

Effect of BA and ovule developmental stages on embryo rescue in Perlette grape (*Vitis Vinifera* L.) cultivar

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Abstract

Production of seedless varieties is one of the major breeding goals in grape vine. Traditional methods result in low percentage of seedless progeny production. Today for producing high percentage of seedless progeny, embryo rescue are used. In this research, the effects of BA concentrations (0, 30, 60 and 100 mg L⁻¹) and the stage of ovule development (25, 35, 45, 55 and 65 days after pollination) were assessed on the success rate of the embryo rescue in Perlette grape (*Vitis vinifera* L.). BA was sprayed 14 days before and 7 days after the anthesis. The berries were harvested at 25, 35, 45, 55 and 65 days post pollination. Ovules were cultured in Nitch and Nitch medium with 0.35 mg L⁻¹ gibberellic acid (GA3) and 1 mg L⁻¹ indolacetic acid (IAA). Characters such as germination, collapsed, callus-formed and enlarged ovules were recorded. The results revealed the effect of BA pre-treating and ovule developmental stage on percentage of germinated embryos, collapsed, callus- formed and enlarged ovules. The highest germination percentage was observed in BA 100 mg L⁻¹ at 65 days post pollination treatment. The highest percentage of the collapsed ovules was observed at 25 days after pollination in all treatments. The highest percentage of the callus formation was observed in the BA treatment of 30 mg L⁻¹ at 55 days after pollination. The highest percentage of the enlarged ovules was observed in the BA treatment of 60 mg L⁻¹ at 45 days after pollination.

Keywords: Benzyladenine, Culture medium, Embryo rescue

Introduction

Due to high diversity of grapes, the extensive cultivation in horticultural crops is important. Depending on the use of specific objective breeding of grapes, Seedless progeny and produce of seedless grape is considered the most important of them (Ebadi et al, 2001). Seedless grapes are classified into two types, stenospermocarpic and parthenocarp.

In stenospermocarpic cultivars pollination and fertilization occurred but for various reasons during the development phases embryo is aborted and in parthenocarp fertilization does not take place and Fruit formed without fertilization. (Tian, 2008; Mejia and Hinrichsen, 2002). Crosses between seedless parent's increases Seedless progeny production, but the problems of this method is embryo abortion. Nowadays through embryo rescue technique seedless progeny is possible to produce (Akkurt et al, 2013), this technique was first proposed in the 18th century and during the year 1890 - 1904 using these techniques widely began.

There are many factors effecting the success of embryo rescue techniques that can be noted as a cultivation time, plant growth regulators and genotype. The cultivation time is very important

and it is crucial that the embryo culture begins before abortion. (Sandra, 2005) Growth regulators have an important role in the success of embryo rescue techniques.

Among these materials, cytokine can be noted. Cytokines have an important role in cell division and preventing cell aging (Yang et al, 2006). Cytokines 4 weeks after pollination has been active in developing seeds and at weeks 5 it stops working and do not have activity until berry ripening. Food balance between seed and fruit components are essential for the development of fruit and seeds. In this matter, cytokines are involved. Foliar Spray of benzyladenine (BA) before flowering, leading to the development of the seeds in grape cultivar and causes enlargement of berries and hypocotyl. (Degirmenci and Marasali, 2001).

Cytokines spread in seed and as a repository for cell division in the ovary, and then act in embryo meristems and therefore are essential for the seed development (Atkins et al., 1998).

Razi et al, (2013) showed that Spraying of BA at a rate of 30 mg L⁻¹ have a significant impact on hybrid embryos resulting from crosses of Ruby seedless and Asgari. Spraying Before full bloom with CPPU, which has cytokines character, resulting in better growth of ovules and embryos. (Nookaraju et al., 2007).

Atkins et al, (1998) observed BA spraying before flowering led to the development of seedless berries, and increase the average berry weight. BA Spraying, two to three weeks before flowering, growth and increase the number of female and full flowers of hermaphrodite and by the increased seeds power to absorb food prevent abortion in the Sultani cultivar (Bharaty et al., 2005).

