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Influence of plant growth regulators, carbohydrate source and concentration on micropropagation and other physiological traits of grape (*Vitis vinifera* L. cv. Shahroudi) under *in vitro* conditions

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Abstract
The grapevine (*Vitis vinifera* L.) is a native of central Asia and has been propagated by various methods, including *in vitro* propagation. Present research was conducted to study the effect of plant growth regulators (BAP, IBA), carbon sources (sucrose, glucose, fructose, table sugar) and their concentrations (30, 60 and 90 g/l) on the proliferation, root induction, protein, chlorophyll and carotenoid contents of the grape (*Vitis vinifera L*. cv. Shahroudi) in *in vitro* conditions based on a completely randomized design with three replications, using nodal explants. The result showed that the highest rate of proliferation (3.04 shoots per explant) occurred at MS medium, containing 1.5 mg/l BAP plus constant amounts of GA3 (0.3 mg/l) and IBA (0.1 mg/l). The highest rate of root induction (88.88%) was obtained at 1/2 MS medium at 0.5 mg/l IBA. Type and concentration of carbon source had a significant effect on some of the measured characteristics. The highest plantlet height was obtained in MS medium supplemented with 30 g/1 table sugar and sucrose. Also, the highest number of shoots per explant (3.5 shoots) belonged to the use of 30 g/l sucrose in the culture medium, followed by 30 g/l table sugar (3.23 shoots). The highest value of chlorophyll *a* was observed for 90 g/l glucose, followed by 30 g/l sucrose and 60 g/l table sugar. The highest chlorophyll *b* content was obtained for 60 g/l table sugar, followed by 30 g/l table sugar and sucrose. In general, with regard to most of the characteristics under study, it could be inferred that the propagation efficiency of 30 g/1 sucrose or table sugar was better than other carbon sources. Considering economic reasons and time, these treatments can be recommended for the commercial micropropagation of the Shahroudi cultivar of grapevine, instead of traditional methods of propagation.

Keywords: Grape; Growth regulators; Physiological traits; Pigments; Regeneration; Tissue culture.

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Introduction

The grape is native to the Mediterranean and central Asia. It is a self-pollinated and dicot plant, which belongs to the *Vitaceae* family. This family has 14 genera Mabberley (1997), but only the *Vitis* genus includes most of the edible species (Talcott *et al.* 2003). Iran is regarded as the home of the original stocks of the grape plant.

Plant tissue culture is a method for accessing mass production with high quality in a shorter space of time. Several reports exist about the grape micropropagation by nodal segments having lateral buds. According to Alizadeh *et al*. (2012), the highest rate of shoot induction in grapes obtained with 2 mg/l benzylaminopurine (BAP) + 0.2 mg/l naphthalene acetic acid (NAA). Lee and Wetzstein (1990) and Jaskani *et al*. (2008) reported the best rate of grape proliferation from the MS medium (Murashige and Skoog 1962) supplemented with 10 and 5 μM benzyl adenine (BA), respectively.

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Based on Abido *et al.* (2013), the highest rate of shoot induction was obtained at the MS medium, including BAP (3 mg/l) + NAA (0.2 mg/l). Also, the maximum rate of root induction (87%) attained with 1 mg/l indolebutyric acid (IBA) + 0.5 mg/l NAA. Singh *et al.* (2004) obtained the greatest rate of root induction at ½ MS with 2 mg/l IBA.

In natural conditions, plants convert the solar light energy into chemical energy through photosynthesis, making it usable to its organs to provide the required carbon. However, in *in vitro* conditions, a carbon source is needed for the plant to survive without the need of sunlight and photosynthesis. Carbon is the essential component of the tissue culture medium. Sucrose, as the most common carbon source and osmotic regulator, provides energy to support the *in vitro* growth of plant tissues (Yaseen *et al*. 2013). In a review paper, Gautheret (1955) summarized the efficiency of different carbon sources in tissue culture. Sucrose was found to be the best carbon source, followed by glucose, maltose and raffinose; fructose was less effective and mannose and lactose were the least suitable. According to Gopal *et al.* (2004), higher concentration of sucrose (60-80 g/l) improved microtuber production, biomass and microtuber dry matter content in potato. However, in a study on the common ninebark plant (*Physocarpus opulifolius* L.), fructose was proposed as the best carbon source for the in vitro shoot proliferation and rooting in this plant (Ilczuk *et al.* 2013).

