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In vitro Embryo Rescue of Flame Seedless Grape

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Abstract: The present study was carried out through (2018-2019) seasons to apply embryo rescue technique for Flame Seedless grape cultivar development to increase breeding efficiency. The ovules excised from self-pollinated berries were harvested at four developmental stages; 35, 42, 49 and 56 days after flowering (DAF) and then *in vitro* cultured on four different embryo formation media; MS, Nitsch, B5 and WPM. After 8 weeks from ovule culture, all developed embryos from the micropylar end of ovule were excised and then were placed on WPM (embryo germination medium) supplemented with 20 gl^{-1} sucrose, 0.5 mgl^{-1} BA and 1 gl^{-1} AC. B5 medium had the highest average percentage of ovule direct germination at 42 DAF, while MS medium had the highest average percentage of ovule producing callus and developed ovules at 49 and 56 DAF, respectively and Nitsch medium recorded the highest average percentage of undeveloped ovule at 35 DAF. Several swelling ovules presented well developed embryos which showed normal developmental patterns. At 49 DAF, the highest percentage of embryos formation on MS medium was (39.2 and 32.7%) in both seasons, respectively. The percentage of embryo germination was increased with increasing developmental stage of berry until reached to (46.4 and 42.7%) in both seasons, respectively at 49 DAF. The highest number of roots (3.5 roots) and length of roots (21.0 cm) of seedlings derived from embryos germination were obtained from the combination treatment (0.1 mgl^{-1} IBA + 0.4 mgl^{-1} BA).

Key words: Seedless grapes · Embryo rescue · Stenospermocarpy · *In vitro* culture · Breeding

INTRODUCTION

Grapevine (*Vitis vinifera* L.) is one of the most valuable fruit crops around the world and 'Flame Seedless' is one of the most important seedless grapevine cultivars grown. Because of its excellent fruit characteristics and high yields, 'Flame Seedless' is suitable for grapevine breeding and improvement programmes.

Seedlessness in *Vitis vinifera* L. cv. Flame Seedless is due to stenospermocarpy in which natural pollination and fertilization occur, but embryo development stops soon after and thus ovules do not develop into mature seeds. Hence, conventional breeding methods are limited and cannot be followed for its improvement.

Emershad and Ramming [1] reported stenospermic grapes could generate plants via ovule culture. It enhanced the application of this *in vitro* method in seedless grape breeding. Since then, this embryo rescue technique has been widely applied to embryo germination of seedless grape cultivars in cross-breeding program [2].

To address the low efficiency of the embryo rescue technique, recent studies have investigated the effects of factors such as genotype, culture medium, the timing of embryo and ovule separation and plant growth regulators. A variety of different culture media including Woody Plant Medium (WPM); Nitsch; Murashige and Skoog (MS) and B5 have been studied in order to improve the efficiency of the embryo rescue technique [3-8, 2].

The objective of present investigation is to determine the most appropriate sampling time for embryo rescue of Flame Seedless cultivar via *in vitro* culture techniques to increase the efficiency of Flame Seedless breeding program.

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MATERIALS AND METHODS

Plant Material: Six year-old trees of Flame Seedless (*Vitis vinifera* L.), grown in a private orchard at Sadat City were used as a source of plant material through (2018-2019) seasons.

Sampling Time: The sampling time refers to the number of days after flowering (DAF), also known as embryo rescue time. Before opening flower cluster, the cluster were enclosed in paper bags (Fig. 1). The self-pollinated berry clusters were harvested at four developmental stages; 35, 42, 49 and 56 days after flowering (DAF) (Fig. 2). The diameter and weight of berry and number of ovules per berry were recorded at all developmental stages.

***In vitro* Culture Conditions:** All the media were adjusted to pH 5.7 ± 0.1 using Hcl or NaOH and autoclaved at 100 K.pa (15 P.S.I) and 121°C for 20 minutes. Ovule cultures were incubated in a growth room at 25±1°C for 8 wks. in the dark, then the isolated embryos were placed under cool white fluorescent lamps (4 lamps per shelf) for the following subcultures to provide light intensity of 4200-4400 lux at explants level (30 cm from the light).

***In vitro* Ovule Culture:** The berries were subjected to continuous flow of tap water about 20 min. then surface sterilized in the laminar flow hood by immersion for 30 s in 70% (v/v) ethyl alcohol, followed by immersion in sodium hypochlorite solution (about 0.5% active chlorine in water) containing a few drops of Tween 20 for 20 minutes. Finally, it rinsed three times for 5 min. for each with sterile distilled water. The berries were longitudinally cut by a scalpel blade afterwards the complete immature ovules were detached using a forceps and distributed on the culture media surface.

