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# Effect of Paternal Genotypes Sprays with BA and IAA Concentration on Embryo Rescue of F1 Progenies from 'Askari' (*Vitis vinifera* L.) Cultivar

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## ABSTRACT

**The major objective of seedless grape breeding program is production of grapes with high quality. Breeding of seedless grapes is difficult because the embryos are aborted after fertilization. Embryo rescue technique is utilized to obtain plants from crosses between seedless cultivars. In the present study, the effect of paternal genotypes and different IAA concentrations in presence of BA applications were investigated on embryo rescue in Askari cultivar in randomized complete block design with three replications. Sprays with BA were performed two times at 18 E-L and 27 E-L stages. Hybrid embryos were produced from the cross between Askari cultivar as a female parent and Ruby Seedless, Bidane Sefid, and Bidane Ghermez as male parents. Forty days after pollination (at 29 E-L stage), ovules were cultured on NN medium with three different concentrations of IAA (1, 1.75, 3 mg $L^{-1}$). The results showed that male genotypes and different concentrations of IAA on the media had a significant effect on embryo germination. BA treatment did not affect the embryo germination solely. But, the effect of BA and different concentrations of IAA was significant on embryo germination. The best concentration of IAA with BA treatment was 1 mg $L^{-1}$ and without BA was 3 mg $L^{-1}$. The highest embryo germination rate was observed in 'Askari×Ruby Seedless' cross that was pretreated with BA and cultured on medium with 1 mg $L^{-1}$ IAA concentration.**

**Keywords**: Benzyladenine, Culture medium, Embryo rescue, Grapevine, Seedlessness.

## INTRODUCTION

Grapevine is one of the most important fruit crops in the world. Seedless grapes are a type of grapevine that is preferred by most fruit consumers. Seedless grapevines are induced via either stenospermocarpy or parthenocarpy. In stenospermocarpy, fertilization occurs but embryo development is aborted in earlier stages (Winkler *et al*., 1997). By *in vitro* techniques, one can rescue embryos and orient them into the mature plants (Cain *et al*., 1983; Emershad and Ramming, 1984; Gray *et al*., 1987; Barlass *et al*., 1988). In previous seedless grapevine breeding programs, seeded cultivars were used as a female parent and seedless ones as male parent. Seedless cultivars can be used as female parent if embryo rescue techniques are integrated in breeding programs (Spiegel-Roy *et al*., 1985; Valdez and Ulanovsky, 1997; Ponce and Tizio, 2002; Valdez, 2005). By this means, higher percentage of seedless progenies can be obtained (Emershad and Ramming, 1984; Ponce and Tizio, 2002).

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Stout (1936) reported that stenospermocarpy is an inheritable trait. According to the hypothesis of Bouquet and Danglot (1996), development of seeds of grapes is governed by *a1, a2, a3* and *i* genes. The last one is a regulator gene. When *a1, a2*, and *a3* are homozygous as recessive, and the regulator gene is homozygous as '*II*' or is heterozygous '*Ii*', the seedlessness expression occurs. Cain *et al*. (1983) used embryo rescue technique for the first time and, subsequently, this technique was improved by other researchers (Aguero *et al*., 1995). Different factors such as genotype, medium compositions, and plant growth regulators influence embryo rescue programs (Aguero *et al*., 1995; Galleta and Himlric, 1989). Male parent genotype is one of important factors that affect the embryo rescue process (Gray *et al*., 1987). The seedless male parent affects the rescue or abortion of embryos in hybrids (Cain *et al*., 1983).

Plant growth regulators are among other factors that have an important role in embryo rescue. Adding GA3 and IAA to the medium culture increased embryo germination in hybrid between seedless and seedless (Spiegel- Roy *et al*., 1985). A study of Bharathy *et al*. (2003) showed that one of the main reasons of stenospermocarpy is deficiency of cytokinins. Also, cytokinins as growth regulators have an important role in cell division (Nookaraju *et al*., 2007) and they enhance initially sink strength of ovary (Atkins *et al*., 1998).

Due to its special ecological environment and habitat, Iran is a suitable area for growing various grape cultivars. The objectives of the present study were to investigate the effect of pre-bloom spraying with benzyladenine, male parent genotypes, and the different concentrations of IAA in media on percentage of hybrid embryo germination and callus formation in Iranian grapevine cultivar 'Askari'. This work is a part of 'Askari' breeding program in order to produce hybrid berries with high yield, firmer flesh, red skin, and long shelf life.

