MICROPROPAGATION OF GRAPEVINE (VITIS VINIFERA L.) CVS. RED GLOBE AND SUPERIOR

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ABSTRACT

This study was conducted to develop a protocol for micropropagation of grapevine (*Vitis vinifera* L.) cultivars Red globe and Superior by investigating their response to three media Murashige and Skoog (MS), Chee and Pool (C2D), and Woody Plant Medium (WPM) during the establishment stage besides study the impact of four cytokinin types 6-benzylaminopurine (BA), 2-isopentyladenine (2iP), kinetin (Kin) and thidiazuron (TDZ) at various concentrations on the shoot multiplication as well as acclimatization of neoformed plantlets on several media. A completely randomized design (CRD) was used and data were analyzed by SASS 9.1. The results revealed that the WPM medium was optimum in terms of shoot length and leaf number (4.00 cm, 5.10) respectively. On the other side, Red globe gave the best results on both C2D and WPM media in average shoot length (4.38, 4.46 cm) and average leaf number (6.00, 5.42) respectively. In the multiplication stage and at 1 mgL^{-1}, BA showed the longest shoot and leaf number (2.3, 6.67) respectively. While kin gave the maximum shoot length with a mean of 3.57 cm. Moreover, BA significantly at 1 mgL^{-1}, 2iP at 2 mg L^{-1}, Kin at 5 mgL^{-1} , and TDZ at 1 mgL^{-1} gave the highest values compared to other concentrations. Plantlets acclimatization results revealed that sterilized 1:1:1 (v:v) peatmoss+perlite+sand mix gave the highest survival rate (100.00%) and showed the best vegetative growth.

Key words: in vitro, grapes, medium types, cytokinins, acclimatization.

الإكثار الدقيق للعنب (VITIS VINIFERA L.) صنفي ريد جلوب وسوبيريور

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مدرس

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المستخلص

أجريت هذه الدراسة بهدف تطوير بروتوكول للإكثار الدقيق لصنفي العنب، ريد جلوب وسوبيريور من خلال دراسة مدى استجابتهما لثلاثة أوساط نمو هي موارشيج و سكوج (MS)، تشي وبول (C2D) و وسط النباتات الخشبية (WPM) وذلك خلال مرحلة التأسيس إلى جانب دراسة تأثير أربعة أنواع من السيتوكينينات هي بنزيل أدنين (BA)، أيزو بينتيلادينين (2iP)، كينيتين (Kin) و ثيديازورون (TDZ) بتركيزات مختلفة في تضاعف الأفرخ، كما تم دراسة أقلمة النبيتات الناتجة على أوساط زراعة مختلفة. تم استخدام التصميم العشوائي الكامل (RCD) وتم تحليل البيانات باستخدام SASS 9.1 . أوضحت النتائج أن وسط WPM كان أفضل من حيث طول النموات وعدد الأوراق (4.00 سم، 5.10) على الترتيب. من ناحية أخرى، أعطى الصنف ريد جلوب أفضل النتائج على كل من الوسطين C2D و WPMبمتوسط طول الفرخ (4.38 ، 4.46 سم) ومتوسط عدد الأوراق (6.00 ، 5.42) على التوالي. في مرحلة التضاعف و عند تركيز 1 ملجم/ لتر، أعطى BA أعلى عدد من الأفرخ والأوراق (2.3 ، 6.67) على التوالي. بينما أعطى Kin أقصى طول للفرخ بمتوسط 3.57 سم. علاوة على ذلك أعطى BA عند 1 مجم/ لتر، 2iP عند 2 ملغم/ لتر، Kin عند 5 ملغم/ لتر و TDZ عند 1 ملغم/ لتر أعلى القيم مقارنة مع التركيزات الأخرى. أظهرت نتائج أقلمة النبيتات أن الخليط المعقم المكون من البيتموس + البيرلايت + الرمل 1: 1: 1 (حجم/حجم) أعطى أعلى نسبة بقاء للنبيتات (100.00%) وأفضل نمو خضري.

الكلمات المفتاحية: الإكثار بالأنابيب، العنب، الأوساط المغذية، سيتوكينينات، أقلمة

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INTRODUCTION

Grape is a worldwide crop with great applications in food and industries. Among thousands of grape cultivars, Red globe and Superior are used for both local market and export. Grapevine can be propagated in several ways, but probably dormant cuttings are usually used. However, this method of propagation is slow and not effective especially when there is a high demand by the growers. Tissue culture propagation or disease elimination technique is being widely used for large scale plant multiplication. Successful explants culture requires high-quality and dependable culture media with balanced nutrients that provide optimum growth. Media selection is considered very important in tissue culture for the establishment stage. MS medium had been widely used in many works. Mozafire et al **(**42) stated that MS medium was more proper than WPM medium for the growth and regeneration of explants of the studied grape cultivars. Kinfe et al(33) revealed that culturing on basal MS medium supplemented with 0.5 mgL^{-1} BAP was effective for shoot initiation. Other researchers Others (5) used MS medium in the micropropagation of four different rootstocks: Dogridge, SO4 (*V. berlandieri* x *V. riparia*), H-144 (*V. vinifera* × *V. labrusca*), and 3309 Couderc (3309C). (46) used MS medium for the propagation of new grape Sunder khani. Others(16) stated that using of MS medium improved the average weight of plants as well as WPM medium increased the number and length of roots. Krizan et al (35) studied *in vitro* propagation of grape rootstocks; Kober 5BB, Kober 125AA, and Teleki 5C. The results showed that using Driver and Kuniyuki Walnut medium for the establishing and multiplication cultures was better than MS medium. Others (7) used MS medium with 1mg L-1 BA for the propagation of *Vitis champini*. In another work, Lu (39) found that WPM medium was more proper for culturing of *Vitis thunbergii* than MS or NN (48). On the other side, several explants such as shoot apical meristems, axillary-bud, or adventitious buds have been used in the tissue culture studies (2 (2 , 32) developed a protocol for micropropagation of grapevine (*Vitis vinifera* L. cv Muscat of Alexandria) from shoot tips and internode segments.Kurme et al (36) showed that nodal segment explants were excellent for shoot proliferation and other micropropagation stages. Cytokinins play a vital role in the multiplication stage. Scientists are trying to find out the suitable cytokinin with the optimum concentration for each genotype. Others (25) reported that among other studied cytokinins (2ip, Kin and TDZ), culture medium supplemented with BA produced the best results especially at 6.67 and 8.9 μM. (30) have shown that BA and 1 mgL^{-1} IBA induced the best shoot formation of two Iranian grape cultivars, Bidane Sefid and Shahroodi. Khan et al (32) revealed that the best number of shoots was obtained on a medium supplemented with high levels of both BAP and NAA (1.5 and 0.5 mgL^{-1}, respectively).Other researchers Others (43) found that BAP at 1.0 mgL^{-1} added to MS medium showed the best proliferation level. Kurmi et al (36) reported that TDZ was found more effective at lower concentrations as compared to BA, and Kin due to its impact on the accumulation of internal cytokinins**.** Abido et al(2) illustrated that the best shoot proliferation was obtained on MS medium supplemented with 3.0 mgL^{-1} BAP + 0.2 mgL^{-1} NAA. This study aimed to develop a protocol for micropropagation of grapevine by investigating the performance of two grape cultivars Red globe and Superior during the establishment and micropropagation stages and to find out the best medium and proper cytokinin with an optimum concentration that achieves the best shoot proliferation.

