ニホンカボチャの未受精胚珠培養における不定胚形成

誌名	園藝學會雑誌
ISSN	00137626
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巻/号	57巻1号
掲載ページ	p. 34-42
発行年月	1988年6月

J. Japan. Soc. Hort. Sci. 57(1) : 34—42. 1988.

Somatic Embryogenesis in Cultured Unfertilized Ovules of *Cucurbita moschata*

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Summary

Unfertilized ovule culture for the production of somatic embryos and its histological observations were carried out in *Cucurbita moschata*. About 17% ovules produced somatic embryos when ovules, excised from the ovaries at anthesis which were pretreated at 5°C for 2 days, were cultured on a liquid half-strength Murashige and Skoog (MS) medium supplemented with MS organics and 30 g/*l* sucrose. At the initiation of culture, the embryo sac began to degenerate. From about 10 days of culture, some of the nucellar cells became conspicuous by large nuclei, dense cytoplasm and thick walls, and divided to give rise to proembryos. Further cell division and differentiation of proembryos led to globular, heart and cotyledonary stages similar to zygotic embryogenesis. Among the obtained embryos, only a few showed normal morphology and many developmental variants were observed, such as embryos with secondary embryos or abnormal cotyledons. Upon transfer of the somatic embryos to the same nutrient medium supplemented with 5 g/*l* sucrose and 8 g/*l* agar, most of them underwent callusing, and only a few developed normal shoots and roots. The regenerated plants were diploid (2n=40) or tetraploid. Our histological and cytological investigations indicate that the embryos from cultured unfertilized ovules of *C. moschata* develop directly from single cells of the nucellus.

Introduction

Plant regeneration from tissue culture has a potential for plant breeding. When regenerated plants originate from the somatic cells, they were very useful for clonal propagation or as a novel source for agriculturally promising variations, according to their genetic stability. In addition, when morphogenesis occurs in the gametophytic cells, it is remarkably efficient for the rapid production of homozygous lines and the detection of mutation. *In vitro* embryogenesis has been reported in cultures of vegetative and reproductive tissues of many plants belonging to widely different families of both dicotyledons and monocotyledons(1, 6, 29, 33). In the case of unpollinated ovary and ovule culture, embryogenesis occurs in the somatic cells, or the embryo sac cells, or both(35). Somatic embryos originate from single cells (10, 20), or group of cells(35), or both(21). The determination of the origin of embryos — whether from the somatic cells or the game tophytic cells and in addition, whether from single cells or group of cells — is considered very important for the use of embryogenesis in plant breeding.

In *Cucurbita pepo*, *in vitro* embryogenesis has been reported from fruit pericarp(26), hypocotyls and cotyledons(13, 14) and from unfertilized ovules(4). However, there are few reports on somatic embryogenesis from cultured tissues of other *Cucurbita* species.

In the present study, we investigated the effects of culture conditions on the development of somatic embryos and its ontogeny in cultured unfertilized ovules of *Cucurbita moschata*.

Received for publication October 3, 1986.

Materials and Methods

Two clutivars, 'Seoul-Dadagi' and 'PM

143', of *Cucurbita moschata* were used as plant materials. 'PM 143' is very similar to 'Seoul-Dadagi' in most characteristics, except for its high female/male flower ratio.

For investigating the effects of flower bud stage, cold treatment of ovaries and the concentration of Murashige and Skoog salts(24) or sucrose on somatic embryogenesis in unfertilized ovules, 'Seoul-Dadagi' was grown in a plastic house. In these experiments, each treatment was applied singly. Unpollinated flower buds were collected at anthesis, or one or two days before anthesis. In cold treatment, ovaries containing unfertilized ovules were excised from flower buds one day before anthesis and pretreated at 5 or 10°C for 2 or 5 days before the excision of ovules. The concentration of MS salts or sucrose was varied. Controls for these treatments were as follows : Unfertilized ovules were dissected from no-cold treated ovaries from flower buds one day before anthesis and cultured on a MS medium supplemented with 60 g/*l* sucrose, 8 g/*l* agar (unless otherwise stated) and pH 5. 8.

