Obtaining Triploid Muscat Grapes by *in vitro* Culture of Ovules and Embryos After Crossing Between Diploid and Tetraploid Cultivars

Goro OKAMOTO, Ken HIRANO, Naomi TANAKAMARU and Naoki OMORI

(Department of Eco-physiology for Crop Science)

Received July 1, 1993

Summary

To breed new seedless grape cultivars we crossed 'Muscat of Alexandria' (2x) and its 4x mutant reciprocally and cultured the immature ovules and embryos *in vitro*. Ovules sampled 50 days after crossing could develop embryos on a liquid medium of half strength of MS supplemented with 2 ppm of IAA, 0.4 ppm of GA_3 and 0.1 % of activated charcoal. After 55 days culture, normally developed embryos were obtained from 20 to 30 % of the ovules from 4x×2x and 50 to 70 % of those from 2x×4x. Embryos taken out from the cultured ovules were planted on an agar medium of MS or N&N for 55 to 65 days. Ten to twenty % of embryos from 2x×4x crossing grew to rooted plantlets successfully. They were proved to be triploid after the measurement of chromosome number. Embryos from 4x×2x, however, could not grow to plantlets because of the failure of rooting.

Introduction

Seedless grape demand by consumers is increasing in all parts of the world, which has turned the development of seedless varieties into an important activity[1,2,3,11,12]. We also made reciprocal crosses between 'Muscat of Alexandria' (2x) and its 4x mutant 'Cannon Hall Muscat', with the objective of creating a new seedless cultivar which possesses the similar vigour and the same high qualities as 'Muscat of Alexandria'[10]. Germination tests were done on the seeds obtained from these crosses and the results achieved were that very few seeds from 2x×4x had good germinability while those from 4x×2x had lost their germinability and no seedlings were obtained. Due to these germination problems, ovules and embryos were cultured *in vitro* after hybridization to obtain triploid plants in an efficient way. This paper reports the results of experiments done to determine suitable conditions for the *in vitro* culture process.

Materials and Methods

Florets of 11-year-old 'Muscat of Alexandria' vines grown in a non-heated greenhouse at Okayama Univ. (Tsushima, Okayama city) and 6-year-old 'Cannon Hall Muscat' vines grown in a sideless plastic house in HANAZAWA Inst. for Grape Breeding (Seto-cho, Akaiwa-gun) were used as diploid and tetraploid parents.

In March, 1991, four to ten clusters were selected from both varieties appoximately two to three days before blooming based on past experience. Florets born in the basal part of the clusters were emasculated and then covered with white paper bags. After two to three days mutual crosses were conducted. Clusters used as a control were not emusculated and self-pollinations were allowed to occur. Berries sampled at 30 and 50 days after crossing were surface sterilized in a 1.5 % sodium hypochloride solution for 10 minutes, then washed with distilled water. Ovules were taken out from them under aseptic condition and placed in groups of 10 in 100 ml-Mayer flasks containing 10 ml of MS medium[5] at half strength supplemented with 3 % of sucrose, 0.1 % of activated charcoal, 2 ppm of IAA and 0.4 ppm of GA_3 and adjusted to pH 5.5 with 0.1N-NaOH. The same formula was supplemented with 0.8 % of agar when used as a solid medium. They were cultured at 25 °C under illumination of 3 klux for 16 hours of daylength.

After 55 and 65 days of culturing the ovules were dissected and the embryo was removed using a binocular. Groups of 3 embryos were placed in Mayer flasks containing 10 ml of MS or N&N[6] medium added with 5 ppm of BA, 3 % of sucrose and 0.8 % of agar. Embryo culture was made under the same conditions as that for ovule culture. After germination the embryos were transplanted to the media containing no BA for rooting. The polyploidy state of the resultant plants was accertained by counting the cromosome number of the cells of root tips by enzymatic maceration method shown by Zhuang et al.[15].