MATERIALS AND METHODS

Plant materials and explants preparation One-year-old transplants were used as mother plants for two grape cultivars named Red globe and Superior as explant sources**.** Single node 1-2 cm in length from both cultivars were used as explants. The leaves were removed, and a short part of the petiole left. The explants were washed with water and a few drops of soap then put under a slow flow of tap water for an hour. Explants were surface sterilized in 1% sodium hypochlorite with 2-3 drops of Tween-20 for 10, 15, and 20 minutes as a surfactant. Then, the nodal cuttings were rinsed 3 times with sterilized water then

divided into separate stem node explants which were used as experimental materials.

The basal media

Three basal media are utilized in the micropropagation, MS (44), WPM (38), and C2D (10), and only MS medium within the multiplication stage. Carbon and energy source was Sucrose at 3% in all studied media and propagation stages. However, it had been adjusted to pH 5.8 ±1 before being solidified with purified Agar (Agar-Agar) at 7 g/l, then autoclaved at 100 psi and 121° C for 20 min. The hormonal supplements differed according to experiments. Glass jars (400 ml) contained nearly 40 ml medium per jar have been used during the microprpagation experiments. The glass jars were washed with detergent then soaked in 5% solution of sodium hypochlorite (NaOCl) for an hour then rinsed with water before using them.

Culture establishment

In order to find out the best basal medium for establishing the studied grape varieties, stem node explants of Superior, and Red Globe have been prepared and inoculated on three free hormone media at full strength MS, WPM, and C2D. Average shoot number, average shoot length (cm) and leaf number per were recorded after 4 weeks.

Shoot multiplication

Cytokinin type: The objective of this experiment was to determine the suitable cytokinin type for the multiplication of Red Globe and Superior. Well explants from the initiation stage were subcultured on full strength MS medium supplemented individually with BA (benzyl adenine), 2ip (Isopentylladenine), kin (6-furfurylaminopurine) and TDZ (Thidiazuron) at 1 mgL^{-1} for each in four treatments. The multiplication measurements were recorded 4 weeks after the subculture date.

Cytokinin concentration

This experiment was carried out to determine the best concentration of BA at 5 concentrations (0.2, 0.4, 0.6, 0.8 and 1 mgL^{-1}) ,2ip (Isopentylladenine) at 5 concentrations (1, 2, 3, 4 and 5 mgL^{-1}), Kin (6-furfurylaminopurine) at 5 concentrations (1, 5, 10, 15, and 20 mgL^{-1}) and TDZ (Thidiazuron) (0.5, 1, 2 and 3 mgL^{-1}) for the multiplication of the cultures of Red globe and Superior cultivars on full strength of MS medium. The multiplication measurements were recorded 4 weeks after subculture date.

Incubation conditions

The cultures were incubated at 25±2°C with 16 hours of light using fluorescent lamps (2 lamps per shelf) and 8 hours dark of 2000-2500 lux light intensity at cultures level.

Acclimatization

Neoformed plantlets were removed from the culture tubes then washed with running tap water to remove any medium residual then treated with a fungicide solution (Prochloraz 45% EC) $1gL^{-1}$, then potted into a mixture of Peatmoss, perlite, and sand 1:1:1 (v/v) and kept inside a glass chamber covered with transparent plastic in the incubation room for 4 weeks and watered constantly before being transferred to the glasshouse.

Data analysis

A completely randomized design (CRD) was used and data were analyzed using SASS 9.1 and means were compared by Duncan's multiple range test at $p \leq 0.05$ level of confidence**.** (14).

RESULTS AND DISCUSSION

Grape micropropagation

Establishment stage: Effect of surface sterilization duration and cultivars on contamination and survival explants percentages

The results presented in **Table 1** illustrate the effect of surface sterilization duration and cultivars on contamination and survival percentage. The results showed that there have been significant differences among the sterilization duration means in contamination percentage. It had been clear that the minimum contamination achieved in 10 min compared to the other durations and the maximum contamination percentage was found in 20 min. On the other hand, the results revealed that there was an insignificant difference between the cultivars' means in contamination percentage. Interaction between sterilization durations and cultivars was significant as the surface sterilization for 10 min obtained the minimum contamination percentage for both cultivars (10.00, 0.00%) respectively. On the contrary, the maximum contamination percentage was in 20 min for both cultivars (30.00%). On the other hand, the results have

shown that there were significant differences among the means of the sterilization duration and the studied cultivars in survival percentage. The maximum survival percentage was obtained in 10 min (96.94%), while there was an insignificant difference between the other durations. The results showed that there was a significant difference between the studied cultivars as Superior achieved the maximum survival percentage (91.07%). Moreover, the interaction between the sterilization duration and the cultivars was significant in survival percentage as the sterilization for 10 min achieved the maximum levels (93.88, 100.00%) respectively.

Table 1. Effect of duration of surface sterilization (min) using 1% Sodium hypochlorite (NaOCl) on contamination and survival percentages of the grape cultivars explants

Duration	Contamination %			Survival %		
	Re	Su	Mean	Re	Su	Mean
10 min	10.00c	0.00d	5.00C	93.88a	100.00a	96.94A
15 min	20.00b	20.00b	20.00B	86.50b	87.50b	87.00 B
20 min	30.00a	30.00a	30.00A	85.71b	85.71b	85.71B
Mean	20.00A	16.66A		88.69B	91.07A	

Re: Red globe, Su: Superior. Means followed by the same letter (s) in each column are not significantly different at p≤0.05 level.

The contamination with microorganisms extremely occurs in the plants growing in the external environment leading to plant tissue culture failure. These external contaminants can be easily removed by washing in running water and treating with surface sterilizing agents (40). *In vitro* propagation provides optimum conditions for fungus and bacteria growth. Therefore, failed sterilization obstructs the success of micropropagation studies (52). On the other side, species, cultivars, and tissue explants differ in their response to the same disinfection method because they have different sensitivity (41). It is well known that Sodium hypochlorite (NaOCl) has antimicrobial activity and effective for the disinfection of plants grown outside (15). In agreement, NaOCl solutions showed good results in several studies (2, 4, 28, 29). In the present study, using 1% NaOCl for 10 min revealed the best results for both Red globe and Superior explants that gave less percentage of contamination and more percentage of survivals that went in parallel with the findings of (6, 40) who reported that treating the explants with sodium hypochlorite+distilled water was more effective in reducing explants contamination. In the same direction, (33) stated that the sterilization of explants using 1% of NaOCl for 7 min duration was optimum. (54) reported that the surface sterilization regimes should aim to use the lowest concentration of NaOCl for the smallest amount of time. High concentrations and long application periods of disinfectants have a negative impact on the explants' growth (8, 52). (11) found that low concentrations of sodium hypochlorite increased the contaminated explants, whereas high concentrations of sodium hypochlorite made the explants lose their viability. Similar results were reported that a high concentration of sodium hypochlorite proved to be toxic resulting in 100% necrosis and death of explants (13). Probably the plant source and age of explants could be some causes of contamination in the obtained results.