The effects of agar concentration and the pH of the medium on the production of somatic embryos in unfertilized ovules were investigated using 'PM 143' as plant materials. Based on the above experiments, controls for these treatments were applied as follows : Ovaries from flower buds at anthesis were pretreated at 5°C for 2 days and unfertilized ovules were dissected out ; they were cultured on a half-strength MS medium supplemented with MS organics, 4 g/*l* agar and pH 5. 2.

In all experiments, the pH of the medium was adjusted with 0. 1 N NaOH or HCl before autoclaving at 120°C for 15 min. Twenty

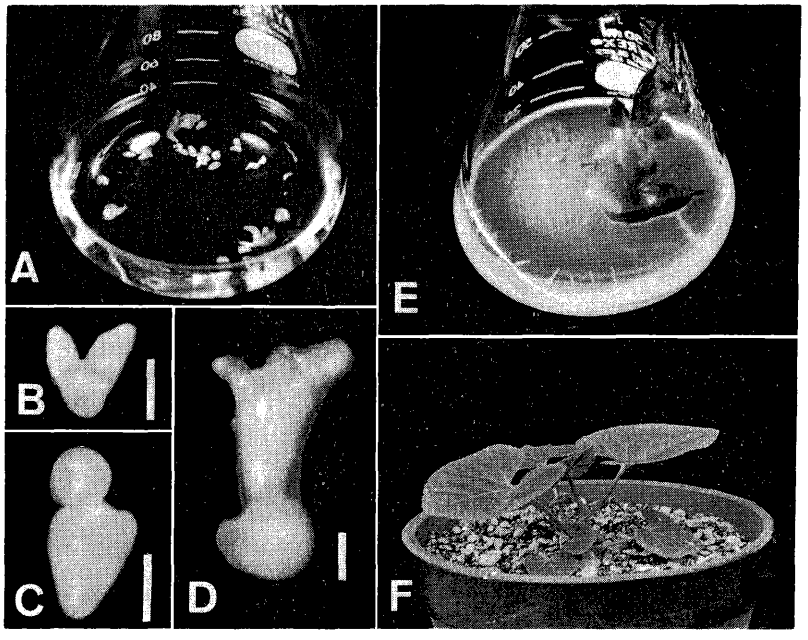

Fig. 1. Somatic embryogenesis and plant regenration from cultured unfertilized ovules of *Cucurbita moschata* (Scale : Plates B-D=1 mm).

A. Development of somatic embryos in liquid medium. Note two precociously germinated embryos.
B. Embryo with normal morphology, from liquid medium.
C. Embryo with secondary embryo due to budding, from liquid medium.
D. Embryo with fused cotyledons attached to the ovule, from agar medium.
E. A plantlet, showing normally developed shoot and root.
F. A regenerated plant in a pot.

or twenty-five ovules were cultured in a 50 ml flask containing 20 ml medium or a 100 ml flask containing 30 ml medium. The cultures were maintained at 25°C under a 16 h light regime (approximately 1,000 lx). Morphogenetic responses were observed after 60 days of culture.

For plant regeneration, somatic embryos were transferred to a secondary medium consisting of half strength MS salts, MS organics, 5 g/*l* sucrose and 8 g/*l* agar, which was reported as a suitable medium for the germination of small zygotic embryos of *Cucurbita maxima*(19).

For chromosome counts, excised roots of regenerated plants were pretreated with 2 mM 8-hydroxyquinoline, fixed in aceto-alcohol, hydrolyzed with 1 N HCl at 60°C for 6 min and stained with 1% aceto-orcein.

For histological observations, unfertilized ovules of 'PM 143' were cultured in a liquid half-strength MS medium. Other culture methods were the same as controls for investigating the effects of agar concentration and the pH of the medium. About fifty ovules were daily fixed in CRPF (0.5 g chromic trioxide : 5 ml propionic acid : 15 ml formalin : 80 ml H_2O) for 24 h at room temperature and then transferred to 70% ethanol for storage at 4°C until use. Fixed materials were dehydrated through a graded series of ethanol-n-butanol in an Autokinate, and embedded in paraffin (58°C melting point). Sections were cut at 8-12 μm and stained with Heidenhain's iron hematoxylin.

