葯培養と花粉培養によるセルリ花粉からのカルスと胚様体の形成

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著者	銅谷, 徳夫 松原, 幸子 村上, 賢治
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Callus Formation and Regeneration of Adventitious Embryos from Celery Microspores by Anther and Isolated Microspore Cultures

Norio Dohya, Sachiko Matsubara and Kenji Murakami

Department of Agricultural Sciences, Faculty of Agriculture, Okayama University, Tsushima, Okayama 700

Summary

Anthers and microspores of *Apium graveolens* L. (celery) were cultured *in vitro* to obtain haploid plants.

Anther culture: Immature anthers of 'Cornell 619' and 'Cornell 19' at three stages of development were cultured on eight different media containing a half strength of Murashige and Skoog (1/2 MS) and B5 media supplemented with combinations of 2, 4-D, BA, NAA, and zeatin plus sucrose and Gelrite from May to June in 1994.

Calli developed from 'Cornell 19' microspores at tetrad stage on 1/2 MS medium supplemented with 2, 4-D and B5 medium supplemented with 2, 4-D. In 'Cornell 619', calli developed from tetrad microspores on B5 medium supplemented with 2, 4-D, and from early uninucleous microspores on B5 medium supplemented with 2, 4-D. Adventitious embryos regenerated from calli transferred to MS medium with or without NAA and BA; plantlets grew after 40 to 60 days.

Isolated microspore culture: Celery microspores at different developmental stages were cultured in liquid B5, NLN, and 1/2 MS media supplemented with 2, 4-D and BA, with or without glutamine and with serine at the density of 2.5×10^3 microspores/m*l* medium. In 'Cornell 619', many microspores of early uninucleate stage developed to colonies in the B5, NLN, and 1/2 MS media; the colonies in B5 and 1/2 MS media developed into heart-shaped embryos; microspores, plated in modified 1/2 MS medium supplemented with 2, 4-D and BA, developed colonies and calli.

Introduction

Production of new celery cultivars by hybridization is tedious work, because the flowers are very small. Haploid plants have the advantage of the possibility of accelerating the production of homozygous lines and detecting recessive mutations. Although anthers and microspore cultures have been used successfully to produce haploid plants in many plant species (Bajaj, 1983), there are few successes in the *Umbelliferae* family. The formation of haploid plants in carrot by anther culture has recently been reported by Andersen et al. (1990), Hu et al. (1993) and Matsubara et al. (1995), but that in celery has not.

This study reports the production of plantlets by anther culture, and haploid embryo and callus formation by microspore culture in celery.

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Materials and methods

Anther culture

Anthers of celery 'Cornell 619' and 'Cornell 19' were obtained from plants grown in the field of Okayama University by forcing inflorescences from May 6 to June 23, 1994. Immature anthers of center florets at the tetrad, early uninucleate and late uninucleate stages from young inflorescences (Fig. 1) were collected in the morning. They were surface-sterilized for 10 sec in 70% ethanol, immersed in sodium hypochlorite (1% available Cl) for 15 min, rinsed thrice with sterile distilled water, and plated on a basal media consisting of a half strength-MS (Murashige and Skoog, 1962) (1/2 MS) and B5 (Gamborg et al., 1968) (B5), supplemented with 30 $g\cdot liter^{-1}$ sucrose, 2 $g\cdot liter^{-1}$ Gelrite, and 0.5 or 1.0 $mg\cdot liter^{-1}$ 2, 4-D and 0 or

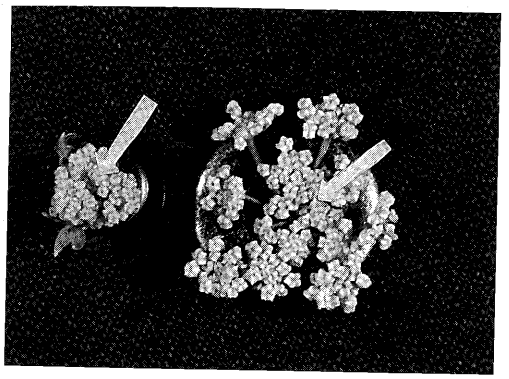

Fig. 1. Stages 1 (left) and 2 (right) of celery inflorescence. Arrows indicate center florets used.

