

Production of triploid grapes by *in ovulo* embryo culture

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S u m m a r y : Triploid grapes were produced by *in ovulo* embryo culture. Ovules were obtained from 5 crosses: Rosario Bianco (*V. vinifera* L. 2x) x Kyoho (*V. vinifera* x *V. labrusca* L. 4x), Katta Kourgan (*V. vinifera* L. 2x) x Kyoho, Kyoho x Rosario Bianco, Kyoho x Sekirei (*V. vinifera* L. 2x) and Kyoho x Rizamat (*V. vinifera* L. 2x). Most embryos were obtained from ovules cultured at veraison. The addition of indole-3-acetic acid (2.0 mg·l⁻¹) and gibberellic acid (0.4 mg·l⁻¹) to 1/2 MS (half strength macro-elements) medium promoted the formation and growth of embryos. Although only a few embryos germinated directly from ruptured ovules, embryos excised from cultured ovules germinated successfully by *in vitro* culture. This paper demonstrates that *in ovulo* embryo culture is expected to be an efficient method to produce triploid grapes.

K e y w o r d s : tissue culture, *Vitis* spp., table grape, breeding, embryo rescue, seedlessness, triploid.

Introduction

Seedless grapes are preferred as table grapes by consumers around the world. Seedlessness of table grapes (*Vitis* spp.) is caused by either stenospermocarpny or parthenocarpny (STOUT 1936).

Breeders have developed seedless grapes by using stenospermocarpic grapes. Another strategy to breed seedless grapes is the production of triploid grapes which, because of their unbalanced chromosome sets, are highly sterile and seedless. However, breeding of triploid grapes is difficult because of the low success rate of hybridizations between tetraploid and diploid. This is because embryo abortion and/or endosperm breakdown often occur within 9 weeks after pollination. If these embryos were allowed to develop triploid progeny is expected to be obtained more efficiently (YAMASHITA *et al.* 1993).

In previous experiments (YAMASHITA *et al.* 1995) an embryo culture technique was successfully utilized to obtain triploid grape seedlings from crosses between tetraploid and diploid cultivars.

Techniques for *in ovulo* embryo culture have been reported for stenospermocarpic grape (CAIN *et al.* 1983; EMERSHAD and RAMMING 1984; SPIEGEL-ROY *et al.* 1985; GRAY *et al.* 1990), early ripening seeded grapes (RAMMING *et al.* 1990) and interspecific crosses (GOLDY *et al.* 1988).

Using these techniques, triploid grapes may be produced more efficiently. We report here a successful *in ovulo* embryo culture to produce triploid hybrids from crosses between tetraploid and diploid grapes.

Material and methods

Rosario Bianco (RB), Katta Kourgan (KK), Sekirei (SR) and Rizamat (RZ) were used as diploid *V. vinifera* L. varieties and Kyoho (KH), *V. vinifera* x *V. labrusca* L. as tetraploid

variety. All of them are cultivated in the grape cultivar collection of the Nagano Fruit Tree Experiment Station in Suzaka, Nagano, Japan.

***In vivo* observation:** Berries from 4x x 2x crossings (KH x RB, KH x SR, KH x RZ) were collected 60-70 d, those from 2x x 4x (KK x KH, RB x KH) 70-80 d after pollination. After these period immature embryos began to degenerate (YAMASHITA 1998). Ovules were excised and examined for embryo under a stereo-microscope. Seeds were obtained from berries at maturity; they were stratified at 4 °C for 180 d and then allowed to germinate in the greenhouse.

