fixed, two or three weeks of culturing in dark induced higher regeneration rate and mean of shoots than the other durations of the dark period. If the leaf explant was cultured directly in light, no adventitious shoots were developed. Four weeks in dark also induced quite high regeneration rate, but, among the adventitious shoots thus developed, only a few survived the long darkness.

DISCUSSION

In the present study, the effects of different hormones and their concentration combinations of the type of the plant explants and of dark periods on the regeneration of the grape 'Wink' were investigated for the purpose of establishing a protocol of regeneration.

Firstly, different placement of leaf explants on the MS medium was studied, and the result was that, when the adaxial side contacted the medium, the explants developed a higher rate of regeneration (Table 1). This was consistent with some previous studies (Li et al., 2002, 2007). However, the rate of regeneration in our study (78.74%±1.60) was obviously higher than those of previous studies, where 33% was observed in Li et al. (2002) and 13.48% in Li et al. (2007). This improvement was attributed to our optimization of hormone combination in the research design.

Secondly, the effect of two cytokinins TDZ and BA on regeneration was compared by experiment. It was observed that TDZ induced higher regeneration rate than BA (Tables 1 and 2), which generally agreed with the reports by Yuan et al. (2007) and Fang et al. (2007).

Thirdly, different types of explants have significant effect on the rate of regeneration (Thomas et al., 2000). The regeneration of leaf, petiole, internode and radicle explants was investigated in the present research. It was found that the leaf explant had the highest regeneration rate but the petiole and internode explants produced stronger adventitious shoots (Tables 3 to 5). The radicle

Table 4. Adventitious shoot regeneration response of the grape 'Wink' via *in vitro* petiole explants as affected by thidiazuron (TDZ) and indole-3-butyric acid (IBA).

TDZ (µM)	IBA (µM)	Callus coverage(0-4)	Regeneration (%) (±SE)	Mean no. shoots (±SE)
4.55	0.05	4	8.03±0.46^{gh}	1.23±0.17^{h}
4.55	0.49	4	9.44±1.30^{g}	1.35±0.14^{h}
4.55	0.99	4	7.27±0.14^{h}	1.20±0.18^{h}
4.55	1.48	4	0^{k}	0^{i}
4.55	2.47	4	0^{k}	0^{i}
9.10	0.05	4	29. 55±1.00^{p}	2.85±0.10^{bc}
9.10	0.49	3-4	39.33±1.47^{a}	3.55±0.50^{a}
9.10	0.99	3-4	26.77±1.60^{c}	3.10±0.15^{b}
9.10	1.48	3-4	21.99±0.49^{d}	2.49±0.02^{de}
9.10	2.47	3-4	16.79±2.19^{e}	2.12±0.70^{fg}
13.65	0.05	3-4	6.96±0.45^{hi}	2.01±0.31^{fg}
13.65	0.49	3-4	11.82±0.87^{f}	2.79±0.14^{bcd}
13.65	0.99	3-4	7.87±0.16^{gh}	2.55±0.04^{cde}
13.65	1.48	3-4	5.31±0.39^{ij}	1.32±0.06^{h}
13.65	2.47	2-3	0^{k}	0^{i}
18.20	0.05	3-4	5.41±0.40^{ij}	1.36±0.16^{h}
18.20	0.49	3-4	9.54±0.86^{g}	2.27±0.21^{ef}
18.20	0.99	3-4	4.20±0.78^{j}	1.80±0.11^{g}
18.20	1.48	2-3	0^{k}	0^{i}
18.20	2.47	2-3	0^{k}	0^{i}

Classification of callus coverage on petiole explants: 0 = 0%, 1 = 25%, 2 = 50%, 3 = 75%, and 4 = 100%. Callus coverage observed on leaf explants for each plant growth regulator combination. Different letters in the same column denote significant differences at P<0.05 by Duncan's test.

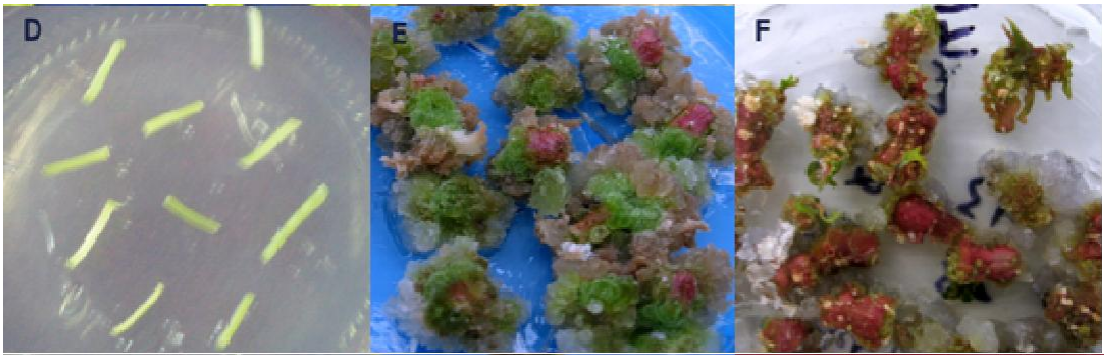

Figure 1 D to F. Adventitious shoot regeneration from *in vitro* explants of the grape 'Wink'. D to F: The process of regeneration of the petoile explant with the adaxial side in contact with the MS medium supplemented with 9.10 µM TDZ and 0.49 µM IBA and in dark for two weeks.

explant showed no regeneration rate (leaves 78.74%±1.60, petiolets 39.33%±1.47, internodes 41.37%±1.13, radicles 0%). These results were similar to those of the study by Quan and Chang (2005) (leaves 17.9%, petiolets 2.3%, internodes 2.6%, radicles 0%).

