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Embryoid and Callus Formation from Microspores by Anther Culture of Pepper (*Capsicum annuum* L.)

JO, Man Hyun* · MATSUBARA, Sachiko[1] · KANG, Tae Jin · LEE, Eun Mo · WOO, In Sik

Chungchongnam-Do Agricultural Research and Extension Services, Taejon, 305-345, Korea: and
[1]Faculty of Agriculture, Okayama Universty, Tsushima, Okayama, 700, Japan *Corresponding author.

고추의 약배양에 의한 캘러스 및 배상체형성

조만현* · 松原幸子[1] · 강태진 · 이은모 · 우인식
충청남도 농업기술원, [1]일본 오카야마대학 농학부

Anthers contanining uninucleate microspores of eight cultivars of pepper were cultured on MS medium supplemented with 0.004 mg/L 2,4-D and 0.1 mg/L kinetin, 3% sucrose and 0.2% Gelrite, kept at 35° C for 24 h, and then cultured at 25° C with a photoperiod of 16 h daylight for 40 days. Frequency of embryoid and callus formation was varied with cultivars. Embryoid formation was found in Cheongyang and Fushimi Amanaga, while callus formation was in California Wonder, Fushimi Amanaga and Geoseong. In anther culture medium supplemented with 1% activated charcoal, embryoid formation was found with 0.5% of frequency only in Cheongyang, while no callus formation was found. In 1/2MS medium, frequency of embryoid formation in Shishitou, Yatsufusa and Taka no Tsume was 1.2%, 0.4% and 0.4%, respectively. On the other hand, in 1/2 B5 medium, frequency of callus formation in Yatsufusa and Taka no Tsume was 2.8% and 2.7%, respectively. Embryoids transferred to hormone-free MS medium were developed to plantlets and acclimatized. The number of chromosomes in the root tip cells of the haploid plant was 2n=x=12 in cv. Cheongyang.

Key words: anther culture, callus, embryoid, haploid, pepper

Anther culture is usually used for production of haploid plants from microspores and is utilized for a rapid large scale production of homozygous lines from which superior lines for hybrid seed production might be selected. Ever since the first report was made by Guha and Maheshiwari(1964) on pollen embryogenesis by anther culture of *Datura innoxia*, the production of haploids in *Capsicum annuum* has been reported by many researchers(Dumas de Vaulx et al., 1981: Harn et al., 1975: Matsubara et al., 1992, 1998: Sakata et al., 1991: Wang et al., 1973: Eun et al., 1994).

Recently, it has been reported that numerous factors including donor plant environment affect microspore embryogenesis(Bajaj, 1990). Temperature and photoperiod (Kristansen and Andersen, 1993), thermal shock(Sakata et al., 1991), and differences among cultivars(Sakata et al., 1991) have been found to influence anther culture response in pepper. In addition, activated charcoal was also used to enhance plant production in cucumber(Lou and Kakao, 1994).

The purpose of the present investigation was to study effects of activated charcoal and different media on microspore embryogenesis, callus formation and different responses to culture among cultivars in pepper.

MATERIALS AND METHODS

Plant materials

Pepper(*Capsicum annuum* L.) native varieties, California Wonder, Fushimi Amanaga, Shishitou, Yatsufusa, Taka no

Tsume and Korean F1 cultivars such as Cheongyang, Sinhong and Geoseong grown in the field of Okayama University in Japan from July to September in 1993 and 1995 were used for anther culture.

Flower buds sterilization

Flower buds were collected when the anther contained microspores at the mid- to late-uninucleate stages. The buds were soaked in 70% ethanol for 30 seconds. Subsequently, they were sterilized for 15 min with 15% sodium hypochlorite containing two drops of Tween 20 as a spreader followed by rinsing three times with sterile distilled water.

Culture media for anther culture

MS medium(Murashige and Skoog, 1962) was supplemented with 0.004 mg/L 2,4-D, 0.1 mg/L kinetin, 3% sucrose, adjusted to pH 5.8, and solidified with 0.2% Gelrite. Media autoclaved at 1.2 kg/cm^2, 120°C for 15 min were dispensed in 5 mL aliquots into petri dishes (ø 60×15 mm), and the petri dishes were sealed with parafilm in clean bench. Hormone-free MS medium was composed of MS basal medium supplemented with 3% sucrose, which was adjusted to pH 5.8, and solidified with 0.2% Gelrite. Ten mL of medium was dispensed into the test tube of 2.5 cm diameter and 12 cm length, and autoclaved at 120°C for 15 min. B5 medium was diluted to half-strength of original concentration of inorganic macroelements.