All ovules cultured for 8 weeks. on four different embryo formation media based on the basal salts and vitamins of MS [7]; Nitsch [6]; B5 [8] and Woody Plant Medium (WPM) [4]. These media were compared as an independent experiment to develop ovules and induce embryos. The previous media were supplemented with both of 30 gl^{-1} sucrose, 2 gl^{-1} activated charcoal (AC), 1.5 mgl^{-1} filter-sterilized indole-3-acetic acid (IAA), 0.5 mgl^{-1} filter-sterilized gibberellic acid (GA_3) and 7 gl^{-1} agar for solidification. The experiment was comprised in a completely randomized design with 16 treatments in a factorial experiment (4 media × 4 developmental stages) in 10 replicates each one consisted of one glass jar (containing 20 ml of medium for each) with 9 ovules.

Afterwards, the ovules were sub-cultured to the same media for further 4 wks. After the second subculture (8 wks. of culturing ovule on the induction medium), the percentages of ovule direct germination, ovule producing callus, developed ovules, undeveloped ovules (unresponsive) and embryo formation at different developmental stages (days after flowering) were recorded.

***In vitro* Embryo Rescue and Culture:** After 8 wks. from culture, the developed ovules under aseptic conditions were placed on a sterile Petri dish and cut in halves longitudinally with a sterile scalpel blade (No. 11) and a forceps, then examined for embryo development and the percentage of all developed embryos was recorded. All developed embryos from the micropylar end of the ovule were excised with a histological needle and the aid of a binocular, then were placed on WPM (embryo germination medium) supplemented with both of 20 gl^{-1} sucrose, 0.5 mgl^{-1} benzyl adenine (BA), 1 gl^{-1} activated charcoal (AC) and 7 gl^{-1} agar for solidification. The experiment comprised 4 treatments (1 medium × 4 developmental stages) in 5 replicates each one consisted of one glass jar (containing 20 ml of medium for each) with 5 embryos. After 4 wks. of culture, the percentage of embryo germination was recorded for each developmental stage of cultured embryos.

Root Proliferation of Seedlings and Acclimatization: To proliferate roots of the seedlings that derived from rescued embryos, the seedlings were sub-cultured on 20 ml of the basal salts and vitamins of MS medium at half strength supplemented with 30 gl^{-1} sucrose, either IBA at (0.1 or 0.5 mgl^{-1}) alone or in combination with 0.4 mgl^{-1} BA and 7 gl^{-1} agar were added as well as the control treatment (free hormone). The experiment was arranged in a completely randomized design with 5 treatments in a factorial experiment with 10 replicates for each. Each replicate contained one seedling. After 4 wks. from subculture, the number and length of roots per seedling were recorded.

Acclimatization: The rooted seedlings were selected and then transferred to plastic pots filled with peat-moss and sand (1:1). The pots were covered with polyethylene bags and placed in the glasshouse for acclimatization. Irrigation was applied every 3 days with a volume of nutrient ½ MS medium without sucrose.

Duncan's multiple range tests at 5% level was used in all aforementioned experiments to differentiate means [9].

Fig. 1: Self pollinated berry clusters of Flame Seedless grape
a: The inflorescences before opening flower buds.
b: The inflorescences after enclosed in paper bags.

Fig. 2: Self pollinated berry clusters and ovules at different developmental stages
a: 35, b: 42, c: 49 and d: 56 DAF.

RESULTS AND DISCUSSION

Effect of Sampling Time (DAF) on the Average of Berry Diameter, Weight and Ovule Number: The average of berry diameter, weight and ovule number was affected by the sampling time or developmental stage of berry (Table 1). In both seasons (2018 and 2019), the berries harvested 35 days after flowering (DAF) recorded the lowest average values for both diameter and weight (9.63 mm, 0.59 g) and (10.35 mm, 0.64 g), during the studies seasons respectively. Progressively, these values increased until reached to (14.17 mm, 1.80 g) and (14.22 mm, 1.54 g) at 56 DAF, respectively. In opposite, the average of ovule number in (2018 and 2019) seasons was

Table 1: Effect of sampling time (DAF) on the average of berry diameter, weight and ovule number in (2018 and 2019) seasons

	Season 2018			Season 2019		
Sampling Time (DAF)	Average berry diameter (mm)	Average berry weight (g)	Average ovule number	Average berry diameter (mm)	Average berry weight (g)	Average ovule number
35	9.63 d	0.59 d	2.20 a	10.35 c	0.64 d	2.10 a
42	11.15 c	0.88 c	1.78 b	11.94 b	0.96 c	1.37 b
49	13.89 b	1.51 b	1.47 b	12.60 b	1.18 b	0.90 c
56	14.17 a	1.80 a	0.93 c	14.22 a	1.54 a	0.57 d

Means with different letter (comparison within each column) are significantly different (Duncan's Multiple Range at 0.05).