***In ovulo* embryo culture:** Berries were collected 50 d (KH x RB, KH x SR and KH x RZ), 60 d (KK x KH) and 65 d (RB x KH) after pollination. 20, 35, 50, 60 d after pollination (KK x KH) or 20, 35, 50 d after pollination (KH x RZ) the effects of treatments on the recovery of embryos were evaluated. Berries were surface-disinfected for 30 s in 70 % ethanol and for 15 min in 1.3 % sodium hypochlorite containing a drop of detergent, and rinsed three times in sterile deionized water. Ovules were aseptically excised and then cultured in 100 ml Erlenmeyer flasks containing 10 ml of liquid medium. The basal culture medium was a half strength macro-element medium (1/2 MS; MURASHIGE and SKOOG 1962). Ovules from all cross combinations were cultured on 1/2 MS + 2.0 mg·l⁻¹ IAA + 0.4 mg·l⁻¹ GA. The effect of auxins and gibberellin on embryo recovery was evaluated by culturing ovules of KH x RB with or without 2.0 mg·l⁻¹ IAA and 0.4 mg·l⁻¹ GA. All media contained 0.1 % (w/v) activated charcoal. The ovules were cultured statically under "daylight" fluorescent light (35-40 μmol·m⁻²·s⁻¹) with a 16-h photoperiod at 25 °C. Ovules were excised and embryos were observed after 100 d of culture. According to HORIUCHI *et al.* (1991) the developmental stage of each embryo was classified as mature, torpedo-shaped, heart-shaped and globular embryo.

Table 1

Embryo formation rate *in vivo* and germination of the seeds from various cross combinations

Cross combination	Number of ovules examined ¹⁾	Embryo formation (%)	Number of seeds sown	Germination (%)
2x x 4x				
Rosario Bianco x Kyoho	112	8.9	106	2.8
Katta Kourgan x Kyoho	107	5.6	105	1.9
4x x 2x				
Kyoho x Rosario Bianco	108	41.7	108	14.8
Kyoho x Sekirei	119	31.9	111	8.1
Kyoho x Rizamat	120	33.3	123	7.3

¹⁾Ovules were examined at 70-80 d (2x x 4x) or 60-70 d (4x x 2x) after pollination.

Embryos excised from cultured ovules did not germinate. Details of embryo culture and plant establishment are described in YAMASHITA *et al.* (1995). Root tips were sampled from shoots of RZ, KH, RB x KH, KK x KH, KH x RB, KH x SR and KH x RZ cultured on MS medium at 10 a.m. They were stored in water at 2 °C for 24 h and then fixed in ethanol (99 %): acetic acid (3:1 v:v) for 24 h. After hydrolysis in 1 N HCl for 1 min at 60 °C the root tips were stained with Feulgen for 8-9 h and squashed in 1-2 drops of acetocarmine (1 % carmine in 45 % acetic acid).

Results and Discussion

In vivo observation: Embryos were found in ovules of all cross combinations at low percentage (5.6-41.7 %). The germination frequency of seed obtained from all cross combinations was also low. When diploid grapes, KK or RB, were used as female parental plants both embryo generation and seed germination were lower as compared to the tetraploid cultivar KH (Tab. 1). A similar result was obtained by reciprocal crosses between Muscat of Alexandria 2x and Muscat of Alexandria 4x (YAMASHITA 1998).

In ovulo embryo culture: Embryos were white and situated at the micropyle end of the ovule (Fig. 1). In ovules of KK x KH cultured 20 and 35 d after pollination,

embryos were found at low percentages (4.6 and 2.0 %, respectively) and no mature or torpedo-shaped embryos were found. 50 and 60 d after pollination the rate of embryo formation increased significantly (9.2 % and 22.9 %, respectively; Tab. 2) and embryos at all developmental stages were recovered from the ovules.

20 d after pollination, no embryos were recovered from the ovule culture of KH x RZ. However, 35 and 50 d after pollination, embryo production rates increased dramatically (46.0 % and 69.2 %, respectively; Tab. 2).

These results suggest that *in ovulo* embryo culture at a later stage is superior to that at an earlier stage since most embryos were recovered from ovules cultured 60 d (KK x KH) or 50 d (KH x RZ). These periods approximately coincided with veraison. Similar results were obtained with regard to the recovery of embryos from *in ovulo* embryo culture of Orlando Seedless crossed with 4 pollen parents (GRAY *et al.* 1990).

Phytohormones affected embryo formation of KH x RB: with IAA and GA embryo formation was 78.8 % as compared to 61.7 % without and the rate of matured and torpedo-shaped embryos was 73.1 % with IAA and GA but 64.8 % without (Tab. 3).