Effect of medium types and cultivars on the criteria of establishment: Data in **Table 2** shows that there were no significant differences among the means of media, the means of cultivars, and the interaction between the media and the cultivars in shoot number per explants. Concerning the average proliferated shoot length, it was clear that WPM gave the best shoot length (4.00 cm), while MS medium gave the shortest shoot length with a significant differences (2.04 cm). In the same field, Red globe explants achieved the longest proliferated shoot length with significant differences compared to Superior (3.52 cm). On the other side, the interactions between the media and the cultivars were significant as Red globe explants gave the longest shoot length when cultured on both C2D and WPM (4.38, 4.46%), while Superior explants achieved the longest shoot length on WPM medium with a significant difference compared to MS and C2D. On the other hand, the results have shown that there were significant differences among means of studied media as WPM medium gave the

highest average of leaf number followed by C2D medium without significant difference between them (5.10, 4.97) respectively. It was observed that there is a significant differences between the cultivars' means in the proliferated leaf number as Red globe explants gave the maximum leaf number (5.22). Regarding the Interaction, Red globe cultivar gave on both C2D followed by WPM the highest leaf number without significant difference between them (6.00, 5.42) respectively compared to MS which gave the minimum leaf number. In the same way, Superior explants gave the highest leaf number on WPM medium with significant difference (4.74).

Table 2. Effect of medium type on the establishment criteria of Red globe and Superior grape cultivars

Medium type	Shoot number/explants			Shoot length (cm)			Leaf number/shoot		
	Re	Su	Mean	Re	Su	Mean	Re	Su	Mean
MS	1.00a	1.00a	1.00A	1.72c	2.36c	2.04C	4.24bc	3.38d	3.81C
C2D	1.00a	1.00a	1.00A	4.38a	2.00c	3.19B	6.00a	3.93cd	4.97AB
WPM	1.00a	1.00a	1.00A	4.46a	3.54b	4.00A	5.42ab	4.74bc	5.10A
Mean	1.00A	1.00A		3.52A	2.63B		5.22A	4.02B	

Re: Red globe, Su: Superior. Means followed by different letters are significantly different at p≤0.05 level

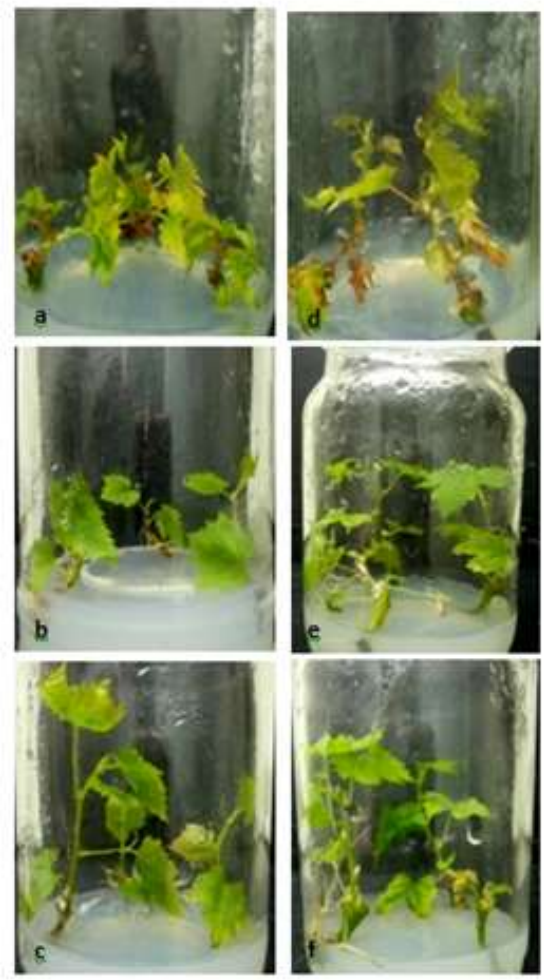

Figure 1. Superior and Red globe on different media during the establishment stage

a. Superior on MS. b. Superior on C2D. c. Superior on WPM. d. Red globe on MS. e. Red globe on C2D. f. Red globe on WPM

The purpose of *in vitro* culture medium is to provide optimum conditions for the explants That determined largely by physical and chemical factors, such as the composition of the nutrient medium (which includes growth regulators, salt, sugars, vitamins) pH, and its physical state in addition to light and temperature. Each species, cultivars, or even explants from different parts of the same plant may have different requirements for optimum growth. Bhan et al (7) used MS media with 1mgL^{-1} BA for the propagation of *Vitis champini*. It is also possible to find contradictory information from published articles. Lu (39**)** declared that WPM medium is more proper for *Vitis thunbergii* than MS or NN (48) which supports this result**.** The obtained results demonstrated that the composition of basal medium affects the growth performance of *in vitro* cultured grape cultivars. In general, it has appeared that the cultivars responded remarkably to lower basal salts in WPM which scored the highest shoot length and leaf number.

Multiplication stage

Effect of cytokinin types and cultivars on some proliferation characteristics during the first subculture: Data presented in **Table 3** indicates the effect of cytokinin type at the concentration of 1 mgl^{-1} and cultivars on some multiplication parameters of superior and Red globe grape cultivars during the first subculture. It was observed that there were significant differences among the means of

cytokinin types, as explants subcultured on BA medium achieved significantly the highest average shoot number (2.30) compared to other experimented cytokinins. On the other hand, the results showed that there was an insignificant difference between the means of Superior and Red globe cultivars in the average shoot number. Regarding the interaction, Red globe explants achieved the highest average shoot number when subcultured on BA medium followed Superior explants without a significant difference at the same medium, while the other treatments gave the lowest shoot number equally with no significant differences among them. Concerning the proliferated shoot length, the obtained results indicated that there was a significant effect of Kin which gave the highest shoot length (3.57 cm) compared to other cytokinins, while 2ip gave the lowest shoot length (2.29 cm). Regarding the cultivars effect, no significant differences were observed between the two studied cultivars in this parameter. Concerning the interaction, Superior explants on Kin medium gave the longest proliferated shoots followed by the Red globe ones on the same medium without significant difference between them (3.80, 3.34 cm) respectively, while the explants of the two studied cultivars gave the shortest proliferated shoots on 2ip medium insignificantly compared to BA and TDZ media and between them. Regarding the average leaf number, it was obvious that the proliferation shoots on BA gave significantly the highest average number of leaves (6.67) followed by those of Kin medium then finally those of 2ip and TDZ media insignificantly between them. Regarding the cultivars, the results showed that an insignificant difference was recorded between Superior and Red globe cultivars in average leaf number. Concerning the interactions, BA achieved the highest leaf number in Red globe followed by Superior without a significant difference between them (7.07, 6.27) respectively.