0.1 mg·liter^{-1} BA. A factorial design with eight combinations was employed. After adjusting the pH of the media to 5.8, they were autoclaved for 15 min at 120 °C. About 40 anthers were plated on 5 m*l* of medium in a petri dish (60 × 15 mm). Three dishes with 80-120 anthers were sealed with Parafilm per treatment. Cultures were incubated at 25°C under 16-hr photoperiod provided with 20 μmol·sec^{-1}·m^{-2} fluorescent light for 40 ~ 60 days. Calli, formed on each medium, were dissected and plated in individual test tubes containing thirty-eight kinds of media. The media consisted of MS medium supplemented with 30 g·liter^{-1} sucrose, 2 g·liter^{-1} Gelrite and 24 combinations of 0, 0.05, 0.1, and 1.0 mg·liter^{-1} NAA and 0.1, 0.2, 0.4, 0.8, 1.0, and 1.6 mg·liter^{-1} BA, 12 combinations of 0.1 and 1.0 mg·liter^{-1} NAA and 0.1, 0.2, 0.4, 0.8, 1.0, and 2.0 mg·liter^{-1} zeatin and without phytohormones, and B5 medium supplemented with 30 g·liter^{-1} sucrose, 2 g·liter^{-1} Gelrite, and 1 mg·liter^{-1} 2, 4-D.

Embryo culture: Embryos were transferred to MS 8 g·liter^{-1} agar solidified medium under the same condition as the anther culture. Regenerated plants were grown in vermiculite for about a month, then transplanted in soil in a plastic house.

Isolated microspore culture

Buds containing microspores at early and late uninucleate stages were surface-sterilized as were the anthers. Buds were macerated in a test tube containing 1/2 MS liquid culture medium supplemented with 3 g·liter^{-1} (w/v) sucrose and 1.0 mg·liter^{-1} 2, 4-D and BA at pH 5.8. Microspores which were isolated by filtration through a 38 μm stainless screen were centrifuged three times at 300 rpm for 3 min with fresh culture medium. The microspores were suspended in B5, Lichter's medium (NLN) (Lichter, 1982) and a half strength MS supplemented with 10 g·liter^{-1} sucrose and 1 mg·liter^{-1} 2, 4-D and BA, with or without 800 mg·liter^{-1} glutamine and 100 mg·liter^{-1} serine at pH 5.8 at a density of 2.5 × 10^3/m*l*. Two milliliters of the microspore suspension were plated onto a 60 × 15 mm plastic petri dish and incubated at 25°C under dark until many colonies were formed. Each treatment consisted of at least five replicates. Two m*l* of the same fresh medium minus sucrose were added a month. Microspores of an early uninucleate stage were plated in modified 1/2 MS containing three combinations of KNO_3 and NH_4NO_3 (KNO_3: NH_4NO_3 (mg·liter^{-1})); 3,000 : 0 (Medium I), 2,161 : 344 (Medium II), and 1,515 : 600 (Medium III). Molarity of NO_3 and NH_4 was 60 mM : 0 (Medium I), 51.4 mM : 8.6 mM (Medium II), and 45 mM : 15 mM (Medium III), and their molar ratios were 6 : 0; 6 : 1; and 3 : 1, respectively. These modified 1/2 MS media were also supplemented with 1.0 mg·liter^{-1} 2, 4-D and BA at a density of 2.5 × 10^3 microspores/m*l* medium.

Results

Anther culture

In 'Cornell 619', microspores at tetrad stage developed to a uninucleate stage in 6.7 ~ 26.6% of anthers after a 10 day culture; these initiated cell division to from calli. More mature microspores at plating time did not form callus, except 5 out of 96 anthers containing microspores at early uninucleate stage on B5 medium supplemented with 1.0 mg·liter^{-1} 2, 4-D (Table 1, Fig. 2). In 'Cornell 19' microspores, calli formed only from tetrad microspores on B5 medium supplemented with 1.0 mg·liter^{-1} 2, 4-D and 1/2 MS medium supplemented with 0.5 and 1.0 mg·liter^{-1} 2, 4-D.