Results

1. Ovule culture

Ovules cultured in agar media hardly grew at all, though they initiated callus formation on the outer integument.No callus formation, however, was observed in ovules when cultured in liquid media. Cultured of ovules from berries sampled 30 days after crossing retained a light-green color. In most of them the interior parts became hollow or turned into a jellified state. Ovules cultured from berries sampled 50 days after crossing became brown and hardened with puffy endosperm soon after culture started. This observation was most remarkable in 2x×4x crosses. Hardly any embryos were obtained in ovules that were cultured 30 days after crossing. In ovules cultured 50 days after crossing the percentage of embryos obtained was about 50 to 70 % (Table 1). In 4x×2x crosses the percentage was about 10 to 20 % and 20 to 30 % for ovules cultured at 30 and 50 days after crossing, respectively. The effect of added IAA was clear both in 2x×4x and 4x×2x combinations (Table 2).

2. Embryo culture

The basal media used for embryo culture were MS and N&N. Between mature embryos there was no difference in rooting and germination (Table 3). However, in embryos taken from 2x×4x ovules cultured over 65 days, rooting was not observed on both media used.

3. Polyploidy of resultant plants

Analysis of cells from the root apex of plants obtained by culture of 2x×4x ovules and embryos (Fig. 1) revealed that they all seem to be 3x (Fig. 2).

Table 1 Effect of the time and duration of ovule culture on development of the embryos[a)]

Cross Combination	Ovule age (Days[b)])	Duration of culture (Days)	No. of ovules planted	No. of embryos developed	% of embryos obtained
4x×2x	30	55	30	7	23.3
		65	30	3	10.0
	50	55	25	6	24.0
		65	25	8	32.0
2x×4x	30	55	30	1	3.3
		65	30	0	0.0
	50	55	35	19	54.3
		65	40	29	72.5

a) Cultured on 1/2MS basal medium supplemented with 2ppm of IAA and 0.4 ppm of GA_3.
b) Days after pollination.

Table 2 Effect of phytohormones added to MS medium on embryo development during ovule culture[a)]

Cross Combination	Phyto-hormones added[b)]	No. of ovules planted	No. of embryos developed	% of embryos obtained
2x×4x	IAA+GA	40	11	27.5
	—	40	4	10.0
2x×4x	IAA+GA	20	5	25.0
	—	20	3	15.0

a) After 55 days culture of ovules sampled 50 days after pollination.
b) IAA ; 2 ppm, GA_3 ; 0.4 ppm.

Table 3 Effect of basal medium on *in vitro* growth of embryos excised after ovule culture

Cross combination	Duration of ovule culture[a)]	Culture medium used[b)]	No. of embryos		
			planted	shooted	rooted
4x×2x	55	MS	3	1	0
		N&N	3	2	0
	65	MS	8	4	0
2x×4x	55	MS	12	4	0
		N&N	10	3	0
	65	MS	19	19	4
		N&N	10	10	1

a) Ovules sampled 50 days after pollination were cultured on 1/2MS medium supplemented with 2 ppm of IAA and 0.4 ppm of GA_3.
b) Supplemented with 5 ppm of BA.

Disscussion

In recent years, ovule and embryo culture techniques have been practised in many countries of the world with the objective of successful grape breeding. Emershad *et al.*[1)]