In ovulo embryo culture greatly enhanced the development of hybrid embryos in all cross combinations. Embryo formation rate of RB x KH, KK x KH (2x x 4x), KH x RB, KH x SR and KH x RZ (4x x 2x) *in vivo* were all low (5.6-41.7 %; Tab. 1).

Table 2

Recovery of embryos from ovules cultured at various dates in Katta Kourgan (KK) x Kyoho (KH) and Kyoho (KH) x Rizamat (RZ)

Ovule culture date (days after pollination)	KK x KH		KH x RZ	
	Number of ovules cultured ¹⁾	Ovules with embryos (%)	Number of ovules cultured	Ovules with embryos (%)
20	65	4.6a ²⁾	50	0.0a
35	50	2.0a	50	46.0b
50	65	9.2b	65	69.2c
60	70	22.9c	—	—

¹⁾ Ovules were cultured on 1/2 MS + IAA (2.0 mg·l⁻¹) + GA (0.4 mg·l⁻¹) for 100 d.²⁾ Mean separation within the columns by LSD at P = 0.05.

On the other hand, using *in ovulo* embryo culture, they were increased about two or three times (Tab. 4). Only one embryo from ovules of RB x KH and three from those of KK x KH germinated directly from ruptured ovules (Fig. 2), while none of KH x RB, KH x SR and KH x RZ did (Tab. 4). However, when embryos were excised from cultured ovules and cultured *in vitro*, they began to germinate (Fig. 3).



Fig. 1: Zygotic embryo of Kyoho x Rizamat after 100 d of *in ovulo* embryo culture.



Fig. 2: Germination of embryo from ruptured ovule (Katta Kourgan x Kyoho).

Table 3

Effect of IAA (2.0 mg·l⁻¹) and GA (0.4 mg·l⁻¹) on recovery of embryos from ovules cultured in Kyoho x Rozario Bianco

Media	Number of ovules cultured ¹⁾	Ovule with embryos (%)	Efficiency of developmental embryos (%) ²⁾			
			Mature	Torpedo	Heart	Globular
1/2 MS	60	61.7	40.5	24.3	24.3	10.8
+ IAA + GA	66	78.8	48.1	25.0	13.5	13.5

¹⁾ Ovules at 50 d after pollination were cultured for 100 d on each medium.

²⁾ Percentage of embryos per total embryos.

Table 4

Results of *in ovulo* embryo culture in various cross combinations

Cross combination	Number of ovules examined ¹⁾	Ovules germinated	Embryo formation (%)	Embryo status ²⁾	Number of embryos cultured	Number of embryos germinated (%)	Number of established plants	Established plants per ovule (%)
2x x 4x								
Rosario Bianco x Kyoho	136	1	22.1	M I	26 4	69.2 0.0	17 0	12.5
Katta Kourgan x Kyoho	109	3	15.6	M I	8 9	75.0 0.0	8 0	7.3
4x x 2x								
Kyoho x Rosario Bianco	132	0	78.8	M I	80 24	75.0 16.7	56 0	42.4
Kyoho x Sekirei	121	0	74.4	M I	43 47	86.0 17.0	35 4	32.2
Kyoho x Rizamat	116	0	72.4	M I	64 20	62.5 20.0	39 2	35.3

¹⁾ Ovules at 50 d (4x x 2x), 60 d (Katta Kourgan x Kyoho) and 65 d (Rosario Bianco x Kyoho) after pollination were cultured on MS medium + IAA (2.0 mg·l⁻¹) + GA (0.4 mg·l⁻¹) for 100 d.

²⁾ M = mature and torpedo-shaped embryo, I = heart-shaped or globular embryo.

