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*Full Length Article*

# Obtaining Viable Explants from Immature Embryos of Seedless Grape Cultivars using Embryo Rescue Method

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## Abstract

The embryo rescue method is widely used to develop new seedless varieties. We applied this method to indigenous Kishmish cultivars. In this work, we studied the days after pollination (DAP) intervals of Kishmish cultivars, optimal artificial media and embryo development, as well as the levels of recovering mature plants from them. Measures have been taken to apply the embryo rescue method for local seedless varieties. For this, Kishmish Sugdiyona (KS), Kishmish Rozoviy (KR) and one seeded Toyfi varieties were selected. Embryos were collected from seedless × seedless, seedless × seeded, self-pollinated and naturally pollinated grape clusters at intervals of 0, 20, 30, 40, 50, 60, 70 and 80 DAP. Murashige and Skoog's (1962) artificial media were used to grow mature plants from embryos. After stratification, embryos were recovered under an XSP-500SM digital microscope in a laminar flow box (HFsafe LC). Statistical analyses were performed in RStudio 2023.12.0 + 369 with ANOVA test $P \leq 0.05$ model. Germination did not occur in embryos separated between 0, 20, 30 and 40 DAP intervals of KS and KR varieties. In each of the two cultivars, the germination of embryos in the form of torpedoes and obtaining mature plants from them was more. Embryos entered the torpedo shape at 70 DAP intervals in both cultivars. Over the next 10 days, 80 DAP, the embryos entered an unknown shape and were degraded. When their amount of $KNO_3$, $NH_4NO_3$ was 400 and 480 mg/L, the levels of embryo and germination number dropped. Both varieties showed effectiveness in pollination and embryos obtained from self-pollinated clusters (cleistogamy) showed development potential. In the practice of reciprocal hybridization, when KR variety was used only as a female, 78 (25%) out of 306 mature plants obtained in total were related to KR × KS combination and 26% (152) of the 585 mature plants obtained from the variant KS resulted from the combination of KS × KR. In conclusion, the recovery, germination and plantlet rates of embryos of seedless grape varieties were studied in the tested artificial media. The embryos were transparent during the initial day intervals and then their colours darkened. In the following days (80 DAP), the embryos were degraded and some of them became discoloured. Moreover, the extraction of embryos at the veraison stage was more efficient. The state of germination and plantlet rates of more embryos in the form of torpedoes was observed. The method of embryo rescue can be used in the selection of local seedless varieties.

**Keywords:** *In vitro* culture; Seedlessness; Embryo; Stratification; Ovule; Plantlet; Cleistogamy

## Introduction

The embryo rescue method is widely used in the breeding program of seedless grape varieties. The advantages and effectiveness of this method have been studied by researchers (Li *et al*. 2015). Ramming *et al.* (2020) crossed two species with a chromosome number of 2n = 38 (*Vitis vinifera*) and 2n = 40 (*V. rotundifolia* ) and developed a stenospermacarpic seedless "C41-5" hybrid generation by embryo rescue. Crossbreeding species with different chromosome numbers is known to result in hybrid offspring with poor reproduction (Delame *et al.* 2019). Another advantage of embryo rescue is that two seedless stenospermacarpic varieties can be crossed to produce hybrid offspring (Tian and Wang 2008; Tian *et al*. 2008). Some scientists have identified a high heterozygosity feature in grape varieties (Zhou *et al*. 2019). However, Singh *et al*. (2011) reported that the "seedlessness" trait in the hybrid progeny obtained by this method could be as high as 85%.

To date, embryo rescue is considered more effective than traditional breeding of seedless grapes. Using this method, a number of selection programs are being

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implemented to develop cold (Moreira and Clark 2019) and disease-resistant (Tian and Wang 2008) varieties from cultivated varieties and new disease-resistant varieties (Li *et al.* 2013) using wild *Vitis* species. DAP intervals, culture medium and cross-breeding combinations have been investigated in embryo rescue studies (Li *et al*. 2015; Xu *et al*. 2022). Amaral *et al*. (2001) reported that grape embryos have four forms: globular (1), heart (2), torpedo (3) and the last one is undescribed. Differences in embryo germination and plant development at different DAP intervals in artificial nutrient media containing growth regulators were determined (Xu *et al*. 2022; Amaral *et al*. 2001).

According to the International Organization of Vine and Wine, the production of table grapes in Uzbekistan is 1,118,596.8 MT, of which about 81% (905,836.6 MT) is consumed. The grape genetic resources conserved in the Republican Scientific Research Organization consists of 1300 samples (Mirzaev and Djavacynce 2004). Using the embryo rescue method in breeding programs increases the diversity of grape varieties. Therefore, we used the embryo rescue method for the selection of Central Asian seedless grape varieties and conducted a two-year study in order to select the optimal DAP interval, hybridization combination and optimal artificial media for the selection of embryos from local Kishmish varieties.

## Materials and Methods

### Experimental material

The study was carried out with three repetitions in 2021 and 2022. In order to carry out seedless grape breeding, local (*V. vinifera*) Kishmish Sugdiyona (KS) and Kishmish Rozoviy (KR) varieties were selected for reciprocal hybridization. At the same time, the single-seeded Toyfi variety was also used as a pollinator. All selected varieties were stored in the grape gene pool at the branch of the Scientific Research Institute of Horticulture, Viticulture and Winemaking, Samarkand (39°32'18.0"N 67°00'58.1"E). The cultivars were trained on a trellis system and the planting system was 3 × 3 m (planting year 2010).

### Hybridization

Before emasculating for 3-4 days, we used paper bags to isolate the inflorescences of female forms and marked them with numbers. The inflorescences of the male form clusters were washed with sterile water and sterile Petri dishes were used for pollen conservation. Until hand pollination, Petri dishes with placed pollens were left at 22°C overnight and then at 4°C until use. The anthers from female parents were removed from the grape cluster with sterile forceps and isolated with a paper bag. After transparent mucus was secreted from the stigma, we brushed the stored pollen onto the stigma with a sterile makeup brush. Furthermore, the pollen was poured into the bags to cover the stigma with pollen grains. Once during the five days, the paper bags were shaken mechanically. The emasculation procedure continued from 6:00 to 9:00 am. For the self-pollination variable, inflorescence of grapes was allowed to pollinate into paper bags without hand pollination. At each DAP interval, marked grape clusters were harvested and taken to the laboratory for surface sterilization. We rinsed the grape clusters under running tap water for 1 min to remove any loose debris, then immersed them in an initial washing solution consisting of 1% soap, one drop of Tween 20 and 1% Ridomil Gold in sterile water. The washed grape clusters were dried in a laminar flow box (HFsafe LC) on a sterile paper surface, followed by rinsing for 30 s. in 70% $C_2H_6O$, 10 min 10% (w/v) NaClO in the end sterile water three times for 4 min. Finally, the grape clusters were placed on the surface of sterile papers to dry for 2 min and the grape berries were carefully placed into a glass bottle (0.5 L), in which several sterile papers were placed to facilitate moisture absorption. The cap of the bottle was hermetically sealed with a parafilm until it was used.

### Media

Four different culture media are given in Table 1, M1 (Murashige and Skoog), M2 (Eriksson), M3 (Nitsch & Nitsch) and M4 (Anderson), were evaluated for their efficacy in supporting all stages of embryo development. For stratification of ovules, media (for both pH 5.8) were divided into solid medium (SM) supplemented with sucrose (30 g/L), activated charcoal (4.0 g/L) and liquid medium (LM) free of sucrose and activated charcoal. Both were prepared and SM/LM autoclaved at 121°C for 20 min. To place sterilized ovules for inoculating in the medium, first, SM medium was poured into a 100 mL conical flask up to 25 mL, followed by pouring LM medium up to 50 mL. Stratification of ovules continued for 65 days at 24°C. In the post-inoculation period of ovules, all naked embryos under the XSP-500SM digital microscope were transferred to SM for germination. The basal media listed in Table 1 were used continuously for the germination phase, specifically in the SM form. Twenty milliliters of germination medium were poured into Petri dishes (100 × 20 mm) and rescued embryos were placed. These Petri dishes were then kept in the environmental chamber (BIOBASE). At biweekly intervals, we refreshed the medium where embryos were grown. The germinated embryos, both normal and abnormal, were recorded. Plants with good shoot/root formation were planted in 500 mL pots before acclimatization.