Despite valuable researches conducted about the tissue culture, it has had limited use in viticulture. Similar to majority of woody perennials, considerable difficulties were encountered and percentages of recovery were low (Chee *et al.* 1984). Furthermore, there is a possibility of somaclonal variation in the tissue culture (Banilas and Korkas 2007). Therefore, the purpose of this study was to evaluate the effects of plant growth regulators, carbon sources and concentrations on the proliferation, root induction, chlorophyll *a*, chlorophyll *b*, carotenoids, total protein content, and other growth characteristics of grape, cv. Shahroudi, grown *in vitro* conditions.

Materials and Methods

Plant material and sterilization

To evaluate the effect of plant growth regulators on micropropagation of *Vitis vinifera* L. cv. Shahroudi under *in vitro* conditions, a completely randomized design with three replications was carried out on the MS medium in ABRII (Agriculture Biotechnology Research Institute of Iran). Some stems *of the* Shahroudi cultivar was collected from the city of Malekan, East Azarbaijan province, Iran. The healthier stems were selected and cut through a single bud. After disinfection of the explants with 70% ethanol for one minute and 0.7 mg/l mercury hypochlorite for 4 minutes with gentle shaking under the laminar hood, the sterile explants were transferred into jars containing the MS medium with different levels of plant growth regulators, and 12.5 mg/l gentamycin and 100 mg/l vancomycin (Tarinejad 2013) for the contamination control at the establishment stage. The disinfected explants stayed at the establishment stage for four weeks (Figure 1).

Culture establishment and shoot multiplication

Similar concentrations were used for both

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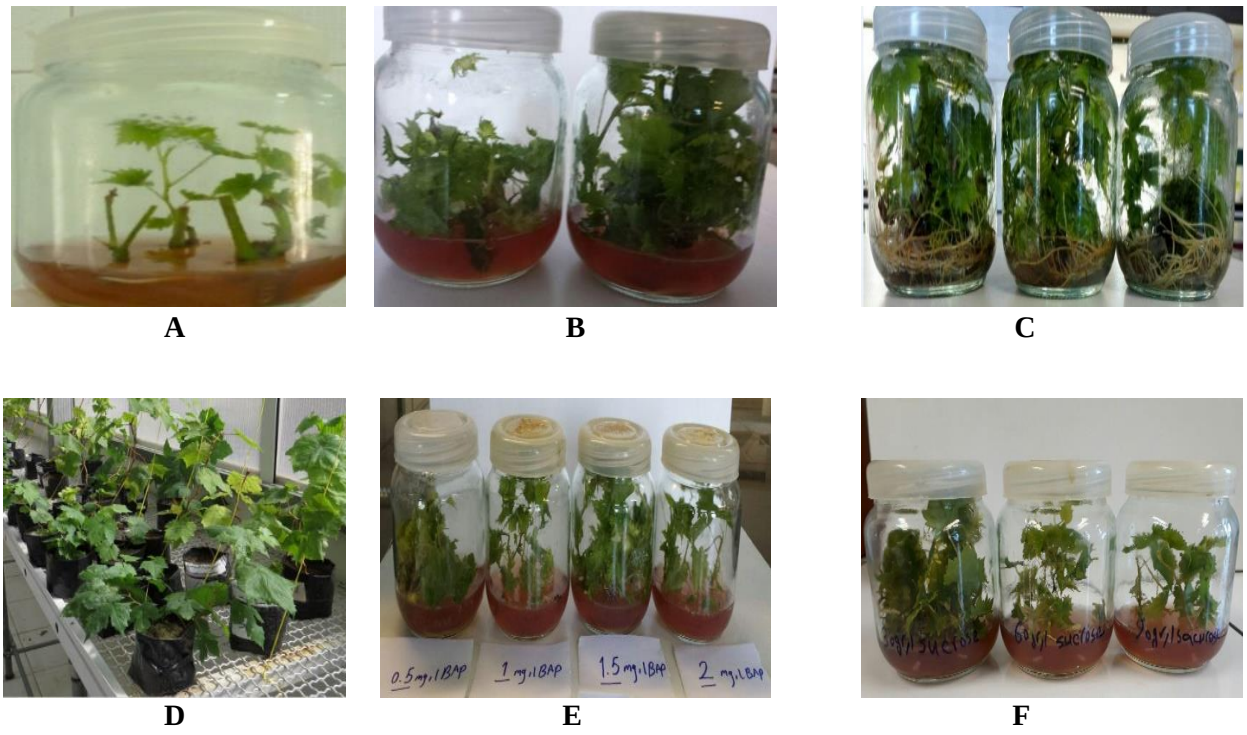

