

***In vitro* embryo rescue for the production of hypotetraploids after cross between hypotetraploid and tetraploid grape cultivars**

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Abstract

Consumer demand for seedless grape with high quality and large berry has been increasing. Breeding of hypotetraploid grape was suggested as one of promising methods to satisfy it, but low frequency of hypotetraploid occurrence and low seed germination by abortive embryo were indicated as the major problem to hamper the development of hypotetraploid grape. Hence, this study was carried out to evaluate the basic efficiency of *in ovulo* embryo culture after the cross between hypotetraploid ('Hanareum') and tetraploid ('Honey Black' and 'Kyoho') grape cultivars on the establishment of hypotetraploid grapes. Embryos and plantlets were hardly obtained in ovules cultured at six after the cross pollination (WAP), but ovules inoculated at 10 WAP produced more embryos as well as plantlets regardless of cross combination. Furthermore, we found that embryo formation was not affected by the basal media in ovules cultured at six WAP, but utilization of specific medium can be more beneficial for embryo formation when ovules were cultured at 10 WAP. A total of 17 plants were obtained from ovules cultured at 10 WAP, and above 50% of plants were identified as hypotetraploid grapes. These results indicate that *in vitro* embryo rescue after cross pollination between hypotetraploid and tetraploid grape can enhance the efficiency for the breeding of hypotetraploid grapes.

Keywords: aneuploidy; ovule; seedless grape; tissue culture

Introduction

Breeding of seedless grapes with large berries is gaining importance because of the increase in consumer demand. Seedless grapes have been mainly developed by using characters of parthenocarpy, stenospermocarpy or triploidy found in grapes (Stout, 1936; Pratt, 1971; Heo *et al.*, 2007). Recently, production of hypotetraploid ($2n = 4x-2$ or $4x-1 = 74$ or 75) grapes have also been suggested as one of promising methods to develop seedless grapes with large berries. They exhibit growth characteristics as stable as those of tetraploid grapes, and they have extremely low fertility of pollen (Park *et al.*, 2015). They, therefore, can produce large seedless fruits via a one-time treatment of gibberellic acid during the full blooming period.

It has been reported that hypotetraploid seedlings were obtained from cross, open, or self-pollination of tetraploid grapes (Park *et al.*, 2010), but the extremely low frequency rate of hypotetraploid seedlings has been acted as the main problems in breeding of hypotetraploid grape cultivars. It was found that cross between

hypotetraploid and tetraploid grapes generates hypotetraploids more easily compared to previously used breeding methods. However, low seed germination caused by abortive embryos after cross pollination has remained a problem that hinders the breeding of hypotetraploid grape cultivars (Park *et al.*, 2016). *In vitro* embryo rescue technique has been successfully applied to generate hybrid seedlings that can hardly be obtained by artificial cross combination of grape cultivars such as stenospermic \times stenospermic or tetraploid \times diploid grapes (Liu *et al.*, 2016; Jiao *et al.*, 2018). It has been well known that several factors affect the efficiency of *in vitro* embryo rescue, but there is no information on *in vitro* embryo rescue system to generate hypotetraploid grapes. Hence, this study was conducted to investigate the effects of ovule removal age and media type for *in vitro* embryo rescue after cross pollination between hypotetraploid and tetraploid grape cultivars on the efficiency of embryo rescue and establishment of hypotetraploids.

Materials and Methods

Plant materials

The hypotetraploid ($2n=4x-1=75$) grape cultivar, 'Hanareum' (HR) developed by our research team was used as a female parent (Park *et al.*, 2020); and tetraploid ($2n=4x=76$) grape cultivars, 'Honey Black' (HB) and 'Kyoho' (KH) were used as male parents. HB, HR, and KH were 9-year-old vines, grown in the research orchard of Gangwondo Agricultural Research and Extension Services at Chuncheon, Korea.

Embryo rescue procedure

Emasculation was carried out five days before anthesis, followed by immediate bagging of the inflorescence. Artificial cross was made using a cotton swab with pollens collected earlier from the male parents. The pollinated clusters from a cross combination of 'HR' \times 'HB' and 'HR' \times 'KH' were collected at 6 and 10 weeks after cross pollination (WAP). Harvested immature fruits were soaked in 70% ethanol for 1 min, following which they were soaked in 2% sodium hypochlorite containing one drop of Tween 20 for 15 min, and then rinsed four times with sterile distilled water. Three different basal media were tested to evaluate the effect of embryo formation and establishment of plants; ovule of more than two mm in length were cultured on Emershad and Ramming (ER), Murashige and Skoog (MS), and Nitsch and Nitsch (NN) media. 30 g l⁻¹ sucrose, 2.0 g l⁻¹ activated charcoal (AC) and 8.0 g l⁻¹ agar were added into all embryo formation medium. The pH of all media was adjusted to 5.7 prior to addition of agar and sterilized at 121 °C for 15 min. Each condition for embryo formation consisted of seven replications each with five ovules. After 8 weeks of *in ovulo* culture, the percentage of embryo formation was investigated, and then each ovule was incubated on Woody Plant (WP) medium supplemented with 1.0 μ M 6-benzyladenine, 30 g l⁻¹ sucrose, 2.0 g l⁻¹ AC and 8.0 g l⁻¹ agar. All cultures were maintained at 20 \pm 1 °C under cool-white fluorescent lamps providing 16-h photoperiod (Figure 1).