Finally, the necessary dark periods of culturing were compared, and two weeks were found to be enough to achieve the optimal regeneration rate (Table 6), which was confirmed in the papers by Korban et al. (1992), Toreegrasa et al. (2001) and Deng et al. (2009).

The adventitious shoots obtained in the present experiment were transferred to the ¾ MS + 1.722 µM IBA medium and they were rooted there. The plantlets thus obtained were acclimatized, transplanted to pots in the greenhouse. Their survival rate was as high as 90% suggesting that the whole experiment was successful.

In conclusion, we have found an optimal hormone combination and an ideal dark period for higher regeneration rate of explants of the grape 'Wink'. The shoots thus obtained displayed a high survival rate (Figure 1 J to L). Our findings might be of significance to the similar studies of other varieties of grapes, which is

Table 5. Callus induction and adventitious shoot regeneration response of the grape 'Wink' via *in vitro* internode explants as affected by thidiazuron (TDZ) and indole-3-butyric acid (IBA).

TDZ (µM)	IBA (µM)	Callus coverage (0-4)	Regeneration (%) (±SE)	Mean no. shoots (±SE)
4.55	0.05	4	10.23±1.25^{fg}	1.31±0.07^{e}
4.55	0.49	4	13.35±1.16^{e}	1.50±0.04^{e}
4.55	0.99	4	9.48±0.84^{fg}	1.15±0.11^{e}
4.55	1.48	4	0^{i}	0^{f}
4.55	2.47	4	0^{i}	0^{f}
9.10	0.05	4	25.48±2.77^{b}	3.46±0.17^{bc}
9.10	0.49	4	41.37±1.13^{a}	4.74±0.64^{b}
9.10	0.99	3-4	24.71±0.96^{b}	5.15±0.61^{a}
9.10	1.48	3-4	21.72±0.34^{cd}	3.20±0.29^{bc}
9.10	2.47	3-4	12.01±2.72^{ef}	2.25±0.39^{d}
13.65	0.05	3-4	23.30±1.40^{bc}	2.95±0.16^{cd}
13.65	0.49	3-4	25.81±1.57^{b}	3.30±0.44^{bc}
13.65	0.99	3-4	23.78±0.56^{bc}	3.23±0.76^{bc}
13.65	1.48	3-4	19.48±1.36^{d}	2.37±0.09^{d}
13.65	2.47	2-3	0^{l}	0^{f}
18.20	0.05	3-4	5.22±1.34^{h}	1.18±0.27^{e}
18.20	0.49	3-4	8.80±0.73^{g}	1.16±0.13^{e}
18.20	0.99	3-4	4.97±1.90^{h}	1.25±0.37^{e}
18.20	1.48	2-3	0^{i}	0^{f}
18.20	2.47	2-3	0^{l}	0^{l}

Classification of callus coverage on leaf explants: 0 = 0%, 1 = 25%, 2 = 50%, 3 = 75%, and 4 = 100%. Callus coverage observed on leaf explants for each plant growth regulator combination. Different letters in the same column denote significant differences at P<0.05 by Duncan's test.

Figure 1 G to F. Adventitious shoot regeneration from in vitro explants of the grape 'Wink'. G to I: The process of regeneration of the internode explant with the adaxial side in contact with the MS medium supplemented with 9.10 µM TDZ and 0.49 µM IBA and in dark for two weeks.

Table 6. Effects of dark periods on shoot organogenesis from leaf explants of grape 'Wink'.

Days in darkness(week)	TDZ (µM)	IBA (µM)	Regeneration (%) (±SE)	Mean no. shoots (±SE)
0	18.20	0.49	0^{d}	0^{c}
1	18.20	0.49	67.16±0.92^{c}	4.01±0.85^{b}
2	18.20	0.49	78.68±1.38 ^{a}	6.70±0.79^{a}
3	18.20	0.49	78..66±0.96^{a}	6.73±1.06^{a}
4	18.20	0.49	72.36±2.23^{b}	3.72±1.25^{b}

Different letters in the same column denote significant differences at P<0.05 by Duncan's test.

Figure 1 J to L. Adventitious shoot regeneration from *in vitro* explants of the grape 'Wink'. J to L: The process of transplant of the plantlets regenerated from the internode explant.

being confirmed by the study of the grape 'Manicure Finger' in our laboratory.

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