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Production of Haploid Plants of Melon by Pseudofertilized Ovule Culture

Norio KATOH, Manabu HAGIMORI and Sumio IWAI

Japan Tobacco Inc. Applied Plant Laboratory 1900 Idei, Oyama, Tochigi, 323 Japan

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Haploid melons were obtained from 12 cultivars when ovules pollinated with irradiated pollen were cultured on either MS medium without plant growth substances or E20A medium with 0. 01 mg/*l* IAA. All the plants obtained were haploid. The chromosome doubling occurred only in the root tips of haploid plants, not in other organs.

Introduction

The production of haploids has been tried in quite a few species since the late 1920's by pollinating the target plant with the pollen of distantly related species or inactivated pollen[1,2]. However, these efforts yielded no efficient and reliable method usable in haploid breeding until Guha and Maheshwari[3] succeeded with an anther culture of *Datura*. Since then, haploids have been obtained efficiently in many species by anther culture. However, in some species such as onion, sugar beet and melon, it is quite difficult to obtain haploids by anther culture. In these species, ovules are considered to be possible alternative as the material for haploid production. And haploids have, in fact, been obtained from ovules in onion[4,5] and sugar beet[6].

In melon, there is only one report of dihaploid production by anther culture[7]. However, the technique is too inefficient for use in haploid breeding. Using ovules, Fujishita *et al.*[8] reported production of plants by unpollinated ovule culture. However, they did not observe haploid plants. In contrast, Sauton and Dumas[9,10] succeeded in producing haploid plants in several cultivars of melon. They cultured ovules fertilized with pollen irradiated with 30 krad of gamma-rays from Co^{60}.

In our preliminary experiment, unpollinated ovule culture was applied to induce haploid or dihaploid plants of melon but was unsuccessful. Hence, we investigated haploid production from the ovules of melon by pseudofertilized ovule culture to establish a method that is more accessible and applicable to a wider range of cultivars.

Materials and Methods

1. *Plant materials*

The plant mainly used was *Cucumis melo* cv. Andes (Sakata Seed Crop. Yokohama, Japan). To investigate differences in response among cultivars, the following 11 cultivars of *C. melo* were also tested: "Earl's Favorite" (Watanabe Seed Co., Ltd. Miyagi, Japan), "F1 Hamiuri" (Kaneko Seed Co., Ltd. Gunma, Japan), "Alice" (Takii & Co., Ltd. Kyoto, Japan), "Bonus No. 2" (Takii), "Cossack" (Sakata), "Honeydew PF" (Sakata), "Elizabeth" (Mikado Seed Co., Ltd.) "Sweet heart" (Suttons Seeds Ltd. Torguay, England), "Hamiuri", "Mauri" and F1 between "Mauri" and "Bird

nest". The seeds of "Mauri" and "Bird Nest" were obtained from The National Institute of Agrobiological Resources (Tsukuba, Japan). F1 plant between "Mauri" and "Bird nest" was heterozygous at two marker gene loci. One marker is a gene "*dwarf*" that is recessive to the dominant gene "*normal*" and causes short internodes when it exists in a homozygous state. The other marker is a gene "*monoecy*" which is dominant to a recessive gene "*andromonoecy*". All the plants were grown in a greenhouse.

2. *Pollination with irradiated pollen*

Flowers were emasculated one day before anthesis and bagged. *C. melo* cv. Andes was mainly used as the pollen donor. Fresh male flowers were collected at 9-10 o'clock in the morning, the petals were removed, and the flowers were placed upright on wet cotton in a Petri dish. Then the Petri dish was transferred to an X-ray unit (OHMIC OM-100R) and irradiated with 100kR of X-rays (1252 R/min). Flowers were pollinated with the irradiated pollen and re-bagged. The pollen obtained from 3 male flowers was applied to one stigma.