Observation for occurrence frequency of hypotetraploids

Plants established from ovules at 10 WAP were transplanted to pots filled with a commercial substrate mix and perlite (1:1) contained in plastic sundae cups with lids, and were hardened by gradually lowering the humidity over a period of one month (Kwon *et al.*, 2019). Hardened plants were transferred to a commercial substrate mix in 1 L plastic pots and maintained in the glasshouse. Chromosome observation in root tip cells from successfully established plants was performed according to the method described by Park *et al.* (1999).

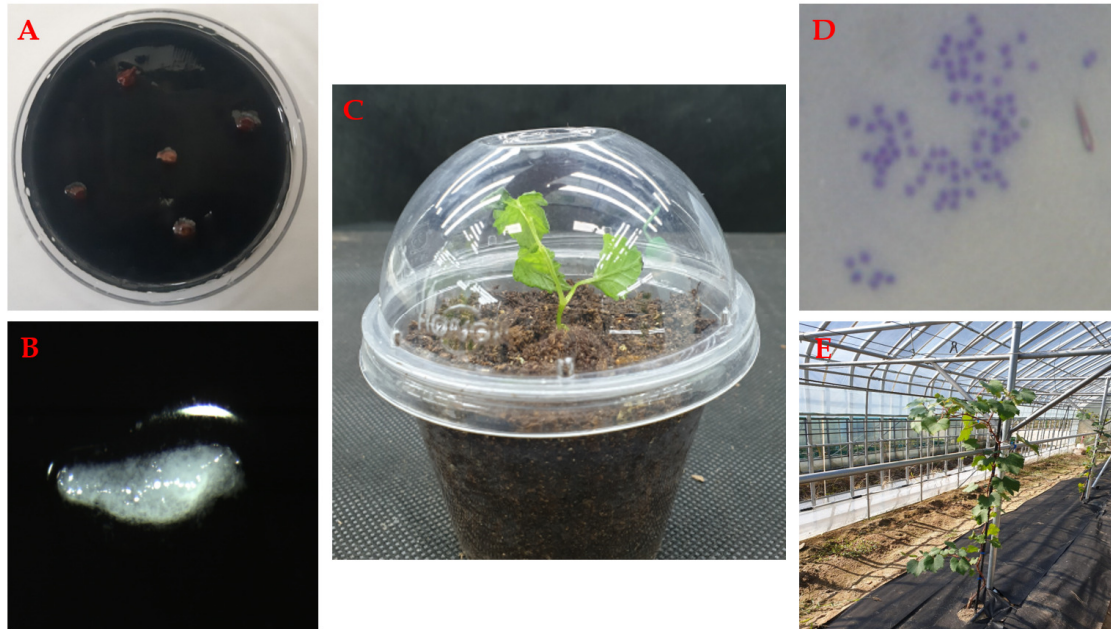


Figure 1. Embryo rescue procedure for production of hypotetraploid hybrid grapes. (A) ovules cultured in solid medium; (B) embryo cultured in solid medium; (C) whole plantlet developed from germinated embryos; (D) chromosome observation of hybrid seedling; (E) growth of seedlings identified as hypotetraploids in greenhouse

Results and Discussion

Efficiency of *in vitro* embryo rescue can be affected by several factors such as ovule removal age, basal medium, and genotype. Of these, removal age for ovule culture has been regarded as one of the most important factors. This is attributed to the fact that early culture, before the optimum ovule removal age, can result in imperfect development of the embryo. Similarly, extremely late culture, beyond the optimum ovule removal age, can cause abortion of the hybrid embryo or physiological dormancy (Guo *et al.*, 2011). It has been reported that the optimum removal age for ovule culture usually ranges from 6 to 10 WAP (Ji *et al.*, 2013). In this study, only a few embryos and plants were obtained from ovules cultured at 6 WAP, whereas a higher number of embryos and plants were obtained from ovules inoculated at 10 WAP, regardless of cross combination, indicating that relatively late ovule culture can be efficient for cross combination. It has been suggested that the optimum removal age for ovule culture mainly depends on the maturity stage of the female parent (Table 1) (Yang *et al.*, 2007). As 'HR' is a medium maturing grape cultivar, its 10 WAP approximately coincides with its version; thus, our result was consistent with previous results.

