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# Embryoid and Callus Formation from Microspores by Anther Culture from July to November in Pepper (*Capsicum annuum* L.)

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Embryoid and callus were regenerated from microspores of six cultivars of pepper (*Capsicum annuum* L.) by culture of anthers obtained from July to November.

Anthers containing microspores at the uninucleate stage were planted in MS media supplemented with 0.004 mg · liter$^{-1}$ 2,4-D, 0.1 mg · liter$^{-1}$ kinetin, 30 g · liter$^{-1}$ sucrose and 2 g · liter$^{-1}$ Gelrite, kept at 35 ℃ for 24 h, and then incubated at 25 ℃ under 16 h daylength for 40 days.

Frequency of embryoid and callus formation varied with cultivars and period of initiation. Embryoid formation was more effective from September to October. A temperature of between 15 to 25 ℃ in the months of September and October was effective for higher embryoid formation. Even though embryoid and callus formation was obtained in all cultivars, the frequency of embryoid formation was higher in Cheongyang and Fushimi Amanaga, while callus formation was higher in Shishitou and California Wonder.

Embryoid transferred to MS medium developed to plantlets, and were acclimatized. The number of chromosomes in the root tip cells was 12 (haploid) in 19 plants, 24 (diploid) in one plant and 20 to 23 (aneuploid) in 3 plants.

Key words : pepper, anther culture, embryoid, haploid

## Introduction

Several advantages in plant breeding are given with haploid plants. These include the rapid production of homozygous lines as well as the possibility of the detection of recessive mutations. Ever since the first report was made by Guha and Maheshwari[3] on pollen embryogenesis by anther culture of *Datura innoxia*, numerous attempts have been made to produce haploid plants in many plant species[1]. The production of haploids in *Capsicum annuum* has been reported by many researchers[2,4,9,12]. Recently, it was reported that numerous factors including environment and donor plant characteristics affected embryogenesis from microspores[1]. Temperature and photoperiod[8], thermal shock[12], and differences among cultivars[12] have been found to influence anther culture response in pepper. Interactions between the genotypes and environment of donor plant have also been documented[6]. Furthermore, a higher androgenic capability of microspores from young plants compared to older plants has been stated as a general phenomenon[11].

In the present paper, we report on the variation in the production of haploid embryoids, calli and plants during different months of the year in

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some Japanese and Korean $F_1$ cultivars.

## Materials and Methods

Anthers of pepper (*Capsicum annuum* L.) cvs. California Wonder (CW), Fushimi Amanaga, Shishitou, Taka no Tsume and Korean $F_1$ Cheongyang grown in the field of Okayama University and whose buds developed during the period from July to November, 1994 were used as explants. Flower buds were collected at the middle of each month when the anthers contained microspores at the mid- to late-uninucleate stages. The buds were then surface-sterilized for 10 sec in 70 % ethanol, immersed in sodium hypochlorite (1 % of available Cl) for 10 min, and rinsed three times with sterilized distilled water. Two different types of basal media were used. In 1994, the basal medium consisted of Murashige and Skoog (1962) (MS)[10] supplemented with 30 g · liter$^{-1}$ sucrose, 2 g · liter$^{-1}$ Gelrite, 0.004 mg · liter$^{-1}$ 2,4-D and 0.1 mg · liter$^{-1}$ kinetin. After adjusting the media pH to 5.8, they were then sterilized in an autoclave for 15 min at 120 ℃. During culturing, about 6 to 9 anthers were planted on 5 ml of the medium in a petri dish (60×15 mm), and sealed with paraffin membrane. The cultures were then incubated at 35 ℃ for 24 hr under dark conditions, followed by incubation at 25 ℃ under 16 hr daylength provided with 20 $\mu$mol/s/m$^2$ of fluorescent light for 40 days.

The cotyledonary embryos grown from the embryoids were transferred to MS medium solidified with 2 g · liter$^{-1}$ Gelrite under the same conditions as with the anther culture. Regenerated plants were grown in pots packed with vermiculite for about a month before being transferred to soil in a plastic house. The ploidy level of the regenerated plants was estimated by examining chromosome numbers in root tips. Fruits and seeds obtained from the regenerated plants were also observed.

## Results

The results of embryoid and callus formation are shown in Table 1. Embryoids regenerated from microspores in opened anthers (Fig. 1) and developed into heart-shaped and torpedo embryos after 40 days. While callus formed from both the microspores and/or filaments and anther walls, its growth was slower and less vigorous in the former than the latter, and it was transparent white (Fig. 2). In Table 1, calli from microspores were selected by origin and colour.