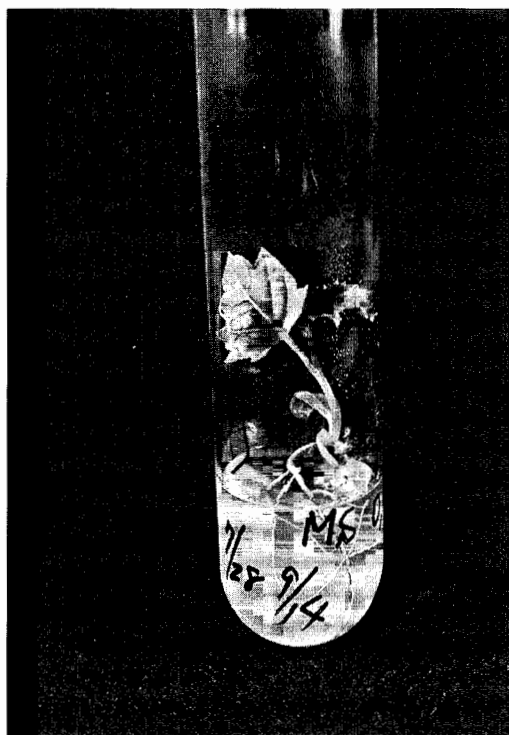


Fig. 3: Seedling from excised embryo of Kyoho x Rizamat after 100 d of *in ovulo* embryo culture.

and RZ, developed to plants at a higher percentage than those from heart-shaped and globular embryos. However, only a few plants were obtained from heart-shaped and globular embryos (Tab. 4).

We confirmed that hybrids had 57 chromosomes by counting 20-30 mitotic metaphase figures of the root-tip cells in each hybrid of RB x KH, KK x KH, KH x RB, KH x SR and KH x RZ (Fig. 4, C).

HORIUCHI *et al.* (1991) observed that grape zygotic embryos of cv. Kosyu initially develop slowly to a heart-shaped embryo stage with a little increase in diameter but rapidly from the heart- to the torpedo-shaped embryo stage with drastic increases in diameter. The latter period almost coincides with the period during which embryos of KK x KH and KH x RZ were successfully recovered by *in ovulo* embryo culture. This suggests that the growth of normal embryos and/or endosperms could be inhibited and embryo abortion and endosperm breakdown could occur during this period.

In ovulo embryo culture of stenospermocarpic grapes excision of embryos from ovules is recommended to obtain rooted plants efficiently (GINA *et al.* 1991). Accordingly, similar results were obtained in our experiment especially when a tetraploid was used as a female parent.

Grape embryos have a dormancy period (HORIUCHI *et al.* 1991) which has also been observed in stenospermocarpic embryos obtained from *in ovulo* embryo culture (GRAY *et al.* 1990).

In contrast, in our experiments most of the mature and torpedo-shaped embryos germinated successfully and dormancy was not clearly exhibited. However, some of the embryos kept white and failed to germinate, indicating possibly dormancy. The application of 6-benzyl adenine (BA) was

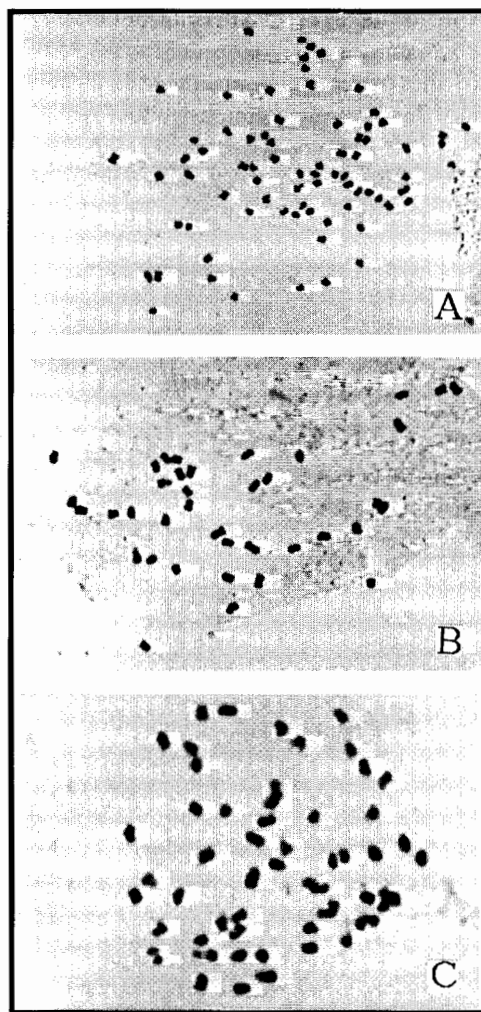


Fig. 4: Somatic metaphase chromosomes in root-tip cells of cuttings *in vitro*. A: Kyoho, B: Rizamat, C: Kyoho x Rizamat.