Table 2: Effect of sampling time (DAF) and medium type on the percentage of ovule direct germination in (2018 and 2019) seasons

	Season 2018					Season 2019				
	Media					Media				
Sampling Time (DAF)	MS	Nitsch	B5	WPM	Mean (A)	MS	Nitsch	B5	WPM	Mean (A)
35	0.0 k	3.7 fg	4.0 ef	1.2 j	2.2 C	0.0 k	3.9 f	4.1 f	1.3 i	2.3 C
42	1.1 j	6.5 d	9.9 a	7.4 c	6.2 A	1.2 i	6.9 d	10.4 a	7.8 c	6.6 A
49	3.0 h	3.2 gh	6.2 d	2.1 i	3.6 B	3.2 g	3.4 g	6.5 d	2.7 h	3.9 B
56	0.2 k	1.4 j	9.1 b	4.4 e	3.8 B	0.7 j	1.5 i	9.7 b	4.7 e	4.1 B
Mean (B)	1.1 C	3.7 B	7.3 A	3.8 B		1.3 C	3.9 B	7.7 A	4.1 B	

Means with different letter (comparison within each factor) are significantly different (Duncan's Multiple Range at 0.05).

the highest (2.20 and 2.10) at 35 DAF and decreased until reached to (0.93 and 0.57) at 56 DAF, respectively. Generally, with increasing developmental stage of berry, the average of berry diameter while weight increased and ovule number decreased, significantly.

Increases in berry weight and diameter during development are associated with a double sigmoid curve and is typically divided into three phases [10]. During Phase I, increase in the size of a berry is initially caused by pericarp cell division, with subsequent growth because of cell enlargement [11]. Little or no pericarp growth occurs in Phase II (lag phase); however, the seed continues to mature. Phase II is an artificial division physiologically, as it reflects a slowing of the Phase I growth period, prior to the abrupt change in growth at the start of Phase III and the onset of ripening [12].

Spiegel-Roy *et al.* [13] reported the linear increased berry weight till 46 DPA in different cultivars of grapes. Singh and Brar [14] recorded linear increase in the diameter of berries up to 30 and 45 days post anthesis (DPA) in Thompson Seedless and Beauty Seedless, respectively. Concerning number of ovule, they reported that the number of viable ovules per berry started declining 20 days post anthesis (DPA) in Thompson Seedless and Beauty Seedless. The decline in the total number of developing ovules in seedless cultivars may be attributed to the degeneration of the embryo; eventually the process led to ovules collapsing and/or seed abortion. In Flame Seedless grape, the gradually decreasing in ovule number with berry growth progresses due to endosperm degeneration at 29 days after flowering (DAF) followed by embryo abortion at 39 DAF [15].

Effect of Sampling Time (DAF) and Medium Type on the Percentage of Ovule Direct Germination: Directly, ovule germination at different developmental stages was observed. In Table (2), at 42 DAF the highest significant mean percentage observed in (6.2 and 6.6%) and the lowest was (2.2 and 2.3%) of the respondent ovules at 35 DAF in 2018 and 2019 seasons, respectively. Concerning medium type, B5 medium had the highest significant mean percentage of ovule direct germination (7.3 and 7.7%) and MS had lowest significant percentage (1.1 and 1.3%) in (2018 and 2019) seasons, respectively. The interaction between the two studied factors, B5 medium with 42 DAF recorded higher significant values than other media in both seasons (Fig. 3a). Generally, with increasing grape development in growth, the average of berry diameter and weight is increasing and ovule number is decreasing.

Similar results obtained by Ji *et al.* [16] achieved the best sampling times for ovule germination rate from different combinations, in days after flowering (DAF), were as follows: 'Flame Seedless × Beichun' (39 DAF); 'DA7 × Blush Seedless' (44 DAF); 'DA7 × Jingyou' (44 DAF) and 'Flame Seedless × Fujiminori' (39 DAF). Xu *et al.* [17] reported that grape ovules of interspecific sternospermocarpic hybrids were excised from berries

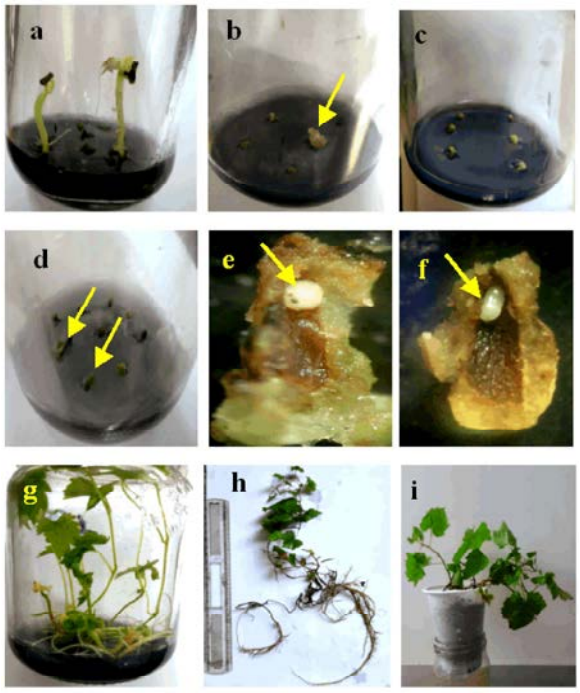

Fig. 3: a Ovule direct germination on B5 medium at 42 DAF.
b Ovule producing callus on MS medium at 49 DAF.
c Developed ovules on MS medium at 49 DAF.
d Undeveloped ovules on WPM medium at 35 DAF.
e, f Developed globular and torpedo embryos, respectively; located in the central position at the micropylar apex of the embryo sac (28× magnification).
g Seedlings derived from embryos germination at 49 DAF.
h Effect of IBA and BA concentrations on root proliferation of seedlings.
i Complete zygotic seedling derived from embryo rescue of Flame Seedless grape.