In the anthers planted in July, the frequency of embryoid formation was 1 % only in Cheonyang. However, these embryoids did not develop into plantlets. During the same period, the frequency of callus formation in CW and Cheongyang was 2 % and 4 %, respectively. In August, embryoid formation from the anthers in Taka no Tsume was 1.6 %, while in Fushimi Amanaga and Cheongyang, the percentage was 2.1 %. A single embryoid regenerated from an anther, and some of embryos developed into plants. The frequency of callus formation was 0.9 % in CW and 5.2 % in Shishitou, 2.7 % in Fushimi Amanaga, and 7.4 % in Cheonyang. More than two embryoids regenerated from an anther in Shishitou and Cheonyang. Most of these embryos rooted, and developed into plants (Fig. 3). Thus, anthers obtained in September may be good explants for embryoid production. On the other hand, callus formation was only observed in Shishitou. In October as well as September, high frequency of embryoid formation was obtained in 3 cultivars such as Fushimi Amanaga (4.5 %), Taka no Tsume (0.8 %), and Cheongyang (6.1 %). At the same time, 3 embryoids from one anther regenerated in Cheongyang. These embryos developed into plants at high frequency. Therefore, anthers obtained in October were also good explant for embryoid formation. Calli formed from 0.8 % of anthers in Fushimi Amanaga and Taka no Tsume and 2.7 % of anthers in CW. In November, plants producing

Table 1 Embryoid and callus formation from microspores by anther culture in every month

| Cultivar | No. of anthers | | | No. of plants |
|---|---|---|---|---|
| | planted | formed callus | formed embryoid | acclimatized[a] |
| | | July | | |
| California Wonder | 50 | 1 (2.0%)[b] | 0 | 0 |
| Fushimi Amanaga | 69 | 0 | 0 | 0 |
| Shishitou | 103 | 0 | 0 | 0 |
| Yatsufusa | 129 | 0 | 0 | 0 |
| Taka no Tsume | 82 | 0 | 0 | 0 |
| Cheongyang | 100 | 4 (4.0%) | 1 (1.0%) | 0 |
| | | August | | |
| California Wonder | 110 | 1 (0.9%) | 0 | 0 |
| Fushimi Amanaga | 97 | 0 | 2 (2.1%) | 2 (2.1%) |
| Shishitou | 115 | 6 (5.2%) | 0 | 0 |
| Yatsufusa | 113 | 0 | 0 | 0 |
| Taka no Tsume | 124 | 0 | 2 (1.6%) | 1 (0.8%) |
| Cheongyang | 97 | 0 | 2 (2.1%) | 1 (1.0%) |
| | | September | | |
| California Wonder | 117 | 0 | 3 | 2 (1.7%) |
| Fushimi Amanaga | 146 | 0 | 4 | 1 (0.7%) |
| Shishitou | 77 | 4 (5.2%) | 2 | 2 (2.6%) |
| Yatsufusa | 156 | 0 | 0 | 0 |
| Taka no Tsume | 151 | 0 | 0 | 0 |
| Cheongyang | 121 | 0 | 9 | 4 (3.3%) |
| | | October | | |
| California Wonder | 37 | 1 (2.7%) | 0 | 0 |
| Fushimi Amanaga | 133 | 1 (0.8%) | 6 | 3 (2.3%) |
| Yatsufusa | 123 | 0 | 0 | 0 |
| Taka no Tsume | 124 | 1 (0.8%) | 1 | 1 (0.8%) |
| Cheongyang | 131 | 0 | 8 | 4 (3.1%) |
| | | November | | |
| California Wonder | 12 | 3 (25.0%) | 0 | 0 |
| Shishitou | 115 | 0 | 0 | 0 |
| Cheongyang | 124 | 0 | 5 | 2 (1.6%) |

[a] Number of plants grown from embryoids.

[b] Percentage of anthers forming calli or embryoids to all anthers planted.

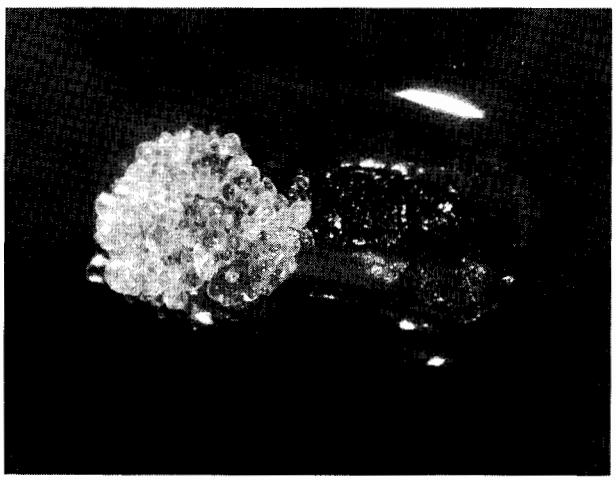

Fig. 1 Callus formed from cv. Shishitou anther.