### Statistical analysis

The data were analyzed by ANOVA method (mean and standard deviation). To test the significance level of treatment factors comparing between DAP intervals and crossing combination groups, ANOVA test $P \leq 0.05$ was used by RStudio 2023.12.0 + 369.

**Table 1:** Medium type for embryo rescue method

| Constituents | Murashige and Skoog (M1) | Eriksson (M2) | Nitsch and Nitsch (M3) | Anderson Rh-ododendron (M4) |
|---|---|---|---|---|
| $KNO_3$ | 1900 | 1900 | 950 | 480 |
| $NH_4NO_3$ | 1650 | 1200 | 720 | 400 |
| $CaCl_2 \cdot 2H_2O$ | 440 | 332 | | 332.20 |
| $KH_2PO_4$ | 170 | 340 | 68 | |
| $MgSO_4 \cdot 7H_2O$ | 180.7 | 180.69 | 90.34 | 180.69 |
| FeNaEDTA | 36.70 | 37,300 | 37.25 | 74.5 |
| $FeSO_4 \cdot 7H_2O$ | 27.8 | 27,800 | 27.85 | 55.7 |
| $MnSO_4 \cdot 4H_2O$ | 22.3 | 16.89 | 18.94 | 16.9 |
| $ZnSO_4 \cdot 7H_2O$ | 8.6 | 9.145 | 10 | 8.6 |
| $H_3BO_3$ | 6.2 | 0.63 | 10 | 0.025 |
| KI | 0.83 | 0.830 | | 0. 3 |
| $CoCl_2 \cdot 6H_2O$ | 0.025 | 0.003 | | |
| $Na_2MoO_4 \cdot 2H_2O$ | 0.25 | | | 6.2 |
| $CuSO_4 \cdot 5H_2O$ | 0.25 | 0.003 | 0.025 | |
| $Na_2MoO_4 \cdot 2H_2O$ | | | 0.25 | 0.213 |
| $NaH_2PO_4$ | | | | 330.39 |
| *myo*-inositol | 100 | | 100 | 100 |
| Nicotinic acid | 5 | 0.500 | 5 | |
| Pyridoxine·HCl | 5 | 0.500 | 0.5 | |
| Glycine | 2 | 2 | 2 | |
| Thiamine·HCl | 1 | 0.500 | 0.5 | 0.4 |
| Indoleacetic acid | 30 | | | |
| Kinetin | 10 | | | |
| Adenine sulfate | | | | 35.1 |
| Biotin | | | 0.05 | 0.3 |
| Folic acid | | | 0.05 | |
| Preservative nail (mL) | 2 | 2 | 2 | 2 |

## Results

The embryos, germination and palnlet numbers were studied each 10 day after pollination (DAP) intervals. The color of ovule was mostly green up to 50 DAP (Fig. 1a, b, c). No embryo formation was observed in ovules obtained from all pollinated combinations of all tested cultivars up to 50 DAP (Fig. 1d, e, f). At 50 DAP, the embryos became more transparent and became globular shaped (Fig. 1g). Their entry into the heart and torpedo-shaped corresponded to 60 and 70 DAP. Embryos were found to be covered by endosperm (Fig. 1h, j). The absorption of the endosperm occurred at 80 DAP and the shape of embryos changed from torpedo to another shape due to increasing size and preserved aliveness without endosperm in the rudiment (Fig. 1k, l).

Embryo development stages of Kishmish cultivars were tested at different DAP intervals and artificial media. Among the 50, 60, 70 and 80 DAP intervals embryos number did not show significant differences (Table 2). High number of germinated embryos were observed at 70 DAP intervals in M1 and M2 artificial media recovered from KR × KS and KR × Toyfi cross combinations. When embryos of Kishmish varieties were recovered at 50 DAP and placed in M 4 medium the number of germinated embryos decreased. Embryos of KR × KS, KR self-poll.combinations did not germinate when they recovered at 50 DAP and were placed in M2 and M4 media. Only M3 medium condition demonstrated plantlet formation from germinated embryos, that recovered among all from DAP intervals and crosses (Table 2).

In the second year of the experiment, in response to the embryo number rate among DAP (50, 60, 70, 80) intervals and crosses repeated the former result ($P > 0.05$). Embryos of KR nat. poll.germinated more when they recovered at 70 DAP interval and were placed in an M2 artificial medium. The excision at 50 DAP and placing in M4 medium resulted in a decrease of KR nat. poll in embryo germination number (Table 2). Once more, the M3 medium, utilized in this experiment promoted embryo germination excised from any cross combinations and DAP intervals (Table 3).

KS nat. poll., KS × KR, KS × Toyfi combinations had no significant ($P > 0.05$) difference in the embryos number among the 60, 70 and 80 DAP intervals. However, 50 DAP showed a lower number of embryos. Embryos recovered form KS nat. poll., KS self-poll., KS × KR, KS × Toyfi and all DAP intervals showed the formation of plantlets when were placed in M3 medium (Table 4). In the 2022 study, embryo number rates peaked at 70 DAP in M 1–3 media. No significant ($P > 0.05$) differences were observed between 60 and 70 DAP. Embryos recovered from KS self-poll., KS × KR, KS × Toyfi showed lower embryo number in M4 at 70 DAP (Table 5). In the first two stages of embryo development (embryo number, germination number), M4 artificial nutrient medium showed a lower embryo number at 80 DAP.

## Discussion

When applying the embryo rescue method to the breeding of seedless grapes, factors such as DAP intervals, parental forms, and compositions of artificial media are important. Abortion of embryos in seedless varieties occurs after a certain period after flowering (Korkutal 2005). Kishmish varieties, studied here showed no embryo formation until 40 DAP. At the veraison stage of grape berry, embryos transited towards torpedo-shape (Fig. 3). It is reported that the 7–14 days pre-veraison stage of the grape berry development is the best period to place embryos in a medium (Hiramatsu *et al.* 2003), while others considered the veraison stage (Yamashita *et al*. 1998; Notsuka *et al*. 2001). We found that Kishmish cultivars in this experiment reached the veraison stage at 70 DAP and formed most torpedo-shaped embryos, which formed more plantlets. The endosperm provides all essential nutrients to the embryo developing and is absorbed after cellularization itself (Gehring *et al*. 2004). Nutrition of embryos during development consists of two stages: heterotrophic, the first stage, and autotrophic, the second stage. When embryos take on a globular shape, it indicates a time of heterotrophic stage and a heart-shaped is autotrophic stage (Raghavan 1966). We observed that torpedo-shaped embryos turned into plantlets more often than globular-shaped ones. The appearance of globular-shaped embryos was found at 50 DAP in Kishmish cultivars in this study.