Table 3: Effect of sampling time (DAF) and medium type on the percentage of ovule producing callus in (2018 and 2019) seasons

	Season 2018					Season 2019				
	Media					Media				
Sampling Time (DAF)	MS	Nitsch	B5	WPM	Mean (A)	MS	Nitsch	B5	WPM	Mean (A)
35	12.3 f	2.5 h	11.1 f	3.7 h	7.4 D	13.1 g	2.6 k	11.8 h	3.9 j	7.8 D
42	21.0 d	7.1 g	7.4 g	7.1 g	10.6 C	22.2 d	7.4 i	7.8 i	7.5 i	11.2 C
49	48.5 a	20.6 d	45.7 b	32.2 c	36.8 A	51.3 a	21.8 d	48.3 b	34.1 c	38.9 A
56	16.7 e	12.3 f	15.3 e	12.3 f	14.2 B	17.6 e	13.0 g	16.2 f	12.9 gh	14.9 B
Mean (B)	24.6 A	10.6 D	19.9 B	13.8 C		26.0 A	11.2 D	21.0 B	14.6 C	

Means with different letter (comparison within each factor) are significantly different (Duncan's Multiple Range at 0.05)

6 to 8 weeks after pollination and then cultured on ER medium supplemented with 0.2 mgl^{-1} BAP or Nitsch medium supplemented with 0.2 mgl^{-1} NAA and 0.5 mgl^{-1} GA_3 direct germinated and produced few hybrid plants. On the other hand, Guo *et al*. [18] showed that the highest germination rate of selfed ovules of 'Venus Seedless' grape appeared when ovule inoculation was done 55 days after flowering.

Effect of Sampling Time (DAF) And Medium Type On The Percentage Of Ovule Producing Callus: Medium type and different sampling time affected in mean percentage of ovule producing callus. After about 4 wks., some of the swollen ovules started to produce callus. Data in Table (3) showed that at 49 DAF, the ovules gave the highest mean percentage of calli (36.8 and 38.9%) and the lowest was (7.4 and 7.8%) at 35 DAF in (2018 and

2019) seasons, respectively. Concerning medium type, MS medium had highest mean percentage of callus (24.6 and 26.0%) and Nitsch had lowest significant percentage (10.6 and 11.2%) in (2018 and 2019) seasons, respectively. The interaction between the two studied factors, MS medium with 49 DAF recorded the highest significant values than other media in both seasons (Fig. 3b).

Similar results obtained by Yang *et al.* [19] who achieved the highest percentage of callus from Fujiminori × Muscat Hamburg and Jingxiu × Kyoho crosses reached to 48.99 % and 35.45%, respectively on MS medium supplemented with 0.5 mg l^{-1} IAA and 0.5 mg l^{-1} GA_3. Ovules of Barbera cv. grape were collected 40 and 55 days after anthesis (DAA) and at fruit maturity. Samples of the first two dates were cultured on Nitsch medium with 10^{-5} M IAA and 10^{-6} M GA_3. Ovules collected 40 (DAA) developed only callus [20]. Gray and Hanger [21] reported that ovules sampled at 20, 30, or 40 days post-anthesis (DPA) were similar but produced significantly more embryogenic cultures than those sampled at 10 DPA in muscadine grape cultivars. Razi *et al.* [22] obtained the highest percentage of callus formation in ovules of Askari × Ruby Seedless cross on Nitsch medium supplemented with 1 mgl^{-1} IAA.

Effect of Sampling Time (DAF) And Medium Type On The Percentage Of Developed Ovules: After about 2 wks. from culture of ovules, some of them started to be swell with green color, these features indicated as developed and viable ovules (Fig. 3c). Data in (Table 4) showed the highest mean percentage at 56 DAF (45.0 and 47.6%) in 2018 and 2019 seasons, respectively. The lowest was (35.5 and 36.3%) in 2018 season and (37.6 and 38.4%) in 2019 season at (49 and 42 DAF), respectively and without significant difference between them. Regading medium type, MS medium had highest mean percentage of swelling ovule (47.8 and 50.5%), while B5 and WPM had lowest significant percentage (34.3 and 35.4%) in 2018 season and (36.2 and 37.5%) in 2019 season, respectively and without significant difference between them. The interaction between the two studied factors, MS medium with 56 DAF recorded higher significant values than other media in both seasons.