## MATERIALS AND METHODS

### Plant Materials

The F1 progenies were produced from the crosses between three stenospermocarpic grapes (Ruby Seedless, Bidane Sefid, and Bidane Ghermez) as a male parents and 'Askari' as a female parent. Askari is an important and widely cultivated table grape in Iran. This cultivar has a short shelf life with green and tender skin (Table 1). Ruby Seedless, Bidane Sefid and Bidane Ghermez have long shelf life and firmer flesh (Table 1). All cultivars (Ruby Seedless, Bidane Sefid, Bidane Ghermez and Askari) were grown in Kahriz Agricultural Station, 44° 58' E longitude and 37° 4' N latitude at Urmia, Iran, and were 10 years old at the time of the experiments. The vine spacing was 2 to 3 m. All cultivars were managed in the same experimental vineyard according to standard vineyard management. Plants were pruned to bilateral cordon.

### Spraying with Benzyladenine

Benzyladenine (BA) (30 ppm) (6-Benzylaminopurine, N6 Benzyladenine, Duchefa Biochemie 99%, Netherlands) were applied two times: first, 14 days before emasculation of 'Askari' flowers (in 18 E-L stage) and second, 7 days after emasculation (in 27 E-L stage) (Tang *et al*., 2009). For

**Table 1**. Characteristics of male parents (Abbas *et al.,* 2006; Jalili Marandi, 2012).

| Cultivars | Characteristic |
|---|---|
| Askari | Green and tender skin, soft pulp, short shelf life, sensitive to transporting, medium-ripening |
| Ruby Seedless | Red skin, stiff pulp, long shelf life, late ripening |
| Bidane Sefid | Yellow and tender skin, stiff pulp, long shelf life, medium-ripening |
| Bidane Ghermez | Red skin, stiff pulp, long shelf life, medium-ripening |





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better absorption of BA solution, it was mixed with 0.1% Tween 20 and was then sprayed on 'Askari' flower clusters at 19:00 to 21:00 PM (Tang *et al*., 2009). Pure water was applied as control.

### Pollination

Pollen grains of male parents (Ruby Seedless, Bidane Sefid, and Bidane Ghermez) were collected in 25 E-L stage and stored at 4°C until pollination. Three days before flowering (in 20 E-L stage), inflorescences of 'Askari' cultivar were emasculated and pollinated by pollen grains of the male parents. Hand pollination was carried out two times with hairbrush in the morning. Clusters were covered with bags to prevent fertilization by external pollens. Two weeks after pollination, bags were removed (Bharathy *et al*., 2003; Samaan *et al*., 1981).

### Ovule Culture

Immature berries were collected 40 days after pollination (in 29 E-L stage) and transferred to the laboratory. Berries were washed with tap water carefully for 20 minutes, disinfected with 2.5% sodium hypochloride (5% active chlorine) for 15 min and rinsed three times with sterile distilled water. Ovules were removed from berries and cultured in petri dishes (10 ovules per petri dish) containing Nitsch and Nitsch medium supplemented with 30 g $L^{-1}$ sucrose [(+) Saccharose, $C_{12}H_{22}O_{11}$, M= 342.30, Scharlau-Spain], 2 g $L^{-1}$ activated charcoal (Charcoal vegetal activated, MERCK Germany), 7 g $L^{-1}$ agar (Bacteriological agar, micro media, Hungary), 0.35 mg $L^{-1}$ $GA_3$ (Gibberelic Acid, $C_{19}H_{22}O_6$, SIGMA Germany) and three different concentrations of IAA (1, 1.75, 3 mg $L^{-1}$) (Indole-3-Acetic Acid, Assay 99%, $C_{10}H_9NO_2$= 175.2, Duchefa Biochemie, Netherlands). Medium pH was adjusted to 5.8 before autoclaving. Cultured ovules grew under fluorescent light (3,000 lux) with 16 hours photoperiod at 25±2°C day temperature and 22±2°C night temperature. Number of germinated and callused ovules was recorded according to Yang *et al.* (2007) method. Induction of callus at every size was considered as callused ovule. Embryo was considered germinated when radical and plumule emerged from ovules. We recorded ovule germination and callus formation every 20 days. Figure 1 shows an ovule germinated after 10 weeks and Figure 2 shows induced callus from hybrid ovules after 3 weeks. Callus formation was observed around ovules 2 weeks after culture.