**Table 2:** Effects of media (M1, M2, M3, M4), crossing combinations and DAP intervals on embryo, germination and plantlet numbers of seedless grape embryos (mean ± SD). The results belong to 2021

| | | 50 DAP | | | 60 DAP | | | 70 DAP | | | 80 DAP | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Embryo number | Germin. Number | Plantlet number | Embryo number | Germin. number | Plantlet number | Embryo number | Germin. number | Plantlet number | Embryo number | Germin. number | Plantlet number |
| M1 | KR nat. poll. | 7.0±2.0$^{a}$ | 1.3±0.6$^{ab}$ | 0.3±0.6$^{ab}$ | 9.7±0.6$^{a}$ | 2.0±0.0$^{ab}$ | 0.7±0.6$^{ab}$ | 7.7±3.1$^{a}$ | 4.0±1.0$^{a}$ | 0.7±1.2$^{ab}$ | 4.3±0.6$^{a}$ | 2.7±0.6$^{ab}$ | 1.0±0.0$^{ab}$ |
| | KR self-poll. | 3.3±3.2$^{a}$ | 0.3±0.6$^{b}$ | - | 6.0±1.7$^{a}$ | 0.7±0$^{ab}$ | - | 4.3±2.5$^{a}$ | 0.7±0.6$^{ab}$ | 0.7±0.6$^{ab}$ | 3.7±2.1$^{a}$ | 1.0±0.0$^{ab}$ | 1.3±0.6$^{ab}$ |
| | KR ×K.Sugd. | 5.3±0.6$^{a}$ | 1.0±0.0$^{ab}$ | - | 8.0±3.0$^{a}$ | 1.3±0.6$^{ab}$ | 0.3±0.6$^{ab}$ | 7.3±1.5$^{a}$ | 4.0±2.0$^{a}$ | 2.0±0.0$^{ab}$ | 5.3±5.9$^{a}$ | 1.3±0.6$^{ab}$ | 0.6±0.6$^{ab}$ |
| | KR × Toyfi | 6.0±0.0$^{a}$ | 1.0±1.0$^{ab}$ | - | 8.3±6.1$^{a}$ | 1.7±1.2$^{ab}$ | 0.3±0.6$^{ab}$ | 6.7±3.8$^{a}$ | 3.3±1.5$^{ab}$ | - | 3.7±1.5$^{a}$ | 2.3±1.5$^{ab}$ | - |
| M2 | KR nat. poll. | 8.0±3.6$^{a}$ | 2.0±1.1$^{ab}$ | 0.6±0.5$^{ab}$ | 11.0±1.7$^{a}$ | 3.0±1.0$^{ab}$ | 1.3±0.6$^{ab}$ | 9.0±1.0$^{a}$ | 4.0±0.0$^{a}$ | 2.0±1.0$^{ab}$ | 5.0±3.6$^{a}$ | 2.3±1.5$^{ab}$ | 1.3±0.6$^{ab}$ |
| | KR self-poll. | 3.0±2.6$^{a}$ | 1.0±1.7$^{ab}$ | 0.3±0.0$^{ab}$ | 5.3±5.8$^{a}$ | 0.3±0.0$^{b}$ | - | 3.7±1.2$^{a}$ | 1.0±0.0$^{ab}$ | 0.3±0.6$^{ab}$ | 3.0±2.0$^{a}$ | 1.3±1.5$^{ab}$ | 0.3±0.5$^{ab}$ |
| | KR × K.Sugd. | 6.0±7.8$^{a}$ | - | - | 10.0±7.2$^{a}$ | 2.3±1.5$^{ab}$ | 1.0±1.0$^{ab}$ | 8.3±5.7$^{a}$ | 3.3±1.2$^{ab}$ | 1.3±0.6$^{ab}$ | 2.7±2.1$^{a}$ | 1.7±1.5$^{ab}$ | 1.7±1.5$^{ab}$ |
| | KR × Toyfi | 7.0±3.5$^{a}$ | 1.3±0.6$^{ab}$ | - | 10.0±7.2$^{a}$ | 2.3±0.6$^{ab}$ | 0.7±0.6$^{ab}$ | 8.3±4.7$^{a}$ | 4.0±2.6$^{a}$ | 1.7±2.1$^{ab}$ | 3.0±2.0$^{a}$ | 1.3±0.6$^{ab}$ | 1.0±1.0$^{ab}$ |
| M3 | KR nat. poll. | 7.3±2.5$^{a}$ | 2.0±1.0$^{ab}$ | 0.7±0.6$^{ab}$ | 10.3±0.6$^{a}$ | 3.0±1.0$^{ab}$ | 1.3±0.6$^{ab}$ | 9.0±1.7$^{a}$ | 3.3±1.2$^{ab}$ | 2.3±1.2$^{a}$ | 4.3±3.5$^{a}$ | 2.3±1.5$^{ab}$ | 1.3±0.6$^{ab}$ |
| | KR self-poll. | 3.0±2.0$^{a}$ | 0.3±0.6$^{b}$ | 0.3±0.6$^{ab}$ | 5.0±5.3$^{a}$ | 0.3±0.6$^{b}$ | 0.3±0.6$^{ab}$ | 3.0±0.0$^{a}$ | 1.0±0.0$^{ab}$ | 0.7±0.6$^{ab}$ | 2.7±2.1$^{a}$ | 1.0±0.0$^{ab}$ | 0.7±0.6$^{ab}$ |
| | KR × K.Sugd. | 5.0±7.0$^{a}$ | 1.7±2.1$^{ab}$ | 0.3±0.6$^{ab}$ | 8.3±5.9$^{a}$ | 2.7±1.5$^{ab}$ | 1.0±1.0$^{ab}$ | 7.3±4.6$^{a}$ | 2.7±1.5$^{ab}$ | 1.3±0.6$^{ab}$ | 2.7±2.1$^{a}$ | 1.3±0.6$^{ab}$ | 1.0±1.0$^{ab}$ |
| | KR × Toyfi | 6.7±3.8$^{a}$ | 1.3±0.6$^{ab}$ | 0.3±0.6$^{b}$ | 9.7±7.4$^{a}$ | 3.0±1.0$^{ab}$ | 1.3±0.6$^{ab}$ | 8.0±5.3$^{a}$ | 3.3±2.1$^{ab}$ | 1.7±1.2$^{ab}$ | 3.0±2.0$^{a}$ | 1.7±0.6$^{ab}$ | 1.0±0.0$^{ab}$ |
| M4 | KR nat. poll. | 3.7±1.5$^{a}$ | 0.7±0.6$^{ab}$ | - | 5.7±0.6$^{a}$ | 2.0±1.0$^{ab}$ | 0.3±0.6$^{ab}$ | 4.0±3.6$^{a}$ | 1.7±0.6$^{ab}$ | 0.7±0.6$^{ab}$ | 2.3±2.3$^{a}$ | 1.0±0.0$^{ab}$ | 0.7±0.6$^{ab}$ |
| | KR self-poll. | 2.0±1.0$^{a}$ | - | - | 3.0±2.0$^{a}$ | 0.3±0.6$^{b}$ | - | 1.7±1.2$^{a}$ | 0.7±0.6$^{ab}$ | 0.3±0.6$^{ab}$ | 1.7±0.6$^{a}$ | 0.7±0.6$^{ab}$ | 0.7±0.6$^{ab}$ |
| | KR × K. Sugd. | 2.7±1.5a | 0.3±0.6$^{b}$ | 0.3±0.6$^{ab}$ | 5.0±2.6$^{a}$ | 2.0±0.0$^{ab}$ | 0.7±0.6$^{ab}$ | 4.0±2.6$^{a}$ | 1.7±0.6$^{ab}$ | 0.7±0.6$^{ab}$ | 2.0±1.0$^{a}$ | 1.0±0.0$^{ab}$ | 0.7±0.6$^{ab}$ |
| | KR × Toyfi | 3.0±1.7 a | 0.3±0.6$^{b}$ | - | 5.0±1.7$^{a}$ | 1.7±0.6$^{ab}$ | 0.7±0.6$^{ab}$ | 4.0±2.0$^{a}$ | 2.0±1.0$^{ab}$ | 0.7±0.6$^{ab}$ | 2.3±0.6$^{a}$ | 1.3±0.6$^{ab}$ | 1.0±0.0$^{ab}$ |

