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Improving micropropagation of some grape cultivars via boron, calcium and phosphate ☆

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ABSTRACT

Background: An efficient regeneration protocol is a priority for the successful application of plant biotechnology. Grape nodal explants were used to develop a micropropagation protocol for Thompson Seedless and Taify cvs. Explants were cultured on MS medium supplemented with Kinetin or benzylaminopurine (BA) and indolebutyric acid (IBA).

Results: For both cultivars, axillary buds were grown, only, on a medium enriched with kinetin, moreover, shoot tip necrosis and callus formation were observed on Thompson Seedless cv. cultures grown on a medium with BA. Supplementing the growth medium with 100 mM (boron) B and 2.5 mM (calcium) Ca successfully help overcome these phenomena. The highest regenerated shoot numbers (14 and 6.2 explant^{-1}) for Taify and Thompson Seedless cvs., respectively, were on media supplemented with 13.2 μM BA + 4.9 μM IBA and BA 13.2 μM + 5.8 μM IBA, respectively. Moreover, these media supported the developing shoots to have the heaviest dry weights (1.46 and 0.72 mg explant^{-1}) for Taify and Thompson Seedless cvs., respectively. Thompson Seedless cv. regenerated shoot numbers and their dry weights were significantly increased by increasing the MS medium PO_4^- concentration. However, these two parameters were significantly decreased for Taify cv. Developing shoots were elongated and rooted on MS medium enriched with 4.9 μM, IBA 100 mM B and 2.5 mM Ca. Plantlets were acclimatized and successfully transferred to the greenhouse conditions.

Conclusions: A novel promising protocol for Thomson Seedless and Taify cvs. micropropagation using single nodes has been developed.

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1. Introduction

The grapevine (Vitis vinifera L.) plant which is native to the warm and temperate zones is considered as one of the most globally cultivated commercial fruit crops. It is consumed as fresh and dried fruit or used for making jam, vinegar, juice, jelly, and wine [1]. Grapefruits are a good mineral and vitamin source, so it could alleviate various human diseases through its antioxidant activity. Moreover, it shows antitumor activities by blocking carcinogen-induced DNA adducts formation [2]. The value of the minor Saudi ancient highly local important grapevine cv. Taify which originated on Azerbaijan [3] has been investigated by Fahmi et al. [4].Thompson Seedless cv. which is a Sultana grape, belonging to the Sultanina family. Due to its high productivity, seedless fruit and versatility for use it is the dominant grape variety grown in many countries [5].

In vitro culture has many advantages over the conventional propagation techniques. Depending on the genotype and the *in vitro* conditions, micropropagation could enable the elite genotype mass production which can be acclimatized in a short period with a low cost [6]. Grape micropropagation can be carried out by culturing shoot apical meristems, axillary-buds or through adventitious bud formation [7–9]. Thomas et al. [7] described the grape cultivars' diversity and suggested the specificity of plant reactions under *in vitro* conditions. Various grape species, cultivars or hybrids respond differently to the culture conditions [9]. Gisbert et al. [10] stated that grapevine genetic resource preservation, selection and/or development cultivars which well adapted to

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the upcoming climate changes are important. Therefore, the preservation of grapevine minor cultivar that is on extinction risk is a major interest in grapevine germplasm preservation.

There are some reports about *in vitro* shoot tip necrosis (STN) which associates with many factors and physiological conditions. Thomas [11] found that vigorous rooting in grape plantlets has been affected by STN due to their ability to produce many axillary branches. This phenomenon could be reduced by improving culture vessel ventilation and proper explant selection. The significance of optimizing Ca and B concentrations in the culture medium to overcome STN problem has been discussed [12,13]. Previously, Harris and Stevenson [14] suggested reducing subculturing intervals to eliminate STN, but, no doubt this is not an economical method. Recently, Surakshitha et al. [15] investigated the subculturing interval role and elevating Ca levels on grape cv. Red Globe STN occurrence. They suggested the significant Ca role on maintenance cellular integrity leading to strong recovery from STN. Their results showed that using half-strength MS medium [16] enriched with 180.18 mg/L Ca and 1.08 mg/L B with 2 weeks subculture interval could efficiently manage the STN incidence.