Embryo culture; Four adventitious embyros were generated from calli which originated from tetrad microspores in 'Cornell 619' (Fig. 3), and an adventitious embryo developed from a callus from an early uninucleate microspore after 40 ~ 60 days (Table 2). The embryos upon transfer to MS basal medium grew into normal plantlets (Fig. 4). In

Table 1. Callus formation from tetrad and uninucleate microspores by celery anther culture on 8 different media.

Age of microspore	Basal medium	Phytohormones 2,4-D	Phytohormones BA	No. anthers plated	Anthers formed callus No.	Anthers formed callus Frequency (%)
			cv. Cornell 19			
Stage 1 (Tetrad)	B5	1.0 mg/l	0	86	4	4.7
		0.5	0	98	0	0
		1.0	0.1	96	0	0
		0.5	0.1	93	0	0
	1/2MS	1.0	0	79	1	1.3
		0.5	0	99	2	2.0
		1.0	0.1	87	0	0
		0.5	0.1	82	0	0
			cv. Cornell 619			
	B5	1.0	0	115	7	6.1
		0.5	0	110	1	0.9
		1.0	0.1	90	0	0
		0.5	0.1	92	0	0
	1/2MS	1.0	0	89	0	0
		0.5	0	99	0	0
		1.0	0.1	100	0	0
		0.5	0.1	92	0	0
Stage 2 (Uninucleate)	B5	1.0	0	96	5	5.2
		0.5	0	102	0	0
		1.0	0.1	106	0	0
		0.5	0.1	104	0	0
	1/2MS	1.0	0	96	0	0
		0.5	0	102	0	0
		1.0	0.1	108	0	0
		0.5	0.1	108	0	0

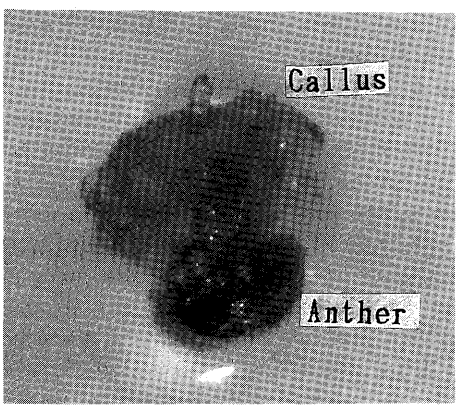

Fig. 2. Callus formation in anther culture.

'Cornell 19', one and three adventitious embryos differentiated from calli on MS basal medium and MS supplemented with 0.1 mg·liter^{-1} NAA and

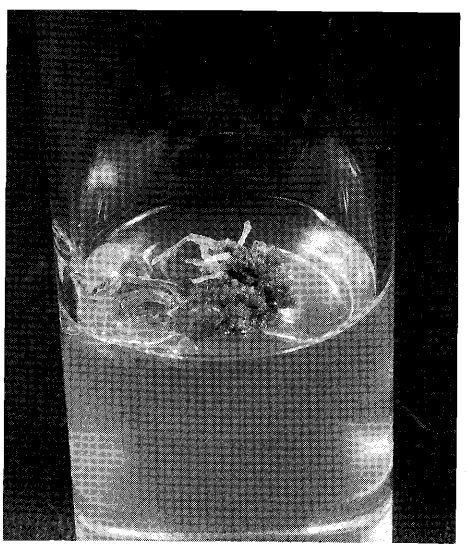

Fig. 3. Adventitious embryos regenerated from callus.

Table 2. Generation of embryoids from microspore-derived callus on 23 different media.

