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Production of Triploid Plants from Crosses between Diploid and Tetraploid Grapes (*Vitis* complex) through Immature Seed Culture and Subsequent Embryo Culture

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To develop efficient embryo–rescue methods in breeding triploid grapes, a combination of immature seed culture and embryo culture, and embryo culture without the preceding culture of immature seeds were applied during different developmental stages of seeds from an interploid cross between tetraploid 'Red Pearl' and diploid 'Muscat Bailey A'. Embryos grew larger than 1mm in their length after three months of the immature seed culture initiated between 24 and 56 days after pollination (DAP). Overall, the embryos through subsequent culture germinated and then grew into plantlets at the higher rates than did those cultured without the preceding immature seeds culture. The highest rate of seedling recovery was 31.5% per cultured seed when immature seeds were collected 52–56 DAP, though none of four growth regulators added separately to the medium of the preceding immature seed culture were effective for development of embryos cultured in the same period. Results from our present and recent studies on breeding triploid grapes indicated that a combination of immature seed culture and subsequent embryo culture initiated one to two weeks before veraison (the beginning of fruit–softening period) is the best method for efficient production of triploid grapes.

INTRODUCTION

Production of seedless fruits with high quality is one of the major objective in breeding table grapes. Hypo– and hypertetraploid from hybridization among tetraploid cultivars (Park *et al.*, 1999b) and, especially, triploid from hybridization between diploid and tetraploid cultivars (Yamashita *et al.*, 1993; Park *et al.*, 2002; Wakana *et al.*, 2002) have been indicated to be the alternative sources for new types of seedless grapes in addition to the stenospermocarpic grapes (Winkler *et al.*, 1962; Einset and Pratt, 1975; Mullins *et al.*, 1992).

Generally, in interploid crosses between diploid and tetraploid plants, seed abortion due to the endosperm degeneration during early embryogenesis is a serious problem for production of triploid plants (Esen and Soost, 1973; Sanford, 1983). In grapes, the germi-

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nation rate of sown seeds from 2x×4x and 4x×2x crosses was 1.9% and 2.7%, respectively (Wakana, *et al.*, 2002), indicating seed sowing was not an efficient method for breeding triploid grapes. An another fact that the germination rate of triploid embryos cultured *in vitro* decreased with decreasing in their size (Wakana *et al.*, 2003) may suggest that *in vitro* culture at the earlier stages of seed development is necessary for improving efficiency in breeding triploid grapes.

In crosses with stenospermocarpic cultivars as seed parents, seedlings were successfully derived through *in ovulo* embryo culture with high frequencies (Ramming and Emershad, 1982: Cain *et al.*, 1983; Emershad and Ramming, 1984; Spiegel–Roy *et al.*, 1985; Goldy *et al.*, 1988; Emershad *et al.*, 1989; Gray *et al.*, 1990). Application of same embryo–rescue techniques was also successful in aneuploid production from triploid grapes (Park *et al.*, 1999a) and triploid production from hybridization between diploid and tetraploid grapes (Yamashita *et al.*, 1998). The cases have, however, been very few to date. Thus, further detailed studies on conditions of *in vitro* techniques should be needed to increase efficiency in breeding triploid and aneuploid grapes.

The purpose of the present study is to clarify 1) effectiveness a combination of immature seed culture and subsequent embryo culture in comparison with sole embryo culture without culturing immature seed, 2) an appropriate seed–development stage for initiation of *in vitro* culture, 3) effect of abscisic acid, adenine sulfate, gibberellin and indole–3–acetic acid on development of embryos and seedlings, using seeds from artificial interploid crosses between tetraploid and diploid grape.

MATERIALS AND METHODS

Plant materials and interploid cross

Diploid cultivar 'Muscat Bailey A' and tetraploid cultivar 'Red Pearl' were used for the interploid cross. They are intercontinental hybrid cultivars (*Vitis* complex) with *Vitis vinifera* and North American *Vitis* species in the pedigrees. Ten– to 15–year–old trees grown in the experimental orchard of University Farm, Kyushu University, Fukuoka were used in the cross. The interploid cross 4x×2x was carried out according to the procedure reported previously (Wakana *et al.*, 2002).