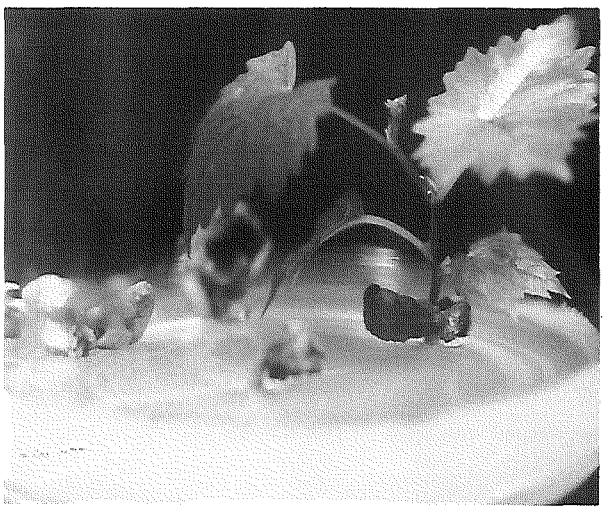

Fig. 1 Triploid Muscat plantlet obtained from *in vitro* embryo culture. Embryos were taken from ovules developed after 2x×4x crossing.

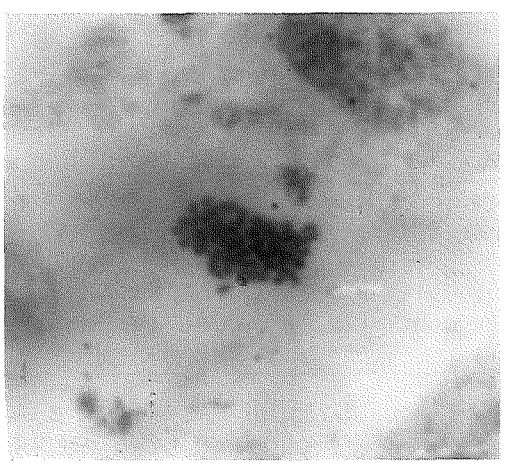

Fig. 2 Chromosomes in root tip cells developed from 2x×4x embryos.

reported that, in embryos obtained from immature fertilized ovules of 'Thompson Seedless' grapes and cultured on liquid medium, germination was unsatisfactory. Spiegel-Roy[10] reported that germination was observed in embryos inside fertilized ovules when cultured in agar medium. Other reports[2] state that embryo germination is better in agar medium than in liquid. Reports on callus formation are few. It is not yet known if callus formation can be related to embryo development hindrance inside the ovule. Horiuchi[4] and Yamashita[13,14] reported success in culture of seedless grapes and 4x×2x hybrid ovules using liquid medium.

The experiment in question was conducted based in other reports[3,7,11,12,14], as well as on the efficiency of the activated charcoal used in ovule culture and phytohormones in the embryo culture. Rates of added activated charcoal and phytohormones and also planting times of ovules and embryos were similar to earlier reports above mentioned. However, contrary to findings of Yamashita[13] embryo development was better when 2x were female plants and 4x were male plants. It is not clear whether this was due to differences in culture conditions. It is known that the 4x mutant 'Cannon Hall Muscat' shows many ovules with morphological abnormalities and imperfect growth at blooming stage[9], and this can be associated with difficulties in normal development. Experiment conducted by Okamoto *et al*[10] aimed to obtain seedlings by seed from reciprocal crosses of 2x and 4x plants showed that, with the combination 4x×2x the embryos developed only until the globular stage. The seeds obtained had no germination ability.

In the present experiment, culture of ovules comming from the 4x×2x combination provided 20 to 30 % of mature embryos, but their rooting was extremely difficult. Further results on media with appropriate formulas and combinations of phytohormones is needed. It seems possible to culture embryos taken off directly from ovules at 20 to 40

days after fertilization. Further research should pursue this line of embryo culture.