### Statistical Analysis

Analysis of variance was performed using general linear model (GLM) procedure in the SAS software. The main effect of male genotypes, BA spraying, and IAA concentration as well as their interactions was determined. The significant interaction effects were sliced in proper manner in the SAS software. When significant treatment effects were found in the analysis of variance, mean comparison were performed with the LSD test.

## RESULTS

### Effect of Male Parent Genotypes on Embryo Germination and Callus Formation

Male parent genotypes had significant effect on embryo germination, so that the cross combination 'Askari×Ruby Seedless' induced appropriate embryo germination (2.05%) (Figure 3). Male parents did not have significant effect on callus induction, but interaction between the male parent genotypes and BA pre-treatment had significant effect on callus formation.





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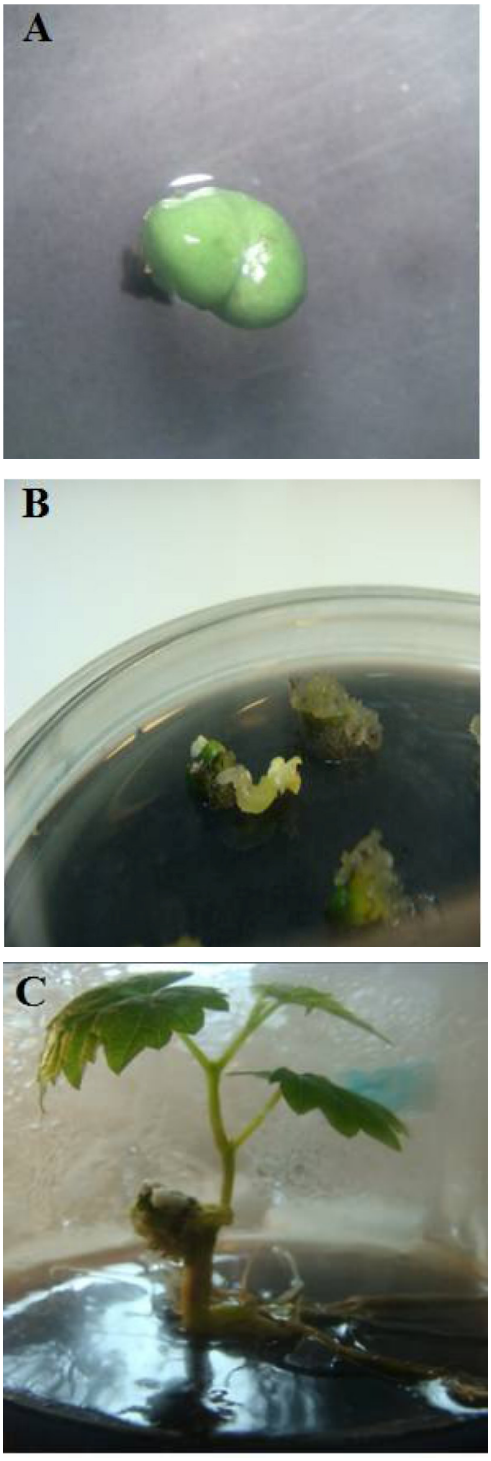


**Figure 1**. *In vitro* embryo rescue and plant development from the cross 'Askari×Ruby Seedless'. (A) An ovule cultured in NN medium; (B) An ovule germinated after 10 weeks, (C) A whole plantlet developed from germinated ovule cultured on half strength Murashige Skoog after 2 weeks.