3. *Ovule culture*

Ovaries were collected 3 weeks after pollination. They were surface sterilized by submersion in 70% ethanol for 15 min, and cut into two pieces. Ovules were picked out of the ovaries and placed on E20A[9] or MS[11] medium supplemented with 2% sucrose and 0.8% agar. The pH was adjusted to 5.8 with 0.1 N KOH before autoclaving. Cultures were kept at 24°C under a 12h-light/12h-dark cycle (White fluorescent lamp, 6 $W \cdot m^{-2}$) for 8 weeks.

The plantlets obtained were transferred to fresh MS medium and cultured for 4 weeks. Then, the plants were potted using vermiculite as the substrate, acclimatized in a growth chamber (25°C, 12h-light/12h-dark cycle) for 2 weeks, and finally transplanted to soil and kept in a greenhouse.

4. *Ploidy level*

The number of chromosomes in the regenerated plants was counted using root tip cells and pollen mother cells by the standard method of aceto-orcein staining. Also, the ploidy level was estimated by counting the number of chloroplasts in guard cells. The relative DNA content of the nuclei of leaf mesophyll cells was analyzed by flow cytometry (Coulter EPICS 753). To isolate nuclei, non-fixed leaves were submerged in a buffer (10mM Tris-HCl, 100mM NaCl, 10mM EDTA, 1% Triton) and chopped up with a sharp razor blade in a Petri dish. The nuclei were stained with DAPI up to a final concentration of 1 ug/m*l*.

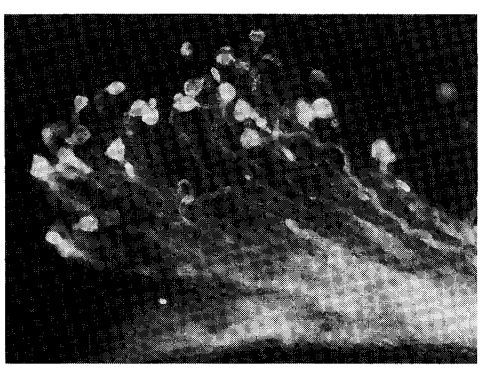

Fig. 1 Germination of irradiated pollen on a stigma of melon.

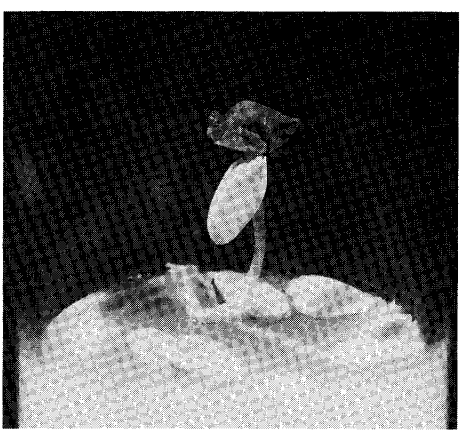

Fig. 2 Plantlet regenerated from cultured ovule.

Results and Discussion

1. *Pseudofertilized ovule culture*

After pollination, the irradiated pollen germinated, pollen tubes elongated into styles (**Fig. 1**), and then ovaries began to swell. After 2 or 3 weeks of culture, some ovules germinated and plantlets were obtained from them (**Fig. 2**). Most of the plantlets grew from ovules via embryogenesis. After germination, shooting and rooting were observed simultaneously on the initial medium. These plantlets matured and flowered in a greenhouse while malformed plantlets which ceased growing barely observed. All of the flowers of the regenerated plants were smaller than those of diploid plants and none had fertile pollen (**Fig. 3**). Regenerated plants originating from ovules of the F 1 plant "Mauri" × "Bird nest" exhibited segregation of the marker genes. Among 13 plants, the internode length phenotype segregated into 11 (normal): 2 (dwarf). And that of floral characteristics segregated into 6 (andromonoecy): 5 (monoecy). These results indicate that the regenerated plants had only one set of the genomes of the mother plants.