Data analyzes were performed by ANOVA (mean value from 90 berries of three replications) by R studio. The significance was marked with a letter and was used multiple comparisons Tukey's test $P < 0.05$

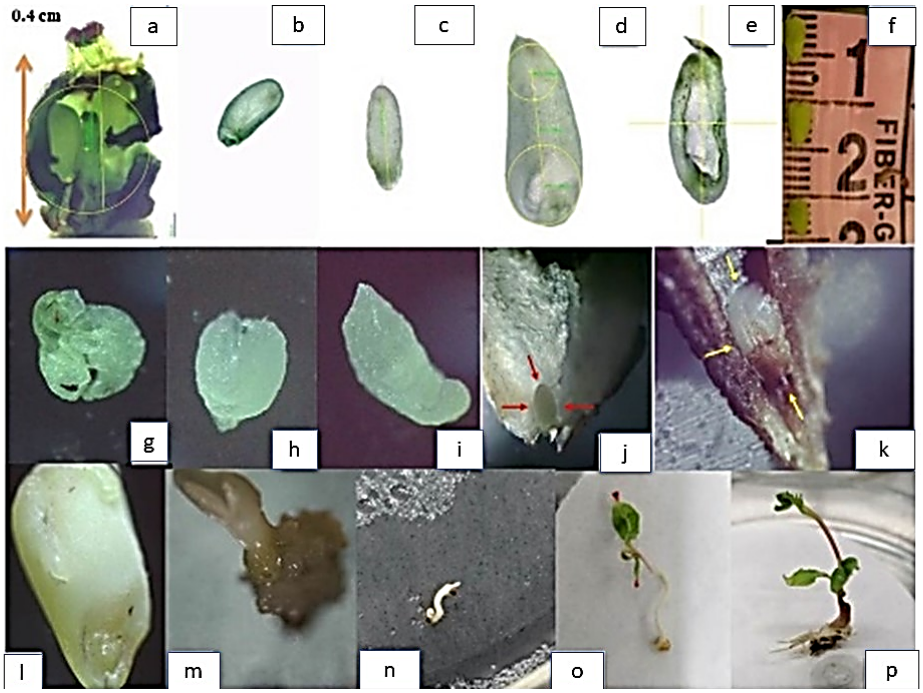


**Fig. 1:** Ovule and embryo development stages of KS seedless grape. (**a**) Ovule training, inflorescence before blooming, (**b**) ovule before blooming, (**c**) ovule at 10 DAP, (**d**) ovule at 30 DAP, € cut away form of ovule (no embryo formation), (**f**) ovules at 40 DAP, (**g**) embryo *globular* shape, (**h**) *heart* shape, (**i**) *torpedo* shape, (**j**) *torpedo* shape embryo inside of endosperm, (**k**) embryo *large cotyledon* after endosperm absorption, (**l**) recovered large cotyledon embryo, (**m**) germinated embryo, (**n**) embryo shoot growth, (**o**) shooting phase, (**p**) rooting phase

Transition to plantlet from globular-shape was lower due to deformities and death (Table 2–5). The transformation of torpedo-shaped embryos into plantlets was higher than other forms. Bharathy *et al*. (2003) reported that embryos of the Thompson Seedless × *V. tilifolia* combination had the lowest plantlet formation in the "globular" form. Li *et al*. (2018) observed that the germination of globular embryos was lower than torpedo-shaped embryos, while our results were in harmony to the results of Li *et al*. (2018). It was observed that the shape of the embryos changed in the artificial medium during development stages (Babadjanova *et al.* 2023). Along the life cycle, the embryos failed to progress due to imbibition (Fig. 2).

**Table 3:** Effects of media (M1, M2, M3, M4), crossing combinations and DAP intervals on embryo, germination and plantlet numbers of seedless grape embryos (mean ± SD). The results belong to 2022

