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In Vitro Propagation of Two Grapes, *Vitis Vinifera* L. cvs. Superior And Red Globe.

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A B S T R A C T

The study aimed to investigate PGR's effect on *in vitro* grapes *Vitis Vinifera* L. cvs. Superior and Red globe single nodes propagation. During the surface sterilization stage (10, 20, and 30%), commercial bleach solution was used as a disinfectant agent for (5, 10, and 15 minutes) immersion periods. In the multiplication stage, explants were planted in Murashige and Skoog (MS) medium enriched with (1, 2, 3, and 4 mg L^{-1}) of Benzyl Adenine (BA) concentrations and the control treatment was (BA-free). The rooting stage included cultivating plantlets from the multiplication stage on half-salt strength MS media supplied with (0, 0.5, 1, and 1.5 mg L^{-1}) indole-3- butyric acid (IBA). The results showed that the highest percentage of uncontaminated explants was achieved in 20% of commercial bleach solutions used for a 10-minute immersion period (90 and 80% for Superior and Red Globe cultivars, respectively). The results of the multiplication stage showed that the highest values of newly formed shoots number and leaves number (4.50 and 20.90, respectively, for the Superior) and (4.10 and 18.50, respectively, for the Red Globe) were recorded in media containing 2 mg L^{-1} BA. The Superior variety showed the highest rooting percentage (90%) and significant root number (3.55) in a medium containing 1 mg L^{-1} IBA, while the Red Globe variety had the highest percentage (100%), and the root number (3.50) in the medium contained 0.5 mg L^{-1}. The results of this study provide a rapid and effective propagative propagation system for selected grapes.

Introduction

Grape *Vitis vinifera* L. is a perennial plant that grows in tropical, subtropical, and temperate regions [1]. belongs to the *Vitaceae* family and the genus Vitis and is classified within European grapes under the *V. vinifera* Sativa subspecies, which includes all cultivated varieties. These varieties are sourced from the direct wild grapes selection in different regions or the subsequent hybridization of cultivated varieties with each other or as a result of mutations [2]. Almost all grapes' varieties are propagated vegetatively by cuttings, layering, or grafting, but this does not prevent the cultivated varieties from being exposed to disease-causing agents (microbes, mites, insects, nematodes, fungi, bacteria, viruses, and, most notably the phylloxera [3]. Although commercially exploited, these vegetative methods are stressful, time-consuming, and highly season-specific. A nursery using woody cuttings for propagation may produce about ten plants per year from a single vine [4].

In vitro propagation techniques have several advantages, such as the possibility of large-scale production of desired genotypes that can be acclimatized quickly at low cost [5]. Moreover, in vitro propagation allows the production of high-quality, disease and pathogens-free plants in large quantities, in a limited space, and in a short time, without being restricted to the season [6]. Several authors have indicated that the *in vitro* propagation efficiency of *Vitis spp* can vary significantly depending on interactions between the species or genotype and the plant growth regulators type or concentration used [7][8].

Numerous studies have been conducted on grapes micropropagation over the past years and worldwide on selected genotypes of the genus Vitis using the cultivation of developing apices, axillary buds, or adventitious buds' formation. [9] studied the effect of different types of cytokinins (Benzyl adenine (BA), isopentenyl adenine 2ip, kinetin, and thiadazuron (TDZ)) in *in vitro* single nodes propagation of two grape varieties (Superior and Red globe), results showed that the largest shoots number (2.02 and 2.57) for both cultivars, respectively, were obtained when 1 mg L^{-1} BA was used, Also, the largest number of leaves/shoots was obtained in the same treatment, with an average leaves number (6.27 and 7.07 leaves), respectively. [5] used solid MS media supplemented with 0.2 mg L-1 benzyl adenine BA for the multiplication stage of grapes Traminer Pink, where the biomass of explant increased from 40 to 60 mg. in the multiplication stage shoot tops and lateral buds of two grape varieties (Bart and Augustine). [11] observed that the addition of plant growth regulators to the MS medium is an important factor for multiplying the branches, mainly when lateral buds are used, so the addition of BA It led to an increase of 5-6 times in the rate of the number of new shoots formed. This study aimed to develop an effective and efficient protocol for propagating Superior and Red Globe grape varieties using tissue culture techniques, PGR Benzyl Adenine (BA), and indole-3- butyric acid (IBA).