In various reports, ovules as early as 10 days and as late as 100 days after bloom were used, the optimal time were being 40-60 days after bloom [23]. When ovules are cultured at early stages of development, their survival was poor or lead to callusing under *in vitro* conditions while culture at late stages is not useful as the ovule shrivelled, indicating breakdown of embryo. Brar *et al.* [24] studied the ovule culture in seedless grapes. They found that maximum survival of ovules was observed in Perlette cv. grape when cultured 30 days after anthesis on MS medium. Guimei and Handfeng [25] obtained developmental rate of ovule of Flame × Ruby Seedless grape hybrid 48.2% and 56.3% on Nitsch and B5 media, respectively, with 1.7 mgl^{-1} IAA and 0.4 mgl^{-1} GA_3 and this rate increased dramatically at 35 to 49 days after pollination.

Effect of Sampling Time (DAF) And Medium Type On The Percentage Of Undeveloped Ovules: The great percentage of undeveloped ovules (unresponsive) was observed. These ovules distinguished with shrinking and browning (Fig. 3d). In table (5), at 35 DAF, the highest mean percentage of undeveloped ovule was (48.3 and 46.1%) and the lowest was (29.3 and 25.7%) at 49 DAF in (2018 and 2019) seasons, respectively. Regading medium type, Nitsch medium had highest mean percentage of unresponsive ovules (47.7 and 44.7%) and MS had lowest significant percentage (26.5 and 22.1%) in (2018 and 2019) seasons, respectively. The interaction between the two studied factors, Nitsch medium with 35 DAF recorded higher significant values than other media in both seasons.

Gray *et al.* [26] cultured ovules of seedless grapes on Nitsch medium with lAA, GA_3, sucrose and charcoal. They noticed that more ovules cultured at 10 days or at 60-70 days after pollination became brown compared to those cultured at 20-40 days. The number of shrivelled ovules started increasing 20 days after anthesis and at the same time, the number of viable ovules also started declining in all the seedless cvs. Perlette, Thompson Seedless and Beauty Seedless [14].

Effect of Sampling Time (DAF) And Medium Type On The Percentage Of Embryo Formation: Several swelling ovules presented well developed embryos which showed normal developmental patterns: globular, heart and torpedo stages (Fig. 3e, f). Data in (Table 6) showed that the highest mean percentage of embryo formation was (29.7 and 29.1%) at 49 DAF and the lowest was (10.3 and 11.2%) at 35 DAF in (2018 and 2019) seasons, respectively. MS medium showed the highest mean percentage of embryo formation (27.8 and 24.3%), while WPM medium showed the lowest (15.8 and 11.2%) in (2018 and 2019) seasons, respectively. The interaction between the two studied factors, MS medium with 49 DAF recorded higher significant values than other media in both seasons.

Table 4: Effect of sampling time (DAF) and medium type on the percentage of developed ovules in (2018 and 2019) seasons

	Season 2018					Season 2019				
	Media					Media				
Sampling Time (DAF)	MS	Nitsch	B5	WPM	Mean (A)	MS	Nitsch	B5	WPM	Mean (A)
35	50.6 b	29.1 hi	35.8 gh	39.5 def	38.8 B	53.5 b	30.7 i	37.9 gh	41.8 ef	41.0 B
42	45.6 c	32.9 gh	33.3 gh	33.3 gh	36.3 C	48.2 c	34.8 h	35.3 h	35.4 h	38.4 C
49	39.4 def	46.3 bc	29.6 hi	26.7 i	35.5 C	41.7 ef	49.0 c	31.3 i	28.2 i	37.6 C
56	55.6 a	43.8 cd	38.3 ef	42.2 cde	45.0 A	58.8 a	46.3 cd	40.5 fg	44.7 de	47.6 A
Mean (B)	47.8 A	38.0 B	34.3 C	35.4 C		50.5 A	40.2 B	36.2 C	37.5 C	

Means with different letter (comparison within each factor) are significantly different (Duncan's Multiple Range at 0.05)

Table 5: Effect of sampling time (DAF) and medium type on the percentage of undeveloped ovules in (2018 and 2019) seasons

	Season 2018					Season 2019				
	Media					Media				
Sampling Time (DAF)	MS	Nitsch	B5	WPM	Mean (A)	MS	Nitsch	B5	WPM	Mean (A)
35	37.0 h	64.7 a	49.1 d	55.6 b	48.3 A	33.4 f	62.8 a	46.3 d	53.0 b	46.1 A
42	32.3 i	53.5 c	49.4 d	52.2 c	45.9 B	28.4 g	50.9 c	46.5 d	49.3 c	43.4 B
49	9.1 m	29.9 j	18.5 l	39.0 g	29.3 D	3.8 k	25.8 h	13.8 j	35.0 f	25.7 D
56	27.6 k	42.5 e	37.4 h	41.0 f	41.1 C	22.9 i	39.2 e	33.8 f	37.7 e	38.1 C
Mean (B)	26.5 D	47.7 A	38.6 C	46.9 B		22.1 D	44.7 A	35.1 C	43.7 B	