| Media | | 50 DAP | | | 60 DAP | | | 70 DAP | | | 80 DAP | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Embryo number | Germin. number | Plantlet number | Embryo number | Germin. Number | Plantlet number | Embryo number | Germin. number | Plantlet number | Embryo number | Germin. number | Plantlet number |
| M1 | KR nat. poll. | 9.0±6.0$^{a}$ | 1.3±0.6$^{a-c}$ | - | 10.3±1.5$^{a}$ | 2.3±1.5$^{a-b}$ | 1.0±1.0$^{ab}$ | 8.0±3.0$^{a}$ | 4.3±1.2$^{ab}$ | 1.3±1.2$^{ab}$ | 4.0±3.0$^{a}$ | 2.0±1.0$^{a-c}$ | 1.0±0.0$^{ab}$ |
| | KR self-poll. | 4.7±3.1$^{a}$ | 0.7±0.6$^{bc}$ | - | 6.3±4.0$^{a}$ | 1.3±1.5$^{a-c}$ | - | 4.7±2.1$^{a}$ | 1.3±0.6$^{a-c}$ | 0.3±0.6$^{a}$ | 2.0±1.7$^{a}$ | 0.7±0.6$^{bc}$ | 1.3±0.6$^{ab}$ |
| | KR × KS | 7.7±3.8$^{a}$ | 1.3±1.2$^{a-c}$ | - | 9.0±3.0$^{a}$ | 2.3±0.6$^{a-c}$ | 0.3±0.6$^{ab}$ | 8.3±6.1$^{a}$ | 2.3±1.2$^{a-c}$ | 1.0±0.0$^{ab}$ | 3.0±1.0$^{a}$ | 1.7±0.6$^{a-c}$ | 0.7±0.6$^{ab}$ |
| | KR × Toyfi | 8.0±6.6$^{a}$ | 1.3±1.2$^{a-c}$ | - | 9.0±1.0$^{a}$ | 2.3±1.2$^{a-c}$ | 0.7±0.6$^{a}$ | 8.0±3.6$^{a}$ | 4.3±1.5$^{ab}$ | - | 3.7±3.1$^{a}$ | 2.0±1.7$^{a-c}$ | - |
| M2 | KR nat. poll. | 10.0±2.0$^{a}$ | 2.7±1.2$^{a-c}$ | 0.6±0.6$^{ab}$ | 11.7±1.5$^{a}$ | 3.3±0.6$^{a-c}$ | 1.3±0.6$^{ab}$ | 9.7±1.5$^{a}$ | 4.7±1.2$^{a}$ | 2.0±1.0$^{ab}$ | 5.3±3.2$^{a}$ | 3.3±2.1$^{a-c}$ | 1.3±0.6$^{ab}$ |
| | KR self-poll. | 4.7±1.5$^{a}$ | 1.0±1.0$^{a-c}$ | 0.3±0.6$^{a}$ | 6.3±2.9$^{a}$ | 1.3±0.6$^{a-c}$ | - | 5.0±0.0$^{a}$ | 1.7±1.2$^{a-c}$ | 0.3±0.6$^{ab}$ | 2.7±1.5$^{a}$ | 0.7±0.6$^{bc}$ | 0.3±0.6$^{ab}$ |
| | KR × KS | 7.0±7.9$^{a}$ | 0.3±0.6$^{c}$ | - | 10.0±7.2$^{a}$ | 3.7±3.1$^{a-c}$ | 1.0±1.0$^{ab}$ | 8.3±5.7$^{a}$ | 3.7±2.3$^{a-c}$ | 1.3±0.6$^{ab}$ | 2.7±2.1$^{a}$ | 1.0±0.0$^{a-c}$ | 1.7±1.5$^{ab}$ |
| | KR × Toyfi | 8.3±3.2$^{a}$ | 0.7±1.2$^{bc}$ | - | 10.0±7.2$^{a}$ | 3.3±2.3$^{a-c}$ | 0.6±0.6$^{ab}$ | 8.3±4.7$^{a}$ | 3.3±1.5$^{a-c}$ | 1.7±2.0$^{ab}$ | 4.0±1.7$^{a}$ | 2.0±0.0$^{a-c}$ | 1.0±1.0$^{ab}$ |
| M3 | KR nat. poll. | 8.7±2.1$^{a}$ | 2.3±0.6$^{a-c}$ | 0.7±0.6$^{ab}$ | 10.7±1.2$^{a}$ | 3.3±0.6$^{a-c}$ | 1.3±0.6$^{ab}$ | 9.3±1.2$^{a}$ | 4.3±1.5$^{ab}$ | 2.3±1.2$^{a}$ | 5.0±2.6$^{a}$ | 3.3±1.5$^{a-c}$ | 1.3±0.6$^{ab}$ |
| | KR self-poll. | 4.3±1.2$^{a}$ | 0.7±0.6$^{bc}$ | 0.3±0.6$^{ab}$ | 5.7±1.5$^{a}$ | 1.7±1.5$^{a-c}$ | 0.3±0.6$^{ab}$ | 5.0±0.0$^{a}$ | 2.0±1.0$^{a-c}$ | 0.7±0.6$^{ab}$ | 2.7±1.5$^{a}$ | 1.3±0.6$^{a-c}$ | 0.6±0.6$^{ab}$ |
| | KR × KS | 7.3±6.8$^{a}$ | 1.3±0.6$^{a-c}$ | 0.3±0.6$^{ab}$ | 10.0±7.0$^{a}$ | 2.7±0.6$^{a-c}$ | 1.0±1.0$^{ab}$ | 8.3±5.5$^{a}$ | 4.0±2.6$^{a-c}$ | 1.3±0.6$^{ab}$ | 2.7±2.1$^{a}$ | 1.3±0.6$^{a-c}$ | 1.0±1.0$^{ab}$ |
| | KR × Toyfi | 7.0±3.5$^{a}$ | 2.0±1.0$^{a-c}$ | 0.3±0.6$^{ab}$ | 10.0±7.2$^{a}$ | 2.7±0.6$^{a-c}$ | 1.3±0.6$^{ab}$ | 9.0±5.2$^{a}$ | 3.0±1.0$^{a-c}$ | 1.7±1.2$^{ab}$ | 3.0±1.0$^{a}$ | 2.0±1.0$^{a-c}$ | 1.0±0.0$^{ab}$ |
| M4 | KR nat. poll. | 4.7±3.2$^{a}$ | 0.3±0.6$^{c}$ | - | 5.7±2.1$^{a}$ | 2.0±0.0$^{a-c}$ | 0.3±0.6$^{ab}$ | 5.3±3.2$^{a}$ | 2.3±1.2$^{a-c}$ | 0.7±0.6$^{ab}$ | 4.0±2.6$^{a}$ | 2.0±1.0$^{a-c}$ | 0.7±0.6$^{ab}$ |
| | KR self-poll. | 3.7±1.2$^{a}$ | 0.3±0.6$^{c}$ | - | 4.3±2.5$^{a}$ | 0.7±0.6$^{bc}$ | - | 3.7±1.5$^{a}$ | 1.0±0.0$^{a-c}$ | 0.3±0.6$^{a}$ | 3.3±1.5$^{a}$ | 1.0±1.0$^{a-c}$ | 0.7±0.6$^{ab}$ |
| | KR × KS | 4.3±0.6$^{a}$ | 1.0±1.0$^{a-c}$ | - | 6.0±2.6$^{a}$ | 2.7±1.2$^{a-c}$ | 0.7±0.6$^{ab}$ | 5.3±2.3$^{a}$ | 3.0±1.7$^{a-c}$ | 0.7±0.6$^{ab}$ | 3.7±1.5$^{a}$ | 2.0±1.0$^{a-c}$ | 0.7±0.6$^{ab}$ |
| | KR × Toyfi | 3.7±1.2$^{a}$ | 0.7±0.6$^{bc}$ | - | 5.3±2.5$^{a}$ | 2.0±0.0$^{a-c}$ | 0.7±0.6$^{ab}$ | 4.7±1.5$^{a}$ | 2.3±0.6$^{a-c}$ | 0.7±0.6$^{ab}$ | 3.7±0.6$^{a}$ | 2.0±0.0$^{a-c}$ | 1.0±0.0$^{ab}$ |

Data analyzes were performed by ANOVA (mean value from 90 berries of three replications) by R studio. The significance was marked with a letter and was used multiple comparisons Tukey's test $P < 0.05$

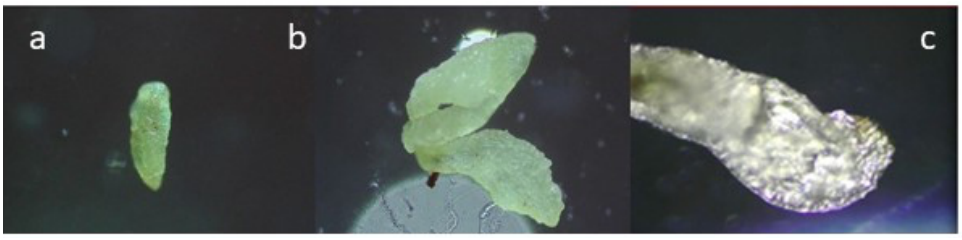


**Fig. 2:** Embryo differentiation during development stages, (**a**) normal torpedo shape embryo, (**b**) abnormal embryo abnormal, (**c**) imbibed embryo

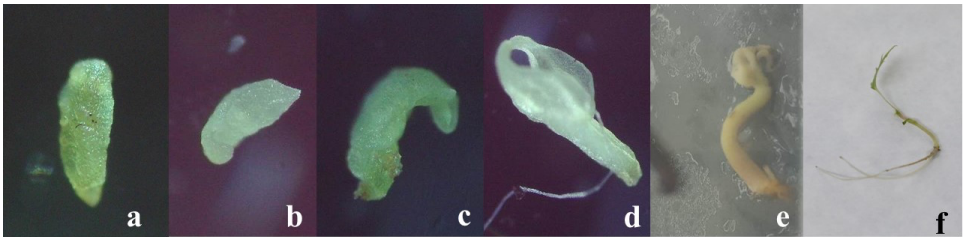


**Fig. 3:** Development stage of torpedo shape KS. embryos of, (**a)** normal torpedo shape, (**b**) enlargement of torpedo embryo, (**c**) twisting due to growth (crescent - shaped), (**d**) formation of the first cotyledon, (**e-f**) development into a mature plant

One of the advantages of embryo rescue is reciprocal hybridization to overcome decreasing seedlessness among hybrid populations. Kismish cultivars demonstrated a valuable rate of reciprocal hybridization. Almost 25% of plantlets were grown (78 plants out of 306) by embryos excised from KR × KS and 26% (152 out of 585) from KS × KR combinations. When these results were compared with the open-pollinated variants and those pollinated with the Toyfi variety, no statistical difference was detected. It is possible to get viable plantlets from embryos by crossbreeding two Kishmish cultivars. We have demonstrated the advantages of reciprocal hybridization from out data. However, plantlet formation from embryos may differ among selected parental forms. Similar results have been previously reported. For instance, Lee *et al.* (2018) reported that the embryo number rate in Flame Seeds × Ruby Seedless combinations was 21.8%, plantlet 8.5%, Flame Seeds × Crimson Seedless embryo number 24.6% and plantlet 14.8%. Giancaspro *et al.* (2022) showed that, Luisa seedless × Thompson seedless combination reached 22.6% plantlet, while Luisa × White seedless combination showed no plantlet formation, but Luisa seedless × Crimson seedless hybridization recorded 13% plantlet. The results obtained from reciprocal hybridization applied to some cultivars were consistent with the results of Flame Seeds × Crimson Seedless and Luisa seedless × Thompson seedless combinations obtained by Li *et al*. (2015) and Giancaspro *et al.* (2022).