Table 3. Effect of cytokinin types at concentration of 1 mgL^{-1} and cultivars on some multiplication parameters

Cytokinin type	shoot number/explants			Shoot length (cm)			leaf number/shoot		
	Re	Su	Mean	Re	Su	Mean	Re	Su	Mean
BA	2.57a	2.02ab	2.3A	2.91bc	2.83bc	2.87B	7.07a	6.27ab	6.67A
2ip	1.00c	1.00c	1.00B	2.25c	2.21c	2.29C	4.75cd	4.5d	4.63C
Kin	1.00c	1.00c	1.00B	3.34ab	3.80a	3.57A	5.58bc	5.33bcd	5.46B
TDZ	1.00c	1.00c	1.00B	2.75bc	2.88bc	2.81B	4.84cd	4.67cd	4.75C
Mean	1.39A	1.26A		2.81A	2.93A		5.56A	5.19A	

Re: Red globe, Su: Superior .Means followed by the same letter (s) in each column are not significantly different at p≤0.05 level.

Figure 2. Red globe on cytokinin types in the multiplication stage.
a. Red globe on BA. b. Red globe on 2ip. c. Red globe on Kin. d. Red globe on TDZ

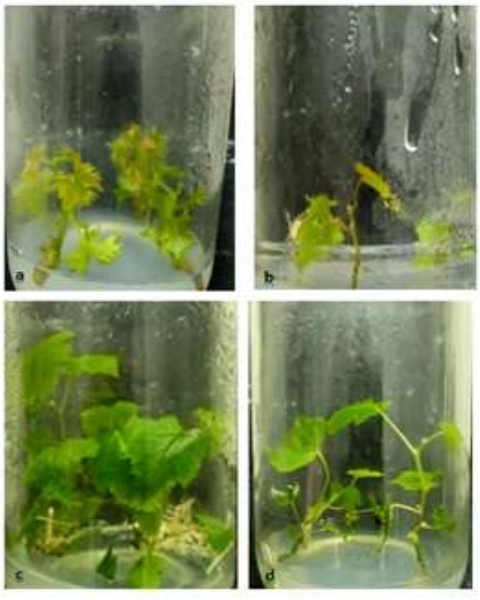

Figure 3. Superior cultivar on cytokinin types in the multiplication stage
a. Superior on BA. b. Superior on 2ip. c. Superior on Kin. d. Superior on TDZ

Effect of Benzyl adenine (BA) concentrations on some proliferation haracteristics of the cultivars during the first subculture: Table 4 illustrates the effect of BA on the shoot number, shoot length, and leaf number per shoot of Superior and Red globe cultivars. The results showed that BA addition to the multiplication medium induced the explants to form callus masses and gave abnormal and hyperhydrated shoots. However, there were significant differences among the means of BA concentrations in the shoot number per explants. It was clear that 1 mgL^{-1} gave significantly the highest number of shoots per explants (2.30) compared to control and other BA concentrations. On the other side, there were insignificant differences between means of Superior and Red globe in this parameter. Interaction between BA concentrations, and cultivars had significant differences and appeared clearly in Red globe explants when cultured on MS medium supplemented with 1 mgL^{-1} BA (2.57). Similarly, Superior explants gave the highest number of shoots on 1 mgL^{-1} BA (2.02), while no significant differences were recorded among other concentrations. The results revealed that there were significant differences among means of BA concentrations in shoot length which appeared obviously in 1 mgL^{-1} BA concentration that gave the longest shoots per explants (2.87 cm), while no significant differences appeared among other BA concentrations. Regarding means of cultivars, Superior scored the longest shoot length (2.08 cm) followed by Red globe (1.99 cm) without any significant differences between them. Concerning the interaction, the results showed that Red globe and Superior gave the highest shoot length on 1 mgL^{-1} BA (2.91, 2.83 cm) respectively without a significant difference between them. It was clearly shown that there were significant differences among means of BA concentrations in average leaf number as the maximum leaf number was achieved on 1 mgL^{-1} BA (6.90). On the other side, no significant differences were observed between Red globe and Superior in leaf number. Interaction between BA concentrations and the cultivars was significant and appeared clearly in Red globe on 1 mgL^{-1} BA (7.10) which gave the maximum leaf number followed by Superior on 1 mgL^{-1} BA (6.69) without any significant difference between them.

Table 4. Effect of Benzyl adenine (BA) concentrations on some proliferation characteristics of the studied cultivars

BA mgL^{-1}	Average proliferated shoot number/explants			Average proliferated shoot length (cm)			Average leaf number/ proliferated shoot		
	Su	Re	Mean	Su	Re	Mean	Su	Re	Mean
0.0	1.00c	1.00c	1.00C	2.13cd	1.71cdef	1.92B	4.72cd	4.25cd	4.49C
0.2	1.25c	1.25c	1.25BC	1.30f	1.25f	1.75BC	3.29e	4.33cd	3.81C
0.4	1.17c	1.25c	1.21BC	1.59def	1.73cdef	1.66BC	3.84de	4.75cd	4.30C
0.6	1.17c	1.17c	1.17BC	2.18cd	1.97cde	2.08B	5.04cd	5.52bc	5.28B
0.8	1.08c	1.25c	1.16BC	2.42b	2.36b	2.39B	4.56cd	5.3cd	4.93B
1.0	2.02ab	2.57a	2.30A	2.83ab	2.91a	2.87A	6.69ab	7.10a	6.90A
Mean	1.28A	1.42A		2.08A	1.99A		4.69A	5.21A	

Re: Red globe, Su: Superior .Means followed by the same letter (s) in each column are not significantly different at p≤0.05 level.

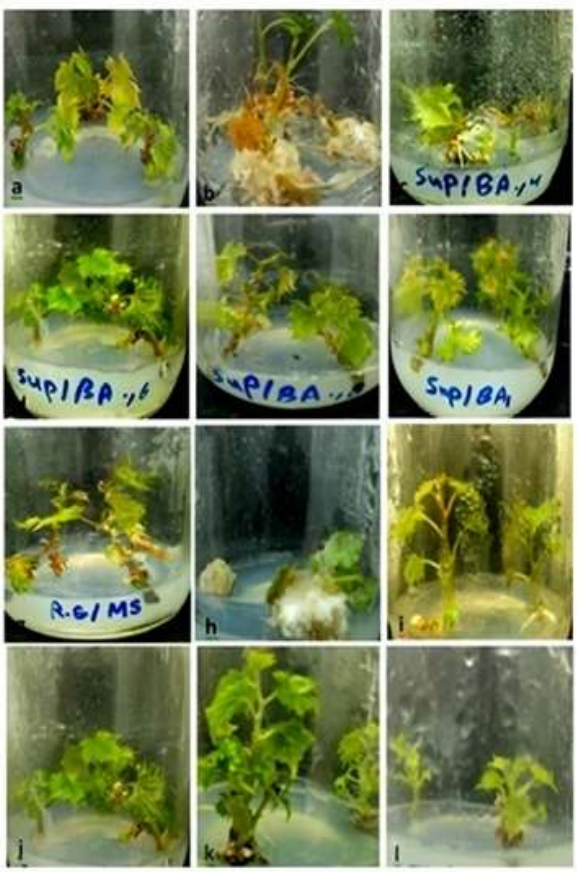

Figure 4. Superior and Red globe on BA concentrations in the multiplication stage

