# ハクサイの葯培養による半数体の育成



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# Production of Haploid Plants via Microspore Embryogenesis in Chinese cabbage Anther Culture

Etsuo MATSUMOTO, Motohiro FUJIMORI and Fusao ŌTANI

## Summary

Some factors affecting microspore embryogenesis in anther culture of chinese cabbage *(Brassica campestris)* were investigated.

Embryoid formation was observed in case of culturing anthers with early uninucleate microspores. Auxin was found to be a key component of the anther culture medium for induction of embryoids. Higher frequency of embryogenesis was obtained in the medium containing 1.0mg/lNAA or 1.0mg/lNAA, 1.0mg/l 2,4-D, 0.1mg/l kinetin. Sugar had a great effect on microspore embryogenesis. Depression of callus formation and stimulation of embryoid production were observed at high sugar concentrations. More than 10% of the anthers cultured produced embryoids at 0.2-0.3M gulcose. Almost the same frequency of embryogenesis was observed in dark and in the light.

All the embryoids were transferred to the embryo culture medium with low sucrose level, but a number of embryoids showed abnormal growth. Frequency of haploids among regenerates was estimated to be 64%. Chromosome number of root tip cells of some plants showed haploidy, 2n=10.

Anther culture can be now utilized as a practical breeding tool in the breeding program of chinese cabbage.

## Introduction

Haploid can be beneficially utilized in the breeding program. First, it shortens the breeding cycle because homozygous diploids can be rapidly obtained by chromosome doubling. Secondly, it facilitates the detection of recessive genes, which is particularly favorable in mutation breeding.

Anther culture is an effective method for induction of haploids. A number of reports on the occurrence of haploids by anther culture have been published in recent years. In *Brassica* species anther culture has been drastically developed since 1975 when pollen embryogenesis was first reported by Keller et al. (2). Haploid plant production from cultured anthers has been reported in *Brassica napus* (4,10,11,13,15), *B. campestris* (5), *B. oleracea* (6, 7), *B. juncea* (1, 12) and *B. hirta* (9). Various factors, such as growth conditions of donor plants, donor genotype, developmental stage of the pollen, culture medium composition, physical culture conditions, have been shown to influence androgenesis in these reports. Enhancement of haploid formation efficiency has made the haploid method of breeding practicable.

We have been trying to breed the disease-resistant variety of chinese cabbage *(B. campestris)* using the haploid method. In this study some factors, pollen developmental stage, culture medium, and environmental culture conditions, affecting microspore embryogenesis in anther culture of chinese ca-

bbage were investigated.

## Materials and Methods

The materials used in this study were two clubroot resistace lines developed at this station. The donor plants were grown in the green house under natural conditions. Unopened floral buds of appropriate size were selected from main and side inflorescences prior to flower emergence. Surface sterilization of collected buds was achieved in 7% (w/v) calcium hypochlorite solution for 10 minutes, followed by rinsing with sterile distilled water. Anthers were aseptically detached from filaments and were plated on solidified culture media.

The basic composition of the culture medium was that of Keller et al. (2). The basal medium was supplemented with 800mg/l L-glutamine, 100mg/l L-serine which were beneficially used in *B. campestris* and *B. napus* anther cultures (2, 3). Four kinds of sugars (sucrose, glucose, fructose, mannitol) were tested at various concentrations. Growth substances employed were 2, 4-dichlorophenoxyacetic acid (2, 4-D), α-naphthaleneacetic acid (NAA) and kinetin.

Cultured anthers were incubated at 35℃ in darkness for 1 day and were then transferred to 25℃, also in darkness except for an experiment on light effect. Anthers producing visible embryoids were kept in the same medium in light for several days. Embryoids excised from anthers were transferred to LS medium and were cultured under the light condition of 3,000 lux, 16h photoperiod.

The regenerated plants were potted in vermiculite and then transplanted to soil.

The ploidy level of the regenerates was examined in root tip cells. Root tips were fixed in 3 : 1 (ethanol : acetic acid) and then stained with acetoorcein.