Masoomi et al, (2010) showed that the effect of genotype and different concentrations of BA on germinated ovules percentage was significant. Pommer et al, (1995) grouped grapevines in three late, medium and early groups, and reported late cultivars have the lowest rate of germination compared with average cultivars. In this study the effect of BA spraying and ovules developmental stage of embryo rescue in Perlette grape cultivar were investigated. Perlette cultivar due to dense clusters of grape among the cultivars used in breeding programs (Bharathy et al., 2005).

Materials and Methods

Plant material

Age of studied cultivar plants (Perlette) was 10 which were trained as a stand Cordon System, 5 plants were selected with same growth and position vigor.

Benzyladenine foliar sprays

Sprays were performed twice (14 days before anthesis and 7 days anthesis). Control clusters were sprayed with distilled water. In order to better compounds absorb, sprayed during the cool evening. Solutions were prepared at spraying time. Chemical compounds were weighed and dissolved in appropriate solvent(NaOH). A few drops of tween 20 were added as a Surfactant.

Ovule Culture

Berries at different times 25, 35, 45, 55 and 65 days after pollination were randomly selected and transferred to the laboratory. Berries were washed with tap water carefully for 30 minutes, disinfected for 15 min in 2/5% sodium chlorite (0/5% active chlorine) containing a few drops of a surfactant (Tween 20) and rinsed three times with sterile distilled water. Ovules were removed aseptically from the berries and then 15 ovules cultured in each per petri dish containing Nitch and Nitch medium supplemented with 30 g L⁻¹ sucrose [(+) Saccharose, C₁₂H₂₂O₁₁, 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 IGA3(Gibberlic Acid C₁₉H₂₂O₆, SIGMA Germany) and 1 mg L⁻¹ IAA (Indole-3-Acetic Acid Assay 99%, C₁₀H₉NO₂=175.2, DuchefaBiochemie, Netherland). Medium pH was adjusted to 5.7 before autoclaving. The petri dishes were sealed with Parafilm then maintained in a growth chamber under cool-white fluorescent

illumination (3000 Lux) with $22 \pm 2^\circ\text{C}$ day / $27 \pm 2^\circ\text{C}$ night and photoperiod of 16 light (or, 8 hours darkness). The number of germinated, collapsed, callus formed and enlarged ovules were recorded every 15 days.

Plant adaptation to Non-sterile environmental conditions

Germinated Ovule for better growth were transferred to MS $\frac{1}{2}$ Medium and non-germinated Ovules in the previous environment (NN) were sub cultured. Seedlings in Five-to six-leaf stage were transferred to pots containing perlite sterile. Approximately three to four weeks later the seedlings were transferred to larger pots containing garden soil and sterile leaf mold and were kept in the greenhouse.

Statistical Analysis

Analysis of variance (ANOVA) was performed using the general linear model procedure (GLM) In the SAS software version 9.2 (SAS Institute Inc.). Mean comparisons performed with Duncan test.

Results

The Effect of BA sprays and ovule developmental stages of embryo germination

After 11 weeks of ovules cultured in NN Medium, embryo germination was observed (Figure 1). Analysis of variance (Table 1) showed that the ovule developmental stage of the Ovules and BA pretreatment has an effect on the percentage of germinated embryos. The interaction between the Ovules developmental stage and pre-treatment of BA on embryo germination was significant at the 1% level. The results showed with increasing developmental stage the percentage of germination increased so that the highest germination percentage (29/33%) obtained from 100 mg L-1 BA at 65 days after pollination, and the lowest percentage of germination was observed in all BA treatments at 25 days after pollination (Figure 2).

The Effect of BA Sprays and ovules developmental stages on collapsed ovules.