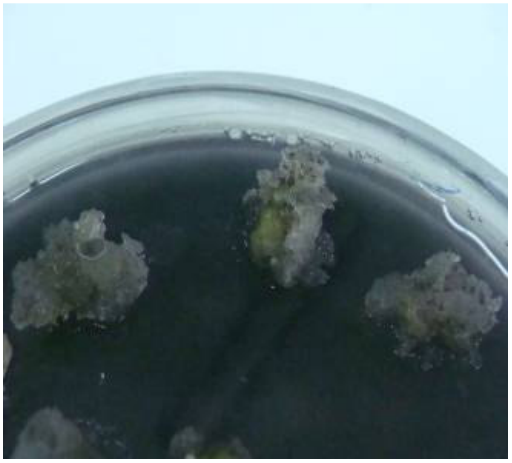

**Figure 2**. Callus induction from hybrid ovules after 3 weeks.

### 12Effect of BA Sprays on Embryo Germination and Callus Formation

Pre-treatment of flower clusters of the female parent by benzyladenine (BA) did not have significant effect on embryo germination. BA×IAA interaction significantly influenced the embryo germination (Figure 4). The highest percentage of embryo germination was observed in ovules treated by BA and cultured in medium supplemented by 1 mg $L^{-1}$ IAA (3.62%) and ovules without BA treatment and cultured in medium supplemented by 3 mg $L^{-1}$ IAA (1.72%), respectively (Figure 4). On the other hand, the effect of BA on callus induction was significant and the highest callus induction (97.99%) was observed in 'Askari×Bidane Sefid' cross that were pre-treated with BA and the lowest one (35.67%) was observed in 'Askari×Bidane Sefid' cross without BA treatment (Figure 5).

### Effect of IAA Concentration in Medium on Embryo Germination and Callus Formation

IAA concentration had a significant effect on embryo germination. Development of





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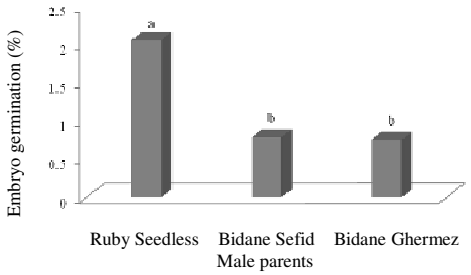


**Figure 3**. Effect of male parents on embryo germination rate.

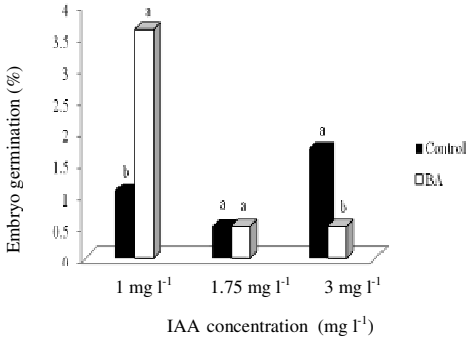


**Figure 4**. Effect of IAA concentration and BA sprays on germination rate of embryos.

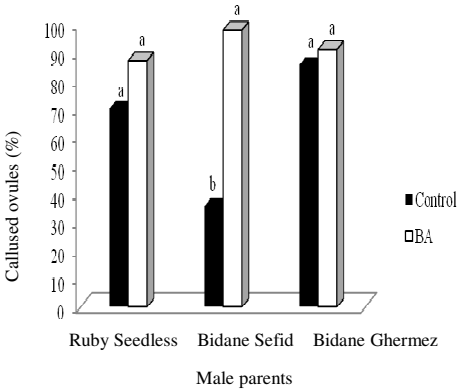


**Figure 5**. Effect of interaction between male parents and pretreatment of BA on callus induction

hybrid immature ovules was influenced by BA pre-treatment and IAA concentration. Percentage of germination was different across the studied crosses, as the highest

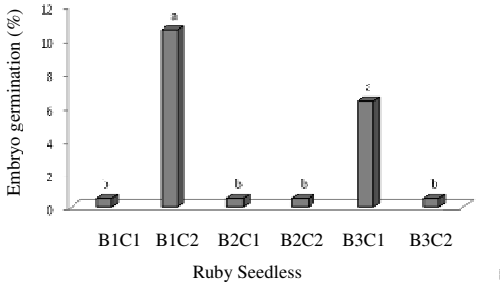


**Figure 6.** Effect of interaction between male parents, pretreatment of BA and IAA concentration in media on embryos germination of Askari×Ruby Seedless. $B_1$: 1 mg $L^{-1}$ IAA, $B_2$: 1.75 mg $L^{-1}$ IAA, $B_3$: 3 mg $L^{-1}$ IAA, $C_1$: Control, $C_2$: BA.