Phosphorus is a vital element in plant biochemistry and can be a limiting factor especially in the active growth cultures when huge tissues or organs grow on a little medium volume [17]. George and De Klerk [18] suggested regulating media phosphate component to enhance micropropagation. The MS medium phosphate content could be absorbed more rapidly than other ions, and almost all PO_4^- are taken up in the first 2 weeks of the culture [18,19]. Dantas de Oliveira et al. [20] and Mohamed [21] highlighted the rapid uptake of PO_4^- which acted as a limiting growth factor in prolonged *in vitro* culture. Nevertheless, definitely the explant response to *in vitro* conditions is highly dependent on the genotype [22].

To the best of our knowledge there is only one published protocol for Taify cv. micropropagation [23]. Nevertheless, this study recorded 1.5–2.5 shoot explant^{-1} which could be considered inefficient for commercial production. On the other hand, there are many reports regarding *in vitro* culture of Thompson Seedless cv. using nodal explant. Botti et al. [24] cultured them on MS medium enriched with 2 mg/L BA and achieved 19.49 shoot explant^{-1} after the 3rd subculture, but the study did not show the number of regenerated shoots after the 1st one. Mostafa et al. [25] obtained 4.95 shoot explant^{-1} using MS medium with 1 mg/L BA + 0.01 mg/L NAA. However, Chapman and Pratt [26] suggested that 1/2 MS medium with 2 mg/L BA was the best condition for micropropagation as 3.6 shoot explant^{-1} was regenerated. Therefore, this study aimed to develop an efficient regeneration protocol for the important endangered Taify cv. using Thompson Seedless cv. as a reference.

2. Materials and methods

2.1. Plant material

Taify and Thompson Seedless cv. (two-year old grapevine) grown under open field conditions were used for this study. Taify cv. plants were obtained from a local farm at Taif district, Saudi Arabia, whereas, Thompson Seedless cv. ones were obtained from the Derab Experimental Farm, King Sauid University, Riyadh, Saudi Arabia. The initial explants (nodal sections) were taken from the 2nd and 3rd bottom nodes of the new developing shoots by the end of March. The explants were washed thoroughly for about 10 min under running tap water, before surface sterilized with 96% (V/V) ethanol for 10 s followed by 20% (V/V) commercial Clorox bleach (Clorox Co., Jeddah, Saudi Arabia) containing few drops of Tween-20 for 15 min [14]. The explants were rinsed several times with sterile distilled water. The damaged tissues were cut off from both ends before culturing into the growth. During explant preparation for *in vitro* regeneration leaves were excised and dried at 70°C for 24 h to determine their P content colorimetrically by the molybdenum-blue method [26] using three samples (500 mg each) of the dried material.

The MS medium [16] supplemented with 3% sucrose was used as the basal medium for all cultures. The medium was solidified with 0.8 % (W/V) Agar (Sigma) and the pH was adjusted to 5.8 prior to autoclaving. All cultures were maintained at 22°C with a 16-h photoperiod under 50 μmol $m^{-2}s^{-1}$ illumination provided by cool-white fluorescent lamps.

The initial explants as described above were cultured on a medium supplemented with 3% (W/V) sucrose and 5.8 μM IBA for 8 weeks with 4 weeks intervals. The healthy developing plantlets were used as a source of explants for the following experiments.

2.2. Shoot regeneration

For adventitious shoot regeneration the nodal explants were cultured on a medium enriched with 4.4, 8.8 and 13.2 μM BAP or Kinetin and 0, 4.9 and 5.8 μM IBA. For each treatment, there were 5 magenta boxes each containing 5 explants. The cultures were transferred into a fresh medium twice every 3 weeks. By the end of the first culture, callus was developed on the base of all explants especially when the growth medium supplemented with 5.8 μM IBA were used. Moreover, STN was observed on the Thompson Seedless cv. proliferated shoots.

2.3. Overcome shoot tip necrosis

To overcome the callus formation and STN, the experiment was repeated using a medium with the same plant growth regulators in addition to 100 mM B (as H_3BO_3) and 2.5 mM Ca (as $CaCl_2$). The cultures were incubated under the previous conditions and transferred onto a fresh medium each 3 weeks for 12 weeks.