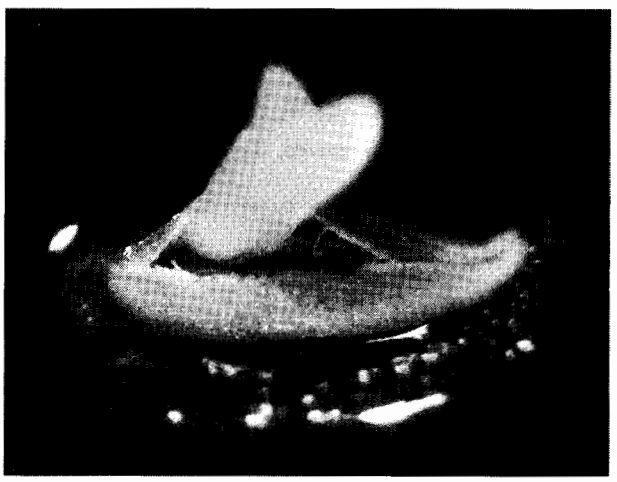

Fig. 2 Embryoid formed from cv. Cheongyang anther.

flower buds decreased, and flower buds were obtained in only CW, Shishitou and Cheongyang. In Cheongyang, however, 4 % of anthers regenerated embryoids, and 2 embryoids regenerated from one anther, while 25 % of the anthers produced calli.

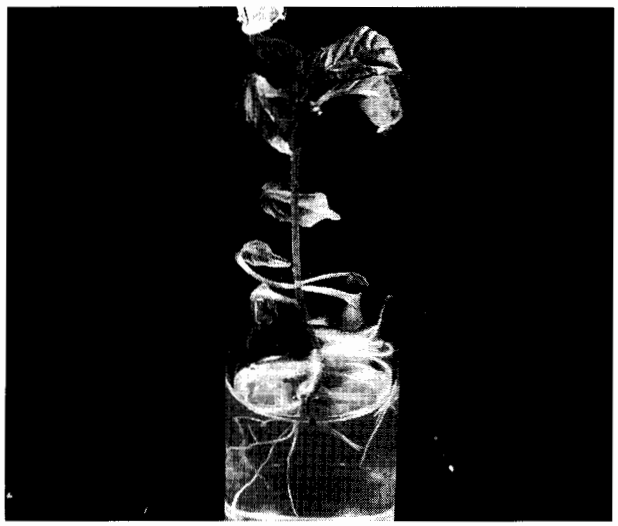

Fig. 3 Plantlet grown from embryoid after transferring to MS medium in cv Cheongyang.

The results of the embryoid and callus formation of the cultivars for 1994 are summarized in Table 2. All cultivars except Yatsufusa formed embryoids and calli. The highest frequency of embryo formation was obtained Cheongyang through the period from July to November, followed by Fushimi Amanaga.

The embryoids which were transferred to MS medium without phytohormones regenerated shoots and some of them rooted. After acclimatization of the regenerated plantlets in plastic boxes, followed by transplanting into pots, all of the plantlets grew normally. Haploid numbers of chromosomes (2n=x=12) (Fig. 4) were observed in the root tip cells of 19 plants regenerated from 19 embryoids including 5 cultivars, while diploids (2n=24) and aneuploids (2n=20~23) were observed in one and three plants, respectively.

The flowers and fruits of the haploids were smaller than those of the diploids. In addition, the

Table 2 Embryoid and callus formation from microspores by anther culture in sixteen cultivars of pepper

| Cultivar | No. of anthers | | | |
|---|---|---|---|---|
| | planted | formed callus | formed embryoid | plants grown[a)] |
| California Wonder | 326 | 6 (1.8%)[b)] | 3 (0.9%) | 2 (0.6%) |
| Fushimi Amanaga | 445 | 1 (0.2%) | 12 (2.6%) | 6 (1.3%) |
| Shishitou | 410 | 10 (2.4%) | 2 (0.5%) | 2 (0.5%) |
| Yatsufusa | 521 | 0 | 0 | 0 |
| Taka no Tsume | 481 | 1 (0.2%) | 3 (0.6%) | 2 (0.4%) |
| Cheongyang | 573 | 4 (0.7%) | 25 (1.0%) | 11 (1.9%) |

[a),b)] See Table 1.