Materials and Methods

The study was conducted in the plant cell and tissue culture laboratory / Department of Horticulture / Agriculture Engineering Science College / Dohuk University / Kurdistan Region of Iraq.

Preparing the nutrient media:

The nutritional medium MS [10] (prepared by Caisson LTD, U.S.A.) was used with 7 g agar (agar and agar) and 30 g-1 sucrose during all study stages. Plant growth regulators were added as required for each study stage, and the pH was adjusted at 5.75 before autoclaving for 20 minutes at a temperature of 121 °C and a pressure of 1.04 bar.

Plant material and surface Sterilization:

The shoot tips growing in spring containing 5-7 buds were taken from two grapevine varieties, Superior and Red globe, from private orchards in the Bakirat region, north of Dohuk Governorate / Kurdistan Region of Iraq, and placed in polyethylene bags.

Explants of each variety were placed separately under running tap water for an hour to remove dirt and dust attached to them from the field, then under the laminar air flow cabinet conditions, both cultivars' explants were immersed separately for ten seconds in 96.6% Ethyl alcohol. The explants were divided into groups to conduct surface sterilization treatments, which included testing the effect of a commercial bleach solution (containing 5.25% sodium hypochlorite (NaOCl)) at concentrations of (10, 20, and 30%) and for (5, 10, and 15 minutes) immersion periods, then, explants were rinsed three times in sterilized distilled water for 15 minutes each to remove the harmful effect of the disinfectant agent, the damaged ends of the parts were removed, and the parts were divided into approximately 1 cm stem cuts containing at least one bud and planted in MS medium free of plant growth regulators. After two weeks of cultivation, the Percentage of uncontaminated explants was recorded.

Initiation stage:

Shoot segments containing 5-7 buds were immersed for ten minutes in a 20% commercial

bleach solution, depending on previous sterilization experiment results, which gave high values regarding the percentage of uncontaminated explants.

The stem cuts were divided into pieces approximately 1 cm long containing at least one bud and cultured on full-strength MS (Murashige and Skoog, 1962) salt. Containing 1 mg L-1 BA (Benzyl Adenine).

Multiplication stage:

Cultivars' explants from the initiation stage were planted on MS medium containing different concentrations (0, 1, 2, 3, and 4 mg L^{-1}) of Benzyl Adenine (BA). Number of newly formed shoots (shoot $explant^{-1}$) and leaves number (leaf $explant^{-1}$), eight weeks after cultivation were taken.

Rooting stage:

During the rooting stage, shoots from the multiplication stage were planted on a half-strength MS medium containing (0, 0.5, 1, and 1.5 mg L^{-1}) indole-3- butyric acid (IBA). After four weeks, the rooting Percentage and number of roots formed on the rooted shoot (root $xplants^{-1}$) were recorded.

Cultivation conditions:

In all study stages, the cultures were incubated in growth chamber conditions under an illumination period of 16 hours of cold white light prepared from fluorescent lamps, 8 hours of darkness, and a temperature of 1 ± 23 Cº.

Acclimatization stage:

Rooted plants were taken out of the vessels, and their roots were washed with running water to remove the remnants of the nutrient media attached to them. Then, the rooted plants were planted on a culture medium consisting of a mixture of peat moss, perlite, and river soil at 1: 1: 1. The plants were covered with polyethylene to maintain moisture, and the cover was gradually lifted until it was completely removed after ten days and then transferred to the greenhouse.

Experiment design:

The experiment was a factorial trial in a completely randomized design (CRD). Data were analyzed using SAS 9.1, and the comparison between the means was performed according to Duncan's multiple range test at a ($p < 0.05$)[12].