In this work, pollen was inactivated by 100 kR of X-rays and it was found that the irradiated pollen germinated and the pollen tubes penetrated the ovules. However, no zygotic embryo developed after pollination. This result indicates that the dose of 100 kR was sufficient to inactivate pollen for pseudofertilization.

In all the reports that have appeared so far on haploid production by the culture of ovules pollinated with inactivated pollen, pollen was inactivated by gamma-ray irradiation from a Cobalt 60 source[9,12-14]. However, gamma-ray irradiation needs special facilities which meet high regulatory standards. In contrast, X-ray irradiation is far more accessible. Therefore, X-ray irradiation was

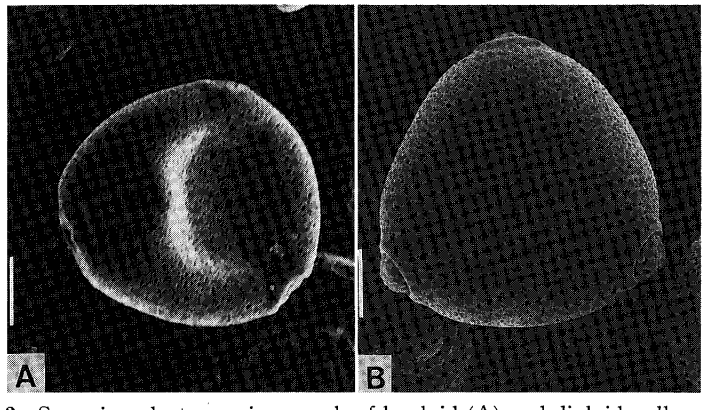

Fig. 3 Scanning electron micrograph of haploid (A) and diploid pollen (B).

Table 1. Effect of mineral salts on induction of regenerated plants from ovules.

Medium	No. of ovules cultured	No. of regenerated plantlets	Efficiency (%)
E20A	684	5	0.73[a]
N6	708	3	0.44[a]
MS	1200	16	1.33[a]

Data followed by the same letter are not significantly different by χ^2-test ($p < 0.05$).

Table 2. Effect of IAA on plantlet induction from ovules.

Exp.	IAA (mg/*l*)	No. of ovules cultured	No. of regenerated plantlets	Efficiency (%)
I	0	972	21	2.16[a]
	0.01	1056	22	2.08[ab]
II	0.01	1248	11	0.88[b]
	0.02	1404	19	1.35[ab]
	0.04	1344	14	1.04[b]
	0.08	1198	10	0.83[b]

Data followed by different letters are significantly different by the χ^2-test ($p<0.05$). Periods of experiment I and II were August to September and May to June, respectively.

Table 3. Embryogenesis in pseudofertilized ovule culture of 11 cultivars of *Cucumis melo* L.

Cultivar	Group	Ovary no.	No. of ovules cultured	No. of plantlets regenerated	Efficiency (%)
E. favorite	reticulatus	1	405	4	1.0
		2	345	2	0.6
		3	390	1	0.3
Hamiuri	inodorus	1	400	8	2.0
		2	360	6	1.7
		3	464	2	0.4
F 1 Hamiuri	〃	1	264	5	1.9
		2	500	3	0.6
Honeydew PF	〃	1	285	3	1.1
		2	480	1	0.2
Mauri	conomon	1	325	3	0.9
		2	264	2	0.8
		3	280	1	0.4
Elizabeth	intervarietal	1	460	2	0.4
	hybrid	2	420	1	0.2
	(no net type)	3	560	0	0
Sweet heart	〃	1	400	10	2.5
		2	264	5	1.9
		3	500	3	0.6
Alice	〃	1	375	7	1.9
		2	404	2	0.5
		3	600	1	0.2
Cossack	intervarietal	1	444	15	3.4
	hybrid	2	156	2	1.3
	(net type)	3	525	3	0.6
Bonus No. 2	〃	1	342	2	0.6
		2	378	0	0
		3	375	0	0
Mauri	intervarietal	1	568	9	1.6
× Bird nest	hybrid	2	320	2	0.5
		3	378	2	0.5

Ovules of these cultivars were obtained on E20A medium with 0.01 mg/*l* IAA.

used to inactivate pollen in this work, and proved to be as effective as gamma-rays.

