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## Note

# Improvement of microspore culture method for multiple samples in *Brassica*

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Microspore culture is an important method for production of haploids and doubled haploids. Although the routine protocol of isolated microspore culture of *Brassica* species has been established, the protocol for a large number of genotypes is laborious. In this paper, we report an improvement of the microspore culture method for dealing with multiple samples. The improved method showed the same results as the conventional one in the number of isolated microspores per bud and embryo yield per dish. The improved protocol is easier and can deal with two to four times more genotypes than the conventional one during the same period. This method provides some advantages to plant breeders and/or geneticists.

**Key Words:** *Brassica*, microspore culture, improved technique.

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## Introduction

Haploids and doubled haploids (DHs) produced by *in vitro* culture of gametophytic cells, especially male gametophyte, are of great importance to plant breeding and basic science. In genus *Brassica*, since successful isolated microspore culture of *B. napus* was reported by Lichter (1982), a large amount of research has been carried out to improve this technique and to expand it other *Brassica* species and allied genera (Ferrie and Keller 2004, Palmer and Keller 1999, Takahata 1997). Microspore culture of Brassicas has several advantages; 1) the techniques of microspore culture are simple and easier than those of anther culture, 2) a high yield of embryos can be obtained within a short period of 2–3 weeks, and 3) this technique offers not only the fastest route to homozygosity, but it also provides several valuable tools for genetic engineering and a model system of developmental research because of its single-cell system (Takahata *et al.* 2005).

Thus far, factors influencing microspore embryogenesis have been clarified, that is genotypes, developmental stage of microspores, donor plant physiology, pretreatments of flower buds and/or microspores, culture media, and culture conditions. Of these factors, most of them have been optimized, however, genotypic variations are non-dissolved factors as shown in other tissue cultures. For breeding and basic sciences, it is important to identify high responsive genotypes. This is because breeding experiments reveal that responsiveness to embryogenesis is a dominant trait and can

be transferred from high responsive genotypes to low or non responsive ones (Palmer and Keller 1999, Zhang and Takahata 2001), and high responsive genotypes have been effectively used as a model system of developmental research and development of genetic engineering. Although the routine protocols of microspore culture of Brassicas have been established (Custers 2003, Dias 2003, Ferrie 2003), surveying the response of a large number of genotypes is tedious work. In this paper, we reported improvement of a microspore culture method for dealing with multiple samples. In comparison with a conventional method, the improved method is characterized by 1) the handling of many genotypes in a experiment, 2) easy microspore isolation using a beads cell disruptor and 3) easy handling of samples in a plastic tube.

## Protocol

The protocol described here is used for *B. rapa*. However, it is also used for other *Brassica* species and allied genera, with slight modifications depending on species. For example, optimal bud size (late uninucleate to early binucleate stages of microspores) and sucrose concentration in medium, and the period of elevated temperature treatment in the culture initiation are dependent on species. Some equipment used in this protocol such as plastic tubes (some of which contain cylindrical metal) and CellTrics filters is autoclaved in advance.

## Sterilization

1. Ten to 30 (50 in maximum) flower buds 2–3 mm in length are placed in a 2 ml plastic tube and surface-sterilized in 1–2 ml sodium hypochlorite (2.0% active

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**Fig. 1.** Improvement of microspore culture for multiple samples. (A) Bud sterilization and washing in 2 ml plastic tubes using a Rotator. (B) A 2-ml plastic tube contained buds and cylindrical metal. (C) Buds were macerated in the tube with cylindrical metal using a Beads Cell Disrupter/Micro Homogenizing System. (D) Microspores were obtained by filtration through a 50 µm CellTrics filter. (E) The improved method produced as many embryos as the conventional method did. Bars (B, E) = 1 cm

chlorite) with rotation at 50 rpm for 10 min on a Rotator (RT-50, TAITEC, Japan). Twelve or more genotypes can be examined in one experiment (Fig. 1A).

2. After sterilization, sodium hypochlorite is exhausted by micropipette or pasture pipette, and 1 ml sterile water is added. Buds are washed with rotation of plastic tubes at 50 rpm for 5 min on a Rotator (RT-50, TAITEC, Japan). This washing is repeated three times (Fig. 1A).