Results:

Results in Table (1) after two weeks **o**f sterilizing the two cultivars' explants and cultivating them on MS medium indicated that cultivars had no significant effect on the percentage of uncontaminated explants (free of contamination). The immersion duration of 10 minutes achieved an uncontaminated percentage of 58.3%, significantly outperforming the 5 and 15-minute periods, which recorded a rate of 35 and 28.3%, respectively. A commercial bleach with a 20% concentration had a significantly higher (51.6%) uncontaminated rate than the other concentrations (10 and 30%), which gave an uncontaminated rate of 35%. Concerning the interaction effect of cultivars and the immersion duration, the highest uncontaminated rate (60 and 56.6%) was recorded in the treatments in which the two cultivars, Superior and Red Club single nodules, were immersed for 10 minutes in the disinfectant agent, respectively, which was significantly superior to the rest of the studied interference treatments. Regarding the interaction effect of the disinfectant agent concentration and the immersion duration, the highest values of uncontaminated rate (85%) were achieved in the interaction treatment of a 10-minute immersion period with a concentration of 20%, significantly outperforming all other studied interference treatments. The lowest uncontaminated rate (00%) was achieved in treatments overlapping the immersion duration of 15 minutes with a concentration of 30%.

As for the triple overlap between varieties, the concentration, and the immersion duration, data of the same table indicated that the treatment in which the two cultivars explants, Superior and Red globe, were immersed for 10 minutes in 20% of the disinfectant agent recorded the highest percentage (90 and 80%, respectively) uncontaminated rate without a significant difference from each other While the lowest uncontaminated explants percentage (00%) with a significant decrease from all the studied triple interference treatments were recorded in the treatments of overlapping the two cultivars explants in an immersed period for 5 minutes in 10% concentration of The bleach solution, as well as the treatment of immersing the two cultivars explants for 10 minutes in 30% of The commercial bleach solution and the treatment of immersing the Red globe explants for 20 minutes in 15% of the disinfectant agent.

Table (1) Effect of cultivars, the concentration of sterilization material, immersion period, and their interaction on the percentage of healthy explant (free of contamination) after two weeks of cultivation in the MS medium.

cultivar	Immersion time/min	Concentration (%)			Effect of Cultivars + Immersion Time	Effect of Immersion Duration	Cultivar effect
		10	20	30			
Superior	5	00 d	40 c	60 b c	33.3 b c		43.3 a
	10	40 c	90 a	50 b c	60 a		
	15	60 b c	50 b c	00 d	36.6 b		
Red Globe	5	00 d	50 b c	60 b c	36.6 b		37.3 a
	10	50 b c	80 a b	40 c	56.6 a		
	15	60 b c	00 d	00 d	20 c		
Effect of Cultivars + con.	Superior	33.3 b	60 a	36.6 b			
	Red globe	36.6 b	43.3 b	33.3 b			
Effect of immersion duration + con.	5	00 d	45 b c	60 b		35 b	
	10	45 b c	85 a	45 b c		58.3 a	
	15	60 b	25 c	00 d		28.3 b	
concentration effect		35 b	51.6 f	35 b			

* Numbers with similar letters within one column are not significantly different according to Dunkin's multiple range test at the 5% probability level.

Multiplication stage:

Shoots number (shoots/explant)

Table (2) shows the effect of BA concentrations on shoot numbers formed eight weeks after cultivating the explants resulting from the establishment stage on the MS medium containing different concentrations of Benzyl Adenine (BA). The data showed that the differences in the cultivars' effect did not reach a significant level. At the same time, the BA concentrations used affected this trait, as 2 mg L^{-1} BA concentration significantly increased shoot number (4.30 shoots $explant^{-1}$) over the other studied concentrations (0, 1, 3, and 4 mg L^{-1}), giving 1.25, 3.50, 2.25, and 1.75 shoots $explant^{-1}$, respectively. As for the interaction effect between cultivar and BA concentration, the highest number of newly shoots formed (4.50 shoots $explant^{-1}$) was recorded in the treatment in which the Superior explants were grown on MS media containing 2 mg L^{-1} BA did not differ significantly from the value recorded when the Red Globe cultivar overlapped with the same BA concentration (4.10 shoots $explant^{-1}$); the table showed that BA concentration increased from 0 to 2 mg L^{-1}, the number of newly formed shoots significantly increased (1.20, 3.60, and 4.50 shoots $explant^{-1}$, respectively, for Superior and 1.30, 3.40, and 4.10 shoots $explant^{-1}$, respectively, for Red Globe). In contrast, High BA concentrations (3 - 4 mg L^{-1}) led to a decrease in this trait.