Results

Embryogenesis occurred in cultured unfertilized ovules of *Cucurbita moschata*, together with callus proliferation. Most of the calli were very small and friable, and showed no morphogenetic response. Therefore, we present here mainly the results of somatic embryogenesis.

Among the obtained embryos, those with normal morphology (Fig. 1 B) were very few, and those with abnormal morphology were dominant, such as embryos with secon-

Table 1. Effects of flower bud stage, cold treatments of ovaries and the concentration of MS salts and sucrose on the production of somatic embryos in cultured unfertilized ovules of *Cucurbita moschata* cv. Seoul-Dadagi.

Treatment	No. ovules cultured	No. ovules forming embryos	Percent ovules forming embryos
Flower bud stage			
2 days before anthesis	280	0	0
1 day before anthesis[z]	300	9	3.0
At anthesis	300	15	5.0
Cold treatment of ovaries			
None[z]	240	3	1.3
5°C for 2 days	220	9	4.1
5°C for 5 days	240	6	2.5
10°C for 2 days	120	1	0.8
10°C for 5 days	240	2	0.8
Concentration of MS salts			
1[z]	225	8	3.6
3/4	300	7	2.3
1/2	225	10	4.4
1/4	200	7	3.5
Concentration of sucrose[y]			
10 g/*l*	200	0	0
30	175	13	7.4
60[z]	200	2	1.0
100	200	1	0.5

[z] Control treatment.

[y] Agar concentration was 4 g/*l* for sucrose effect.

dary embryos (Fig. 1 C), fused cotyledons (Fig. 1 D) or multiple cotyledons. Embryos in liquid medium were generally seperated from the ovules and suspended, while those in agar medium were attached to the mother tissues. Regardless of the physical state of the medium, some of the embryos germinated precociously (Fig. 1 A). When these immature and precociously germinated embryos were transferred to the secondary medium,

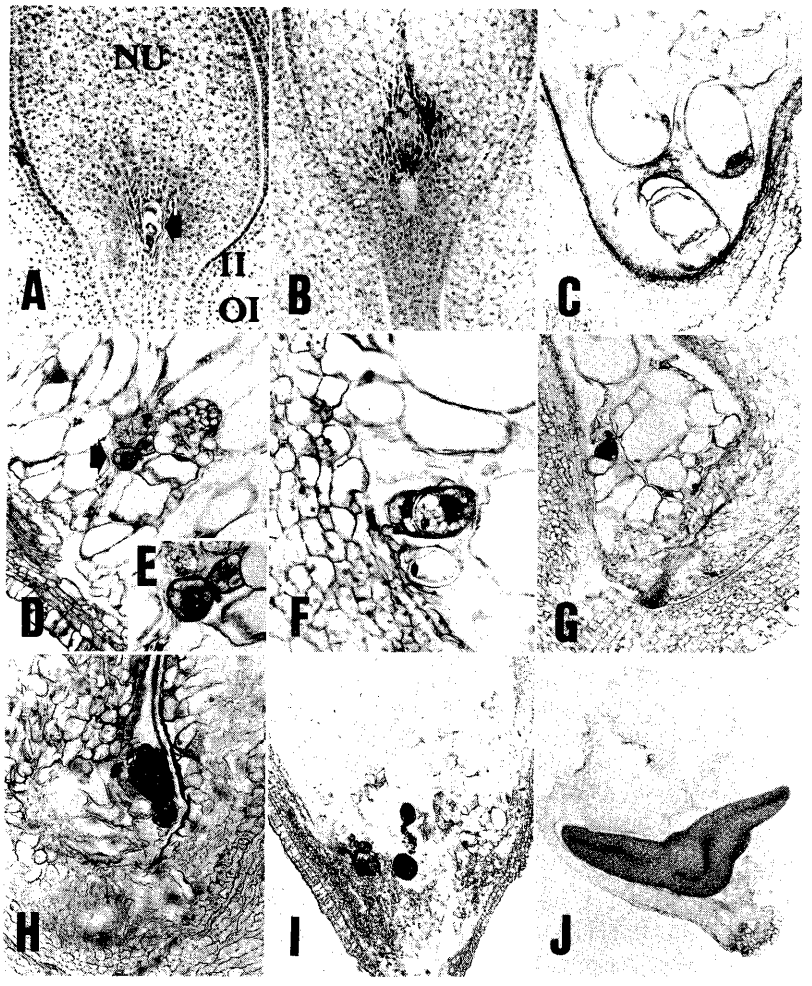

Fig. 2. Ontogeny of somatic embryos from cultured unfertilized ovules of *Cucurbita moschata*.