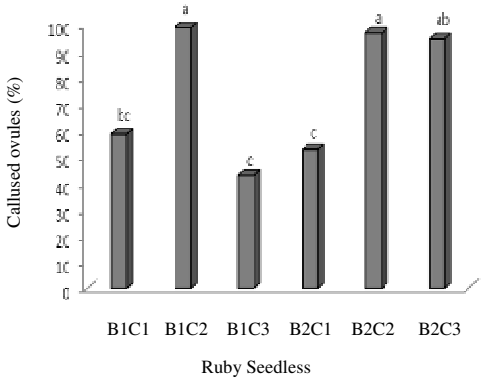


**Figure 7**. Effect of interaction between male parents, pretreatment of BA and IAA concentration on callus induction of Askari×Ruby Seedless. $B_1$: 1 mg $L^{-1}$ IAA, $B_2$: 1.75 mg $L^{-1}$ IAA, $B_3$: 3 mg $L^{-1}$ IAA, $C_1$: Control, $C_2$: BA.

embryo germination was observed in 'Askari×Ruby Seedless' in medium supplemented by 1mg $L^{-1}$ IAA (10.55%) and 3 mg $L^{-1}$ IAA (6.34%) (Figure 6). In 'Askari×Bidane Sefid' the highest germination rate was observed in medium supplemented with 1mg $L^{-1}$ IAA (3.07%) (Table 2). On the other hands, interaction between male parents, IAA concentration in media culture, and pre-treatment with BA had a significant effect on callus induction. The highest percentage of callus formation in Askari×Ruby Seedless cross was observed in medium supplemented with 1 mg $L^{-1}$ IAA and pre-treated by BA (98.88%) and the lowest one was observed in medium





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**Table2**. *In vitro* germination for immature seeds treated with BA sprays on different concentration of IAA in NN medium

| Askari × male parents | Media | Control (without BA treatment) | | | Treated by BA | | |
|---|---|---|---|---|---|---|---|
| | | No. of ovule cultured | PG (%) | PC (%) | No. of ovule cultured | PG (%) | PC (%) |
| Ruby Seedless | 1 | 30 | 0.5[b] | 58.68[bc] | 30 | 10.55[a] | 98.88[a] |
| | 2 | 30 | 0.5[b] | 42.75[c] | 30 | 0.5[b] | 52.73[c] |
| | 3 | 30 | 6.34[a] | 96.98[a] | 30 | 0.5[b] | 94.68[ab] |
| Bidane Sefid | 1 | 30 | 0.5[a] | 52.90[b] | 30 | 3.07[a] | 88.78[ab] |
| | 2 | 30 | 0.5[a] | 46.26[bc] | 30 | 0.5[a] | 97.44[a] |
| | 3 | 30 | 0.5[a] | 12.27[c] | 30 | 0.5[a] | 99.47[a] |
| Bidane Ghermez | 1 | 30 | 0.5[a] | 81.17[b] | 30 | 0.5[a] | 99.66[a] |
| | 2 | 30 | 0.5[a] | 71.81[a] | 30 | 0.5[a] | 49.03[b] |
| | 3 | 30 | 0.5[a] | 97.36[a] | 30 | 0.5[a] | 99.66[a] |

Means followed by the same letter are not significantly different ($p \leq 0.05$) according to the LSD test. PG (%): Percentage of Germination, PC (%): Percentage of Callus. 1, NN + $GA_3$ 0.35 mg $L^{-1}$ + IAA 1 mg $L^{1}$; 2, NN + $GA_3$ 0.35 mg $L^{-1}$ + IAA 1.75 mg $L^{-1}$; 3, NN + $GA_3$0.35 mg $L^{-1}$ + IAA 3 mg $L^{-1}$ .

supplemented by 1.75 mg $L^{-1}$ IAA without BA treatment (42.75%) (Figure 7). In Askari×Bidane Sefid cross, the highest percentage of callus formation was observed in medium supplemented with 3 mg $L^{-1}$ IAA and pre-treated by BA (99.47%) and the lowest one was observed in medium supplemented with 3 mg $L^{-1}$ IAA without BA treatment (12.27%) (Figure 8). In Askari×Bidane Ghermez cross, the highest callus formation was observed in media supplemented with 1 and 3 mg $L^{-1}$ IAA with BA treatment (99.66%) and the lowest one was observed in medium supplemented by 1.75 mg $L^{-1}$ IAA and pre-treated with BA (49.03%) (Figure 9).