Table (2) The effect of cultivars and BA concentrations on the average shoot number of the grape variety eight weeks after cultivation on the MS medium.

cultivar	shoot number (shoots $explant^{-1}$) BA concentration mg L^{-1}					Cultivar effect
	Zero	1	2	3	4	
Superior	1.20 f	3.60 bc	4.50 a	2.50 d	1.80 ef	2.72 a
Red globe	1.30 f	3.40 c	4.10 ab	2.00 de	1.70 ef	2.50 a
concentration effect	1.25 e	3.50 b	4.30 a	2.25 c	1.75 d	

*Numbers with similar letters within the Sunday column are not significantly different according to Dunkin's multiple range test at the 5% probability level.

Leaves number (leaves/explants):

Table (3) reveals significant differences in leaf mean number between Superior and Red Globe cultivars, with Superior having the highest rate (15.14) and Red Globe having (13.56) leaves/explants. As for the BA concentrations effect, it was noted that the most significant opening leaves (19.70 leaves) that occurred in media treated with 2 mg L^{-1} BA did not differ significantly from explant-grown on media supplemented with 1 mg L^{-1} BA was significantly superior to explant grown in media supplemented with 3 or 4 mg L^{-1} BA, which in turn was significantly superior to control (6.70 leaves).

As for the interaction between varieties and the BA concentrations, it was noted from the same table that the interaction of the Superior variety with 2 mg L^{-1} BA achieved the largest leaf opening (20.90 leaves) and did not differ significantly from the treatment of the same cultivar with 1 mg L^{-1} BA, but it significantly outperformed all the other studied

crosses. At the same time, the lowest number of leaves was recorded in the control free of BA (7.20 and 6.20 leaves explant^{-1}, Superior and Red Globe, respectively).

Table (3) The effect of cultivars and BA concentrations on leaves explant^{-1} of the grape variety after eight weeks of cultivation on the MS medium.

cultivar	Leaves number (leaves explant^{-1}) BA concentration mg L^{-1}					Cultivar effect
	Zero	1	2	3	4	
Superior	7.20 f	19.60 ab	20.90 a	15.60 d	12.40 e	15.14 a
Red globe	6.20 f	16.80 cd	18.50 bc	13.10 e	13.20 e	13.56 b
Con. effect	6.70 c	18.2. a	19.70 a	14.35 b	12.80 b	

*Numbers with similar letters within the Sunday column are not significantly different according to Dunkin's multiple range test at the 5% probability level.

Rooting stage:

Rooting percentage (%):

Table (4) shows that cultivars had no significant effect on the rooting percentage after four weeks of planting shoots resulting from the multiplication stage in rooting media. In contrast, IBA concentration recorded significant differences between the studied concentrations, as the shoots cultivated in the medium equipped with 1 mg L^{-1} IBA excelled in rooting percentage (85%) over its peers grown in the rest of the media except for that supplemented with 0.5 mg L^{-1} IBA (70%), which in turn did not differ significantly from that grown on the medium containing 1.5 mg L^{-1}, while IBA-free media recorded the lowest rooting percentage (15%). As for the interaction effect between the cultivar and IBA concentrations, the results of the same table indicate that the highest rooting percentage (100%) was recorded when cultivating Red Globe shoots medium containing 0.5 mg L-1, IBA, which did not differ significantly from the value of 90% that was recorded in the treatment of the Superior variety with 1 mg L^{-1} IBA, while the lowest rooting percentage (10 and 20%) (Superior and Rose Club, respectively) was recorded in control (IBA-free).

Table (4): Effect of cultivars and IBA concentrations and their interaction on the percentage of rooting (%) of grape Vitis Vinifera L. shoots after four weeks of planting.

cultivar	Rooting percentage (%) IBA concentration mg L^{-1}				Cultivar effect
	Zero	0.5	1.0	1.5	
Superior	10 e	40 c-e	90 a	70 a-c	52.5 a
Red globe	20 de	100 a	80 ab	50 b-d	62.5 a
Con. effect	15 c	70 ab	85 a	60 b	

*Numbers with similar letters within the Sunday column are not significantly different according to Dunkin's multiple range test at the 5% probability level.