a. Superior on 0 mgL^{-1}. b. Superior on 0.2 mgL^{-1}. c. Superior on 0.4 mgL^{-1}. d. Superior on 0.6 mgL^{-1}. e. Superior on 0.8 mgL^{-1}. f. Superior on 1 mgL^{-1}. g. Red globe on 0 mgL^{-1}. h. Red globe on 0.2 mgL^{-1}. i. Red globe on 0.4 mgL^{-1}. j. Red globe on 0.6 mgL^{-1}.k. Red globe on 0.8 mgL^{-1}. l. Red globe on 1 mgL^{-1}

Effect of Isopentyladenine (2ip) concentrations on some proliferation characteristics of the cultivars during the first subculture

2ip concentrations affected remarkably the studied proliferation characteristics of Superior and Red globe as shown in **Table 5**. It was obvious that 2ip at all concentrations did not have any effect on the average shoot number for both cultivars. Also, the results showed that there were not any significant differences between the means of Superior and Red globe and the interactions among the 2ip concentrations and the cultivars in this parameter. On the other side, there was the remarkable effect of 2ip at 2 mgL^{-1} on the average shoot length compared to other concentrations (2.94 cm) and it is worth mentioning that increasing 2ip concentration above 2 mgL^{-1} resulted in decrease in average shoot length. Concerning the means of Superior and Red globe, there were no significant differences between them in average shoot length. Regarding the interactions, both Red globe and Superior subcultured on MS contains 2 mgL^{-1} achieved significantly the maximum average of shoot length (3.00, 2.87 cm) respectively. It was clearly shown that no significant differences were observed between Superior and Red globe means of average leaf number, while 2 mgL^{-1} gave significantly the highest leaf number (6.10) compared to other concentrations. Concerning the interactions, Red globe and Superior explants achieved significantly the maximum leaf number at 2 mgL^{-1} 2ip (6.33, 5.83) respectively, while the results showed insignificant differences among the cultivars and other 2ip concentrations.

Table 5. Effect of Isopentyladenine (2ip) concentrations on some proliferation characteristics of the studied cultivars

2ip mg L^{-1}	Average proliferated shoot number/explants			Average proliferated shoot length (cm)			Average leaf number/ proliferated shoot		
	Su	Re	Mean	Su	Re	Mean	Su	Re	Mean
0	1.00a	1.00a	1.00A	2.13b	1.71c	1.92BC	4.72bc	4.25bcd	4.49B
1	1.00a	1.00a	1.00A	2.20b	2.25b	2.23B	4.50bcd	4.75bc	4.63B
2	1.00a	1.00a	1.00A	2.87a	3.00a	2.94A	5.83a	6.33a	6.10A
3	1.00a	1.00a	1.00A	1.66c	1.75c	1.71C	4.10bcd	3.91bcd	4.00B
4	1.00a	1.00a	1.00A	1.70c	1.67c	1.69C	4.91b	3.67d	4.29B
5	1.00a	1.00a	1.00A	1.57c	1.70c	1.64C	4.75bc	3.83cd	4.29B
Mean	1.00A	1.00A		2.02A	2.01A		4.80A	4.47A	

Re: Red globe, Su: Superior . Means followed by the same letter (s) in each column are not significantly different at p≤0.05 level

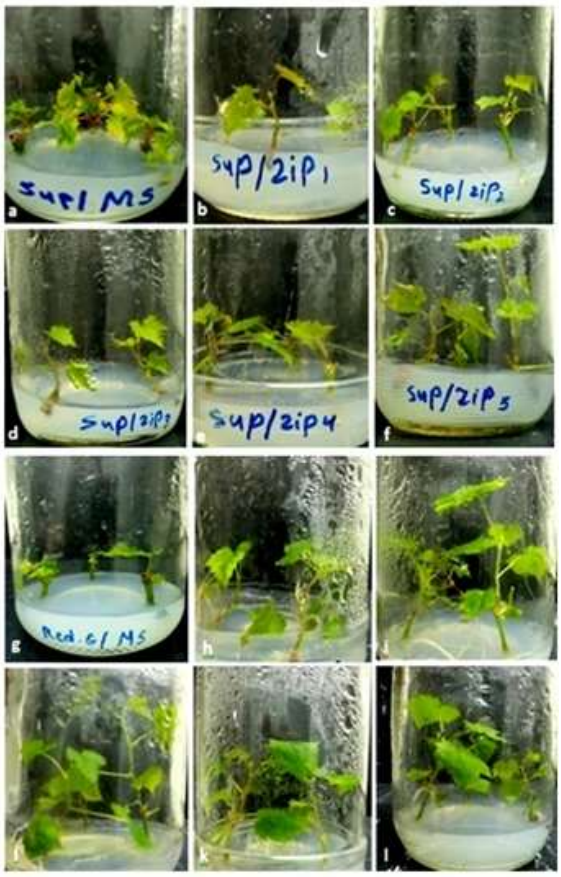

Figure 5. Superior and Red globe on 2ip concentrations in the multiplication stage
a. Superior on 0 mgL^{-1}. b. Superior on 1 mgL^{-1}. c. Superior on 2 mgL^{-1}. d. Superior on 3 mgL^{-1}. e. Superior on 4 mgL^{-1}. f. Superior on 5 mgL^{-1}. g. Red globe on 0 mgL^{-1}. h. Red globe on 1 mgL^{-1}. i. Red globe on 2mgL^{-1}. j. Red globe on 3 mgL^{-1}. k. Red globe on 4 mgL^{-1}. l. Red globe on 5 mgL^{-1}

Effect of Kinetin (6-furfurylaminopurine) concentrations on some proliferation characteristics of the cultivars during the first subculture

The results presented in **Table 6** show the effect of Kin concentrations on some proliferation characteristics of Superior and Red globe. Results revealed that increasing Kin concentrations did not affect the average shoot number. In addition, no significant differences were recorded between means of Superior and Red globe cultivars in average shoot number, besides the interactions which were not significantly appeared in shoot number. On the other side, average shoot length increased remarkably in parallel with increasing Kin concentration from 1 to 5 mgL^{-1} which was the best in 5 mgL^{-1} (4.33 cm) then decreased obviously and constantly by increasing Kin concentration above 5 mgL^{-1} and was at a minimum level at 20 mgL^{-1} Kin. Concerning the interactions, Superior gave significantly the longest shoots at 5 mgL^{-1} (4.42 cm) followed by 1 mg L-1 without a significant difference between them while the shortest shoots were at 20 mgL^{-1} (0.68 cm). The same results were recorded in Red globe which gave the longest shoots at 5 mgL^{-1} (4.23 cm) and the shortest shoots at 20 mg L^{-1}. It was clearly shown that there were significant differences among means of Kin concentrations regarding average leaf number per proliferated shoot. The concentration of 5 mgL^{-1} seemed to be better than other Kin concentrations that gave significantly the maximum leaf number (5.67) followed by 1 mgL^{-1} (5.46) without a significant difference between them while the minimum leaf number was observed in 20 mgL^{-1} (2.04). On the other hand, no significant difference was recorded between means of Superior and Red globe in average leaf number. Concerning the interactions, Red globe and Superior stem pieces at 5 and 1 mgL^{-1} achieved significantly the highest leaf number (5.79, 5.58) and (5.54, 5.33) respectively while the lowest leaf number was at 20 mgL^{-1} (1.91, 2.17) respectively.