The selection of a proper basal medium is regarded as another critical factor for *in ovulo* embryo culture of grapes because it is essential in promotion of embryo formation and prevention of embryo abortion. We observed that embryo formation in ovules cultured at 6 WAP was not affected by basal media. However, utilization of specific medium can be beneficial for embryo formation based on a cross combination of ovules cultured at 10 WAP. NN and ER medium had the highest rate of embryo formation in 'HR × HB' and 'HR × KH', respectively. It appeared that the use of NN and ER media resulted in the enhancement of nutrient absorption in ovules generated from 'HR × HB' and 'HR × KH'. Similarly, research has already shown that the effect of basal medium on embryo formation varies among genotypes (Liu *et al.*, 2003; Sun *et al.*, 2011). These results indicate the importance of optimal basal medium selection in employing embryo rescue

techniques in the production of hypotetraploid grapes. After transferring to WP medium, embryos that originated from ER and NN media showed the highest number of established plants, but many of them failed to germinate or produce root. Therefore, further research on the effect of plant hormones on germination and root formation of embryos is needed to improve the efficiency of *in vitro* embryo rescue system for hypotetraploid production.

A total of 17 plants were established from ovules inoculated at 10 WAP in 'HR × HB' and 'HR × KH', and their chromosome numbers were observed to range from $2n=4x-2=72$ to $2n=4x=76$ (Table 2). In 'HR × HB', four plants were identified as tetraploids, and the other four plants were identified as hypotetraploids. Of the nine plants obtained from 'HR × KH', five and four plants were found to be hypotetraploids and tetraploids, respectively. As a result, the frequency of occurrence of hypotetraploids 'HR × HB' and 'HR × KH' was calculated as 50.0% and 55.6%, respectively.

Table 1. Effect of the time of ovule culture and basal medium on development of the embryos and *in vitro* growth of embryos excised after ovule culture

Ovule age (weeks)	Culture medium used	HR × HB		HR × KH	
		No. of embryos obtained (%)	No. of plants established (%)	No. of embryos obtained (%)	No. of plants established (%)
6	ER	7 (20.0) ^{ab}	1 (2.9) ^{ab}	7 (20.0) ^b	1 (2.9) ^b
	MS	6 (17.1) ^b	0 (0.0) ^b	7 (20.0) ^b	0 (0.0) ^b
	NN	6 (17.1) ^b	0 (0.0) ^b	8 (22.9) ^b	1 (2.9) ^b
10	ER	10 (28.6) ^{ab}	3 (8.6) ^{ab}	15 (42.9) ^a	5 (14.3) ^a
	MS	8 (22.9) ^{ab}	1 (2.9) ^{ab}	10 (28.6) ^{ab}	1 (2.9) ^b
	NN	11 (31.4) ^a	4 (11.4) ^a	12 (34.3) ^{ab}	3 (8.6) ^{ab}

Note: Means followed by the same letter within each column are not significantly different at P0.05.

Table 2. Chromosome number in plants obtained from ovules inoculated at 10 WAP

Cross combination	Culture medium used	No. of plants	Chromosome number				Frequency of hypotetraploids (%)
			$2n=4x-4=72$	$2n=4x-2=74$	$2n=4x-1=75$	$2n=4x=76$	
HR × HB	ER	3	1	-	1	1	66.7
	MS	1		-	-	1	0.0
	NN	4		1	1	2	50.0
HR × KH	ER	5		1	2	2	60.0
	MS	1		-	-	1	0.0
	NN	3		1	1	1	66.7

Previously, 52 mature seeds were obtained from diverse artificial crosses using 'HR' as a female parent (Park, 2005), but only two germinated seedlings, including one hypotetraploid seedling, were obtained. Apart from this fact, we observed that hypotetraploid seedlings obtained from *in vitro* embryo rescue showed stable vine growth. These results suggest that applying *in vitro* embryo rescue techniques based on a well-developed protocol after a cross between hypotetraploid and tetraploid grapes can improve the efficiency of production of hypotetraploid grape hybrids. Further effort to improve the protocol of *in vitro* embryo rescue using a greater number of genotypes for the production of hypotetraploids will facilitate the development of new seedless grape varieties with large berries.

Conclusions

In this study, we investigated the effects of ovule removal age and media type for *in vitro* embryo rescue after cross pollination between hypotetraploid and tetraploid grape cultivars on the efficiency of embryo rescue and establishment of hypotetraploids. As a result, we found that efficiency of *in vitro* embryo rescue was affected by ovule removal age, basal medium and cross combination, and specific condition for *in vitro* embryo rescue can be helpful for production of hypotetraploids.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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