Collapsed Ovules is an ovules that Swollen than others and collapsed over the medium, Then gradually open the walls of the ovules and produce callus (Figure. 1). Analysis of variance (Table 1) showed that the percentage of collapsed ovules under the influence of different concentrations of BA and ovules developmental stage. The interaction between the Ovules developmental stage and BA pre-treatment on the percentage of ovules was significant at the 1% level. Comparisons means of treatment in the ovules developmental stage, showed the highest percentage of collapsed ovules (66/70%) in all BA treatments was at 25 days after pollination and the lowest collapsed ovules (33/33%) was in BA 100 mg L-1 at 65 days after pollination (Figure. 3). The results showed that during the initial sampling of collapsed ovules were higher than any other time, While the use of growth regulators, and rising concentrations of collapsed ovules 25 days after pollination decreased in comparison with control treatment.

The Effect of BA Sprays and ovules developmental stage on callus-formed ovules

In some ovules after 2 to 4 weeks of culture, callus was formed from the ovules wall (Figure. 1). Based on the analysis of variance (Table 1) was observed the percentage of callus production affected by ovules developmental stage and pre-treatment of BA. The interaction between the ovules developmental stage and pre-treatment of BA on callus-formed ovules was significant at 1% level. According to the results of control treatments, over time the percentage of the callus-formed ovules showed a decreasing trend, and this process continued after the application of growth regulators. But BA treatment during certain stages of development in the callus-formed ovules, the percentage of the callus-formed ovules have increased, so the highest percentage of callus-formed ovules (33/21%) at 30 mg L-1 BA treatment at 55 days after pollination was observed (Figure. 4).

The Effect of BA sprays, and ovules developmental stage on ovules enlarged

20 days after ovules culture in NN, number of ovules were swollen (Figure. 1). The results of the analysis of variance (Table 1) showed the effect of BA and ovule developmental stage of ovules enlarged percentage is significant. The interaction between the ovules developmental stage and BA on the percentage of enlarged ovules was significant at the 1% level. Study the Comparisons of mean showed that the highest percentage of ovules enlarged (33/17%) was in BA 60 mg L⁻¹ at 45 days after pollination (Figure 5). According to the results, the percentage of ovules enlarged in the control treatments of BA showed a decreasing trend, This trend was also observed in other treatments, although the use of this substance at some time, a large increase in the percentage of enlarged ovules was observed (Figure 5).

Table 1: Variance analysis of BA pre-treating effect at ovule developmental stage and slicing interactions, on germinated embryos, collapsed, callus-formed and enlarged ovules in Perlette.

Source of variation	degree freedom	Mean of square			Enlarged ovules
		Germinated ovules	Collapsed ovules	callus-formed ovules	
Replicate (A)	2	82/4**	604/82 **	10/67*	2/71 ns
Concentration of Putrescine (B)	3	850/94**	894/34 **	43/70**	1/66 ns
A×B	12	5/54 ns	23/84**	16/00**	5/43*
Time (C)	4	1178/79**	3084/05 **	62/07**	10/64**
B×C	12	76/13 **	86/46 **	30/11**	14/85**
C×A	16	8/03 ns	33/50**	6/22ns	2/43 ns
Experimental Error	48	7/50	5/1	4/16	2/88
B×C sliced by C					
Ab1	4	69/19**	241/78**	16/30**	7/24*
Bb2	4	277/58**	980/53**	203/59**	2/71 ns
Cb3	4	394/89**	944/95**	7/20ns	28/89**
Db4	4	665/54**	1176/18**	15/23**	7/24*
Coefficient of Variation (%)		19/32	7/27	23/85	23/14