2. *Effect of medium composition*

To investigate the effects of various media, ovules were cultured on E20A, MS and N6 media. **Table 1** shows their effects on the induction efficiency of plantlets. The χ^2-test revealed no significant statistical differences in the efficiency among these media, even though the E20A medium used by Sauton[9,10] to induce haploid plants of melon contains more organic matter than the other media. The effects of the IAA concentration in the medium was examined in two independent experiments carried out in different seasons (**Table 2**). The IAA level tested did not affect the frequency of plantlet induction. However, the sampling season had a significant effect. The effect of casamino acids (Difco) was also investigated at concentrations of 0, 200 and 400 mg/*l*. When 400 mg/*l* was added to a medium, the induction frequency became lower than the control. The difference between concentrations of 0 and 200 mg/*l* was not statistically significant (data not shown).

3. *Effect of genotypes*

Eleven cultivars were examined for their potential to regenerate plantlets from pseudofertilized ovules (**Table 3**). They are classified as follows; "Earl's favorite" belongs to var. *reticulatus*; "Hamiuri","F1 Hamiuri" and "Honeydew PF" belong to var. *inodorus*; "Mauri" belongs to var. *conomon*; "Elizabeth", "Sweet heart" and "Alice" are no net type produced by intervarietal cross; "Cossack" and "Bonus No. 2" are net type produced by intervarietal cross; and "Mauri × Bird nest" is also an intervarietal hybrid between var. *conomon* and var. *reticulatus*. Haploids were obtained from all the cultivars tested, though the regeneration frequency of haploids per ovule ranged from 0% to 3.4%. The frequency of induction of haploid plants varied with the genotype as reported in pseudofertilized ovule culture in *Actinidia*[13] and in *Malus*[14]. And the frequency of haploid induction was almost the same as the results of Sauton[10] (maximum 2.9% per ovule).

4. *Ploidy of regenerated plants*

The ploidy level of the regenerated plants was checked by counting the number of chromosomes in the root tip cells. All the root tips of the main root from 7 regenerated plants cultured for 1 week after germination *in vitro* showed the haploid chromosome number of *C. melo* ($2n=12$) (**Fig. 4**). The chromosome number of all roots branching out from the main root of these plantlets doubled spontaneously with growth. However, the above-ground parts of the regenerated plants remained haploid to maturity. The DNA content of mesophyll cells of the regenerated plants was half that of diploid cells (**Fig. 5**). Also, this result showed that some diploid cells were present in the leaves of the obtained plants. But, pollen mother cells of the plants showed a haploid chromosome number

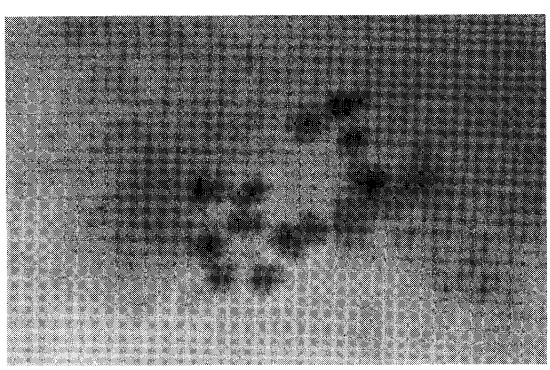

Fig. 4 Root-tip squash of a plantlet derived from cultured ovule after pseudofertilization ($2n=12$).