2.4. Optimizing phosphate concentration for regeneration medium

The previous experiment showed that the optimum plant growth regulators' concentrations for Taify and Thompson Seedless cvs. regeneration were (13.2 μM BA + 4.9 μM IBA) and (13.2 μM BA + 5.8 μM IBA), respectively. So, single node explants of both cultivars were cultured on the MS medium, or MS medium supplemented with an extra PO_4^- (as $NaH_2PO_4.2H_2O$) to achieve 1.5 and 2.0 times that of its original concentration. On all cases the growth medium was enriched with the optimum plant growth regulator concentrations for the specific cultivar. After 3 weeks the cultures were subcultured onto a fresh medium for another 3 weeks before elongation and rooting as described before.

2.5. Shoot elongation and rooting

Developing shoot clumps were transferred onto MS medium containing 4.9 μM IBA for 4 weeks. Then 4 shoot clumps were randomly taken out from each vessel to estimate shoot numbers. Following that, the attached medium was removed and shoot clumps were blotted on a filter paper before drying at 70°C to estimate the individual dry weights. The shoots of the remaining shoot clumps were divided into single nodes and cultured onto the same medium for elongation and rooting for 4 weeks.

2.6. Rooting and plantlet acclimatization

Regenerated shoots which were longer than 3 cm were separated and cultured on MS medium supplemented with 4.9 μM, IBA 100 mM B and 2.5 mM Ca for rooting. The regenerated

plantlets were acclimatized to the greenhouse conditions in plastic cups containing a 1:1 (V/V) soil and vermiculite covered with a plastic wrap for 4 weeks.

Finally, plantlets were potted in soil and subsequently adapted to the greenhouse, with 70–80% humidity, 26 ± 2°C, and 1160 lx luminance.

2.7. Statistical analysis

All experiments were performed with 5 replicates and repeated twice. The results were subjected to the analysis of variance (ANOVA), and the means between each pair of data were compared using Duncan's multiple range test ($p \geq 0.01$) [27]. The analysis was performed using the software (SPSS 16.0.0 release; SPSS Inc., Chicago, IL).

3. Results

Both cultivars regenerated many adventitious shoots on a medium supplemented with BA; however, only axillary buds developed shoots on a medium with Kinetin. So those data regarding regenerated shoot numbers and their dry weights for explants cultured on a medium with Kinetin were not represented. Callus was developed on Thompson Seedless cv. explant bases which cultured on a medium with BA. Shoot tip necrosis has not been observed during the first culture, but Thompson Seedless cv. developing shoots were severely affected with STN following the 1st transfer to a fresh medium. These symptoms coincided with brown callus formation on the explant bases (Fig. 1). Enriching the growth medium with 100 mM B and 2.5 mM Ca successfully eliminated the STN, but, by the end of the rooting and elongation stage purple color and chlorosis were observed on Thompson Seedless cv. plantlets leaf-margin.

Regenerated shoot numbers were significantly affected by BA and IBA concentrations as well as, the cultivar type. Also, the interaction among cultivars, auxin and cytokinin had a significant effect on that parameter. For both cultivars increasing BA concentration under the same IBA concentration significantly increased the regenerated shoot numbers. The Taify cv. regenerated shoot numbers did not differ ($p > 0.01$) when the growth medium supplemented with 4.4 μM BA regardless, the IBA concentration (Table 1). When medium supplemented with 8.8 μM BA the medium concentration of IBA had the lowest regenerated shoot number explant^{-1} (3.6) of Thompson Seedless cv. compared to the other two concentrations. However, when the medium supplemented with 13.2 μM BA increasing IBA from 0.0 to 4.9 μM it significantly increased the regenerated shoot number. Overall, supplementing the growth medium with 13.2 μM BA and 4.9 μM IBA enabled the Taify cv. nodal explant to regenerate the highest shoot numbers (17.0) ($p > 0.01$). However, the highest regenerated shoot numbers (6.2) for Thompson Seedless cv. explants was on a medium supplemented with the same concentration of BA but 5.8 μM IBA. Results may indicate that increasing BA concentration to 13.2 μM may be more efficient for Taify cv. micro propagation using nodal explant.