## DISCUSSION

Media composition, especially plant growth regulators (PGRs) and their concentration, plays an important role in embryo rescue. The same as our study, Gribaudo *et al*. (1993) reported that PGRs have positive effect on embryo formation. Utilization of $GA_3$ and IAA promoted embryo recovery from Seedless×Seedless crosses (Spiegel-Roy *et al*. 1985; Valdez and Ulanovsky, 1997; Liu *et al*. 2003; Valdez, 2005). An earlier report by Yang *et al*. (2007) showed that the addition of 0.35 mg $L^{-1}$ $GA_3$+1.75 mg $L^{-1}$ IAA to NN-1969 medium improved the percentage of embryo germination in 'Fujiminori×Muscat Hamburg' and 'Jingxiu×Kyoho' crosses.

Also, Valdez and Ulanovsky (1997) reported that the higher concentration of IAA (3 mg $L^{-1}$) and $GA_3$ (5 mg $L^{-1}$) are required for embryo germination. In addition to plant growth regulators, male parent genotype has beneficial effect on ovule germination. Effect of genotype on embryo rescue of *V. vinifera* has been confirmed by Burger and Goussard (1996) and Liu *et al*. (2003). The seedless male parent influenced the rate of viable embryos in cross with seedless grapes (Cain *et al*., 1983). Also, Tian *et al*. (2008) reported that the best embryo germination was produced in cross combination of 'Emerald Seedless×Beichun' compared to other crosses. Gray *et al*. (1990) reported that male parents had significant effect on ovules germination, and our observations were in accordance with their reports.

In this study, BA sprays had no positive effect on embryo germination in the cross of 'Askari×Bidane Ghermez' compared to the untreated plants. Spraying with BA had a beneficial effect on embryo germination





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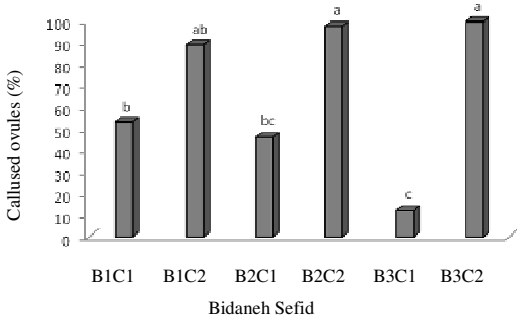


**Figure 8**. Effect of interaction between male parents, pretreatment of BA and IAA concentration in media on callus induction of Askari×Bidane Sefid. $B_1$: 1 mg $L^{-1}$ IAA, $B_2$: 1.75 mg $L^{-1}$ IAA, $B_3$: 3 mg $L^{-1}$ IAA, $C_1$: Control, $C_2$: BA

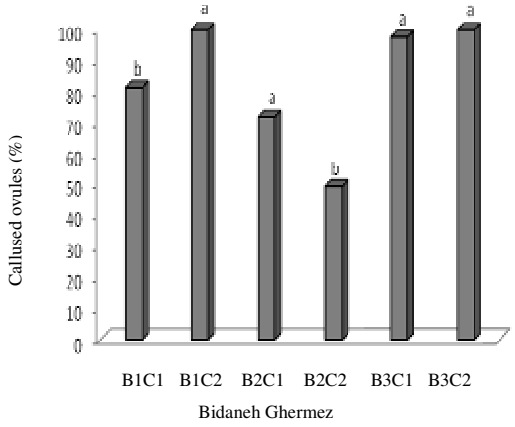


**Figure 9**. Effect of interaction between male parents, pretreatment of BA and IAA concentration on callus induction of Askari×Bidane Ghermez. $B_1$: 1 mg $L^{-1}$ IAA, $B_2$: 1.75 mg $L^{-1}$ IAA, $B_3$: 3 mg $L^{-1}$ IAA, $C_1$: Control, $C_2$: BA.

depending on male parent genotype. The rate of embryo growth was also influenced by male parent genotypes. Bharathy *et al*. (2003) noted that the reason for aborting embryo in stenospermic grapes was not clear exactly, but one common reason was deficiency of cytokinins. Pandy (1982) notified that cytokinins show activity till 4 weeks after anthesis and, then, disappear during the fifth week till ripening of berries. Bharathy *et al*. (2005) reported that BA treatment results in enlarged hypocotyls and cotyledons in germinated embryos. It is known that the BA is a cytokinin that orients the nutrition materials to organs and can establish new source-sink relations in plants (Huang *et al*., 2002). In accordance with our results in Ruby Seedless and Bidane Sefid, Bharathy *et al*. (2005) argued that BA spraying had a significant effect on ovules germination of crosses between 'Flame Seedless' with different seedless grapes as male parents, except 'Flame Seedless×Concord' cross.