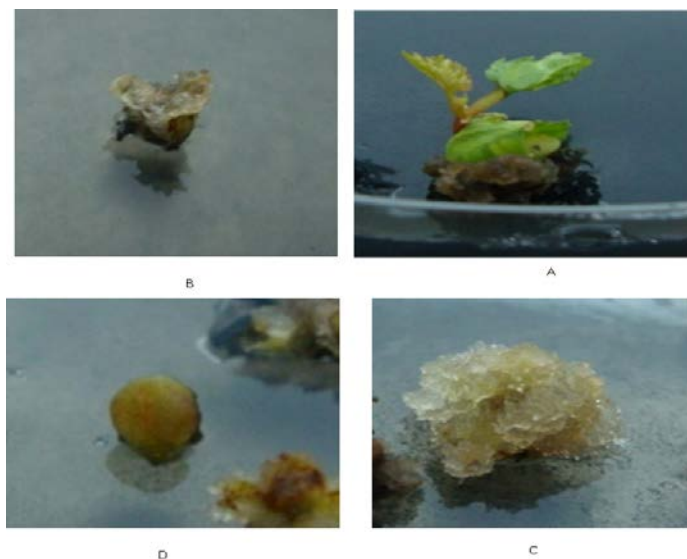


Figure 1: A) germinated embryos B) Collapsed ovules C) callus-formed ovules) D) Enlarged ovules.

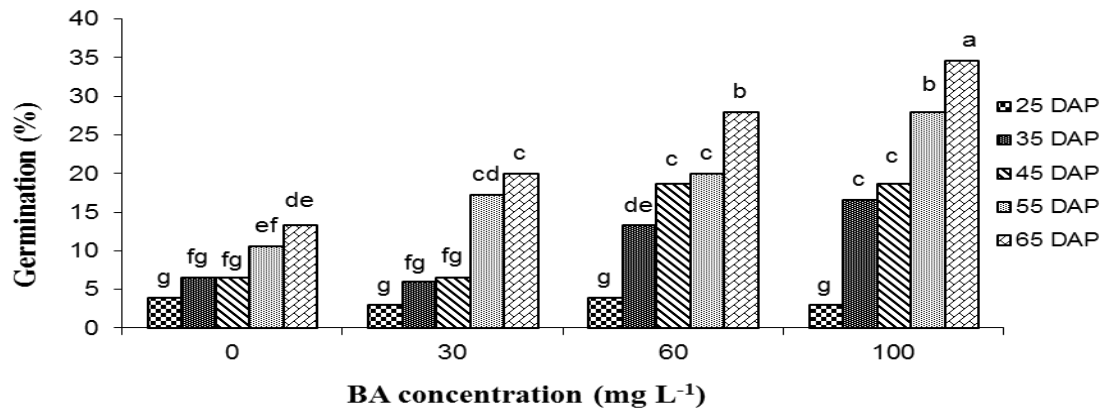


Figure 2: Comparing means of BA pre-treating on the germination percentage of embryos at ovule developmental stage. Different letters in a column indicate significant differences between means separated by Duncan test ($P \leq 0.01$). DAP: day after pollination.

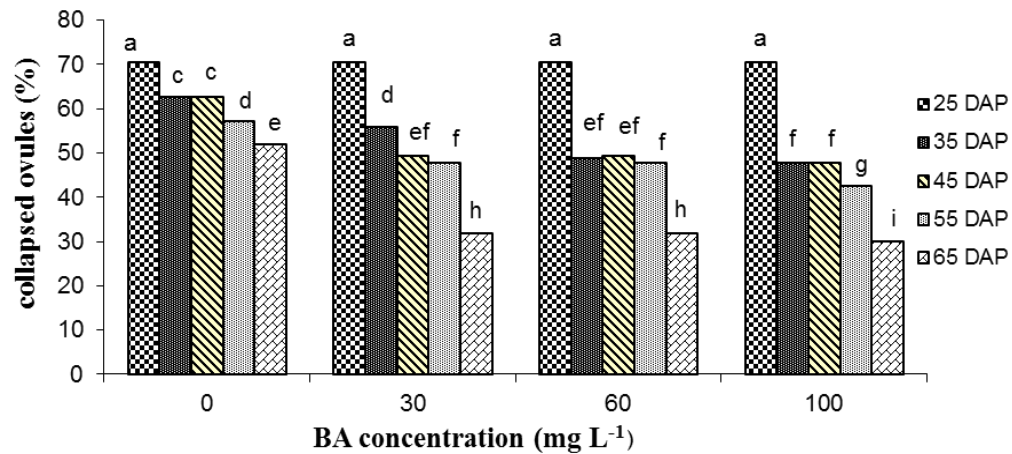


Figure 3: Comparing means of BA pre-treating on the collapsed ovules at ovule developmental stage. Different letters in a column indicate significant differences between means separated by Duncan test ($P \leq 0.01$). DAP: day after pollination.