High temperature treatments

The cultures were then incubated at 35°C for 24 h under dark condition, followed by incubation at 25°C under 16 h daylight with 20 μmol · m^{-2} · s^{-1} of fluorescent light for 40 days.

Plant regeneration from embryoids

The cotyledonary embryos grown from the embryoids were transferred to hormone-free MS medium under the same conditions as the anther culture.

Plant growth

Regenerated plantlets were transferred to Magenta vessels containing a mixture of sterilized vermiculite supplemented with one-fourth strength of MS inorganic salts and acclimatized under 16 h photoperiod at 25°C, 20 μmol · m^{-2} · s^{-1} of fluorescent light. The plantlets were covered with a vinyl film for the first 30 days, after which they were transferred to 9 cm diameter vinyl pots. Plants were transferred to pots containing a mixture of compost : sand (2 : 1, v/v) and grown in a glasshouse.

Number of chromosomes

Plants were used for observation of chromosomes after growing in the glasshouse for one month. Root tips were excised from plants and placed in 0.002 M 8-hydroxyquinoline for 3 h, followed by fixation in FAA solution (formalin: acetic acid: 50% ethanol = 5: 5: 90, v/v/v) and stored in refrigerator at 4°C until observation.

Root tips were washed in distilled water and hydrolyzed in 1 N-HCl for 10 min and stained with orcein acetic acid solution. Chromosomes at intact metaphase stage were counted under a light microscope by squash method.

RESULTS

Embryoid and callus formation from microspores of eight cultivars in pepper

The results of embryoid and callus formation are shown in Table 1. Embryoids derived from microspores in opened anthers were developed into cotyledonary embryoids after 40 days (Figure 1A), while callus was formed from the microspores, filaments and anther walls, and its growth was slower and less vigorous in the microspore than those in the latter transparent white(Figure 1B). In Table 1, calli formed from microspores were selected by origin and colour. The

Table 1. Callus, embryoid and plant formation from microspores of different pepper cultivars harvested in July 1993.

Cultivar	No. of anthers cultured	No. of calli formed	No. of embryoids formed	No. of plants acclimatized
California Wonder	114	3(2.6%)[a]	0	0
Fushimi Amanaga	139	1(0.7%)	1(0.7%)[a]	1(100%)[b]
Shishitou	196	0	0	0
Yatsufusa	142	0	0	0
Taka no Tsume	158	0	0	0
Cheongyang	113	0	2(1.8%)	2(100%)
Sinhong	128	0	0	0
Geoseong	145	1(0.7%)	0	0

[a]Percentage of calli or embryoids formed from anthers.
[b]Percentage of plants derived from embryoids.