## Results

Embryoid emergence was generally detected after 2-3 weeks of culture. Embryoids of various developmental forms, globular, heart, torpedo stage, and mature structure with developed cotyledons breaked through anther walls (Fig. 1). Well developed embryoids often showed trumpet-like structure with fused cotyledons. Usually one or a few embryoids were formed per anther, but twenty or more embryoids were occasionally observed.

Pollen embryogenesis was observed in case of culturing anthers excised from buds of nearly 2 mm length which corresponded to early uninucleate (Table 1). No embryoid was formed in large buds more than 3 mm.

Growth regulator was a key component of anther culture media. Auxin found to be a critical hormone for induction of embryoid. The media of auxin-free and low concentration gave no embryoid. NAA was more effective than 2, 4-D. A good response was obtained in the medium containing 1.0mg/l NAA or 1.0mg/l NAA, 1.0mg/l 2, 4-D, 0.1mg/l kinetin (Table 2). The addition of kinetin at high concentration appeared to have inhibitory effect.

Sugar had a great effect on microspore embyogenesis. The effect of four kinds of sugars was examined (Table 3). Depression of callus formation and stimulation of embryoid production were observed at high sugar concentration. Embryoids were formed in the medium containing sucrose, glucose, and fructose. Higher frequency of embryoid formation was obtained at 0.2-0.3M glucose. Mannitol(sugar alcohol) gave no embryoid. As for sucrose the most effective concentration was found to be nearly 10% (0.29M) (Table 4).

Almost the same frequency of embryogenesis was detected in the dark and in the light, 3,000lux-16h photoperiod (Table 5).

The embryoids failed to grow further if they were maintained on the anther culture medium. So all the embryoids were transferred to the embryo culture medium with low sucrose level. It was difficult for small globular embryoids to develop further. A number of embryoids showed abnormal growth of cotyledon or hypocotyl without the development of shoot apical meristem. About 20-

30% of the transferred embryoids directly developed into plantlets (Fig. 2).

Pollen-derived plants via embryogenesis were grown in the greenhouse (Fig 3). In a population of 36 regenerates, 23 had small, sterile flowers: they were evaluated to be haploid, which was certified by cytological observation (Fig 4). The remainding 13 regenerates were fertile and were considered to be diploid although some tetraploids may have been present (Table 6). Plants grown from self-fertilized seeds of anther-derived diploids were highly uniform in morphological characteristics.

Table 1. The production of embryoids by cultured anthers taken from different size buds.

| Bud length | No. of anthers cultured | No.of anthers producing embryoids | % of anthers producing embryoids | No. of embryoids produced |
|---|---|---|---|---|
| Below 2mm | 100 | 3 | 3.0 | 3 |
| 2mm | 90 | 1 | 1.1 | 1 |
| 3mm | 100 | 0 | 0 | 0 |
| 4mm | 80 | 0 | 0 | 0 |

Pollen developmental stage in each bud size Below 2mm: Tetrad-early uninucleate 2mm: early uninucleate 3mm: late uninucleate 4mm: binucleate

Table 2. Effect of growth regulators on embryogenesis in anther culture of chinese cabbage

| Medium | No.of anthers cultured | No.of anthers producing embryoids | % of anthers producing embryoids | No. of embryoids produced |
|---|---|---|---|---|
| Basal+1.0mg/1 2,4-D | 205 | 20 | 9.8 | 78 |
| Basal+1.0mg/1 NAA | 161 | 31 | 19.3 | 113 |
| Basal+1.0mg/1 2,4-D +1.0mg/1 NAA +0.1mg/1 Kinetin | 190 | 27 | 14.2 | 148 |
| Basal+1.0mg/1 2,4-D +1.0mg/1 NAA +1.0mg/1 Kinetin | 221 | 24 | 10.9 | 48 |

Table 3. Effect of various sugars on embryogenesis in anther culture of chinese cabbage.