#### Microspore isolation and culture

1. After washing the buds, sterile water is discarded and 1 ml B5 medium (Gamborg *et al.* 1968) supplemented with 10% sucrose at pH 6.0 (B5-10) is added into the plastic tube, and for release of microspores buds are macerated in the tube with cylindrical metal (18 mm length/7 mm width) (Yasuikikai, Japan) using Beads Cell Disrupter/Micro Homogenizing System (Micro Smash MS-100, TOMY, Japan) at 2,000 rpm for 10–20 sec (Fig. 1B, 1C). This equipment can process 12 tubes per treatment. Maceration time depends on the number of buds in a tube or species used.
2. The plastic tube was lightly centrifuged spin down the microspores. The contents of the plastic tube were filtered through a 50-µm CellTrics filter (Partec, Germany) into a 2-ml plastic tube (Fig. 1D). The plastic tube and filter were rinsed with 1 ml of B5-10 medium, and poured through the filter.
3. The microspore suspension is centrifuged at  $\times 125$  g for 3 min, the supernatant is exhausted and 1 ml B5-10 medium is added to the microspores pellet for resuspending. This washing and centrifugation are repeated two more times.
4. The microspores pellet is suspended at a density of  $1 \times 10^5$ /ml in NLN medium (Custers 2003, Swanson 1990) with the concentration of major salts reduced by 50% and supplemented with 10% sucrose at pH 6.0 (1/2 NLN-10). In some cases, 6-Benzylaminopurine (BA) is added in 1/2NLN-10 medium (Charne and Beversdorf 1988, Ferrie 2003).
5. Two ml of the microspore suspension is plated in a  $60 \times 15$  mm plastic Petri dish. The dishes are incubated at

32.5°C in the dark for 1 day, and then they are transferred to a 25°C incubator in the dark. After 2 or 3 weeks of culture, the embryo yield is examined (Fig. 1E).

#### Plant regeneration

1. Cotyledonary embryos are transferred to 1.6% agar-solidified B5 medium supplemented with 2% sucrose at pH 5.8 (B5-2) or to a filter paper placed on top of the 0.8% agar-solidified B5-2 medium, and incubated at 22–25°C with a 16 h/day photoperiod ( $50 \mu\text{mol m}^{-2} \text{s}^{-2}$ ).

#### Comparison between improved method and conventional method

The results of comparison between conventional and improved methods are shown in Table 1. Two cultivars, ‘Early White Flat Dutch’ and ‘Nagasaki Aka Kabu’ of *B. rapa* ssp. *rapa*, which were provided by the Institute of Plant Genetics and Crop Plant Research (IPK) in Germany and Noguchi Seed Co. in Japan, respectively, were used. In a preliminary experiment, these two plants showed the marked embryogenesis. These plants were grown in a growth chamber under 13/8°C with a natural photoperiod. Our improved method showed the same results as that of the conventional one. Student t-test showed that the number of isolated microspores per bud and the number of embryos per dish were not significantly different between the two protocols. This result indicates that microspores isolated mechanically using the Beads Cell Disrupter/Micro Homogenizing System have not lost their embryogenesis ability. Such strength of microspores to physical force supported by the results of Nitta *et al.* (1997) who revealed that microspore wall of the reticulate structure is already formed on microspores from the late uninucleate to early binucleate stages by SEM observation. When applying this improved method to other species, we obtained similar results with a number of isolated microspores in *B. napus*, and we obtained embryos in several other *B. rapa* cultivars and in radish (*Raphanus sativus* cv. Sayadaikon) (unpublished).

The improved protocol is characterized by 1) the handling of many genotypes in one experiment, 2) a simple

**Table 1.** Comparisons between conventional and improved methods of microspore culture in *B. rapa*

Content of comparison	Conventional method <sup>c</sup>	Improved method
No. of collected microspore/bud <sup>a</sup>		
Early White Flat Dutch	125,405 ± 29,678	134,412 ± 18,073
Nagasaki Aka Kabu	117,926 ± 15,094	122,733 ± 33,602
No. of embryo/dish <sup>a,b</sup>		
Early White Flat Dutch	71 ± 17	63 ± 9
Nagasaki Aka Kabu	80 ± 36	108 ± 8

<sup>a</sup> Values tabulated are means ± SD of at three replication. The no. of collected microspore/bud and the no. of embryo/dish were not significantly different between the two methods.

<sup>b</sup>  $2 \times 10^5$  microspores were cultured in each petri dish. Microspores were cultured in 1/2NLN-10 supplemented with 0.1 mg/l BA.

<sup>c</sup> The conventional method was carried out according to Zhang and Takahata (2001).

method of sterilization of tools used, and 3) an easy method of microspore isolation. Using the conventional protocol, generally 4–6 genotypes at maximum have been used in one experiment (Ferrie 2003). On the other hand, in the improved one, 12 or more genotypes are usually used in one experiment. In other words, the improved method can deal with two to four times more genotypes than the conventional method during the same period. In addition, sterilization of tools is easier, because only plastic tubes (some which contain cylindrical metal) and CellTrics filters are autocleaved in glass bottles.

The improved protocol in this study is an easier and time-saving method for microspore culture of *Brassica* and can be applied to other genera. This method provides some advantages to plant breeders and/or geneticists in various phases such as finding high responsive genotypes in survey experiments and QTL analysis on microspore embryogenesis.

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