**Table 4:** Effects of media (M1, M2, M3, M4), crossing combinations and DAP intervals on embryo, germination and plantlet numbers of seedless grape embryos (mean ± SD). The results belong to 2021

| | | 50 DAP | | | 60 DAP | | | 70 DAP | | | 80 DAP | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Embryo number | Germin. number | Plantlet number | Embryo number | Germin. Number | Plantlet number | Embryo number | Germin. Number | Plantlet number | Embryo number | Germin. Number | Plantlet number |
| M1 | KS nat. poll. | 5.0±1.0$^{bc}$ | 1.3±0.6$^{d-f}$ | 0.7±0.6$^{ab}$ | 8.0±1.0$^{a-c}$ | 2.3±0.6$^{c-e}$ | 1.3±0.6$^{ab}$ | 10.3±1.5$^{a-d}$ | 4.0±1.7$^{a-c}$ | 2.3±0.6$^{ab}$ | 6.0±2.6$^{a-c}$ | 3.3±0.6$^{a-c}$ | 2.0±0.0$^{ab}$ |
| | KS self-poll. | 3.0±1.0$^{ef}$ | 1.0±0.0$^{f-h}$ | 0.7±0.6$^{ab}$ | 5.7±2.1$^{a-c}$ | 2.0±1.0$^{d-f}$ | 0.7±0.6$^{ab}$ | 7.7±1.5$^{a-d}$ | 3.7±1.5$^{a-c}$ | 1.7±0.6$^{ab}$ | 7.3±1.5$^{a-c}$ | 3.7±1.5$^{a-c}$ | 1.7±1.2$^{ab}$ |
| | KS ×KR | 1.3±1.5$^{g}$ | 0.3±0.6$^{h}$ | - | 6.0±1.0$^{a-c}$ | 2.7±1.2$^{c-e}$ | 1.3±1.3$^{ab}$ | 7.7±1.5$^{a-d}$ | 3.3±1.5$^{a-c}$ | 2.0±1.0$^{ab}$ | 4.7±1.2$^{b-d}$ | 3.0±1.0$^{b-d}$ | 1.7±0.6$^{ab}$ |
| | KS × Toyfi | 4.0±1.0$^{cd}$ | 1.3±0.6$^{d-f}$ | 0.7±0.6$^{ab}$ | 6.0±3.0$^{a-c}$ | 3.7±1.5$^{a-c}$ | 2.3±1.5$^{ab}$ | 10.7±1.5$^{a-d}$ | 5.7±1.2$^{a-c}$ | 3.3±1.2$^{ab}$ | 4.7±1.2$^{a-c}$ | 3.0±1.7$^{b-d}$ | 1.7±0.6$^{ab}$ |
| M2 | KS nat. poll. | 4.7±1.2$^{bc}$ | 1.3±0.6$^{d-f}$ | 0.7±0.6$^{ab}$ | 7.0±2.0$^{a-c}$ | 2.3±0.6$^{c-e}$ | 1.3±0.6$^{ab}$ | 10.0±2.6$^{a-d}$ | 5.3±4.2$^{a-c}$ | 3.0±2.6$^{ab}$ | 6.7±2.1$^{a-c}$ | 4.0±1.0$^{a-c}$ | 2.7±0.6$^{ab}$ |
| | KS self-poll. | 2.7±2.1$^{ef}$ | 0.3±0.6$^{h}$ | *0.3±0.5$^{b}$* | 6.7±2.1$^{a-c}$ | 1.3±0.6$^{ef}$ | *0.7±0.6$^{ab}$* | 3.3±2.1$^{de}$ | 1.0±0.0$^{f-h}$ | *0.3±0.6$^{b}$* | 6.3±2.1$^{a-c}$ | 3.3±2.1$^{a-c}$ | 1.3±0.6$^{ab}$ |
| | KS ×KR | 2.0±1.7$^{fg}$ | 0.3±0.6$^{h}$ | - | 7.0±2.6$^{a-c}$ | 3.7±1.2$^{a-c}$ | 0.7±0.7$^{ab}$ | 9.6±1.5$^{a-d}$ | 7.0±2.0$^{ab}$ | 3.3±2.5$^{ab}$ | 6.0±1.7$^{a-c}$ | 3.3±0.6$^{a-c}$ | 2.0±1.0$^{ab}$ |
| | KS × Toyfi | 2.0±1.0$^{fg}$ | 0.7±0.6$^{f-h}$ | *0.3±0.6$^{b}$* | 6.0±1.0$^{a-c}$ | 3.3±1.5$^{a-c}$ | 1.0±0.0$^{ab}$ | 10.7±2.1$^{a-d}$ | 6.3±2.1$^{a-c}$ | 2.7±0.6$^{ab}$ | 6.0±2.0$^{a-c}$ | 3.7±1.5$^{a-c}$ | 1.7±0.6$^{ab}$ |
| M3 | KS nat. poll. | 5.3±1.5$^{a-c}$ | 2.0±1.0$^{d-f}$ | 0.7±0.6$^{ab}$ | 8.0±1.0$^{a-c}$ | 3.7±1.2$^{a-c}$ | 1.7±1.2$^{ab}$ | 11.3±1.5$^{a}$ | 5.7±0.6$^{a-c}$ | 3.3±1.2$^{ab}$ | 8.0±2.0$^{a-c}$ | 5.0±3.0$^{a-c}$ | 2.0±1.0$^{ab}$ |
| | KS self-poll. | 4.0±1.0$^{cd}$ | 1.7±0.6$^{d-f}$ | 0.7±0.6$^{ab}$ | 6.7±1.5$^{a-c}$ | 3.0±1.7$^{b-d}$ | 1.0±0.0$^{ab}$ | 6.0±2.0$^{c}$ | 2.7±2.1$^{c-e}$ | 1.0±0.0$^{ab}$ | 6.0±2.0$^{a-c}$ | 3.0±1.0$^{b-d}$ | 1.7±0.6$^{ab}$ |
| | KS ×KR | 5.3±3.1$^{a-c}$ | 2.7±1.5$^{c-e}$ | 0.7±0.7$^{ab}$ | 8.3±1.5$^{a-c}$ | 4.7±1.2$^{a-c}$ | 2.3±2.3$^{ab}$ | 9.3±1.5$^{a-d}$ | 5.3±0.6$^{a-c}$ | 3.3±2.9$^{ab}$ | 6.3±1.5$^{a-c}$ | 3.7±1.2$^{a-c}$ | 2.0±1.0$^{ab}$ |
| | KS × Toyfi | 5.0±1.0$^{bc}$ | 2.0±1.7$^{d-f}$ | *0.3±0.6$^{b}$* | 9.7±1.5$^{a-c}$ | 5.0±2.0$^{a-c}$ | 2.3±1.5$^{ab}$ | 10.0±1.0$^{a-d}$ | 7.3±1.5$^{a}$ | 4.0±1.7$^{a}$ | 7.0±1.0$^{a-c}$ | 4.0±1.0$^{ab}$ | 2.3±0.6$^{ab}$ |
| M4 | KS nat. poll. | 4.0±1.0$^{cd}$ | 1.7±0.6$^{d-f}$ | *0.7±0.6$^{b}$* | 8.0±2.6$^{a-c}$ | 3.0±0.0$^{b-d}$ | 1.7±0.6$^{ab}$ | 10.0±1.7$^{a-d}$ | 5.0±1.0$^{a-c}$ | 3.3±0.6$^{ab}$ | 5.7±3.5$^{a-c}$ | 2.7±1.2$^{c-e}$ | 1.7±0.6$^{ab}$ |
| | KS self-poll | 2.7±2.1$^{f}$ | 0.3±0.6$^{h}$ | - | 5.0±2.0$^{b-d}$ | 1.7±0.6$^{d-f}$ | 0.7±0.6$^{ab}$ | 4.0±1.0$^{c-e}$ | 1.7±0.6$^{d-f}$ | 0.7±0.6$^{ab}$ | 2.7±1.2$^{e-g}$ | 1.3±0.6$^{ef}$ | 0.7±0.6$^{ab}$ |
| | KS ×KR | 4.0±1.0$^{cd}$ | 1.7±0.6$^{d-f}$ | 1.0±1.0$^{ab}$ | 7.3±0.6$^{a-c}$ | 3.3±1.5$^{a-c}$ | 2.0±2.0$^{ab}$ | 8.0±2.0$^{a-d}$ | 3.7±1.2$^{a-c}$ | 2.3±2.1$^{ab}$ | 5.7±2.1$^{a-c}$ | 3.3±2.3$^{a-c}$ | 2.0±1.7$^{ab}$ |
| | KS × Toyfi | 5.0±1.0$^{bc}$ | 2.3±0.6$^{c-e}$ | 1.0±1.0 $^{ab}$ | 6.7±2.5$^{a-c}$ | 3.3±1.5$^{a-c}$ | 2.0±1.0$^{ab}$ | 8.0±1.0$^{a-d}$ | 4.0±1.0$^{a-c}$ | 2.3±0.6$^{ab}$ | 7.0±2.0$^{a-c}$ | 4.0±1.0$^{a-c}$ | 2.7±1.2$^{ab}$ |