The regenerated shoot dry weights of both investigated cultivars did significantly differ due to the BA and IBA concentrations meanwhile, the interaction among the cultivar, BA and IBA was significant (Table 2). The lowest shoot dry weight (0.03 g explant^{-1}) beaning for Taify cv. explant cultured on IBA-free medium enriched with BA at 4.4 μM. Whereas, the highest shoot dry weight (1.46 g explant^{-1}) beaning for the same explants grown on a medium with BA at 13.2 μM and IBA at 4.9 μM. When the growth medium contained 4.4 μM BA, the Thompson Seedless cv. explant had significantly higher shoot dry weights than that of Taify cv. ones under the same IBA concentration. On the other hand, Taify cv. explant had higher shoot dry weights than that of Thompson Seedless cv. ones when the growth medium supplemented with BA at 13.2 μM (Table 2). Regenerated shoot fresh weights had a similar trend (data not shown).

Thompson Seedless cv. leaf purple color and chlorosis symptoms were not observed following enriching the growth medium with PO_4^- at 1.5 or 2 times of the original concentration. Moreover, the regenerated shoot numbers were significantly increased from 6 to 8 shoots explant^{-1} by increasing the PO_4^- concentration form original concentration to two-fold. On the other hand, this number was decreased ($p > 0.01$) from 14.6 shoots explant^{-1} on the regular MS medium to 10.4 and 10.6 shoot explant^{-1} for Taify cv. which was maintained on a medium with higher PO_4^- concentrations (1.5 and 2 fold), respectively (Fig. 2). Thompson Seedless cv. regenerated shoots dry weights were significantly increased from 0.71 to 0.90 g explant^{-1} by doubling the PO_4^- concentration. Meanwhile, these values for Taify cv. were decreased from 1.09 g explant^{-1} to 0.72 g explant^{-1} on a medium with the mentioned PO_4^- concentrations.

Almost all nodal explants from regenerated shoots were elongated and rooted on a medium enriched with 4.9 μM IBA regardless of the cultivar type or regeneration media components. Plantlets

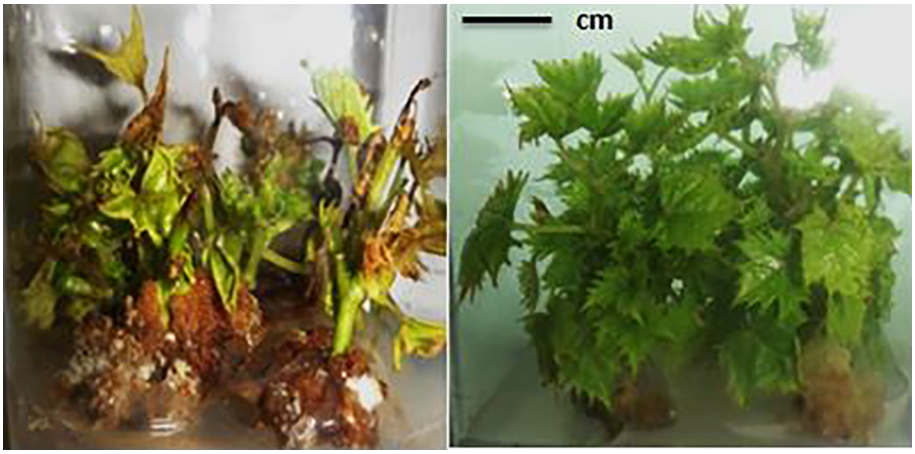

Fig. 1. Thompson Seedless cv. explants showing shoot-tip necrosis and developing brown calli on a medium supplemented 13.2 μM BA and 4.9 μM IBA (left) and normal shoot growth on a medium with 100 mM B and 2.5 mM Ca (right) (scale bar = 1 cm).

Table 1
Effect of plant growth regulators on regenerated shoot numbers nodal explant^{-1} of two grapevine cultivars after culturing for 12 weeks on MS medium enriched with 100 mM boron and 2.5 mM calcium.