Figure 1. Different stages of micropropagation of *Vitis vinifera* L. cv. Shahroudi; A) Establishment of explants; B) Proliferation of explants; C) Rooting of explants; D) Final adaptation stage in the greenhouse; E) Comparison of BAP levels; F) Comparison of carbon concentrations.

establishment and proliferation of the explants as follows: BAP (0, 0.5, 1, 1.5 and 2 mg/l) with the constant concentration of GA3 (0.5 mg/l) and IBA (0.1 mg/l); the only difference was that the GA3 level was changed to 0.3 mg/l at the proliferation stage and antibiotics were omitted. The factorial treatments were arranged as completely randomized design (CRD). The proliferation rate for all treatments was measured in three replications, and each replication consisted of three explants. After optimization of shoot induction within eight weeks, for *in vitro* rooting studies, elongated shoots (3-4 cm long) were transferred to MS, 1/2 MS and 1/4 MS, including IBA (0, 0.5 and 1 mg/l). The percentage of rooted shoots was recorded for each experimental unit. To remove the remnants of agar, the well-rooted plantlets were gently rinsed with tap water. Then, these plantlets were transferred to plastic bags filled with a mixture of autoclaved peat and perlite (1:1). After moistening with the Hoagland liquid medium, the bags were kept in a mist chamber for four weeks under following conditions: 80-70% relative humidity, day/night temperature of 20 ± 2 °C, 16 h photoperiod. Then, plantlets with six leaves were transferred to a greenhouse under controlled conditions. A mixture of autoclaved peat, perlite and field soil (2:1:1) was used at this stage.

Culture media and conditions

After optimization of the micropropagation, a factorial experiment was carried out to investigate the effect of different carbon sources and concentrations on the proliferation, total protein

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and other physiological traits of grape, cv. Shahroudi. At this step, the first factor included four carbon sources (sucrose, fructose, glucose, table sugar) and the second factor had three levels of carbon concentration (30, 60 and 90 g/l). After sub-culturing of the explants in the jars having carbon treatments, they stored in the growth chamber under 16 hours of light and 8 hour of dark at 25 ± 2 °C. At the time the shoots reached the sufficient growth, characteristics such as total protein, chlorophyll a, chlorophyll b, total chlorophyll, carotenoid, proline, plantlet height and shoot induction rate were measured. The protein content was measured based on the Bradford (1976) method. The amounts of pigments and proline concentration were determined according to Lichtenthaler and Wellburn (1983) and Abraham *et al.* (2010), respectively.

Statistical analyses

After analysis of variance, means were compared by Duncan's multiple range test. Analyses were carried out with the help of MSTAT-C software (Alizadeh and Tarinejad 2010).

Results

Analysis of variance (ANOVA) showed a significant difference among the treatments for the establishment and proliferation (table not shown). The highest (96.30%) and lowest (37.03%) rate of explant establishment that occurred in the MS medium containing 1 and 0 mg/l BAP, respectively (Table 1). The maximum (3.04) and minimum (1.18) number of shoot induction was obtained at 1.5 and 0 mg/l BAP, respectively. The highest (88.88%) and lowest (29.63%) rate of root induction was acquired in ½ MS with 0.5 mg/l IBA and MS medium with 0 mg/l IBA, respectively.