Means with different letter (comparison within each factor) are significantly different (Duncan's Multiple Range at 0.05)

Table 6: Effect of sampling time (DAF) and medium type on the percentage of embryo formation in (2018 and 2019) seasons

	Season 2018					Season 2019				
	Media					Media				
Sampling Time (DAF)	MS	Nitsch	B5	WPM	Mean (A)	MS	Nitsch	B5	WPM	Mean (A)
35	16.6 gh	16.4 gh	2.7 l	5.6 k	10.3 D	16.8 h	16.0 h	5.3 k	6.8 j	11.2 D
42	17.5 g	16.3 h	24.8 e	13.9 i	18.1 C	16.2 h	19.8 f	22.1 e	16.1 h	18.5 C
49	39.2 a	19.4 f	27.6 d	32.5 c	29.7 A	32.7 a	26.4 d	30.6 b	26.7 d	29.1 A
56	37.9 b	25.1 e	14.3 i	11.3 j	22.1 B	31.4 b	28.5 c	18.4 g	12.9 i	22.8 B
Mean (B)	27.8 A	19.3 B	17.4 C	15.8 D		24.3 A	22.7 B	19.1 C	15.6 D	

Means with different letter (comparison within each factor) are significantly different (Duncan's Multiple Range at 0.05)

It is important to determine the sampling time for each cultivar as it has a significant effect on embryo formation. Embryos are aborted if sampling time is too late and it is hard to rescue if sampling time is too early [27]. Tsolova [28] obtained 52 viable embryos from the total of 640 ovules cultured of crosses of seedless grapevine varieties. He showed that the early date of ovule cultivation by the 50th day after anthesis proves to be more suitable in Kishmish Moldevski × Seedless hybrid VI-4. Yang *et al.* [19] reported that the optimal removal age of immature seeds for the best development of embryos of Fujiminori × Muscat Hamburg and Jingxiu × Kyoho crosses was 35-45 days after pollination. Li, *et al.* [29] showed that the highest embryo development rate of Emerald seedless grape was 27.3%, so its best sample time was 47 days after pollination (DAP), while for Crimson seedless grape was 24.7%, so its best sample time was 60 DAP after pollination. In other a study, the highest embryo formation percentage of Thompson Seedless, Flame Seedless, Crimson Seedless and Ruby Seedless was 37.5, 35.8, 35.0 and 45.7% occurring at 37, 45, 60 and 65 days after pollination, respectively [30].

The culture medium type is the source of nutrition for grape embryos developing *in vitro* as part of the embryo rescue process and, as such, represents a critical factor in determining the success rate. About 34.0% of embryo formation of seedless grape cultivars from the ovules excised 7 weeks after pollination (WAP) was obtained when these ovules cultured on double-phase ER medium supplemented with 6.0% sucrose and 0.3% activated charcoal [2]. Spiegel-Roy *et al.* [13] recovered thirteen plants out of 27 embryos from open pollinated ovules of cv. Thompson Seedless cultured on Nitsch medium supplemented with 10^{-5} M IAA and 10^{-6} M GA_3. Ji *et al.* [16] obtained the highest embryo formation rate (13.2%) when ovules of crossing between seedless grape cultivars were cultured on modified ER medium with different macronutrients and 500 mgl^{-1} mashed banana.

Table 7: Effect of sampling time (DAF) on the percentage of embryo germination in (2018 and 2019) seasons

	Embryo Germination %	
Sampling Time (DAF)	Season 2018	Season 2019
35	11.7d	9.4 d
42	25.2 c	23.9 c
49	46.4 a	42.7 a
56	38.7 b	36.5 b

Means with different letter (comparison within each column) are significantly different (Duncan's Multiple Range at 0.05).

Table 8: Effect of IBA and BA concentrations on root proliferation of seedlings

Treatment	No. of roots/seedling	Average length of root/seedling (cm)
Control (Free hormone)	1.7 c	9.4 d
0.1 mgl^{-1} IBA	2.6 b	15.0 b
0.1 mgl^{-1} IBA + 0.4 mgl^{-1} BA	3.5 a	21.0 a
0.5 mgl^{-1} IBA	2.3 bc	13.1 bc
0.5 mgl^{-1} IBA + 0.4 mgl^{-1} BA	2.2 bc	11.4 cd

Means with different letter (comparison within each column) are significantly different (Duncan's Multiple Range at 0.05)

Effect of Sampling Time (DAF) on Percentage Of Embryo Germination: Progressively, the percentage of developed embryos germination that derived from detached immature ovules was increased with increasing developmental stage of berry until reached to (46.4 and 42.7%) in (2018 and 2019) seasons, respectively at 49 DAF (Table 7) (Fig. 3g). Then these values started to decrease at 56 DAF (38.7 and 36.5%) in (2018 and 2019) seasons, respectively.