PGR (μM)		Cultivar (Cv.)		Mean
BA	IBA	Taify	Thompson Seedless	
4.4	0.0	1.0 i*	1.9 h	1.45
	4.9	1.0 i	2.6 gh	1.80
	5.8	1.0 i	2.9 fh	1.95
8.8	0.0	2.0 h	4.5 e	3.25
	4.9	6.1d	3.6 f	4.85
	5.8	5.3 d	4.4 e	4.85
13.2	0.0	10.5 c	3.7 f	7.10
	4.9	17.0 a	5.4 d	11.20
	5.8	14.0 b	6.2 d	8.10
Mean		6.4	3.9	

*Values with different letters are significantly different at $p < 0.01$ (Duncan's multiple range test)

Analysis of variance for regenerated shoot number

Source	df	SS	MS	F value	*P*-value
Cv.	1	151.451	151.451	533.8776	<0.01
BA	2	915.287	457.644	1613.2355	<0.01
Cv.*BA	2	456.175	228.088	804.0295	<0.01
IBA	2	71.550	35.775	126.1103	<0.01
Cv.*IBA	2	32.065	16.033	56.5167	<0.01
BA*IBA	4	43.148	10.787	38.0251	<0.01
Cv.*BA*IBA	4	28.057	7.014	24.7255	<0.01
Error	72	20.425	0.284		
Total	89	1718.158			

Table 2
Effect of plant growth regulators on regenerated shoot dry weights nodal explant^{-1} (g) of two grapevine cultivars after culturing for 12 weeks on MS medium enriched with 100 mM boron 2.5 mM calcium.

PGR (μM)		Cultivar (Cv.)		Mean (g)
BA	IBA	Taify	Thompson Seedless	
4.4	0.0	0.03 i*	0.23 h	0.13
	4.9	0.07 i	0.26 h	0.16
	5.8	0.07 i	0.19 h	0.13
8.8	0.0	0.27 h	0.25 h	0.26
	4.9	0.52 f	0.29 gh	0.41
	5.8	0.48 f	0.37 g	0.43
13.2	0.0	1.07 b	0.62 e	0.85
	4.9	1.46 a	0.70 de	1.08
	5.8	0.94 c	0.72 d	0.83
Mean		0.54	0.40	

*Values with different letters are significantly different at $p < 0.01$ (Duncan's multiple range test)

Analysis of variance for shoot dry weights/nodal explant

Source	df	SS	MS	F value	*P*-value
Cv.	1	0.450	0.450	86.6765	<0.01
BA	2	9.624	4.812	927.1363	<0.01
Cv.*BA	2	1.579	0.790	152.1615	<0.01
IBA	2	0.3	0.153	29.4457	<0.01
Cv.*IBA	2	0.183	0.091	17.6145	<0.01
BA*IBA	4	0.295	0.074	14.2163	<0.01
Cv.*BA*IBA	4	0.279	0.070	13.4476	<0.01
Error	72	0.374	0.005		
Total	89	13.090			

were acclimatized and successfully transferred to the greenhouse conditions (Fig. 2)

Leaves which excised during explant preparation were used to estimate its P content. Results showed that Thompson Seedless and Taify cv mother plants contented 0.72 and 1.21% of phosphorus.

4. Discussion

Supplementing the growth medium with BA and IBA enhanced adventitious shoot formation of both cultivars moreover, more callus was developed on the Thompson Seedless cv. explant on a medium enriched with 5.8 μM IBA. On the other hand Taify cv. explants did not develop callus and only axillary shoots were developed from the explant cultured on the medium which was enriched with Kinetin. Also, the effect of plant growth regulators was observed on the regenerated shoot number as well as their dry weights. There are many lines of evidence confirming the dynamic role of the auxins and cytokinins on in vitro growth, callus formation and adventitious shoot development. Meanwhile, these effects depend on the type and concentration of the auxins and cytokinins and the ratio between them [27]. Previously, Heloir et al. [8] and Mostafa et al. [25] found that the in vitro response of other grape cultivar explants depended on the genotype as an inheritable trait [28].