Based on ANOVA, the type of carbon source had a significant effect on all traits measured. Sugar level was also significant for most traits, except for chlorophyll *b* and total protein. Furthermore, the carbon source × carbon level interaction was significant for all of the studied traits, except for plant height and total protein (table not shown).

Comparison of carbon sources showed that sucrose and table sugar obtained the highest plantlet height and were significantly different with glucose and fructose. Also, the carbon concentration of 30 g/l had significantly greater plantlet height than other levels (Figure 2). Among the carbon sources, table sugar showed the highest total protein content followed by fructose. These two treatments were significantly different than glucose and sucrose (Figure 2).

The means of different carbon concentrations for the studied traits, averaged over carbon sources, are shown in Table 2. By increasing the carbon level from 30 to the higher levels, the amount of pigments increased significantly; however, the proliferation rate (number of shoots per explant) was better at the low concentration of carbon (30 g/l).

Means of measured variables for the combinations of different carbon sources with carbon concentrations are presented in Table 3. The highest amount of chlorophyll *a* was obtained at 90 g/l glucose followed by 30 g/l sucrose, 60 g/l table sugar and 90 g/l sucrose. Moreover, the lowest chlorophyll *a* was observed under 30 g/l

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Table1. Means of the traits related to shoot and root induction for different hormone treatments.

BAP (mg/l)	Proliferation		IBA (mg/l)	% root induction		
	% establishment of explants	Shoots per explant		MS	½ MS	¼ MS
0	37.03b	1.18d	0	29.63c	33.33c	40.74c
0.5	77.78a	2.26c	0.5	74.07a	88.88a	81.48a
1	96.30a	2.44bc	1	55.55b	77.77b	62.96b
1.5	88.89a	3.04a				
2	85.18a	2.78ab				

Values with different letters within a column indicate significant difference based on Duncan's multiple range test ($p \leq 0.01$).

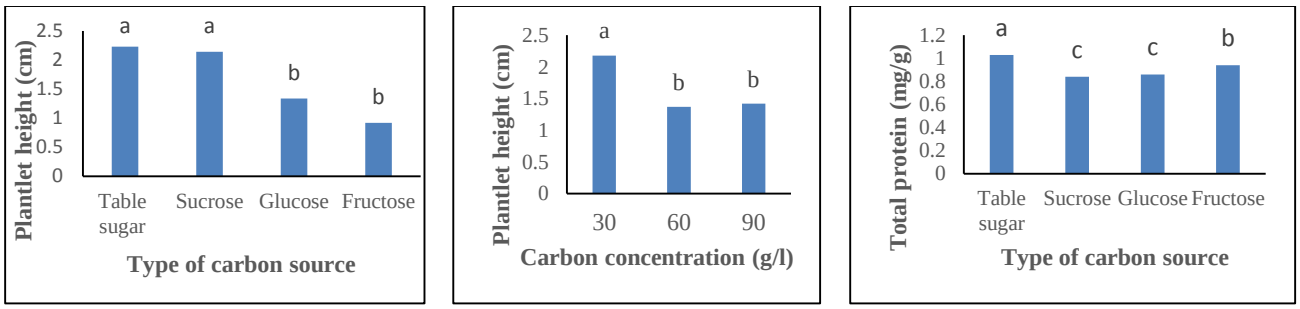

Figure 2. Effect of carbon sources and concentrations on plantlet height and total protein of grape, cv. Shahroudi; means with different letters are significantly different at 0.05 probability level, based on Duncan's multiple range test.

Table 2. Means of the studied traits as affected by different carbon concentrations.