References

1) Emershad, R. L. and D. W. Ramming : In-ovulo embryo culture of *Vitis vinifera* L. cv. 'Thompson Seedless'. Amer. J. Bot. **71**, 873—877 (1984)
2) Gray , D. J., L. C. Fisher nd J. A. Mortensen : Comparison of methodologies for *in ovulo* embryo rescue of seedless grapes. HortScience **22**, 1334—1335 (1987)
3) Gray, D. J., J. A. Mortensen, C. M. Benton, R. E. Durham and G. M. Moore : Ovule culture to obtain progeny from hybrid seedless bunch grapes. J. Amer. Soc. Hort. Sci. **115**, 1019—1024 (1990)
4) Horiuchi, S., J. W. Wang and R. Mochioka : Studies on seedling development of seedless grapes through in-ovulo embryo culture. J. Japan. Soc. Hort. Sci. **59 (Suppl. 1)**, 58—59 (1990). (In Japanese)
5) Murashige, T. and F. M. Skoog : A revised medium for rapid growth and bioassays with tabacco tissue cultures. Physiol. Plant. **15**, 473—493 (1962)
6) Nitsch, J. P. and C. Nitsch : Haploid plants from pollen grains. Science **163**, 85—87 (1969)
7) Nozuka, K., N. Hirakawa and T. Sumi : Efficient breeding of new seedless grape cultivars by ovule culture. Abstr. Japan. Soc. Hort. Sci. Spring Meet. 64—65 (1988). (In Japanese)
8) Nozuka, K., N. Hirakawa and T. Sumi : Efficiency of seedless grapevine breeding by ovule culture. 2. Effect of varietal differences and seasonal change on embryo development. J. Japan. Soc. Hort. Sci. **59 (Suppl. 1)**, 56—57 (1990). (In Japanese)
9) Okamoto, G., K. Yamamoto and K. Shimamura : A study on the difference in the occurence of seedless berries among the tetraploid grapes related to 'Kyoho' variety. J. Japan. Soc. Hort. Sci. **53**, 251—258 (1984). (In Japanese with English summary)
10) Okamoto, G., K. Hirano, N. Tanakamaru and N. Omori : Seed devolopment and seedling growth after reciprocal crossing between diploid and tetraploid Muscat grapes. Sci. Rep. Fac. Agri. Okayama Univ. **80**, 80—91 (1992) (In Japanese with English summary)
11) Spiegel-Roy, P., N. Sahar, J. Baron and U. Lavi : In vitro culture and plant formation from grape cultivars with abortive ovules and seeds. J. Amer. Soc. Hort. Sci. **110**, 109—112 (1985)
12) Tsolova, V. : Obtaining plants from crosses of seedless grapevine varieties by means of in vitro embryo culture. Vitis **29**, 1—4 (1990)
13) Yamashita, H., S. Horiuchi and S. Nakagawa : A study on tetraploid grape breeding. Abstr. Japan. Soc. Hort. Sci. Spring Meet. 62—63 (1984) (In Japanese)
14) Yamashita, H., T. Haniuda and H. Shiba : Studies on embryo culture for breeding of triploid grapes. J. Japan. Soc. Hort. Sci. **59 (Suppl. 2)**, 184—185 (1990) (In Japanese)
15) Zhuang, D. H., A. Kitazima, M. Ishida and Y. Sobajima : Chromosome numbers of *Diospyros kaki* cultivars. J. Japan. Soc. Hort. Sci. **59**, 289—297 (1990). (In Japanese with English summary)

胚珠培養，胚培養による3倍体マスカット個体の育成

岡本五郎・平野　健・田中丸直美・大森直樹
（作物機能調節学講座）

3倍体のマスカット系ブドウ品種を育成するために，'マスカット・オブ・アレキサンドリア'（2倍体）と同品種の4倍体変異を正逆交雑し，その胚珠及び胚を *in vitro* で培養した．交配50日後の胚珠を IAA2ppm 及び GA_3 0.4ppm を含む1/2 MS 液体培地（活性炭0.1%，ショ糖3%加用）で55日間胚珠培養すると，4x×2xでは約20～30%，2x×4xでは50～70%の胚珠から胚が得られた．胚培養は MS または N&N の基本培地に BA5ppm を添加したカンテン培地を用い，55日または65日間行った．成熟胚からの発芽はいずれの組み合わせでも容易であったが，発根は2x×4xの65日間培養でのみ見られ，その個体は根端細胞の染色体数から3倍体であることが認められた．4x×2x の胚からは植物体は得られなかった．