Embryo culture without the preceding immature seed culture

Immature berries set by the artificial hybridization were collected at seven days interval from 49 to 77 days after pollination (DAP). Mature berries collected 120 DAP were also used. The procedure of *in vitro* embryo culture followed that by Wakana *et al.* (2003).

A combination of immature seed culture and embryo culture

Immature berries set by the artificial hybridization were collected 6, 12, 24 and 48 DAP, and surface–sterilized for ten minutes in a solution of 1% NaClO containing one drop of Tween 20 per liter. After rinsing the immature berries twice in sterilized water, immature seeds were extracted from the berries with forceps and a surgical knife under aseptic conditions. After measuring seed length with a micrometer equipped in a stereoscopic microscope, each immature seed was cultured in a 25 mm×100 mm culture glass

vessel containing autoclaved 10 ml Nitsch and Nitsch (NN) medium (1969) supplemented with 20 g/l sucrose and 0.7 g/l agar, and adjusted pH to 5.5. The culture vessels were placed under continuous light condition (60–80 μmol m^{-2} s^{-1}, cool–white fluorescent lights) at 25±2 °C.

To examine independent effect of growth regulators on development of triploid embryos and seedlings, immature seeds collected 52–56 DAP were also cultured by the same procedure, except for supplement of the following regents. The growth regulators supplemented were 1–125 mg/l adenine sulfate (ADS), 0.01–1.25 mg/l, and indole–3–acetic acid (IAA), 0.01–1.25 mg/l abscisic acid (ABA) and 0.01–1.25 mg/l gibberellin (GA_3). IAA and GA_3 were added to the medium through filtration with sterilized cellulose acetate membrane after autoclaving.

After three months of immature seed culture, length of cultured immature seeds was measured. Embryos inside the seeds were extracted and length of them was measured. Then, the embryos were transplanted into a 200 ml conical flask containing autoclaved 40 ml of Murashige and Skoog (MS) medium (1962) supplemented with 30 g/l sucrose, 0.7 g/l agar, 500 mg/l casein hydrolysate and adjusted pH to 5.7. Five embryos were planted per flask. Every one of established plantlets was further transplanted into a conical flask containing autoclaved 40 ml MS medium supplemented with 10 g/l sucrose, 8 g/l agar, 0.01 mg/l NAA, 100 mg/l casein hydrolysate and adjusted pH to 5.7.

RESULTS

Embryo culture without the preceding immature seed culture

In the immature seeds collected 49 DAP embryos could not be cultured since they were too small to be extracted from the seeds. In berries collected 56, 63, 70, 77 and 120 DAP, 72–85% of seeds contained embryos (Table 1). Endosperm degeneration was observed in the micropylar half of these seeds. Mean length of the embryos increased from 56 to 77 DAP and the length at 77 DAP reached almost same as that at 120 DAP. Embryos cultured from 56 DAP germinated at the rate of 5.3%, but none of them grew

Table 1. Growth responses of triploid embryos from 'Red Pearl' (4x)×'Muscat Bailey A' (2x) in embryo culture. The embryos were extracted from seeds within immature and mature berries collected 49–120 days after pollination and cultured on Murashige and Skoog (1962) medium. Embryos were not observed at 49 days after pollination.

Days after pollination	No. of seeds examined	No. of seeds with the embryo (%)	Mean length of embryo at culture initiation (mm)	No. of embryos cultured	No. of embryos germinating (% per cultured embryo)	No. of seedlings established (% per examined seed)
56	49	40 (81.6)	0.51	38	2 (5.3)	0 (0)
63	34	27 (79.4)	0.72	26	3 (11.5)	2 (7.7)
70	20	17 (85.0)	0.83	16	4 (25.0)	1 (6.3)
77	31	25 (80.6)	0.98	25	4 (16.0)	2 (8.0)
120	47	34 (72.3)	0.93	34	2 (5.9)	2 (5.9)

into plantlets. Embryos cultured from 63, 70 and 77 DAP germinated at a relatively high rate (11.5–25.0%) as compared with those cultured from 120 DAP. The germinating embryos grew into plantlets also at a higher rate (5.0–6.5% per seed set) than those cultured from 120 DAP.