| Sugar | No. of anthers cultured | No. of anthers forming callus | No. of anthers producing embryoids | % of anthers producing embryoids | No.of embryoids produced |
|---|---|---|---|---|---|
| 0.2M(6.8%) Sucrose | 105 | 6 | 4 | 3.8 | 19 |
| 0.3M(10.3%) Sucrose | 119 | 4 | 4 | 3.4 | 23 |
| 0.2M(3.6%) Glucose | 81 | 8 | 10 | 12.3 | 42 |
| 0.3M(5.4%) Glucose | 105 | 1 | 11 | 10.5 | 61 |
| 0.4M(7.2%) Glucose | 79 | 0 | 3 | 3.8 | 4 |
| 0.5M(9.0%) Glucose | 103 | 0 | 0 | 0 | 0 |
| 0.3M(5.4%) Fructose | 106 | 3 | 5 | 4.7 | 9 |
| 0.5M(9.0%) Fructose | 116 | 0 | 1 | 0.9 | 11 |
| 0.3M(5.5%) Mannitol | 70 | 0 | 0 | 0 | 0 |
| 0.5M(9.1%) Mannitol | 101 | 0 | 0 | 0 | 0 |

Table 4. Effect of sucrose level on embryogenesis in anther culture of chinese cabbage.

| Sucrose concentration | No. of anthers cultured | No. of anthers producing embryoids | % of anthers producing embryoids | No. of embryoids produced |
|---|---|---|---|---|
| 6% | 300 | 3 | 1.0 | 3 |
| 8% | 302 | 2 | 0.7 | 5 |
| 10% | 301 | 7 | 2.3 | 19 |
| 12% | 300 | 4 | 1.3 | 5 |

Table 5. Effect of light on embryogenesis in anther culture of chinese cabbage.

| Light condition | No.of anthers cultured | No.of anthers producing embryoids | % of anthers producing embryoids | No.of embryoids produced |
|---|---|---|---|---|
| Dark | 158 | 10 | 6.3 | 51 |
| 3,000lux ※ | 130 | 8 | 6.2 | 47 |

※16h photoperiod

Table 6. Ploidy level of anther-derived plant estimated by flower size and fertility.

| Flower size and fertility | Estimated ploidy | No. of plant |
|---|---|---|
| Normal, fertile | Diploid, tetraploid | 13 (36%) |
| Small, sterile | Haploid | 23 (64%) |

Fig. 1 . Embryoid emergence from a cultured anther.

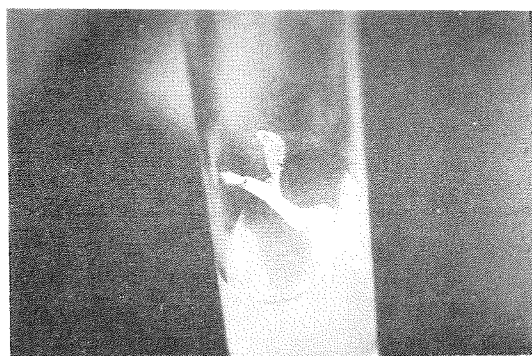

Fig. 2 . An embryoid-developed plantlet.

Fig. 3 . Plants derived from anther culture. left, haploid; right, spontaneos diploid

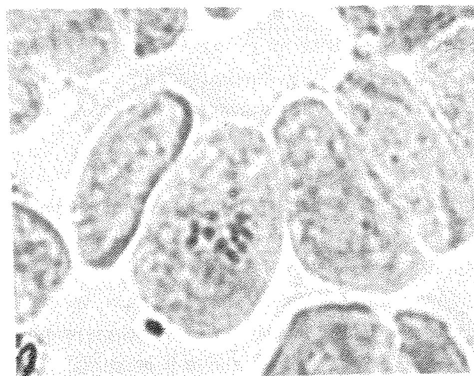

Fig. 4 . Chromosomes of a haploid root tip cell ( 2 n=10).

## Discussion

Microspore embryogenesis was observed in most cases, but frequency of embryo production varied among experiments. It is due mainly to the physiological conditions of donor plants. We could get good results when donor plants had vigorous vegetative growth followed by flower bud formation under moderate temperature and light conditions. Suitable donor growth conditions in *B. campestris* were described in detail by Keller et al.(8).