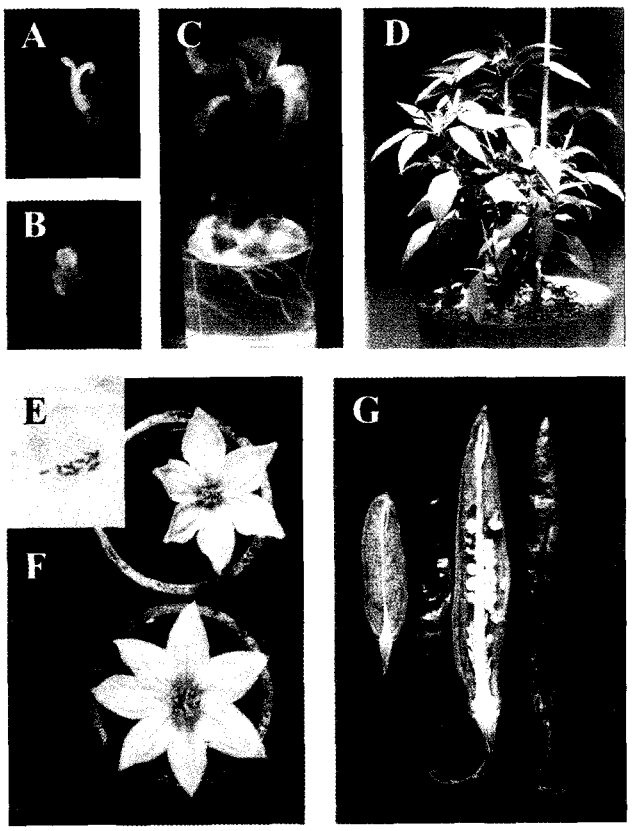

Figure 1. Plant regeneration by anther culture of *Capsicum annuum* L. A: Embryoid derived from anther of cv. Cheongyang: B: Callus derived from anther of cv. Cheongyang: C: Haploid plants of cv. Taka no Tsume derived from embryoids after transferring to hormone-free MS medium: D: Haploid plants of cv. Taka no Tsume in pot: E: Chromosomes from root tip cell of haploid plants of cv. Cheongyang(2n=x=12): F: Flowers of a diploid(lower) and haploid plant(upper) of cv. Cheongyang: G: Parthenocarpic fruit of haploid plant(left) and normal fruit of diploid plant(right) of cv. Cheongyang.

percentage of calli formed from anthers in California Wonder, Fushimi Amanaga and Geoseong was 2.6%, 0.7% and 0.7%, respectively. However, when the calli were transferred to the media prepared for induction of plantlets, no signs of organogenesis were observed in four weeks after the transfer. On the other hand, the percentage of embryoid formed from anthers in Fushimi Amanaga and Cheongyang was 0.7% and 1.8%, respectively. The embryoids were transferred to hormone-free MS medium. Four weeks later, embryoids grew and rooted to become plantlets

Effect of activated charcoal on embryoid and callus formation

The effects of activated charcoal on callus and embryoid formed from anther of both Fushimi Amanaga and Cheongyang are shown in Table 2. In Fushimi Amanaga, the percentage of calli formed from control and anther culture basal medium supplemented with activated charcoal 1% was 10.0% and 1.0%, respectively. However, most of calli had not developed further, moreover, no embryoid formation was found. In Cheongyang, the percentage of embryoid formed from control and anther culture basal medium supplemented with 1% activated charcoal was 0 and 0.5%, respectively. The anthers containing aborted embryoids had not developed further. In addition, no callus was formed.

Effect of media on embryoid and callus formation in different cultivars

Effect of different media on callus and embryoids from anthers is shown in Table 3. In 1/2MS, the frequency of embryoid formation in Shishitou, Yatsufusa and Taka no Tsume was 1.2%, 0.4% and 0.4%, respectively. However, the frequency of callus formation was 2.7% in Shshitou and 1.3% in Yatsufusa. In B5 medium, the frequency of embryoid formation was found only in Shishitou by 0.7%. While, callus was not found in any cultivars. In 1/2 B5, the frequency of callus formation in Shishitou, Yatsufusa and Taka no Tsume was 0, 2.8% and 2.7%, respectively. On the other hand, embryoid was not formed at all.

Chromosome number and morphogenesis of plants from embryoids

The embryoids transferred to hormone-free MS medium were regenerated into plantlets. All embryoids were developed to plantlets and acclimatized successfully. After they were

Table 2. Effect of activated charcoal on callus and embryoid formation from anthers of different pepper cultivars harvested in August, 1993.

Cultivar	Activated charcoal (mg/L)	No. of anthers cultured	No. of calli formed	No. of embryoids formed
Fushimi Amanaga	0	209	21(10%)[a]	0
	1	200	2(1.0%)	0
Cheongyang	0	215	0	0
	1	195	0	1(0.5%)[a]

[a]Percentage of calli or embryoids formed from anthers.

Table 3. Effect of media on callus and embryoid formation from anthers of different cultivars of pepper harvested in September 1995.

Media	Cultivar	No. of anthers cultured	No. of calli formed	No. of embryoids formed	No. of plants acclimatized
1/2MS[a]	Shishitou	339	9(2.7%)[c]	4(1.2%)[c]	4(100%)[d]
	Yatsufusa	238	3(1.3%)	1(0.4%)	1(100%)
	Taka no Tsume	242	0	1(0.4%)	1(100%)
B5	Shishitou	135	0	1(0.7%)	1(100%)
	Yatsufusa	90	0	0	0
	Taka no Tsumel	125	0	0	0
1/2B5[b]	Shishitou	144	0	0	0
	Yatsufusa	143	4(2.8%)	0	0
	Taka no Tsume	147	4(2.7%)	0	0

[a]Inorganic macroelements decreased in MS medium to half-strength.