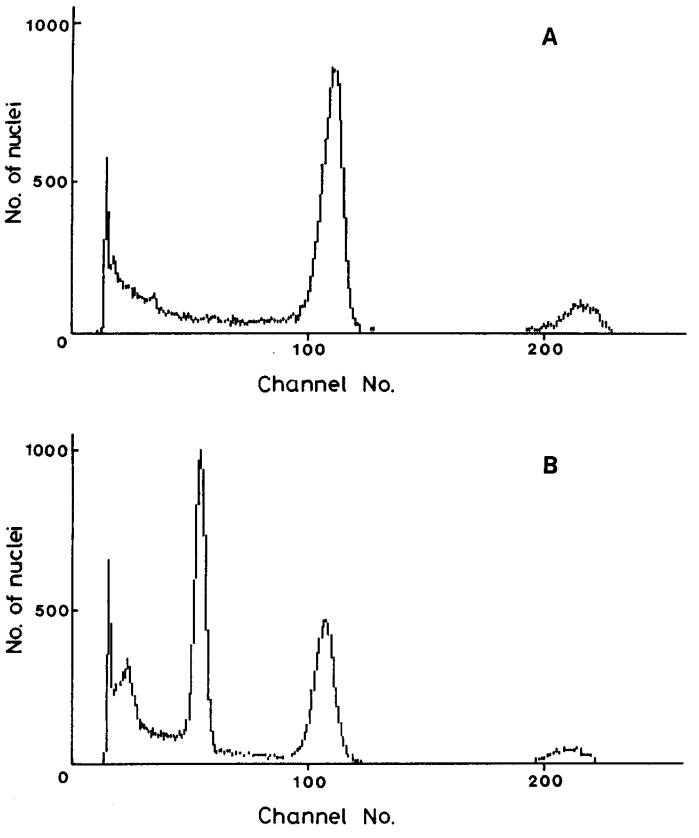

Fig. 5 DNA content of mesophyll cells of a diploid plant (A) and of a plant derived from cultured ovule after pseudofertilization (B).

Table 4. Chloroplast number per guard cell in leaf epidermis of the plants produced via ovule culture and of diploid plants.

	Plant No.	Mean	S. D.
Diploid plant	1	4. 95[a]	0. 76
Plants produced via ovule culture	2	2. 70[b]	0. 57
	3	2. 40[b]	0. 60
	4	2. 45[b]	0. 51
	5	3. 00[b]	0. 79

Data followed by different letters are significantly different by the t-test ($p<0.05$).

and no fertile pollen was produced in all plants obtained. Hence, the obtained plants were estimated as haploid. The chloroplast number in the guard cells of stomata in the lower epidermis of regenerated plants was also checked (**Table 4**). There was a good correlation between the chloroplast number and the ploidy level. The average number of chloroplasts in haploid guard cells was 2. 40-3. 00, while that in diploid cells was 4. 95. These results show that plants induced by the culture of pseudofertilized ovules were ploidy chimera, that is to say, these plants have diploid roots and haploid above-ground parts. To our knowledge, similar results have been obtained only in the haploids of sugar beets regenerated from unpollinated ovules[6]. It is interesting that in both cases, haploid plants were induced from ovules, but not from anthers. To determine the ploidy level,

chloroplast counts of guard cells proved to be the most useful among the various methods investigated because they are reliable enough and simpler than other methods. Experiments are in progress to determine how chromosomes double in haploids.

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《和文要約》

偽受精胚珠培養によるメロン半数体植物の作出

加藤紀夫・萩森　学・岩井純夫

日本たばこ産業株式会社植物開発研究所

メロン，12品種においてX線照射花粉を受粉させ，その後胚珠を摘出し植物成長調節物質を含まないMS培地もしくはインドール酢酸0.01 mg/*l*を添加したE20A培地で培養したところ，すべての品種から植物体が得られた．得られた植物体はすべて半数体であった．得られた半数体植物の根では，自然倍加が高頻度で観察された．しかしながら，地上部での自然倍加は観察されず，倍加処理無しに稔性を回復した個体は認められなかった．