Data analyzes were performed by ANOVA (mean value from 90 berries of three replications) by R studio. The significance was marked with a letter and was used multiple comparisons Tukey's test $P < 0.05$

**Table 5:** Effects of media (M1, M2, M3, M4), crossing combinations and DAP intervals on embryo, germination and plantlet numbers of seedless grape embryos (mean ± SD). The results belong to 2022

| Media | | 50 DAP | | | 60 DAP | | | 70 DAP | | | 80 DAP | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Embryo number | Germin. Number | Plantlet number | Embryo number | Germin. Number | Plantlet number | Embryo number | Germin number | Plantlet number | Embryo number | Germin. Number | Plantlet number |
| M1 | KS nat. poll. | 6.0±1.0$^{fg}$ | 2.0±1.0 $^{d-i}$ | 0.7±0.6$^{c-e}$ | 8.3±1.2$^{a-c}$ | 3.3±1.2$^{a-i}$ | 2.0±1.0$^{a-e}$ | 12.3±0.6$^{ab}$ | 6.0±1.0$^{a-d}$ | 3.7±1.5$^{a-d}$ | 6.3±2.5$^{ef}$ | 3.3±1.5$^{a-i}$ | 2.3±1.5$^{a-i}$ |
| | KS self-poll. | 3.0±1.0$^{km}$ | 1.0±1.0$^{g-i}$ | | 6.0±1.0$^{fg}$ | 2.0±2.0$^{d-i}$ | 1.0±1.0$^{b-e}$ | 8.3±2.1$^{a-c}$ | 4.3±1.2$^{a-d}$ | 2.3±1.2$^{a-e}$ | 7.0±1.0$^{cd}$ | 4.0±1.0$^{a-i}$ | 2.0±1.0$^{a-i}$ |
| | KS ×KR | 4.3±0.6$^{ij}$ | 2.0±0.0$^{d-i}$ | | 6.7±0.6$^{de}$ | 3.0±0.0$^{a-i}$ | 1.3±0.6$^{a-e}$ | 7.0±2.0$^{cd}$ | 3.0±1.7$^{a-c}$ | 1.7±1.2$^{a-e}$ | 4.7±1.2$^{ij}$ | 2.7±0.6$^{b-i}$ | 1.7±0.6$^{b-i}$ |
| | KS × Toyfi | 4.0±1.0$^{ij}$ | 1.3±0.6$^{f-i}$ | 0.3±0.6$^{de}$ | 7.7±3.1$^{a-c}$ | 5.0±3.0$^{a-h}$ | 3.3±2.5$^{a-e}$ | 9.3±1.5$^{a-c}$ | 6.0±1.7$^{ab}$ | 3.7±0.6$^{a-d}$ | 5.0±1.7$^{hi}$ | 3.0±1.0$^{a-i}$ | 2.0±1.0$^{a-i}$ |
| M2 | KS nat. poll. | 5.7±1.2$^{gh}$ | 2.3±0.6$^{c-i}$ | 1.0±0.0$^{b-d}$ | 7.7±2.1$^{a-c}$ | 3.7±1.2$^{a-i}$ | 1.7±0.6$^{a-e}$ | 11.3±3.5$^{ab}$ | 4.0±1.7$^{a-d}$ | 4.7±1.2$^{a}$ | 6.3±1.5$^{ef}$ | 4.0±1.7$^{a-i}$ | 2.3±1.5$^{a-e}$ |
| | KS self-poll. | 3.3±2.1$^{km}$ | 0.3±0.6$^{i}$ | | 7.7±1.5$^{a-c}$ | 1.7±1.2$^{c-i}$ | 1.0±1.0$^{b-e}$ | 12.7±2.1$^{ab}$ | 5.0±1.7$^{a-d}$ | 2.7±0.6$^{a-e}$ | 6.7±1.2$^{de}$ | 3.7±1.5$^{a-i}$ | 2.0±1.0$^{a-e}$ |
| | KS ×KR | 2.3±1.5$^{lm}$ | 1.0±1.0$^{g-i}$ | | 7.3±0.6$^{bc}$ | 4.7±1.5$^{a-i}$ | 2.3±0.6$^{a-e}$ | 11.7±1.5$^{ab}$ | 6.3±1.2$^{a-d}$ | 3.7±0.6$^{a-d}$ | 6.7±1.5$^{de}$ | 4.0±1.0$^{a-i}$ | 2.3±0.6$^{a-e}$ |
| | KS × Toyfi | 3.0±1.0$^{km}$ | 1.3±0.6$^{f-i}$ | 0.7±0.6$^{c-e}$ | 6.3±1.5$^{ef}$ | 3.7±1.5$^{a-i}$ | 1.7±1.2$^{a-d}$ | 12.3±0.6$^{ab}$ | 7.3±0.6$^{a}$ | 4.3±1.2$^{ab}$ | 6.3±1.2$^{ef}$ | 4.0±1.0$^{a-i}$ | 2.3±0.6$^{a-e}$ |
| M3 | KS nat. poll. | 5.7±1.2$^{gh}$ | 2.3±0.6$^{c-i}$ | 0.7±0.6$^{c-e}$ | 8.3±2.1$^{a-c}$ | 4.7±0.6$^{a-i}$ | 2.3±1.2$^{a-e}$ | 13.0±1.0$^{a}$ | 6.7±1.5$^{a-c}$ | 4.0±1.0$^{a-c}$ | 9.0±2.0$^{abcd}$ | 6.0±1.0$^{a-e}$ | 3.7±1.5$^{a-e}$ |
| | KS self-poll. | 5.0±1.0$^{h}$ | 2.0±0.0$^{d-i}$ | 0.7±0.6$^{c-e}$ | 7.7±1.2$^{a-c}$ | 3.7±1.2$^{a-i}$ | 1.3±0.6$^{a-e}$ | 10.3±1.2$^{a-c}$ | 6.0±1.0$^{a-e}$ | 3.0±0.0$^{a-e}$ | 6.7±2.5$^{de}$ | 3.3±1.5$^{a-i}$ | 2.0±1.0$^{a-e}$ |
| | KS ×KR | 5.7±2.1$^{gh}$ | 4.0±1.0$^{a-i}$ | 1.3±0.6$^{a-e}$ | 8.3±1.2$^{a-c}$ | 3.7±2.1$^{a-i}$ | 1.3±0.6$^{a-e}$ | 10.3±0.6$^{a-c}$ | 6.3±1.2$^{a-d}$ | 3.7±0.6$^{a-d}$ | 7.0±1.0$^{cd}$ | 3.7±1.5$^{a-i}$ | 2.3±1.2$^{a-e}$ |
| | KS × Toyfi | 5.7±1.5$^{gh}$ | 2.3±0.6$^{c-i}$ | 1.0±0.0$^{b-d}$ | 10.3±1.5$^{abc}$ | 5.3±0.6$^{a-g}$ | 2.7±1.5$^{a-e}$ | 12.0±1.7$^{ab}$ | 7.0±1.7$^{ab}$ | 3.3±0.6$^{a-e}$ | 7.3±1.5$^{bc}$ | 4.3±1.5$^{a-i}$ | 2.7±1.5$^{a-e}$ |
| M4 | KS nat. poll. | 4.3±1.2$^{ij}$ | 1.7±0.6$^{e-i}$ | - | 8.3±0.6$^{a-c}$ | 3.7±2.1$^{a-i}$ | 2.3±2.1$^{a-e}$ | 10.3±1.2$^{a-c}$ | 5.7±1.2$^{a-f}$ | 2.0±1.7$^{a-f}$ | 5.0±2.6$^{hi}$ | 3.7±1.5$^{a-i}$ | 2.0±0.0$^{a-e}$ |
| | KS self-poll. | 3.7±0.6$^{ij}$ | 1.7±1.5$^{e-i}$ | | 4.3±0.6$^{ij}$ | 2.3±1.2$^{c-i}$ | 1.3±1.5$^{a-e}$ | 5.7±1.2$^{gh}$ | 2.3±1.2$^{c-i}$ | 1.3±0.6$^{c-i}$ | 1.7±1.5$^{m}$ | 1.3±1.2$^{f-i}$ | 0.7±0.6$^{c-e}$ |
| | KS ×KR | 4.3±2.1$^{ij}$ | 0.7±0.6$^{hi}$ | 0.7±0.6$^{c-e}$ | 5.0±0.0$^{hi}$ | 2.0±1.0$^{d-i}$ | 1.7±0.6$^{a-e}$ | 6.0±2.6$^{fg}$ | 2.0±1.0$^{d-i}$ | 1.7±0.6$^{a-i}$ | 3.3±1.2$^{kl}$ | 2.0±0.0$^{d-i}$ | 1.7±0.6$^{a-e}$ |
| | KS × Toyfi | 4.3±1.5$^{ij}$ | 1.3±0.6$^{f-i}$ | | 5.0±0.0$^{hi}$ | 2.7±1.5$^{b-i}$ | 1.3±1.2$^{abcde}$ | 5.7±2.1$^{gh}$ | 4.0±2.6$^{a-i}$ | 2.0±1.0$^{a-i}$ | 4.3±0.6$^{ij}$ | 2.7±0.6$^{b-i}$ | 2.0±0.0$^{a-e}$ |