Table 6. Effect of Kinetin (kin) concentrations on some proliferation characteristics of the studied cultivars

Kin mg L^{-1}	Average proliferated shoot number/explants			Average proliferated shoot length (cm)			Average leaf number/ proliferated shoot		
	Su	Re	Mean	Su	Re	Mean	Su	Re	Mean
0	1.00a	1.00a	1.00A	2.13cd	1.71cd	1.92C	4.72ab	4.25b	4.49B
1	1.00a	1.00a	1.00A	3.80ab	3.33b	3.57B	5.33a	5.58a	5.46A
5	1.00a	1.00a	1.00A	4.42a	4.23ab	4.33A	5.54a	5.79a	5.67A
10	1.00a	1.00a	1.00A	2.46c	2.42c	2.44C	4.10b	4.08b	4.09B
15	1.00a	1.00a	1.00A	1.33de	1.20de	1.27D	3.00c	3.00c	3.00C
20	1.00a	1.00a	1.00A	0.68e	0.58e	0.63E	2.17cd	1.91d	2.04D
Mean	1.00A	1.00A		2.47A	2.25A		4.14A	4.10A	

Re: Red globe, Su: Superior . Means followed by the same letter (s) in each column are not significantly different at p≤0.05 level

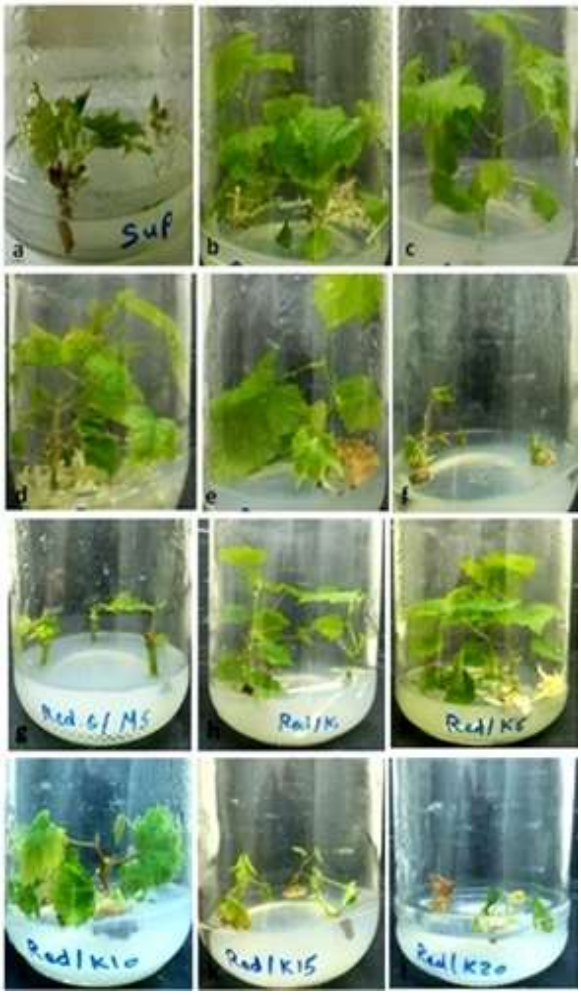

Figure 6. Superior and Red globe on Kin concentrations in the multiplication stage

a. Superior on 0 mgL^{-1}. b. Superior on 1 mgL^{-1}. c. Superior on 5 mgL^{-1}. d. Superior on 10 mgL^{-1}. e. Superior on 15 mgL^{-1}. f. Superior on 20 mgL^{-1}. g. Red globe on 0 mgL^{-1}. h. Red globe on 1 mgL^{-1}. i. Red globe on 5 mgL^{-1}. j. Red globe on 10 mgL^{-1}. k. Red globe on 15 mgL^{-1}. l. Red globe on 20 mgL^{-1}

Effect of Thidizuron (TDZ) concentrations on some proliferation characteristics of the cultivars during the first subculture

The results in **Table 7** shows that an insignificant effect of TDZ was recorded on the average shoot number at all studied concentrations, and the same results obtained on means of Superior and Red globe in addition to the interactions among the TDZ concentrations and the cultivars. On the other side, the TDZ effects were clearly shown on the average shoot length which quite appeared at 1 mg L^{-1} (2.84 cm) while 3 mgL^{-1} TDZ gave the shortest shoots compared to other concentrations (1.08 cm). Concerning the interaction, Superior at 1 mgL^{-1} scored significantly the longest shoots (3.17 cm) followed by Red globe (2.50 cm) without significant difference between them. While 3 mgL^{-1} gave the lowest shoot length for both cultivars. Regarding the average leaf number, the results showed that 1 mgL^{-1} TDZ achieved significantly the highest average leaf number (4.67) compared to other concentrations. On the other side, no significant differences were recorded between means of Superior and Red globe. Concerning the interactions, it was clearly shown that Superior gave significantly the maximum average leaf number when subcultured on MS medium contains 1 mg L^{-1} TDZ (5), while 3 mgL^{-1} gave the lowest leaf number in both cultivars (3, 3.34) respectively. It is worth mentioning that TDZ significantly had a good effect at low concentrations on the average shoot length, average leaf number in addition to the length and thickness of the roots and it seemed that 1 mgL^{-1} TDZ was optimum.