Embryo rescue and germination were affected by Sampling time with different seedless grape cultivars, such as 49 days after pollination (DAP) for Flame seedless [4] and 40 DAP for Thompson seedless [23]. Emershad *et al.* [31] reported that excision and culturing of Flame Seedless ovules 49 days after anthesis gave higher germination percentage. Gray *et al.* [26] showed that more embryos of Flame Seedless and Thompson Seedless grape cultivars were recovered from ovules cultured at 40 or 60 days than at 10 or 20 days after pollination. Tian *et al.* [2] obtained about 91.2% of embryo germination of seedless grape cultivars which removed from the ovules excised from immature fruits 7 weeks after pollination. Also, Guo *et al.* [18] reported the highest embryo germination rate of selfed ovules of 'Venus Seedless' grape reached 41.25% when sampling was done 55 days after flowering.

Effect of IBA and BA Concentrations On Root Proliferation of Seedlings: Data in (Table 8) showed effect of IBA and BA concentrations on root proliferation of seedlings derived from embryos germination. The highest number and length of roots were obtained from the combination treatment (0.1 mgl^{-1} IBA + 0.4 mgl^{-1} BA) reached to (3.5 root/seedling) and (21.0 cm length) (Fig. 3h). The lowest number and length of roots were (1.7 root/seedling) and (9.4 cm length) with control treatment. Greatly, root proliferation enhanced the acclimatization success of regenerated seedlings (Fig. 3i).

Similar results obtained by Li *et al.* [32] recorded well-rooted plant of seedless grapes when germinated embryos transferred to MS medium supplied with 0.1 mgl^{-1} IBA and 0.4 mgl^{-1} BA. While, Mostafaet *et. al.* [33] achieved the best root formation (90, 100, 80 and 70%) for Concord, Thompson Seedless, Beauty Seedless and King ruby cultivars, respectively on half strength MS medium supplied with 1.0 mgl^{-1} IBA.

CONCLUSION

The use of embryo rescue technique is critical for maximizing success when using stenospermocarpic grapes as female parents to breed new seedless grape cultivars. Flame seedless grape seedlings were obtained by *in vitro* culturing the ovules on different four media; MS, Nitsch, B5 and WPM, followed by separating and rescuing the embryos. These embryos were placed on WPM medium for germination. The suitable sampling time was 49 days after pollination for percentage of the embryos formation inside the ovules on MS medium reached to (39.2 and 32.7%) and for percentage of the embryos germination on WPM medium (46.4 and 42.7%) in (2018 and 2019) seasons, respectively. The best rooting of seedlings obtained from the combination treatment (0.1 mgl^{-1} IBA + 0.4 mgl^{-1} BA) reached to (3.5 roots/seedling) and (21.0 cm length).