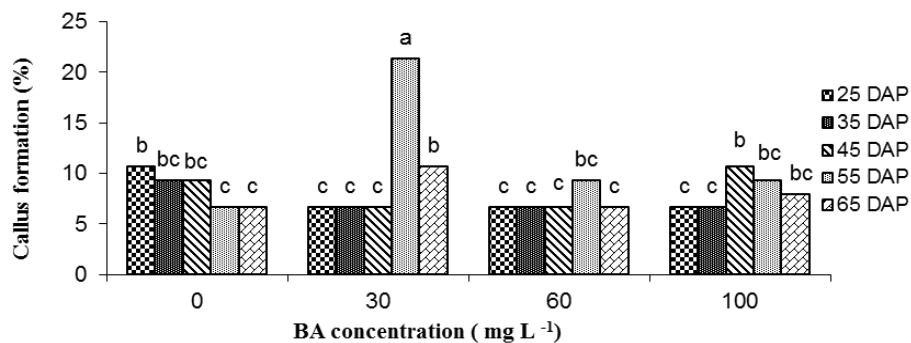


Figure 4. Comparing means of BA pre-treating on the callus formed ovules at ovule developmental stage. Different letters in a column indicate significant differences between means separated by Duncan test ($P \leq 0.01$). DAP: day after pollination.

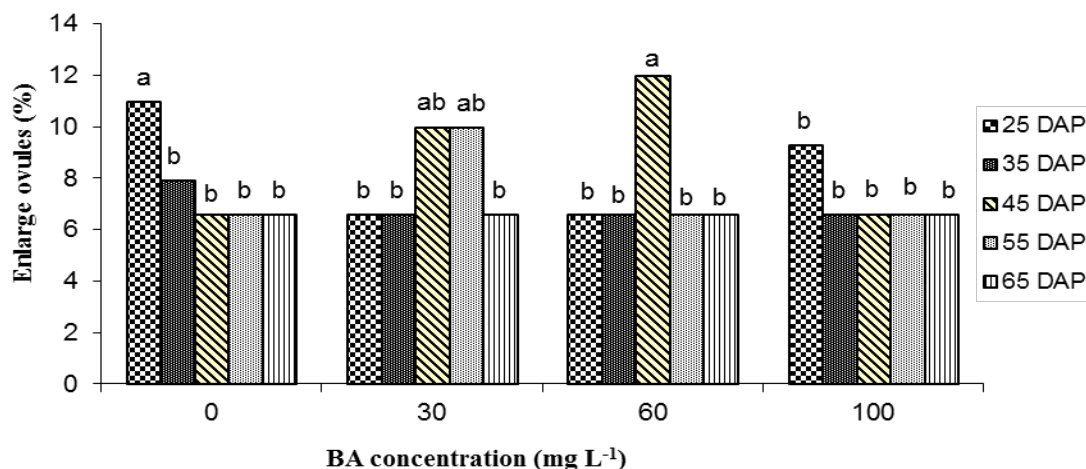


Figure 5: Comparing means of BA pre-treating on the enlarged ovules at ovule developmental stage. Different letters in a column indicate significant differences between means separated by Duncan test ($P \leq 0.01$). DAP: day after pollination.

Discussion

The results of the germinated embryos were under the influence of ovules developmental stage and BA. The obtained results were consistent with the results of Ji et al, (2013). That Reported germination of embryos depends on the stage of ovules development. Pommer et al, (1995) reported the ovules developmental stage and the cultivation time have a significant effect on rescue grape embryo, And the best time for cultivation early grapevines were reported 6 to 10 weeks after full bloom. Ebadi et al, (2002) reported the best time for ovules cultivation for Asgari and keshmeshi cultivars 45 days after pollination.