Fig. 2. Thompson Seedless and Taify cv. nodal explants elongation and rooting (a and b, respectively) on MS medium with two-fold PO_4^- concentration and 4.9 μM IBA. Plantlets after 4 weeks of acclimatized to the greenhouse conditions (c).

Shoot tip necrosis has not been observed during the first culture of both cultivars. Only, Thompson Seedless cv. which regenerated shoots on a medium with BA (unlike those on a medium with Kinetin) showed STN symptoms. *In vitro* culture of grapevine like other woody species is often hampered by browning and necrosis which required an adequate treatment to overcome this obstacle [18]. Bona et al. [28] stated that grape buds became severely oxidized in MS medium and the addition of silver nitrate enabled their development for most tested cultivars. The significant effect of the genotype and plant growth regulators on STN has been well documented [29]. Bairu et al. [12] considered that the lack of roots, which are the main sites of cytokinin biosynthesis, could increase STN. It is worth to see no STN symptoms on Taify cv. shoots developed on a medium with kinetin which did not develop many adventitious shoots or even with the highest BA concentrations which developed the highest shoot numbers (14/explant). Therefore, these results might indicate that STN could be related to the genotypes more than the plant growth regulators.

All Thompson Seedless cv. explants cultured on the medium supplemented with 100 mM B and 2.5 mM Ca did not show STN symptoms. Calcium shortage during the multiplication stage is accompanying with STN in many woody species [30–32]. Calcium is an important element for some changes that could contribute to the physiological aging concomitant with STN, as its deficiency causes serious damage on the cell ultrastructure, causing the rapid deterioration of metabolically active tissues however, reports about the effect of Ca on STN lack consistency with regard to the plant species [12]. Abdulnour et al. [33] reported that the Ca and B influence the uptake of each other therefore, it is important to optimize their concentration in the culture medium while addressing the STN obstacle.

By the end of the rooting and elongation stage purple color and chlorosis were observed on Thompson Seedless cv. leaves. The elevation of PO_4^- concentration to 1.5 and 2.0 of its MS original concentration relieved these symptoms. Increasing PO_4^- concentration improved Thompson Seedless cv. developing shoot numbers and their dry weight. However, these parameters were decreased for the Taify ones which indicate the genotype effect. This genotypic effect could be due to the variation on the active constituents which may vary quantitatively and qualitatively, affecting the reproducibility of results [28]. Many reports indicate that such non-organic MS component may be too low for some micropropagation purposes and should increase up to 3.71 mM to induce adventitious shoot formation or to increase the rate of shoot multiplication in shoot cultures [18].

Results showed that Thompson Seedless cv. mother plant had significantly lower P content (0.72%) than Taify cv. (1.21%). That could explain the role of increasing phosphate content on the growth medium to increase the regeneration rate of Thompson Seedless cv. unlike the Taify one George and De Klerk [18] and Mohamed [21] referred that the phosphate in MS medium is inadequate for some cultures, for example, when many shoots grow together in a static shoot culture. Results suggested that higher PO_4 concentration than that of original MS medium could improve micropropagation of some grapevine cultivar. However, the explant response to the culture components is definitely dependent on the genotype and the mother plant growth conditions which affect its organic and non-organic constitute [22].

Thus, compared with previously published protocols, which only regenerated 2.5 and 4.95 shoot explant^{-1} for Taify and Thompson Seedless cvs. respectively, a novel and efficient protocol for shoot regeneration for these cvs. using single nodes has been developed. The present study achieved 17.0 and 6.2 shoot explant^{-1} using MS medium supplemented with BA ad IBA. Enriching the growth medium with 100 mM B and 2.5 mM Ca successfully overcome the STN phenomena of Thompson Seedless cv. Moreover, results indicated that doubling the MS medium PO_4^- concentration improved the regenerated shoot numbers and their dry weights of only Thompson Seedless cv. which reflect the genotype effects.

So, this protocol may permit the use of micropropagation for the mass production of both important cultivars, and assist the use of cryopreservation technique for short as well as long term preservation. Also, this protocol could use for the transformation technology to improve their traits. It may also form the basis for *in vitro* plants selection for biotic and/or abiotic stresses.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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