REFERENCES

1. Emershad, R.L. and D.W. Ramming, 1982. *In ovulo* embryo culture of seeded and seedless *Vitis vinifera* L. HortScience, 17: 487.
2. Tian, L., Y. Wang, L. Niu and D. Tang, 2008. Breeding of disease-resistant seedless grapes using Chinese wild *Vitis* spp. I. *In vitro* embryo rescue and plant development. Scientia Horticulturae, 117: 136-141.
3. Emershad, R.L. and D.W. Ramming, 1994. Somatic embryogenesis and plant development from immature zygotic embryos of seedless grapes (*Vitis vinifera* L.). Plant Cell Rep., 14: 6-12.
4. Lloyd, G. and B.H. McCown, 1980. Commercially-feasible micropropagation of mountain Laurel, Kalmia latifolia, by use of shoot-tip culture. Combined Proceedings-International Plant Propagator's Society, 30: 421-427.
5. Schenk, R.V. and A.C. Hildebrandt, 1972. Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Canadian Journal of Botany, 50: 199-204.
6. Nitsch, J.P. and C. Nitsch, 1969. Haploid plants from pollen grains. Science, 163: 85-87.
7. Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Plant Physiology, 15: 473-497.
8. Gamborg, O.L., R.A. Miller and K. Ojima, 1968. Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res., 50: 151-158.
9. Snedecor, G.W. and W.G. Cochran, 1982. Statistical Methods. 7th Ed., The Iowa State Univ. Press, Ames, Iowa, USA.
10. Coombe, B.G., 1976. The Development of Fleshy Fruits. Annual Review of Plant Physiology, 27: 507-528.
11. Harris, J.M., P.E. Kriedemann and J.V. Possingham, 1968. Anatomical aspects of grape berry development. Vitis., 7: 106-119.
12. Coombe, B.G. and P.G. Iland, 2005. Grape berry development and winegrape quality. In: Viticulture. Volume 1 – resources. Eds. P.R. Dry and B.G. Coombe (Winetitles: Adelaide), pp: 210-248.
13. Spiegel-Roy, P., N. Sahar, J. Baron and U. Lavi, 1985. *In vitro* culture and plant formation from grape cultivars with abortive ovules and seeds. Journal of the American Society Horticultural Science, Alexandria, 110(1): 109-112.
14. Singh, Z. and S.J.S. Brar, 1992. *In vivo* development of ovule in seedless and seeded cultivars of grapes (*Vitis vinifera* L.) A particular reference to *in ovulo* embryo culture. Vitis, 31: 77-82.
15. Yuejin, W., J. Shuping, L. Xiaoning and Z. Jianxia, 2010. Mechanism of embryo abortion in stenopermocarpic seedless grape. http://europepmc.org/abstract/CBA/645009, 1987-1993.
16. Ji, W., Z.Q. Li, Q. Zhou, W.K. Yao and Y.J. Wang, 2013. Breeding new seedless grape by means of *in vitro* embryo rescue. Genetics and Molecular Research, 12(1): 859-869.
17. Xu, X., L.U. Jiang, D. Dalling, H. Hong, C. Louime, Z. Ren, F. Bradley and R. Reed, 2008. Application of embryo rescue in grape (*Vitis*) cultivar development. Proc. Fla. State Hort. Soc., 121: 38-40.
18. Guo, X., W. Chen, Y. Guo, Z. Liu, H. Lin, J. Tang, K. Li and Y. Zhao, 2015. Influencing factors of embryo rescue in seedless grape. Pak. J. Bot., 47(2): 669-673.
19. Yang, D., W. Li, S. Li, X. Yang, J. Wu and Z. Cao, 2007. *In vitro* embryo rescue culture of F1 progenies from crosses between diploid and tetraploid grape varieties. Plant Growth Regul., 51: 63-71.
20. Vallania, R., 1990. Quaderni della scuola di specializzazione in.viti coltura ed Enologia, Univ. Torino., 14: 319-326.
21. Gray, D.J. and L.A. Hanger, 1993. Effect of ovule maturity on recovery of zygotic embryos and embryogenic cultures from muscadine grape. HortSci., 28: 227.
22. Razi, M., R.J. Marandi, H.D. Baneh, B. Hosseini and R. Darvishzadeh, 2013. Effect of paternal genotypes Sprays with BA and IAA concentration on embryo rescue of F1 progenies from 'Askari' (*Vitis vinifera* L.) cultivar. J. Agr. Sci. Tech., 15: 1023-1032.
23. Emershad, R.L. and D.W. Ramming, 1984. In-ovulo embryo culture of *Vitis vinifera* LCV 'Thompson seedless'. Am. J. Bot., 71: 873-877.
24. Brar, S.J.S., Z. Singh and S.S. Gosal, 1991. *In ovulo* embryo culture in seedless grapes (*Vitis vinifera* L.). Horticulture New Technologies and Applications, pp: 249-254.
25. Guimei, Q. and D. Hanfeng, 2001. Ovules culture and plant formation of hybrid progeny of seedless grape. Journal of Agriculture in the Tropics and Subtropics, 102(2): 147-152.

26. Gray, D.J., J.A. Mortensen and C.M. Benton, 1990. Ovule culture to obtain progeny from hybrid seedless bunch grapes. J. Am. Soc. Hortic. Sci., 115: 1019-1024.
27. Bin, H., B. Sharon, K. Roger, M. Brian and K. Wilf, 1991. Plant regeneration from microspore-derived embryos of *Brassica napus*: effect of embryo age, culture temperature, osmotic pressure and abscisic acid. *In vitro* Cell Dev. Biol.-Plant, 27: 28-31.
28. Tsolova, V., 1991. Obtaining plants from crosses of seedless grapevine varieties by means of *in vitro* embryo culture. Vitis, 29: 1-4.
29. Li, G.R., G. Wang and Y.J. Wang, 2013. Embryo rescue to obtain progeny from seedless grapes (*Vitis vinifera* L.) × wild Chinese *Vitis* species. Journal of Food, Agriculture & Environment, 11(3&4): 1058-1062.
30. Li, G.R., W. Ji, G. Wang, J.X. Zhang and Y.J. Wang, 2014. An improved embryo-rescue protocol for hybrid progeny from seedless *Vitis vinifera* grapes×wild Chinese *Vitis* species. *In vitro* Cell. Dev. Biol. Plant, 50: 110-120.
31. Emershad, R.L., D.W. Ramming and M.D. Serpe, 1989. *In ovulo* embryo development and plant formation from stenospermic genotypes of *Vitis vinifera*. Am. J. Bot., 76: 397–402.
32. Li, Z., T. Li, Y. Wang and Y. Xu, 2015. Breeding new seedless grapes using *in ovulo* embryo rescue and marker-assisted selection. *In vitro* Cell. Dev. Biol. Plant, 51: 241-248.
33. Mostafa, F.M.A., M.M. Shaaban, Doaa S. Elazab and M.T. Kamel, 2015. *In vitro* propagation of four grape cultivars. Assiut J. Agric. Sci., 46(4): 65-76.