A combination of immature seed culture and embryo culture

Cultured immature seeds turned light green during immature seed culture except that those collected 48 DAP turned brown. In the greening seeds, callus was formed on the outer layers of outer integument. The immature seeds collected 24 DAP were relatively large and grew over the size of mature seeds collected 120 DAP after three months of the culture, whereas those collected 6 and 12 DAP were very small and slightly enlarged after the culture (Table 2). The size of immature seeds collected 48 DAP was nearly same as that of mature seeds collected 120 DAP and slightly enlarged after the culture. None of cultured immature seeds germinated during the culture.

Neither the endosperm nor the embryo was detected in the immature seeds cultured from 6 and 12 DAP (Table 3). For the seeds cultured from 24 and 48 DAP, embryos developed in 45.2% and 73.1%, respectively. Endosperm development was observed only

Table 2. The *in vitro* growth of immature seeds extracted from berries at various intervals after pollination in 'Red Pearl' (4x)×'Muscat Bailey A' (2x).

Days after pollination	No. of seeds examined	Mean length of immature seeds (mm)	
		At the initiation of culture	After three months culture
6	25	0.94	1.10
12	20	1.90	1.62
24	31	5.17	6.85
48	26	6.04	6.22
120	47	6.08	–[z]

[z] Mature seeds collected 120 days after pollination were not cultured.

Table 3. Growth response of triploid embryos through immature seed culture and subsequent embryo culture in 'Red Pearl' (4x)×'Muscat Bailey A' (2x). The seeds were extracted from immature berries collected 6–48 days after pollination and cultured for three months on Nitsch and Nitsch (1969) medium.

Days after pollination	Immature seed culture		Subsequent embryo culture			No. of seedlings obtained (% per cultured seed)
	No. of immature seeds cultured	No. of immature seeds with embryo	Mean length of embryo (mm)	No. of embryos cultured	No. of embryos germinating (% per cultured embryos)	
6	25	0 (0)	–	–	– (–)	0 (0)
12	20	0 (0)	–	–	– (–)	0 (0)
24	31	14 (45.2)	1.03	14	3 (21.4)	2 (6.4)
48	26	19 (73.1)	1.24	18	5 (26.4)	4 (15.4)

in the latter treatment, though the endosperm development was imperfect and observed only in the micropylar half of the embryo sac as was in the immature seeds beyond 56 DAP. The reached sizes of embryos after culturing immature seeds collected 24 and 48 DAP were larger than that of embryos within mature seeds collected 120 DAP (Table 1 and 3).

After subsequent culture of the embryos developed by immature seed culture, the rate of germination and seedling production both were higher in embryos cultured from 48 DAP than in those from 24 DAP (Table 3). The highest rate of seedling production (15.4%) in the successive culture was about 2.4 to 3.6 times as high as the rates in sole embryo culture without the preceding immature seed culture (Table 1 and 3).

Effect of growth regulators

Addition of four growth regulators on the medium did not show distinct effect on embryo and seedling development in the successive culture (Table 4). It was noticeable that the production rate of seedlings reached 31.5% without any growth regulators.

Table 4. The effect of growth regulators on growth of triploid embryos in immature seed culture and subsequent embryo culture. The immature seeds were extracted from young berries collected 52–56 days after pollination in 'Red Pearl' (4x)×'Muscat Bailey A' (2x) and cultured for three months on Nitsch and Nitsch (1969) medium.