Number of roots per rooted shoots:

Table (5) shows that cultivars do not significantly affect the number of roots rooted shoots^{-1}. At the same time, IBA concentrations significantly affected this trait, as the shootlets were planted in 0.5 and 1 mg L^{-1} IBA (3.00 and 3.15 root/rooted shoot, respectively) with superior significantly. As for the effect of the cultivar's interaction with IBA concentrations, it is clear from the same table that the most significant number of roots (3.55) formed on Superior shoots when cultivated on media equipped with 1 mg L^{-1} IBA did not differ significantly from the Red Globe interaction treatment of 0.5 mg L-1 concentration gave 3.50 root/rooted shoot, while IBA-free media recorded the lowest number of roots for the two cultivars, Superior and Red Globe (1.00 and 1.50 root/branch, respectively).

Table (5): Effect of cultivars and IBA concentrations and their interaction on the roots Number per rooted shoot of grape *Vitis Vinifera* L. four weeks after planting.

cultivar	roots rooted shoots^{-1} IBA concentration mg L^{-1}				Cultivar effect
	Zero	0.5	1.0	1.5	
Superior	1.00 d	2.50 a-c	3.55 a	2.28 bc	2.33 a
Red globe	1.50 cd	3.50 a	2.75 ab	2.20 bc	2.48 a
Con. effect	1.25 c	3.00 a	3.15 a	2.24 b	

*Numbers with similar letters within the Sunday column are not significantly different according to Dunkin's multiple range test at the 5% probability level.

Figure (1): In vitro propagation stages of grapes *Vitis Vinifera* L. single nodes.
A, healthy explant B, Initiation stage C**,** Multiplication stage D, Rooting stage: Superior cv.
E, healthy explant F, Initiation stage G**,** Multiplication stage H, Rooting stage: Red globe cv.

Discussion

Microorganism contamination during the tissue culture process is one of the main reasons for its failure. The environment surrounding the donor plant is the main source of explant contamination when grown *in vitro*. [13] have indicated that contamination, such as dust, dirt, and microorganisms, such as fungi, can be eliminated by washing explants with running tap water and then treating them with disinfection agents. *In vitro* propagation conditions are ideal for microorganism growth. Therefore, the efficiency of the sterilization process is the most crucial reason for the success of micropropagation studies [14]. [15] stated that the response of the explant to the same sterilization process differs in various species and cultivars. Our results are consistent with many studies on the efficiency of commercial bleach as a sterilizer for explants [16][17][9]

The current study (Table 1) indicated that immersion of grapes explants for 10 minutes in 20% commercial bleach solution containing 5.25% sodium hypochlorite (NaOCl) achieved the best results for both studied cultivars (Superior and Red Globe), which gave the lowest contamination percentage and the most considerable explants survival rate, which agreed with [9] who shown that uses of 1% sodium hypochlorite (NaOCl) for 10 minutes immersed gave the lowest percentage of contamination and the largest survival rate for the two varieties (Red Globe and Superior explant). [18] and[19] reported that explants treated with sodium hypochlorite solution effectively reduced explants' contamination. Also [20] stated that surface sterilization systems should aim to use the lowest concentration of NaOCl for the least period. Higher concentrations and long disinfection immersion periods hurt the excised explants' growth and development [21][14]. Whil [22] found that low concentrations of sodium hypochlorite increase the contamination percentage in excised plants, while high concentrations of hypochlorite Sodium made the excised plants lose their vitality. Using a high

concentration of sodium hypochlorite in the surface sterilization process had a toxic effect, leading to 100% of the explant's death [23].