A. Median longitudial section of ovule at anthesis. Note the mature embryo sac (arrow). OI=outer integument. II=inner integument. NU=nucellus (250×).

B. Ovule three days of culture. Note degeneration of the embryo sac and development of a strand of degenerating cells in the nucellus (400×).

C. Large vacuolated cells containing a large nucleus developed in the nucellus (250×).

D. Two embryogenic cells (arrow) initiated in the nucellus (250×).

E. Magnification of D. Note the large nuclei, dense cytoplasm and thick walls (500×).

F. Two-celled proembryo at the peripheral region of the nucellus (500).

G. A multicellular proembryo in the nucellus (380×).

H. A proembryo in the nucellus. Note the suspensor (400×).

I. Development of many somatic embryos within an ovule. Note two globular embryos and several proembryos (100×).

J. A well-differentiated somatic embryo. Note the closed vascular system (100×).

Table 2. Effects of agar concentration and the pH of the medium on the production of somatic embryos in cultured unfertilized ovules of *Cucurbita moschata* cv. PM 143.

Treatment	No. ovules cultured	No. ovules forming embryos	Percent ovules forming embryos
Agar concentration			
0 g/*l*	325	55	16.9
4[z]	325	30	9.2
8	325	22	6.8
12	325	14	4.3
pH of the medium			
4.9	150	5	3.3
5.2[z]	125	6	4.8
5.5	125	8	6.4
5.8	150	5	3.3
6.1	150	10	6.7

[z] Control treatment

most of them underwent callusing, and only a few developed normal shoot and roots (Fig. 1 E). Thus, we could raise three mature plants, one of which has proved diploid (2 n = 40) and two of which were tetraploid (2 n = 80).

Effects of flower bud stage and cold treatment of ovaries on the production of embryos are presented in Table 1. Ovules from flower buds two days before anthesis produced no somatic embryos. Ovules from flower buds at anthesis resulted in better embryogenic response than those from flower buds one day before anthesis. Of the five cold treatments of ovaries, treatment at 5°C for 2 days was most efficient for embryogenesis, while treatment at 10°C for 2 or 5 days was suppressive for embryogenesis comparing with no treatment.

The concentration of MS salts and the pH of the medium showed little effect on embryogenic response, whereas the concentration of sucrose had a great effect on the formation of embryos (Table 1, 2). The highest percentage of ovules forming embryos occurred in 30 g/*l* sucrose. Lower (10 g/*l*) or higher (60 or 100 g/*l*) concentration of this resulted in no embryogenesis or a low frequency of embryogenesis.

The concentration of agar played also an important role in the production of embryos (Table 2). As the concentration of agar increased, the percentage of ovules forming embryos decreased. In liquid medium, about 17% ovules produced embryos, and showed the highest frequency of embryogenesis in this study.

Ovules from flower buds at anthesis contained mature embryo sacs (Fig. 2 A). At the initiation of culture, the ovules from flower buds pretreated at 5°C for 2 days showed a sign of degeneration in both embryo sac cells and nucellar cells. Three days of culture, the nuclei and cytoplasm of embryo sac cells became loose in their affinity for staining, and a strand of degenerating cells developed in the nucellus from the antipodal side of the embryo sac towards the chalazal part (Fig. 2 B). About 7 days of culture, some of the nucellar cells around the degenerated embryo sac developed into large vacuolated cells being conspicuous by sparse cytoplasm and large neclei with one or more nucleoli (Fig. 2 C). These large vacuolated cells disappeared later. About 10 days of culture, embryogenic cells being conspicuous by their prominent nuclei, dense cytoplasm and thick walls were observed mainly at the micropylar half of the nucellus (Fig. 2 D-E). After 10 days of culture, the embryogenic cells divided to give rise to proembryos (Fig. 2 F-H). Further cell division and differentiation of proembryos led to globular, heart and cotyledonary stages similar to those in zygotic embryogenesis (Fig. 2 I-J). After about 25 days of culture, well differentiated somatic embryos with closed vascular system were observed (Fig. 2 J). Although only normal ontogeny of somatic embryos has been described here, many developmental variations were also observed. Development of some embryos was arrested in the globular stage, and some embryos produced secondary embryos by budding from the superficial cells of the embryo axis and cotyledons.