Carbon Level (g/l)	Chl a (µg/ml)	Chl b (µg/ml)	Total Chl (µg/ml)	Carotenoids (µg/ml)	Shoots per explant
30	0.196 ± 0.05c	0.135 ± 0.02a	0.331 ± 0.07a	19.63 ± 3.82b	2.90 ± 0.20a
60	0.293 ± 0.03b	0.163 ± 0.02a	0.456 ± 0.04b	25.22 ± 3.06a	2.58 ± 0.20b
90	0.346 ± 0.04a	0.150 ± 0.01a	0.496 ± 0.06b	30.72 ± 3.93a	2.48 ± 0.12b

Values with different letters within a column indicate significant difference based on Duncan's multiple range test ($p \leq 0.01$);
Chl a: chlorophyll a; Chl b: chlorophyll b; total Chl: total chlorophyll.

fructose and glucose concentrations. The highest chlorophyll b was obtained at 30 and 60 g/l table sugar, followed by 30 g/l sucrose and 60 g/l glucose.

For the carotenoid pigments, 90 g/l sucrose resulted in the highest production of this pigment, which was significantly different from other treatments. Glucose at the 90 g/l and table sugar at 60 g/l showed the highest carotenoid content after sucrose.

The highest number of shoots per explant was occurred at the MS medium containing 30 g/l sucrose (3.5 shoots per explant) followed by 30 g/l table sugar (3.23 shoots per explant), 60 g/l sucrose and table sugar, and also 30 g/l glucose (3.03 shoots per explant). The lowest number of shoots per explant was observed at 30 and 60 g/l of fructose. Therefore, using sucrose and table sugar as the carbon source, produced more shoots per explant than other sources.

The differences between means of table sugar and sucrose with respect to the studied traits are presented in Table 4. There were no significant differences between the two carbon sources for

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Table 3. Means of chlorophyll a, chlorophyll b, carotenoids and shoots per explant as affected by the interaction of carbon type × carbon concentration.

Trait	Sucrose (30+)	Sucrose (60)	Sucrose (90)	Glucose (30)	Glucose (60)	Glucose (90)	Fructose (30)	Fructose (60)	Fructose (90)	Table sugar (30)	Table sugar (60)	Table sugar (90)
Chlorophyll a (µg/ml)	0.495ab	0.225d	0.444b	0.096fg	0.302c	0.534a	0.045g	0.180de	0.180de	0.148ef	0.463b	0.225d
Chlorophyll *b* (µg/ml)	0.195a	0.174ab	0.172ab	0.128bc	0.179ab	0.194a	0.021d	0.093c	0.106c	0.195a	0.207a	0.129bc
Carotenoids (µg/ml)	32.62c	13.85f	44.80a	6.29g	25.93d	42.17b	7.84g	20.04e	14.70f	31.76c	41.06b	21.21e
Shoots per explant	3.5a	3.03abc	2.77bcd	3.03abc	2.73bcd	2.23de	1.83ef	1.53f	2.33de	3.23ab	3.03abc	2.56cd

Values with different letters within a row indicate significant difference based on Duncan's multiple range test ($p \leq 0.01$); +: gr/l.

Table 4. Results of the t-test for the difference between the means of table sugar and sucrose as carbon sources with respect to various traits measured at the propagation stage.

Carbon source	Plantlet height (cm)	Total protein (mg/g)	Chlorophyll a (µg/ml)	Chlorophyll b (µg/ml)	Total chlorophyll (µg/ml)	Carotenoids (µg/ml)	Shoots per explant
Sucrose	2.14 ± 0.43	0.84 ± 0.01	0.38 ± 0.04	0.18 ± 0.01	0.57 ± 0.04	30.43 ± 4.5	3.10 ± 0.12
Table sugar	2.22 ± 0.26	1.03 ± 0.01	0.28 ± 0.05	0.17 ± 0.02	0.46 ± 0.05	31.34 ± 2.9	2.94 ± 0.16
t-test	-0.177ns	-16.81**	1.69ns	0.182ns	1.59ns	-0.17ns	0.772ns

ns, **not significant and significant at 1% probability level, respectively.

plantlet height, chlorophyll *a*, chlorophyll *b*, total chlorophyll, carotenoids and number of shoots per explant. However, the total protein of table sugar was significantly higher than sucrose.