[b]Inorganic macroelements decreased in B5 medium to half-strength.

[c]Percentage of plants derived from embryoids.

[d]Percentage of calli or embryoids formed from anthers.

transplanted into pots(Figure 1D), all of the plantlets grew normally. The number of chromosomes in root tip cells of the haploid plant regenerated from cv. Cheongyang was 12(Figure 1E). The size of flowers and fruits of the haploids was smaller than those of the diploids. Furthermore, the fruits from haploid plants did not produce any normal seeds(Figure 1F, 1G).

DISCUSSION

An earlier reports on anther culture noted that the elevated temperature treatments at 35°C during the initiation period of anther culture in *C. annuum* resulted in stimulation of the androgenic response(Dumas de Vaulx et al., 1981: Hu et al., 1993). Low temperature treatment such as 4°C also stimulated the androgenesis in pepper(Sakata et al., 1991). However, only high temperature treatment was effective for embryogenesis in anther culture (Matsubara et al., 1992). The best anther culture response was obtained when average temperature was from 16°C to 28°C(Matsubara et al., 1998). Approximately 83% of the regenerated plants were haploids, whereas the remaining plants were diploids with a few aneuploids(Matsubara et al., 1998). Although no supporting evidence was presented here, the diploids may have been derived from haploid microspores which underwent a doubling process during anther culture. Such a process has been found in *Brassica* species(Keller et al., 1975: Keller and Armstrong, 1977).

The cytological test of embryoid-derived plants showed that most of embryoid-derived plants were haploids (data not shown). Ernst(1974) reported that activated charcoal stimulated the growth of orchids. However, it was decreased and/or no callus was formed, and only 0.5% embryoid was formed in Cheongyang in present study. For a number of species, a positive correlation of optimal temperature between plant growth and embryo formation from cultured anther has been indicated(Dunwell et al., 1985: Gaillard et al., 1991). The calli derived from the connective, filament or inner somatic tissue of the anther started to grow very early and grew vigorously. Haploid callus and embryoid were emerged from the anther locule. Their microspore-origin was ontogenetically confirmed(Harn et al., 1975). The present study is in agreement with the previous report that the efficiency of embryogenesis depending on the genotypes was varied from 0.8% to 12.0% in cultured anthers(Eun et al., 1994). There was a little intervarietal differences on the frequency of embryoid formation.

Our present study accords well with reports that embryoids were induced more frequently when the anthers were at late uninucleate stage. Frequency of embryoid formation among cultivars varied, especially in embryoid formation from the anthers in Fushimi Amanaga and Cheongyang compared to the other cultivars. According to Matsubara et al.(1998), embryoid and callus formation was obtained in all cultivars, however, the frequency of embryoid formation was higher in Cheongyang and Fushimi Amanaga. This result accords well with our present study.

적 요

고추(*Capsicum annuum* L.) 8품종을 공시하고, 1핵기의 화분을 포함한 약을 0.004 mg/L 2,4-D, 0.1 mg/L kinetin, 3%

sucrose 그리고 0.2% Gelrite를 첨가한 MS배지에 치상하고, 35°C에서 24시간처리 후, 25°C 16시간 조명에서 40일간 배양하였다.

캘러스와 배상체형성율은 품종에 따라 다양하였다. 배상체는 'Cheongyang'과 'Fushimi Amanaga'에서 형성되었고, 반면에 캘러스는 'California Wonder', 'Fushimi Amanaga' 그리고 'Geoseong'에서 형성되었다. 활성탄 1%를 첨가한 배지에서의 배상체는 'Cheongyang'만 0.5% 형성되었고, 반면에 캘러스는 형성되지 않았다. 1/2MS배지에서 배상체는 'Shishitou', 'Yatsufusa' 그리고 'Taka no Tsume'가 1.2%, 0.4% 그리고 0.4% 각각 형성되었지만, 1/2 B5배지의 캘러스는 'Yatsufusa' 그리고 'Taka no Tsume'가 2.8% 그리고 2.7% 각각 형성되었다. 배상체는 MS배지로 이식하여 생장하였다. 'Cheongyang'의 근단 염색체수는 2n=x=12의 반수체이었다.

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