Because of asynchrony of embryogenesis, embryos at various stages of development were observed within an ovule (Fig. 2 I) and within a flask. Most frequently, one ovule produced one embryo, but in a few cases, ovules producing more than one embryos

were also observed (Fig. 2 I). Sometimes, the nucellus protruded through the integumental enclosure due to cell enlargement, and embryos developed at the superficial cells of the nucellus contacting directly the medium.

Discussion

Somatic embryogenesis has been reported to occur directly from predetermined embryogenic cells, or indirectly from differentiated cells which have dedifferentiated and subsequently redetermined for embryogenic development(7, 28, 29, 33). Plant growth regulators are very important for somatic embryogenesis. Evans *et al.* (7) summarized that in the primary medium for induction of somatic embryogenesis, 2, 4-dichlorophenoxyacetic acid (2, 4-D) is used for 57.7% of the crop species and 57.1% of the non-crop species, kinetin for 50.0% of the crop species and 14.3% of the non-crop species, and naphtaleneacetic acid (NAA) for 26.9% of the crop species and 21.4% of the non-crop species. On the other hand, somatic embryos develop in the medium containing no growth regulators in some species, e. g., *Citrus sinensis*(30), *Petroselinum hortense*(22) and *Ilex aquifolium*(11). In *C. pepo*, indirect somatic embryogenesis has been reported in the culture of fruit pericarp(26) and hypocotyls and cotyledons(13, 14) under the effect of exogenous growth regulators. In our preliminary experiments, however, the growth regulators such as 2, 4-D, NAA, kinetin and benzyladenine suppressed somatic embryogenesis at the concentration of 1 mg/*l*. In the present study, therefore, no growth regulators were supplemented in the medium. Our results of somatic embryogenesis in *C. moschata* show to be another evidence of direct embryogenesis from predetermined embryogenic cells, since nucellar cells developed into somatic embryos even in the medium without any exogenous growth regulators.

Little data are available for the factors influencing somatic embryogenesis in the genus *Cucurbita*. In *C. moschata*, ovules from flower buds at anthesis resulted in better embryogenic response than those from flower buds one or two days before anthesis (Table 1), whereas in *C. pepo*, Chambonnet and de Vaulx(4) reported that ovules from flower buds one or two days before anthesis showed good morphogenetic response. This difference may result from the difference between species used as plant materials. Our results of cold treatment of ovaries showed that the treatment at 5°C for 2 days is advantageous for somatic embryogenesis (Table 1). In anther culture, cold treatment of anthers has been reported to be promotively effective for androgenesis(8, 27). We found that the concentration of agar and sucrose are very important in the development of somatic embryos (Table 1, 2). Although somatic embryos developed in either agar or liquid medium, the yield of embryos increased according to the decrease of agar concentration. This suggests that the agar used in our experiments is suppressive for the development of somatic embryos. In anther culture of *Nicotiana tabaccum*(16), the same conclusion was reported ; i. e., a liquid medium is advantageous for androgenesis comparing with an agar medium ; this is due to inhibitory effects of agar which can be absorbed by active carbon.

Histology of embryogenesis from cultures of unpollinated ovaries and ovules has been carried out in several plant species. The results of the origin of embryos are summarized as follows : 1) from the somatic cells in grapevine(23) and cotton(15), 2) from the embryo sac cells in tobacco(36), and 3) from both in rice(18), barley(12), lily(9) and sunflower(34). Chambonnet and de Vaulx(4) reported that embryos were obtained from the cultured unfertilized ovules of *C. pepo* and the embryos might develop from the embryo sac cells without histological observations. In our study, three lines of evidence indicate clearly that embryos develop from the somatic cells in cultured unfertilized ovules of *C. moschata*, i. e., 1) the embryo sac degenerate in the early time of culture (Fig. 2 B), 2) embryogenic cells and proembryos were observed only in the nucellus (Fig. 2 D-I), and 3) the regenerated plants were diploid or tetraploid.