shown to be effective to stimulate embryo germination in *in ovulo* embryo culture of seedless bunch grapes (GRAY *et al.* 1990). The application of BA to *in ovulo* embryo culture of triploid grapes would be efficient to promote triploid embryo germination.

Obviously the germination frequency of triploid seed obtained from the crosses examined was very low. The yields of all hybrid combinations from *in ovulo* embryo culture could undoubtedly be improved compared with *in vivo*. Thus, our results suggest that *in ovulo* embryo culture is a useful strategy to increase the number of triploids from reciprocal crosses between tetraploid and diploid.

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References

- CAIN, D. W.; EMERSHAD, R. L.; TARAIOLO, R. E.; 1983: *In ovulo* embryo culture and seedling development of seeded and seedless grapes (*Vitis vinifera* L.). *Vitis* **22**, 9-14.

- EMERSHAD, R. L.; RAMMING, D. W.; 1984: *In ovulo* embryo culture of *Vitis vinifera* L. cv. Thompson Seedless. *Amer. J. Bot.* **71**, 873-877.
- GINA, E. F.; CLARK, J. R. MOORE, J. N.; 1991: Effect of seedcoat manipulation on the germination of stenospermocarpic grape embryos cultured *in ovulo*. *HortScience* **26**, 1220.
- GOLDY, R.; EMERSHAD, R. L.; RAMMING, D. W.; CHAPARORO, J.; 1988: Embryo culture as a means of introgressing seedlessness from *Vitis vinifera* to *Vitis rotundifolia*. *HortScience* **23**, 886-889.
- GRAY, D. J.; MORTENSEN, J. A.; BENTON, C. M.; DURHAM, R. E.; MOORE, G.A.; 1990: Ovule culture to obtain progeny from hybrid seedless bunch grapes. *J. Amer. Soc. Hort. Sci.* **115**, 1019-1024.
- HORIUCHI, S.; KUROOKA, H.; FURUTA, T.; 1991: Studies on the embryo dormancy in grapes. *J. Japan. Soc. Hort. Sci.* **60**, 1-7.
- MURASHIGE, T.; SKOOG, F. M.; 1962: A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **15**, 473-497.
- RAMMING, D. W.; EMERSHAD, R. L.; SPIEGEL-ROY, P.; SAHAR, N.; BARON, I.; 1990: Embryo culture of early ripening seeded grapes (*Vitis vinifera* L.) genotypes. *HortScience* **25**, 339-342.
- SPIEGEL-ROY, P.; BARON, I.; LAVI, U.; 1985: *In vitro* culture and plant formation from grape cultivars with abortive ovules and seeds. *J. Amer. Soc. Hort. Sci.* **110**, 109-112.
- STOUT, A. B.; 1936: Seedlessness in Grapes. *New York Agricult. Exp. Stat. Tech. Bull.* **238**, 1-68.
- YAMASHITA, H.; 1998: A study for breeding of triploid grapes. Ph. D. Thesis. Osaka Prefecture Univ., Sakai, Osaka, Japan.
- -; HANIUDA, T.; SIBA, H.; 1995: *In vitro* culture of embryos obtained by crossing tetraploid cultivar Kyoho with diploid cultivars. *J. Japan. Soc. Hort. Sci.* **63**, 719-724.
- -; HORIUCHI, S.; TAIRA, T.; 1993: Development of seeds and the growth of triploid seedlings obtained from reciprocal crosses between diploids. *J. Japan. Soc. Hort. Sci.* **63**, 249-255.

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