The growth characteristics (shoots number and the leaves number in the multiplication stage) (Tables 2 and 4) were significant in most treatments treated with cytokinin compared with the control treatment, may be due to the role of cytokinin in regulating The activity of apical meristems, morphological formation, chloroplast development, and leaf growth [24], as the positive effect of cytokinin used in vegetative propagation events comes from their role in stimulating the growth of lateral buds by eliminating apical dominance and stimulating cell division and cell widening [25]. (As well as its role in attracting and aggregating metabolites at lateral buds and stimulating the transfer of nutrients and other growth materials to start bud growth [26]. In addition to its effect in preventing the degradation of chlorophyll and proteins in cells, it works to reduce the activity of the enzyme ribonuclease (RNAase), which helps prevent the decomposition of RNA and thus increases it and amino acids production since cytokinin regulates the functional work between transporter RNA (tRNA) and messenger RNA (RNA-m) [27], as it was believed that the presence of cytokinin in a part of the RNA-t and near the anti-codon has an essential role in linking RNA-t with RNA-m during the formation of proteins since RNA-t without the Isopentenyl side chain of adenine is inactive. This is what the new evidence indicated about the role of cytokinin in regulating the construction of protein, as it was found that treating explants with cytokinin causes an increase in their content of polyribosomes [28].

The increase in the response of some of the studied traits upon raising the levels of cytokinin in the media can be attributed to the fact that these increases achieved a state of ideal hormonal balance in the explant to give the optimal response. The high concentrations led to an imbalance in the hormonal balance of the explant cells, which negatively affected the conduct of vital processes in it, especially cell division and elongation. They thus decreased the values of the studied traits [29]. Our results agree with [30] in their study, micropropagation of grapes, Thompson Seedless cultivar (cv. Thompson Seedless) who indicated that the best medium for single node multiplication was MS medium containing 2 mg L^{-1} BA with the highest shoot number (3.6 shoots/explant).

As for cytokinin inhibitory effect at high concentrations, [11], in their study to improve the hormonal and mineral composition of the media used for in vitro propagation of grapes, revealed that medium containing cytokinin at high concentrations is not recommended for explant grow because this can cause Inhibition of growth processes.

As for the significant effect of adding IBA to the rooting media in raising the rooting percentage (Table 4) and roots number (Table 5) for each rooted shoot, it may be due to the role of auxins, which are considered the most effective substances for adventitious root formation [31], as well as its role in reducing the time required for rooting, increasing the rooting rate, and stimulating root formation [32]. The improvement in the rooting rate is due to the role of IBA in encouraging the emergence of adventitious roots by stimulating cell division in the cambium and root elongation [33][34].

The number of roots formed on the shoots significantly increased when adding 0.5 and 1 mg L-1 IBA to the nutrient media may be due to the appropriateness of these concentrations to cause the greatest stimulation of cell division and elongation [33][34]. These results are consistent with [5] funding, who indicated that explants obtained from the multiplication stage were placed on MS medium with half-strength salt containing 1 mg L^{-1} IBA and achieved a 100% rooting rate. [35] obtained rooted shoots after four weeks of cultivating them, on a full-strength MS nutrient medium containing 1 mg of salt. L^{-1} Indole-3- Butyric Acid (IBA), during their micropropagation study of two grape varieties (Thompson Seedless and Taify)**.**

Conclusions

The results obtained from this study prove that immersing the explant of grape varieties Superior and Red Globe in 20% commercial bleach solution for 10 minutes effectively reduced the contamination percentage and maintained the explant's vitality during the sterilization stage. The results of the multiplication stage after eight weeks of explant cultivation showed that the highest number of newly formed shoots and leaves was recorded in media containing 2 mg L^{-1} BA. The highest rooting percentage (90%) and the most significant root number (3.55 root/rooted shoots) for the Superior cultivar were achieved in a medium containing 1 mg L^{-1} IBA, while the medium was containing 0.5 mg L^{-1} IBA had the highest rooting percentage (100%) and the largest root number (3.50 root/rooted shoots) for the Red Globe variety. The rooted plants of the two cultivars showed the highest survival rate (100.00%) and the best vegetative growth during the acclimatization stage when they were planted in a mixture of peat moss, pearlite, and river soil in a ratio of 1: 1: 1.

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Conflict of interest

The researcher supports the idea that this work does not conflict with the interests of others**.**

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