Somatic embryos has been reported to develop from single cells and/or group of cells (33). Generally, a multicellular origin appears to produce embryos attached to the mother tissue over a broad area, whereas a unicellular origin is more likely to give embryos attached to the mother tissue by a narrow suspensor. In the culture of unfertilized ovules of *C. moschata*, somatic embryos are considered to originate from single cells since embryogenic cells developed from single cells of the nucellus (Fig. 2 D-E) and the proembryos had suspensors (Fig. 2 H).

Embryogenic cells and proembryos were initiated only in the nucellus of the ovules where large vacuolated cells were also developed (Fig. 2 D-I), so the large vacuolated cells seem to be highly related to the initiation of embryogenic cells. Although the early development of somatic embryos was normal and very similar to that of zygotic embryogenesis, the late development showed many morphological variations. Thus, cultured ovules produced many atypical embryos such as multiple embryos (Fig. 1 C), abnormal cotyledons (Fig. 1 D) and precociously germinated embryos (Fig. 1 A), which are common in somatic embryogenesis (2, 17). Morphological variations in somatic embryogenesis have been reported to be able to be controlled by the manipulation of plant growth regulators in *Carum carui*(2) and *Corylus avellana*(25). Therefore, to increase the yield of normal embryos, it is necessary to investigate the effects of growth regulators on the development of somatic embryogenesis in *C. moschata*.

Genetic and phenotypic variations in regenerated plants from tissue culture has been reported in a large number of cultures(3). However, Vasil(31) reported that in cereals and graases, regenerated plants through somatic embryogenesis showed no evidence of aneuploids, polyploids and phenotypic variations. According to D'Amato(5), nucellar cells may undergo chromosome endoreduplication, most frequently. Ploidy variations in our regenerated plants may be due to originally endoreduplicated cells in the nucellus. Similarly, Chambonnet and de Vaulx(4) reported that most of the regenerated plants were diploid and some were haploid-diploid, aneuploids and polyploids. Genetic and phenotypic stability in regenerated plants from cultured unfertilized ovules of *C. moschata* should be investigated after developing techniques for converting somatic embryos into mature plants at high frequency.

The present series of experiments have demonstrated, for the first time, that somatic embryos develop directly from single cells of the nucellus without the mediation of callus phase when unfertilized ovules of *Cucurbita moschata* are cultured on a modified MS medium without exogenous growth regulators. The production of somaclonal lines through somatic embryogenesis should be very useful for *Cucurbita* breeding.

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ニホンカボチャの未受精胚珠培養における不定胚形成

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摘　要

ニホンカボチャの未受精胚珠培養で不定胚形成とその組織学的研究を行った．

開花当日の子房を 5℃ で 2 日間前処理して摘出した未受精胚珠は，MS（Murashige-Skoog）の 1/2 量無機塩類，全量有機物，しょ糖 3% 組成からなる液体培地での静置培養で，17% が不定胚を形成した．

未受精胚珠の胚嚢は培養の初期に退化が始まった．培養 10 日目ごろから，大きな核と濃厚な細胞質，そして厚い細胞膜をもつ珠心細胞が目立つようになり，これらが分裂して前胚を形成した．その後，この前胚は分裂を続け，接合子胚に似た経過で，球状期，ハート状期をへて子葉期の胚へと発達した．しかし正常胚は少なく，二次胚や奇形子葉を付けた異常胚が多かった．

これらの不定胚を，同じ組成（ただししょ糖 0.5%）の固体培地（寒天 0.8%）で継代培養を行った．その結果，多くはカルス化し，正常に育ったのは数個体にすぎなかった．これら再生植物は 2 倍体（2n＝40）と 4 倍体（2n＝80）であった．

以上のことから，ニホンカボチャの未受精胚珠培養で形成された不定胚は，珠心組織の単細胞起源であることが示唆された．