Growth regulator	Concentration (mg/l)	Immature seed culture		Subsequent embryo culture			No. of seedlings established (% per cultured seed)
		No. of immature seeds cultured	No. of immature seeds with embryo	Mean length of embryo (mm)	No. of embryos cultured	No. of embryos germinating	
Control	0	54	48	1.15	46	23	17 (31.5)
ADS	1	27	21	1.21	19	8	7 (26.0)
	5	27	24	1.16	23	11	6 (23.1)
	25	27	23	1.17	17	5	3 (11.1)
	125	27	22	1.27	22	11	6 (22.2)
IAA	0.01	26	23	1.12	23	10	7 (27.0)
	0.05	26	22	1.49	21	11	6 (23.1)
	0.25	26	21	1.07	21	11	5 (19.2)
	1.25	27	24	1.34	24	9	5 (19.2)
ABA	0.01	27	25	1.33	24	9	6 (22.2)
	0.05	27	25	1.26	22	6	2 (7.4)
	0.25	27	27	1.03	23	11	5 (18.5)
	1.25	27	23	1.25	22	4	2 (22.2)
GA	0.01	28	25	1.26	23	9	6 (21.4)
	0.05	27	23	1.10	19	9	5 (18.5)
	0.25	26	23	1.30	22	9	6 (23.1)
	1.25	27	25	1.41	24	9	7 (26.0)

DISCUSSION

Comparison of a series of our studies on triploid production from 4x 'Red Pearl'×2x 'Muscat Bailey A' by *in vitro* techniques and sowing seeds shows that a combination of immature seed culture and subsequent embryo culture is the most efficient method (Table 5). Combined with results from similar study using triploid grapes by Yamashita *et al.* (1998) and aneuploid grapes by Parks *et al.* (1999a), our results show that the combination culture of immature seeds and embryos extracted from them could be widely applicable to most interploid crosses using diploid, tetraploid and triploid grapes.

The fact that immature seeds collected 48 to 56 DAP were superior in seedling recovery to those collected before 24 DAP indicates that the effective period initiating the immature seed culture is one or two weeks before the beginning of fruit–softening period, namely 'varaison'. In the very early stage of seed development, the tissues inside the seeds such as nucellus and endosperm are supposed to lack the ability of nutritional supply to the immature embryos because of the high degree of their underdevelopment or degeneration.

The sole *in vitro* culture of embryos extracted from immature seeds collected 63–77 DAP showed rather higher recovery rates than that extracted from mature seeds collected 120 DAP. The fact indicates that embryos of immature seeds beyond the veraison stage remained more viable than those of mature seeds. In many cases of the culture, however, embryos smaller than 1mm ceased the growth and could not germinate (see also Wakana *et al.*, 2003). The more immature embryos cultured from 56 DAP were, furthermore, very difficult to grow. These results demonstrate that many of the underdeveloped triploid embryos themselves are supposed to be highly heterotrophic, and impossible to grow further only by supplying artificial nutrients. It is, thus, stressed again that co–cultured nuceller and endosperm tissues are very critical for promoting growth of immature triploid embryos as sources of effective nutritional supply.

The reason why the four growth regulators used in the present study were not effective for the development of immature seeds and the embryos within them is obscure. Addition of these growth regulators on culture media have been reported to be effective for the recovery of hybrid seedlings from crosses with stenospermocarpic grapes through

Table 5. Compariosn of the rates of seedling recovery by *in vitro* techniques and seed sowing in 4x 'Red Pearl'×2x 'Muscat Bailey A' among our recent and the present studies.

Method	Stage of berry development	The optimum time for seed collection (days after pollination)	% of seedlings established per setting or cultured seed
Sowing seed[z]	Mature	>90	0
Embryo culture	Mature	>90	5
Embryo culture	Veraison	60–80	7
Immature seed culture[y]	Before veraison	50–60	32

[z]Wakana *et al.*, 2002; [y]Wakana *et al.*, 2003.

in ovulo embryo culture (Spiegel–Roy *et al.*, 1985; Gray *et al.*, 1990; Tsolova, 1990). The difference in the effect of the growth regulators among the studies might be attributed to the genetic and physiological difference in functions of nutritional supply of the under-developed endosperm and nucellus.

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