Table 3 Number of haploids, diploids and aneuploids of plants developed from embryoids regenerated by anther culture

| Cultivar | No. plants | | | |
|---|---|---|---|---|
| | observed | of haploid | of diploid | of aneuploid[a)] |
| California Wonder | 2 | 1 | 0 | 1 |
| Fushimi Amanaga | 6 | 5 | 0 | 1 |
| Shishitou | 2 | 2 | 0 | 0 |
| Taka no Tsume | 2 | 1 | 1 | 0 |
| Cheongyang | 11 | 10 | 0 | 1 |

[a)] Chromosome number of aneuploids is from 20 to 23 in root tips.

fruits from haploid plants did not produce any normal seeds (Fig. 5).

## Discussion

The highest anther culture response was obtained at 23~28 ℃ in September, followed by October at 16~21 ℃. Thus the temperature in October must have been cooler than the optimum temperature required for vigorous growth, which agrees with the report on eggplant[13].

An earlier report on anther culture has noted that the elevated temperature treatment during the initiation period of *C. annuum* anther culture resulted in stimulation of the androgenic response[2,5]. On the other hand, low temperature treatment such as 4 ℃ stimulated the androgenesis in pepper[12]. From our previous report[9], only high temperature treatment was effective for embryogenesis in anther culture.

Fig. 4 Chromosomes in root tip of haploid plant in cv. Cheongyang.

In order to obtain better growth of the plantlets from embryoids, cultures should be transferred to MS medium without phytohormones. The survival of the plants after acclimatization was relatively high in pepper, and acclimatization was easy. Approximately 80 % of regenerates were haploids, whereas the remaining plants were diploid and aneuploids. Although supporting evidence was not presented here, the diploid may be derived from haploid microspores which may have undergone a doubling process during anther culture. Such a process has been reported for *Brassica* species[7].

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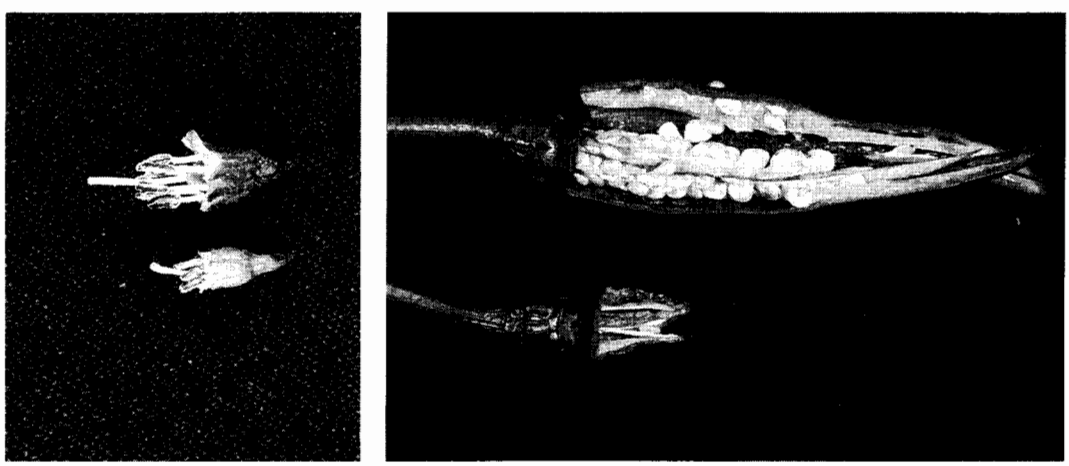

Fig. 5 Flower organs, petals removed (left) and fruits (right). Diploid plant (upper) and haploid plant (lower) in cv. Cheongyang. Fruit length of diploid and haploid; 7.5 cm and 2.3 cm.

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# トウガラシの7月から11月にかけて得られた葯の培養によるカルスおよび胚様体形成

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トウガラシ（*Capsicum annuum* L.）6品種を供試し，7月から11月にかけて葯培養を行った．1核期の花粉を含む葯を，0.004mg・liter$^{-1}$2,4-D，0.1mg・liter$^{-1}$ kinetin，30g・liter$^{-1}$ sucroseおよび2g・liter$^{-1}$ Gelriteを添加したMS培地に植え付け，35℃で24時間処理後，25℃16時間照明で40日間培養した．

カルスおよび胚様体形成率は，品種および葯の採取時期により異なった．胚様体形成率は，平均気温が15から25℃である9月と10月が最も高かった．カルスや胚様体はいずれの品種でも形成されたが，胚様体形成率は‘伏見甘長’と韓国の品種の‘Cheongyang’が高く，カルス形成率は‘シシトウ’と‘カリフォルニアワンダー’が高かった．

形成した胚様体をMS培地に移植すると，植物体に生長した．根端の染色体数は，調査した23個体のうち19個体が2n＝x＝12の半数体で，1個体が2n＝2x＝24の2倍体，3個体が20から23の染色体数で，異数体と考えられた．