Discussion

The explant establishment had a good response to cytokinins, so that the lowest and highest rate of establishment was obtained at 0 and 1 mg/l BAP, respectively; however, there was no significant differences between 1 and 1.5 mg/l BAP in terms of explant establishment. Also, by increasing the BAP level, the shoot induction increased, and the highest shoot induction was observed at 1.5 mg/l BAP. Cytokinins are involved primarily in cell division, apical dominance, shoot initiation and growth, and embryonic development (Kieber and Schaller 2014). In a study on the *in vitro* propagation of grapevine, Abido *et al.* (2013) obtained the maximum number of proliferated shoots on MS medium containing 3.0 mg/l BAP + 0.2 mg/l NAA. Gray and Benton (1991) studied the effect of BA on muscadine grape (*Vitis rotundifolia*). They showed that 5, 10 and 20 µM BA and 5 µM thidiazuron (TDZ) produced the highest number of shoots per apex (3.4-3.8).

In this study, the highest rate of root induction was obtained from 1/2 MS with 0.5 mg/l IBA. Also, other researchers such as Lee and Wetzstein (1990), Lewandowski (1991), Heloir *et al*. (1997), Barreto and Nookaraju (2007), Jaskani *et al.* (2008) and Abido *et al*. (2013) reported the root induction by application of IBA in the tissue culture of *Vitis*.

Our study showed that, sucrose and table sugar had highest values of the plantlet height among carbon sources. Furthermore, sucrose had greatest number of shoots per explant, followed by 30 and 60 g/l table sugar. In the plant tissue culture, sucrose is used as a source of carbohydrates to provide energy for cellular function (Yaseen *et al*.

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2013). Similar results were also observed in pine by Swamy *et al*. (2010), who demonstrated that 20% sugarcane juice resulted in the maximum shoot length and higher number of multiple shoots in the MS medium. Moreover, they obtained maximum fresh weight of shoots on MS medium containing 2% sucrose. Also, in a literature review by Gautheret (1955), sucrose was indicated as the best carbon source, followed by glucose, maltose and raffinose, but fructose was less effective. However, some workers have reported different results than ours in several plants. Abou Rayya *et al*. (2011) studied the effects of carbon sources such as sucrose, glucose and fructose on the tissue culture characteristics of bitter almonds. They concluded that glucose is the most efficient carbon source for stimulating the production of shoots, fresh weight and shoot length, followed by sucrose and fructose. On the other hand, sucrose gave healthier plants than glucose or fructose. Mamiya and Sakamoto (2000) stated the role of sucrose, glucose, fructose, mannitol and sorbitol on germination of somatic embryos in asparagus with no significant difference among these carbon sources. According to Ilczuk *et al.* (2013), fructose was the best carbon source for the in vitro shoot proliferation and rooting in common ninebark (*Physocarpus opulifolius* L.).

Table sugar acquired greatest amount of chlorophyll b and high values of chlorophyll a and carotenoids at 60 g/l concentration. On the other hand, sucrose showed highest carotenoids content at 60 g/l, and high chlorophyll a and chlorophyll b at 30 g/l concentrations. The plant chlorophyll is an important component of photosynthetic capacity and hence plant growth (Li *et al.* 2018). Therefore, the carbon sources that result in higher chlorophyll content, will be more suitable in the tissue culture technique.

Table sugar showed highest amount of total protein on the average of three levels, which was significantly higher than sucrose. According to Swamy *et al.* (2010), the protein content was maximum on the media supplemented with 20% sugarcane juice followed by 2% sucrose.

The t-test showed no significant differences between sucrose and table sugar (on the average of the concentration levels) with respect to the studied traits, except protein content; even table sugar had significantly higher protein content than sucrose (Table 4). Considering concentration levels separately (Table 3), 30 g/l sucrose had highest number of shoots per explant (3.5 shoots); however, it was not significantly different from the value obtained by 30 g/l table sugar (3.23 shoots). Furthermore, at the concentration of 30 g/l, both carbon sources showed similar chlorophyll b and carotenoids contents (Table 3). Therefore, it seems feasible to use 30 g/l table sugar in the medium instead of 30 g/l sucrose, considering cost and availability.