On the other hand, the three male parents had different effects on germination rate: 'Ruby Seedless' and 'Bidaneh Ghermez' had the highest and the lowest effect on germination rate, respectively. Cytological and embryological studies of seedless grapes have shown that the growth of male gametophyte in combination with female gametophyte during fertilization is inhibited (Kovaleva *et al*., 1997). The highest callus formation in the three crosses was observed in ovules treated with BA and cultured on media supplemented with 1 and 3 mg $L^{-1}$ IAA. This indicated that the presence of auxin and cytokinin induced most callus formation. Callus formation from outer integument was reported by Gribaudo *et al*. (1993), Emershad and Ramming (1984), and Gray *et al*. (1990). Notsuka *et al*. (1992) reported that the combination of 1 µM 2,4-D and 0.2 µM CPPU (N1-(2-chloro-4-pyridyl)-N2-phenylurea) caused the highest callus induction on unfertilized ovules of 'Neo Muscat' grape. Our results were in agreement with their findings. Also, in our study, the highest callus induction was observed on ovules treated with BA, compared to those without BA treatment.

## CONCLUSIONS

The results showed that the male parents had an important effect to hybrid embryo rescue. Spraying with BA could have positive effect on hybrid embryo rescue, depending on male parent genotypes. Besides, we found that, in 'Askari' breeding





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programs, using 'Ruby Seedless' as a male parent could cause the highest embryo germination. Bidane Sefid as a male parent can be used for Askari cultivar if BA sprays and 1 mg $L^{-1}$ IAA were used. Spraying BA in any of the three male parents caused the highest callus formation. To get better results on embryo germination, spraying with BA and using 1 mg $L^{-1}$ IAA are proposed.

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## تأثیر والد پدری، محلول پاشی بنزیل آدنین و غلظت ایندول استیک اسید بر نجات جنین نتاج F1 رقم انگور عسکری

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### چکیده

هدف اصلاح انگورهای بیدانه تولید میوه های با کیفیت بالامی باشد. اصلاح انگورهای بیدانه مشکل است، زیرا بعد از عمل لقاح جنین ها سقط می شوند. به منظور تولید گیاه از تلاقی ارقام بیدانه از تکنیک نجات جنین استفاده می شود. تیمارهایی وجود دارد که سقط جنین را در این ارقام به تأخیر می اندازند و باعث توسعه نجات جنین می شوند. در مقاله حاضر اثرات ژنوتیپ پدری و غلظت های مختلف ایندول استیک اسید در حضور بنزیل آدنین بر روی نجات جنین رقم عسگری در قالب طرح بلوک های کامل تصادفی با ۳ تکرار بررسی گردید. محلول پاشی با بنزیل آدنین در دو مرحله انجام گرفت و رقم عسکری به عنوان والدماده و ارقام رابی سیدلس، بیدانه سفید و بیدانه قرمز به عنوان والد نر انتخاب شدند. ۴۰ روز بعد از گرده افشانی تخمک های هیبرید در محیط کشت نیچ و نیچ دارای ۳۰ گرم در لیتر ساکاروز، ۲ گرم در لیتر ذغال فعال و ۳ غلظت متفاوت ایندول استیک اسید (۱ میلی گرم در لیتر، ۱/۷۵





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میلی گرم در لیتر و ۳ میلی گرم در لیتر) کشت شدند. نتایج نشان داد که والد پدری و غلظت های متفاوت ایندول استیک اسید محیط کشت تأثیر معنی داری بر جوانه زنی جنین ها داشتند. محلول پاشی بنزیل آدنین به تنهایی تأثیر معنی داری بر جوانه زنی جنین ها نداشت اما اثر متقابل محلول پاشی بنزیل آدنین و غلظت های متفاوت ایندول استیک اسید محیط کشت تأثیر معنی داری بر جوانه زنی جنین ها داشتند. بیشترین میزان جوانه زنی در جنین های حاصل از تلاقی عسکری × رابی سیدلس که با بنزیل آدنین محلول پاشی شده و در محیط کشت دارای ۱ میلی گرم در لیتر ایندول استیک اسید کشت شده بودند، مشاهده گردید.



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