Table7. Effect of Thidizuron (TDZ) concentrations on some proliferation characteristics of the cultivars during the first subculture

TDZ mgL^{-1}	Average proliferated shoot number/explants			Average proliferated shoot length (cm)			Average leaf number/ proliferated shoot		
	Su	Re	Mean	Su	Re	Mean	Su	Re	Mean
0.0	1.00a	1.00a	1.00A	2.13ab	1.71bc	1.92B	4.72ab	4.25bc	4.49AB
0.5	1.00a	1.00a	1.00A	1.84bc	1.75bc	1.79B	3.25cd	4.42bc	3.84BC
1.0	1.00a	1.00a	1.00A	3.17a	2.50ab	2.84A	5.00a	4.34bc	4.67A
2.0	1.00a	1.00a	1.00A	1.58bc	1.21c	1.4CB	4.00bc	3.50cd	3.75BC
3.0	1.00a	1.00a	1.00A	1.13c	1.04c	1.08C	3.00c	3.34cd	3.17C
Mean	1.00A	1.00A		1.97A	1.64A		3.99A	3.97A	

Re: Red globe, Su: Superior. Means followed by the same letter (s) in each column are not significantly different at p≤0.05 level

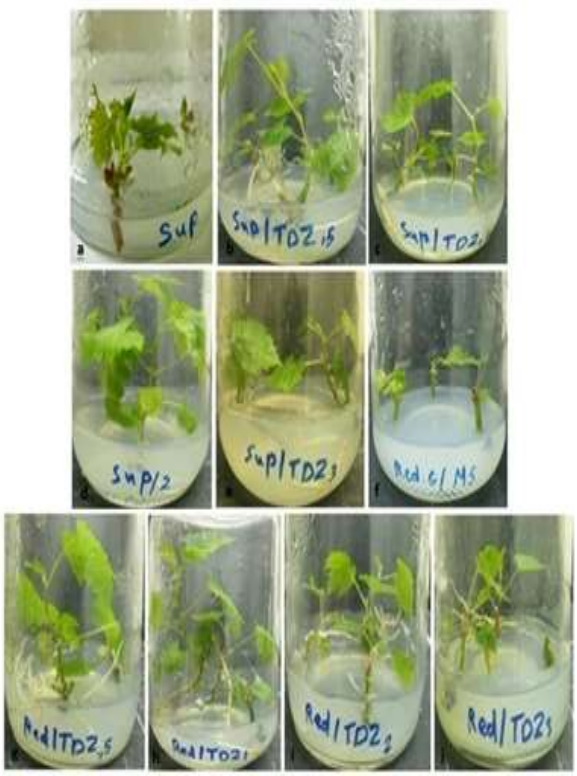

Figure 7. Superior and Red globe cultivars on TDZ concentrations in the multiplication stage

a.Superior on 0 mgL^{-1}. b. Superior on 0.5 mgL^{-1}. c. Superior on 1 mgL^{-1}. d. Superior on 2 mgL^{-1}. e. Superior on 3 mgL^{-1}. f. Red globe on 0 mgL^{-1}. g. Red globe on 0.5 mgL^{-1}. h. Red globe on 1 mgL^{-1}. i. Red globe on 2 mgL^{-1}. j. Red globe on 3 mgL^{-1}

It might be concluded that cytokinins used in the previous experiments affected the growth and development of cultivars' stem nodes. BA gave the highest average of shoot number significantly compared to 2ip, Kin, and TDZ at an equivalent concentration. This finding might be attributed to the mode of action of BA on stimulation both cellular division and enhancement growth of shoots in plant tissue culture as reported by (17, 20). In the earlier studies, BA was also superior for shoot proliferation in grape (5, 12, 41, 51). (39) mentioned that BA was significantly more efficient than 2ip or Kinetin at an equivalent concentration for promoting growth, which resulted in a three to four-fold shoot increase. The undertaken results revealed that BA increased the average number of shoots remarkably with the increasing of BA concentration in the medium. However, it seemed that 1 mgL^{-1} for cultivars that achieved the highest average number of shoots, highest shoot length, and highest average of leaf number.Azami (1) revealed that BA at 1.5 mgL^{-1} had good results within the multiplication stage in both Soltanin' and 'Sahebi' cultivars. (30) declared that 1 mgL^{-1} BA induced the best shoot proliferation of two Iranian grape cultivars, which was in accordance with the present findings. On the other side, BA forced the explants to form callus masses and short shoots on them had hyperhydricity symptoms even at low concentrations which are not recommended for multiplication. The most role of BA was known, however, the mechanisms behind BA requirement have not been understood yet. BA may have an essential role in callus growth by organizing antioxidant enzyme activities (49). Low concentrations of cytokinins like BA and kinetin together with auxins were often used to enhance callus initiation (9). Hyperhydricity of micropropagated shoots resulted from several factors like growth and culture conditions, stress conditions, wounding, and soft culture media. Overall, hyperhydricity might be created by the high ionic strength, with high level of nitrogen and growth regulators combined with a humid and gaseous atmosphere (31). Kin and TDZ had good results on shoots and leaf number in the cultivars at 1 mgL^{-1} compared to BA and 2ip. However, Kin had a good impact at high concentrations that at 5 mgL^{-1} achieved shoots with good appearance and color as gave the highest average shoot length and leaf number. Poudel (49) stated that kinetin was effective on two grape cultivars. This might be associated with the cultivars, as plant growth regulators have a different impact on *in vitro* propagation of various cultivars. Plant growth regulators effective for a cultivar might not be effective for others (49, 51). TDZ was found more effective at low concentrations within the range of 0.5-1.0 mgL^{-1} as compared to BA and Kin (they were found more effective in the range of 1.0 -5.0 mgL^{-1}). The effectiveness of TDZ at a low level perhaps was related to encouraging the accumulation of internal cytokinins (45). In the present study, TDZ at concentrations further than 1.0 mgL^{-1} did not have an acceptable response when supplemented into MS medium as gave thick and short shoots. This result goes along with the findings of (24) who stated that TDZ at high concentrations affects shoot elongation.

Others (21) reported that shoot number and length decreased at the highest concentration of TDZ and causing thickening of the stems. However, the real mechanism of TDZ enhanced shoot proliferation in plants is not clear so far. The use of 2iP only resulted in the production and growth of one shoot per sprouted bud. These shoots were longer than those obtained with BA and less in length to those obtained with Kin and TDZ. It seemed that 2 mg L-1 was optimum for both the cultivars compared to other concentrations. This study revealed that increasing the concentration of 2iP more than the optimal level (2 mg L^{-1}) resulted in low shoot length frequency at 4 mgL^{-1} in agreement with (49).

Effect of subculture number and cultivars on the multiplication characteristics

Table 8 exhibits the effect of subculture number and cultivars on the multiplication characteristics of Superior and Red globe. It is obvious that there were insignificant differences among the three subcultures in average shoot number per explants, the cultivars, and the interaction between the two factors. On the other side, the results showed that there were significant differences among means of subcultures number in average shoot length and appeared clearly in the 1st subculture which surpassed significantly compared to other subcultures (4.14 cm) then the values decreased gradually by the 2nd and 3rd subcultures. Superior achieved significantly the longest shoots (3.15 cm) in comparison to Red globe cultivar. Regarding the interaction, Superior and Red globe achieved the longest shoots at the 1st subculture (4.04, 4.23 cm) respectively, while the lowest value in Superior was in the 3rd subculture and in the 2nd subculture in Red globe (2.38, 1.25 cm) respectively. In terms of average leaf number, the highest values were significantly in the 1st subculture (5.40) then gradual reduction occurred in the 2nd and 3rd subcultures. On the other hand, insignificant differences were observed between the studied cultivars in this parameter although the average leaf number was higher in Superior to in Red globe. Interaction between the two factors revealed that Red globe achieved significantly the highest values in the 1st subculture (5.79) whereas, no significant differences were recorded between Superior and the subculture number in this parameter although the highest average leaf number was in the 1st subculture.