Data analyzes were performed by ANOVA (mean value from 90 berries of three replications) by R studio. The significance was marked with a letter and was used multiple comparisons Tukey's test $P < 0.05$. the letters indicate significance of value and letter sequencing shortened

In our study, cleistogamy (pollination before flower cup fall) occurred in the Kishmish varieties. There was no significant difference in embryo number when KR self-poll and other combinations were compared (Table 2). There are several theories regarding the pollination of grapes and what factors influence on pollination (Kimura *et al*. 1998). Some have observed that cleistogamy occurs in many *V. vinifera* (Wang *et al.* 2023). But, Topale and Roychev (2022) identified eight types of pollen sterility associated with different causes in the grape pollination. Kishmish cultivars demonstrated susceptiblity to high temperatures during blooming period. Some inflorescence of grape clusters failed in progress due to higher temperatures inside isolated paper bags (Fig. 4).

Among the media, M1, M2 and M3 showed potential growth conditions to get valuable embryo, germination and plantlets numbers. Several studies reported the efficiency on plantlet growth of seedless grape embryos in M3 and M1 media condition and percentage of efficiency ranged between 13.8, 33 and 15%, respectively (Gray *et al.* 1990; Tsolova and Atanassov 1994; Ebadi *et al*. 2002*;* Ubaydullaeva *et al*. 2023a; Abdullaev *et al.* 2025). Using M2 medium in the seedless grape breeding is limited and it demonstrated similar result regarding plantlet rate with M1 and M2.

Nitrogen metabolism is essential for tissue differentiation and morphogenesis of somatic embryogenesis. *In vitro* culture $NH_4^+$ is used as the nitrogen source, the main form of nitrogen assimilation by amino acids (Duarte-Ake *et al*. 2022; Ubaydullaeva *et al.* 2023b).


**Fig. 4:** Self-pollination (self-poll.) two Kishmish grape cultivars: KR nat. poll- Kishmish Rozoviy natural (open) pollination, KS. nat. poll - natural (open) pollination

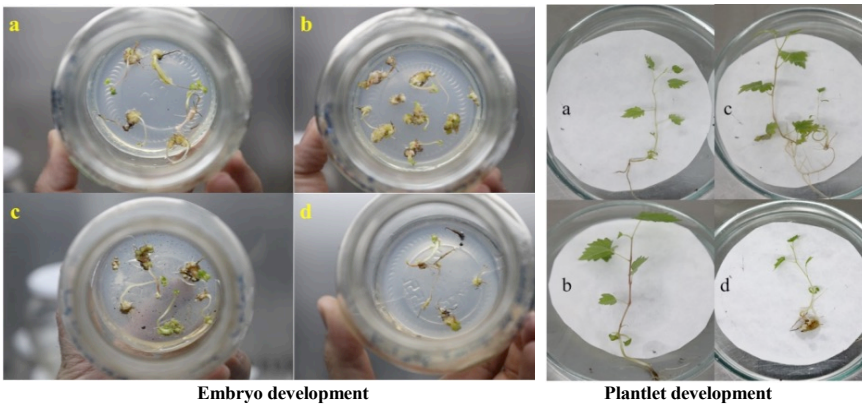


**Fig. 5:** Germinated embryos and plantlet growth (KS) on four different media: a-M1, b-M2, c-M3, d-M4 (embryos recovered at 70 DAP)

When the level of $NH_4NO_3$ ranged from 720 to 1650 mg/L in the media, there was not negative influence on embryo number in KR cultivar at all DAP and in KS at 60, 70, 80 DAP. However, the medium (M4) contained 480 mg/L of $NH_4NO_3$, the embryo germinated and the plantlet number decreased in KS cultivar, embryos recovered at 70 DAP (Fig. 5). The effect of amino acids and vitamins (in M2 and M3) on cell growth has been determined (Tian *et al*. 2008). Additionally, the M1 medium also proved a high potential for success in embryo rescue and as reported by some works (Shasha *et al.* 2022).

## Conclusion

The effectiveness of the embryo rescue method was demonstrated when using the studied varieties as mother plant in breeding programs. A mature plant was obtained from the immature embryos of Kishmish varieties, which were supported by artificial media. We identified the optimal DAP interval, media and embryo shape for two Kishmish cultivars for increasing embryo efficiency. To improve the effectiveness of this method, it is necessary to conduct more extensive research on local Kishmish varieties.

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## Author Contributions

SAA: Formal analysis, data curation, investigation, methodology, visualization, resources, validation, writing original draft. KhAU: Writing, data curation and experiment design. JBE, FIB, ANA and AAB: Conceptualization, investigation, methodology ZTB: Writing – review, editing and project administration.

## Conflict of interest

The authors declare no conflict of interest

## Data Availability

Data presented in this study will be available on a fair request to the corresponding author.

## Ethics Approval

Not applicable to this paper.

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