Nookaraju et al, (2007) reported that using BA overcome the cytokines shortages and thus lead to better development of ovules and embryo. BA treatment have a Positive effect on embryonic development in the Stenospermocarpy cultivar. (Bharathy et al., 2005). Foliar Sprays of 100 mg L⁻¹ BA increase the number of embryonic development in Thomson seedless (Tang et al., 2009). BA spraying before flowering led to the development of grape seeds of Sultani cultivar (Degirmenci and Marasali, 2001).

Razi et al, (2013) reported that Spraying of 30 mg L⁻¹ BA have a Significant effect on the germination of hybrid embryos resulting from crosses of Ruby seedless × Asgari, which corresponded with this research results.

According to the obtained results, the percentage of collapsed ovules is under the influence of the developmental stages of ovules and BA Pre-treatment. In this study the percentage of collapsed ovules of the perlette grape cultivar was higher than the early developmental stages of ovules, and using BA reduced the percentage of collapsed ovules after the first stage in comparison with control treatment which could be due to the role of developmental age of the ovule in the incidence of this trait and role of BA on the development of the ovarian inner wall tissue.

Sari Khani et al, (2000) with study of five stenospermocarpe grapevine cultivars reported that cultivars in terms of collapsed ovules show differences in the culture medium.

In Razi et al, (2011) study the high rates of collapsed ovules in crosses red currants × Asgari with benzyl adenine were reported.

Alifar et al, (2012) showed that the percentage of collapsed ovules not affected by cultivar type and parent. They female parent ovary wall has a decisive role in the occurrence of this trait.

Korkutal, (2005) reported the development of the inner wall of the ovary in the *Stenospermocarpe* cultivars and developed without sclerenchyma tissue

Effects of BA on callus-formed ovules in the Studied (Perlette) cultivars in different developmental stages are different, and increase in the percentage of callus-formed ovules was observed.

Wang et al, (1995) reported callus development in various concentrations of BA is different and corresponded with this research results. Cause of Increase the percentage of callus is due to hormonal role in stimulating the production of callus and Age of maternal tissue. Because During certain times sensitivity of the maternal tissue to the hormone increases.

Based on Ebadi et al, (2002) reports, female parent tissue has a crucial role in the occurrence of this trait.

Masoomi et al, (2010) showed that different concentrations of BA in callus-formed ovules is statistically significant at the 1% level, which is consistent with this study results. Medora et al, (1979) reported that the BA has an inhibitory effect on callus production and which is not consistent with this study results. Jaskani et al, (2008) reported that 5 mg L⁻¹ BA caused the greatest amount of callus production on explants of grape stems.

In ovules development, the ovary wall has the main role and genotype of the maternal parent is responsible for determining this trait (Sari Khani et al., 2008). Results of research conducted by Aguero et al. (2000) on seedless cultivars show that the amount of available gibberellin compounds on berry and seed cultivars in variant genotypes is different.

That can be effective for traits including ovules development. Based on the results the percentage of developed ovules reduced over time in the control Treatments, while in other treatments, the process was stable, except that the concentration of certain developmental stages increase in the percentage of developed ovules was observed.

Iwahori et al. (1968) reported that in the early stages the gibberellic acid level is reduced and reached to zero, therefore, the balance of these hormones with other hormones is an Effective factor in many traits including ovules development. This can be associated with an increase in the use of gibberellin compounds in berry in response to BA in specific developmental stages of perlette grape cultivar.

Conclusion

BA concentration and development stage had significant effect on embryo germination, collapsed ovules, callous formation and enlarged ovules. The highest germination percentage was recorded in BA 100 mg L⁻¹ at 65 days after pollination. Due to the observation of seed remains in collected samples the probable time of embryo abortion was recorded at 70 days after pollination. The best time for ovule culture without BA application is at 65 days after pollination. BA does not delay the embryo abortion time but overcomes on embryo abortion and so increases the rate of embryo germination.

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