In most *Brassica* species anther culture has been carried out using anthers with uninucleate microspores. Our study showed that anthers containing early uninucleate pollen were optimal for embryoid formation. Suitable anthers could be identified by external staging system( 2 , 8 ).

Lichter(10) reported high frequency of embryo formation in a liquid culture. But we couldn't get satisfactory results for the liquid medium, so all the media used were solid.

Auxin was a indispensable component for microspore embryogensis. Although Keller et al.(2) obtained embryoids in the medium containing 0.1mg/l NAA, 0.1mg/l 2 , 4 - D in turnip *(B. campestris)*, it was difficult to induce pollen embryogenesis at such auxin level in chinese cabbage. Ten-fold increase in auxin concentration stimulated embryogenesis. The difference of the response to auxin among genotypes in the same species might be related to the level of endogenous

hormones.

Sugar concentration played an important role in embryoid formation. High concentration at 0.2-0.3M stimulated embryogenesis, on the other hand somatic cell proliferation was inhibited in such concentrations. Sugar concentration seems to be one of key factors controling morphogenesis. As Keller et al. (2) pointed out,the role of high sugar levels is highly specific and not due solely to an osmotic effect. Further study is needed to clarify the mechanism of differentiation control by sugar concentration.

Cultured anthers have been usually maintained in the dark. But in our result culture condition of darkness might be not always necessary for microspore embryogenesis.

All the anthers were treated in high temperature at 35℃ for 1 day prior to incubation at 25℃, since Keller and Armstrong(5) obtained the highest embryo yields in the elevated temperature treatment at 35℃ for 1 day or 3 days. The effect of high temperature shock has been reported in various *Brassica* species (1, 4, 5, 6, 9).

High sugar concentrations were necessary for embryogenesis but not necessary for subsequent embryo development. A large number of embryoids grew abnormally in the transferred medium with low sucrose concentration, which resulted in unsatisfactory regeneration frequency. It would be necessary to establish the effective regeneration method as the secondary embryo formation system (3, 14) to make good use of anther culture.

Ploidy level of regenerated plants could be estimated by the morphology and function of reproductive organs. Frequency of haploids was higher than that of polyploids. A considerable number of regenerates were assumed to be diploids. Polyploidization may have resulted from the process of endoreduplication or nuclear fusion during early culture stages (3).

Progeny of spontaneous diploids and artificial chromosome doubling diploids have been cultivated in the fields and their characteristics and disease resistance have been examined.

Anther cultuer can be now utilized as a practical breeding tool in the breeding program of chinese cabbage. Technical progress in the haploid production method will bring about new breeding systems.

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# ハクサイの葯培養による半数体の育成

松本悦夫・藤森基弘・大谷英夫

## 摘　要

ハクサイの葯培養における花粉起源胚様体の形成に及ぼす諸要因の影響について検討を行なった。

胚様体は花粉発育ステージ１核期前期の比較的若い葯から生じた。培地へのオーキシンの添加は胚様体の形成を著しく促進し、1.0㎎／ℓ　ＮＡＡまたは1.0㎎／ℓ　ＮＡＡ、1.0㎎／ℓ　2,4－D、0.1㎎／ℓカイネチンを添加した培地で高い胚様体形成率が得られた。糖濃度に関しては、高糖濃度でカルス化が抑制され、胚様体誘導が促進される傾向がみられ、0.2－0.3Ｍグルコース添加培地で10%以上の高い胚様体形成率を認めた。培養光条件については、明暗いずれも同程度の胚様体形成率を示した。

胚様体は低糖濃度の胚培養用培地に移したが、正常な発育をするものは少なく、多くは奇型化を呈した。

再生個体の花器の形態、稔性の調査により64%が半数体と推定され、これら個体の根端細胞染色体は半数性（２ｎ＝10）を示した。

ハクサイ葯培養における高率な胚様体形成は半数体育種を現実的なものとした。