Conclusions

MS medium with 1.5 mg/l BAP plus constant amounts of GA3 (0.3 mg/l), IBA (0.1 mg/l) and sucrose (30 g/l), resulted in a good proliferation rate. Although both sucrose and table sugar were effective on growth characteristics of the grapevine explants in our research work, but table sugar is more economic and suitable for the tissue culture of grape, because of lower cost and availability. In conclusion, our micropropagation protocol for

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grape, cv. Shahroudi, is comparable to the traditional propagation methods due to good proliferation (3.5 shoots per explant) and root induction rate (88%). Therefore, with respect to economy and time, we recommend this protocol for micropropagation of the Shahroudi cultivar of grapevine on a commercial scale.

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تأثیر تنظیم کننده‌های رشد گیاه، منبع کربوهیدرات و غلظت آن بر ریزازدیادی و سایر خصوصیات فیزیولوژیکی انگور (*Vitis vinifera* L. cv. Shahroudi) تحت شرایط درون شیشه‌ای

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چکیده

انگور (*Vitis vinifera* L. cv. Shahroudi) بومی آسیای مرکزی است و با روش‌های مختلفی از جمله ریزازدیادی درون شیشه‌ای تکثیر می‌شود. پژوهش حاضر با هدف بررسی تأثیر تنظیم کننده‌های رشد گیاه (BAP، IBA)، منبع کربن (ساکارز، گلوکز، فروکتوز، شکر معمولی) و غلظت آن (۳۰، ۶۰ و ۹۰ گرم در لیتر) بر ریزازدیادی، القای ریشه، مقدار پروتئین، کلروفیل و کاروتنوئید انگور رقم شاهرودی در شرایط درون شیشه ای بر اساس طرح کاملا تصادفی با سه تکرار روی ریزنمونه‌های میان‌گره انجام شد. نتایج نشان داد که بیشترین میزان شاخه زایی (۳/۰۴ شاخه به ازای هر ریزنمونه) در محیط MS محتوی BAP (1.5 mg/l) به اضافه مقادیر ثابت GA3 (0.3 mg/l) و IBA (0.1 mg/l) و بیشترین میزان القای ریشه (۸۸/۸۸ درصد) در محیط MS ½ حاوی IBA (0.5 mg/l) به دست آمد. نوع و غلظت منبع کربن روی برخی از صفات اندازه‌گیری شده، تاثیر معنی‌داری داشت. بیشترین ارتفاع گیاهچه در محیط MS حاوی ۳۰ گرم در لیتر ساکارز و شکر معمولی به دست آمد. همچنین، بیشترین تعداد گیاهچه به ازای هر ریزنمونه (۳/۵ گیاهچه) متعلق به غلظت ۳۰ گرم در لیتر ساکارز بود و در مرتبه بعدی غلظت ۳۰ گرم در لیتر شکر معمولی (۳/۲۳) گیاهچه قرار داشت. بیشترین مقدار کلروفیل a در مورد ۹۰ گرم در لیتر گلوکز مشاهده شد و به دنبال آن ۳۰ گرم در لیتر ساکارز و ۶۰ گرم در لیتر شکر معمولی واقع شدند. بیشترین مقدار کلروفیل b نیز در غلظت ۶۰ گرم در لیتر شکر معمولی و سپس ۳۰ گرم در لیتر شکر معمولی و ساکارز به دست آمد. به طور کلی، با توجه به بیشتر صفات مورد مطالعه، می‌توان بیان نمود که کارآیی پرآوری برای ۳۰ گرم در لیتر ساکارز یا شکر معمولی بهتر از سایر منابع کربنی بود. بنابراین، با در نظر گرفتن دلایل اقتصادی و عامل زمان، این تیمارها را می‌توان برای ریزازدیادی انگور رقم شاهرودی در مقیاس تجاری به جای روش‌های تکثیر سنتی توصیه کرد.

واژه‌های کلیدی: انگور؛ باززایی؛ تنظیم کننده‌های رشد؛ رنگدانه‌ها؛ صفات فیزیولوژیکی؛ کشت بافت.

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