Phytohormones in media (mg/l)		Generation of embryoids	
NAA	BA	cv. Cornell 19	cv. C. 619
0	0	+[z]	+[y]
	0.1	−	−
	0.2	−	−
	0.4	−	−
	0.8	−	−
	1.0	−	−
	1.6	−	−
0.05	0.1	−	−
	0.2	−	−
	0.4	−	−
	0.8	−	−
	1.0	−	−
	1.6	−	−
0.1	0.1	+[x]	−
	0.2	+[x]	−
	0.4	+[x]	−
	0.8	−	−
	1.6	−	−
1.0	0.1	−	−
	0.2	−	−
	0.4	−	−
	0.8	−	−
	1.6	−	−

[z] One embryoid regenerated from microspore callus cultured in 1/2MS+1.0mg/l 2, 4-D medium.
[y] Five embryoids regenerated from microspore callus cultured in B5+1.0mg/l 2, 4-D.
[x] Three embryoids regenerated from microspore callus cultured in B5+1.0mg/l 2, 4-D.

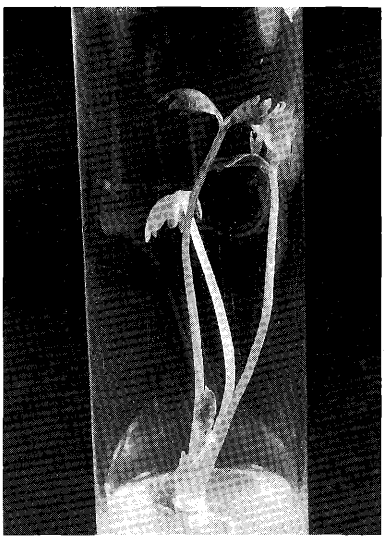

Fig. 4. Normal plantlet from an adventitious embryo.

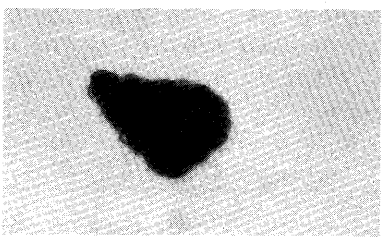

Fig. 5. A heart-shaped embryo from a microspore in isolated microspore culture.

0.1, 0.2 and 0.4 mg·liter^{-1} BA, respectively, after 40 to 60 days. These embryos grew into normal plantlets upon transfer to the same fresh media.

Isolated microspore culture

Cell division of 'Cornell 619' uninucleate microspores was observed in 3, 2, and 3 petri-dishes out of 5 supplied with B5, NLN, and 1/2 MS media, respectively (Table 3). Formation of heart-shaped embryos (Fig. 5) was observed in 2 petri-dishes with B5 medium and 2 petri-dishes with 1/2 MS medium, but the embryos did not grow into plantlets.

Nearly all microspores plated in Medium III formed colonies (Fig. 6) which developed to calli in 3 out of 5 petri-dishes. In microspores plated in other media, less than 30% initiated cell division,

Table 3. Cell division and embryo formation from uninucleat microspores in isolated microspore culture.

Basal medium	No. of petridishes showing	
	Cell division[z]	Embryo formation[y]
B5	3	2
NLN	2	0
1/2MS	3	2
1/2MS amino acids[x]	0	0

[z] Number of petridishes showing cell division in microspores; n=5.
[y] Number of petridishes having heartshaped embryos after 3 months.
[x] A half strength of MS medium supplemented with 800 mg/l glutamine and 100 mg/l serine.

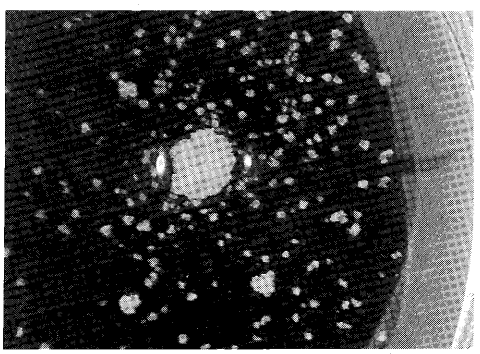

Fig. 6. Colonies and calli formation from microspores in isolated microspore culture.

but they did not develop into calli. Cell division and colony formation in microspores of 'Cornell 19' were not observed on any medium.