Table 8. Effect of subculture number and cultivars on the multiplication characteristics

Sub-culture	Average proliferated shoot number/explants			Average proliferated shoot length (cm)			Average leaf number/ proliferated shoot		
	Su	Re	Mean	Su	Re	Mean	Su	Re	Mean
1	1.00a	1.00a	1.00A	4.04ab	4.23a	4.14A	5.00ab	5.79a	5.40A
2	1.00a	1.00a	1.00A	3.04bc	1.25e	2.15B	4.67abc	3.58c	4.13B
3	1.00a	1.00a	1.00A	2.38cd	1.74de	2.06B	4.17bc	3.83bc	4.00B
Mean	1.00A	1.00A		3.15A	2.41B		4.61A	4.40A	

Re: Red globe, Su: Superior. Means followed by the same letter (s) in each column are not significantly different at p≤0.05 level

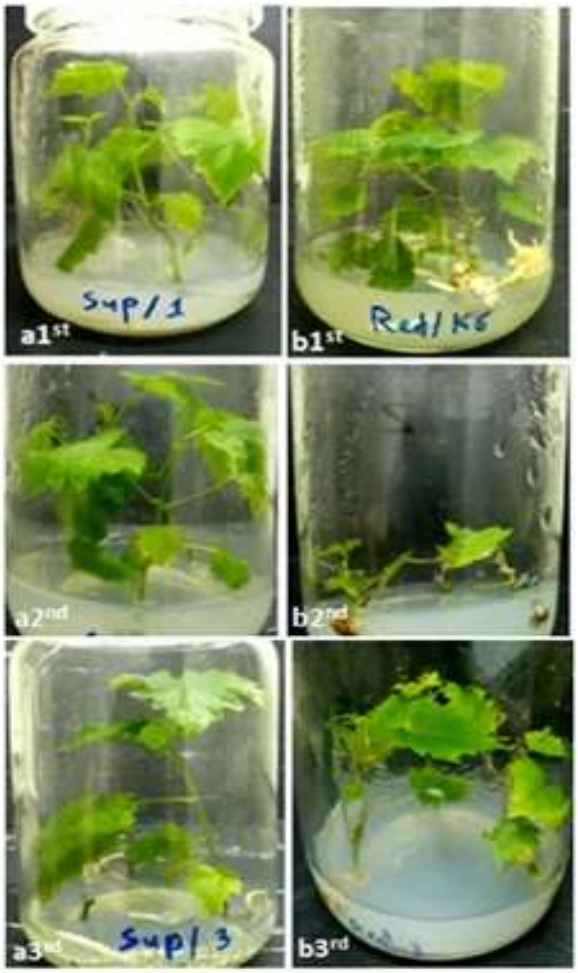

Figure 8. Effect of subculture number on the multiplication rate of the cultivars
a. subcultures in Superior. b. subcultures in Red globe

Successive subcultures of stem nodes cultured on a medium supplemented with Kin at 5 mg L-1 in order to know the number of subcultures might be obtained with a high number of shoots without affecting their appearance and quality. It might be concluded from the previous results that the average shoot number remained stable during three subcultures. The 1st subculture had the highest shoot length and leaf number. The results showed a sharp decrease in shoot length and leaf number from the 1st to 3rd subculture. The obtained shoots were of excellent appearance in terms of colour and size. The 4th subculture in the cultivars showed callus massed formation and hyperhydricity symptoms therefore the experiment was stopped. The observed hyperhydricity might be as consequence of various factors like cytokinin concentration, ethylene presence, the humidity inside the culture jar, the agar quality, the transfer time. (53) revealed that the appearance of hyperhydric shoots was done by increasing the number of transfers more than three, and the propagation capacity of the cultures was reduced obviously. The successive subcultures of Refosk cultivar explants in a medium supplemented with cytokinin led to shoot elongation inhibition (34). In parallel with the results obtained, it might be concluded that the number of subcultures could be shortened to three. On the other side, different concentrations of Kin might increase the subcultures (3, 23), as well as utilizing different cytokinin content of propagation medium during the subcultures (19).

Acclimatization of the cultivar plantlets

Effect of soil mixture on the acclimatization success of Red globe plantlets: The survival rates of Red globe and Superior plantlets after acclimatization planted on different artificial soil mixtures have been recorded**.** It is clear that the plantlets affected by the characteristics of the mixture, which the sterilized 1:1:1 (v:v) peatmoss+perlite+sand mix were found to be effective of *in vitro* plantlets hardening which significantly gave the highest survival (100%) and showed superior vegetative growth compared to other soil mixtures whereas the lowest rate was in 1:1 (v:v) peatmoss+perlite (50%). mixture of organic matter with peatomoss was extremely had a strong impact on the plantlets' survival, whereas, the sand with peatmoss as 1:1 (v:v) may add an addition by modifying the mixture and improved the survival rate (80%)**.** Immediately after transplantation, visible wilting was observed. However, the water content of plants might still stable after some days or weeks**.** Potting media have different physical, and chemical properties and therefore the plantlet survival and growth may be a reflection of how the mixing ratio affected the physical and chemical property of the mix. Therefore, Different workers have suggested different media like soil-vermiculite mixture (18), soil (37), and sand-peatmoss (22). Among various potting mixtures tried, the mixture containing sand+peatmoss+perlite (1:1:1) was found to be the most suitable. The establishment of *in vitro* produced plantlets is really affected by the properties of the potting mixture (27). The better performance of peatmoss could be attributed to its ability to improve the

biological properties of the mixture. Sand could also be responsible for providing sufficient aeration and high water holding capacity besides satisfied water drainage and good ventilation providing by perlite. Hence, mixing sand, peatmoss, and perlite in equal volumes might have helped in giving a better hold for the roots, good ventilation and sufficient organic matter. Similar results have also been obtained by (22, 47, 50).

Figure 9. Plantlets of Red globe planted on peatmoss+perlite+sand mixture (1:1:1) 30 days after the acclimatization

Figure 10. Plantlets of Superior one month after the acclimatization

Figure 11. Red globe and Superior planted in plastic bags in the glasshouse

CONCLUSION

The results of this study showed that 10 min of sterilization in 1% NaOCl was effective for sterilization. The best medium for the average number of both shoots and leaves was WPM medium (4.00 cm, 5.10) respectively. In the multiplication stage and at 1 mgL^{-1},BA showed the highest shoot and leaf number (2.3, 6.67) respectively. While, kin gave the maximum shoot length with a mean of 3.57cm. Regarding cytokinin concentrations, BA significantly at 1 mgL^{-1}, 2iP at 2 mgL^{-1}, Kin at 5 mgL^{-1} and TDZ at 1 mgL^{-1} gave the highest values. The acclimatization results revealed that sterilized1:1:1(v:v) peatmoss+perlite+sand mix gave the highest survival rate (100.00%) and showed the best vegetative growth.

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