Discussion

This is the first report on the production of calli and plantlets of celery by anther culture and embryo and callus formation by isolated microspore culture. In anther culture, anther walls or connecting tissues did not form callus, whereas tetrad microspores grew to an uninucleate stage. Although the chromosome number of plants obtained by anther culture was not counted, we believe that calli were derived from microspores and not from somatic tissues. Roots of plants regenerated by anther culture scarcely grew after transfer to vermiculite or soil; some plantlets which did not root flowered *in vitro* at 25 °C without vernalization. Therefore, observation of the chromosome numbers in root tips was very difficult. A few plants rooted, but the growth of the roots was slow. Methods of acclimatization in regenerated celery plantlets need to be studied.

A specific character in haploid plant formation of celery is that the embryogenesis and callus formation were induced only in very immature microspores just as in carrot, fennel, and *Cryptotaenia japonica* (mitsuba) (Matsubara et al., 1995). In most plant species, embryogenesis was inducible in the uninucleate microspores developed *in vivo* (Bajaj, 1983), but it was induced from tetrad microspores cultures *in vitro* in *Umbelliferae* family including celery. Tetrad microspores matured to uninucleate microspores *in vitro,* then formed embryoids or calli; the stimulus for cell division is not clear. In microspore culture of celery, cell division occurred directly in uninucleate microspores as in carrot and in more mature microspores.

The composition of the media and concentrations of phytohormones are important factors leading to embryoid and callus formation from anthers but also for subsequent regeneration of plantlets. Microspore embryogenesis of celery can be induced on the media with lower concentrations of minerals than MS, such as B5 and 1/2 MS and supplemented with 0.5 or 1.0 mg/*l* 2, 4-D; BA was ineffective. Celery anther required only 2, 4-D for androgenesis as in carrot, fennel, and mitsuba (Matsubara et al., 1995).

In microspore culture, B5 and 1/2 MS media were effective as basal media; microspores required 2, 4-D and BA for androgenesis. Note that colony and callus formation was affected with a ratio between NO_3 and NH_4 in a given molarity, 60 mM. The most effective ratio of NH_4 and NO_3 was 1 : 3, and those of 1/2 MS and B5 media which are useful for microspore culture of other plants were 1 : 2 and 1 : 12, respectively. However, these media were ineffective for androgenesis in celery.

For regeneration of embryoids from calli, or plantlets from embryoids, cultures should be transplanted to MS medium without phytohormones. The frequencies of plant regeneration and survival of celery plants after acclimatization were very low, because roots did not develop well as did those of carrot.

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葯培養と花粉培養によるセルリ花粉からのカルスと胚様体の形成

銅谷徳夫・松原幸子・村上謙治

岡山大学農学部　700　岡山県岡山市津島中 1-1-1

摘　要

セルリの半数体育成のために，葯培養と花粉培養を試みた．供試品種として‘コーネル 619’と‘コーネル 19’を用いた．

若い 3 段階の令の花粉を含む葯を，1/2 濃度の MS と B 5 培地に 2，4-D と BA，3 g·liter^{-1} ショ糖，0.2 g·liter^{-1} ゲルライトを添加した培地に 5 月から 6 月にかけて植え付けた．

‘コーネル 19’では 4 分子期の花粉から，0.5 または 1.0 mg·liter^{-1} 2，4-D を添加した 1/2 MS または 1.0 mg·liter^{-1} 2，4-D を添加した B 5 培地でカルスを形成した．‘コーネル 619’では 4 分子期の花粉から 0.5 と 1.0 mg·liter^{-1} 2，4-D を添加した B 5 培地で，1 核期前後の花粉から 1.0 mg·liter^{-1} 2，4-D を添加した B 5 培地上でカルスを形成した．これらのカルスを NAA と BA を添加または無添加の MS 培地で培養することにより，不定胚を再生した．同じ組成のホルモンフリー培地へ移植することにより 40 日から 60 日後に植物体に生育した．

種々の令の花粉を，2.5×10^3/m*l* 培地の密度で，B 5，NLN，1/2 MS 液体培地で培養した．

‘コーネル 619’では 1 核期前期の花粉からコロニーが形成されたが，B 5 および 1/2 MS 培地でのコロニーからのみ心臓型胚が再生したがそれ以上には生長しなかった．‘コーネル 19’では花粉分裂がみられなかった．