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Plant regeneration from isolated microspore cultures of Chinese cabbage (*Brassica campestris* spp. *pekinensis*)

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ABSTRACT

Isolated microspores of Chinese cabbage (*Brassica campestris* ssp. *pekinensis*) were incubated in modified NN medium containing 10% sucrose in darkness at 33°C for one day followed by culture at 25°C. After 14 days of culture, microspores developed into embryos ranging from globular to cotyledonary stage. Plants were regenerated after transfer of embryos to medium containing 3% sucrose and no plant growth regulators.

Abbreviations
NN: Nitsch and Nitsch ; MS: Murashige and Skoog ; NAA: naphthaleneacetic acid ; BA: 6-benzylaminopurine

INTRODUCTION

Since haploid plants were obtained via isolated microspore culture of *Brassica napus* by Lichter (1982), the technique of microspore culture has been studied intensively to improve the yield of embryos for the application to practical plant breeding of oil seed rape, *Brassica napus* ssp. *oleifera*. Embryo yields from microspores were increased remarkably by using donor plants having high embryogenic capacity (Chuong *et al.* 1988), improving culture medium (Charne and Beversdorf 1988), and careful selection of bud size (Kott *et al.* 1988). Techniques for large scale microspore culture were established for the selection of herbicide resistant lines for breeding programs of oil seed rape (Swanson *et al.* 1987, Swanson *et al.* 1988, Polsoni *et al.* 1988).

Successful plant regeneration from isolated microspores has not been reported so far in *Brassica campestris*, which includes several important crops, such as Chinese cabbage and turnip rape. During the study of anther culture of Chinese cabbage, we identified lines having high embryogenic ability (Sato *et al.* 1989). In this report we show that large numbers of microspore-derived embryos can be obtained from this Chinese cabbage line.

MATERIAL AND METHODS

Brassica campestris ssp. *pekinensis* cv. "Formosa 45 Days", introduced from the Asian Vegetable Research and Development Center (AVRDC), was used as the donor material. Plants were grown in a controlled environmental room at 20°C under 16 h photoperiod of 10,000 lx without any vernalization. Young flower buds 2.0 to 2.5 mm in length were sterilized in 70 % ethanol for 30 sec. and then in sodium hypochlorite solution containing 1 % active chlorine and one drop of Tween 20 for 15 min. The buds were washed three times with sterilized distilled water, then gently macerated in a washing solution of B5 medium (Gamborg *et al.* 1968) supplemented with 13% sucrose (Chuong and Beversdorf 1985) for 1 min., using an 8 cm mortar and 13 cm long pestle. About 50 buds were processed with 5 ml of washing solution at a time, at room temperature. The resulting suspension was passed through a 50-μm nylon mesh and collected in a centrifuge tube, and then washing solution was added (up to 30 ml finally). The filtrate was centrifuged at 200 xg for 3 min. at room temperature followed by three washes with washing solution, and resuspended in culture medium. The culture medium was composed of vitamins and half strength macronutrients of NN medium (Nitsch and Nitsch 1967), and micronutrients of MS medium (Murashige and Skoog 1962), containing 10% sucrose, 30 mg/l glutathione, 800 mg/l L-glutamine, 100 mg/l L-serine, 0.5 mg/l NAA and 0.05 mg/l BA, titrated to pH 6.0 and filter sterilized.

Two milliliters of microspore suspension at a density of $2x10^5$ /ml in culture medium were plated in 60x15 mm plastic petri dishes. These were incubated at 33°C for 1 day and then maintained at 25°C in darkness. After 2 to 3

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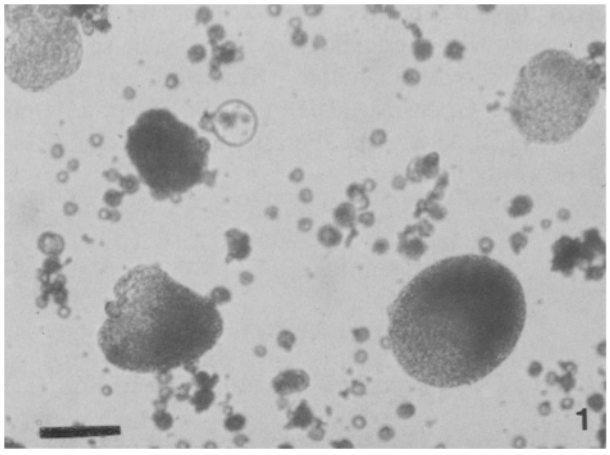

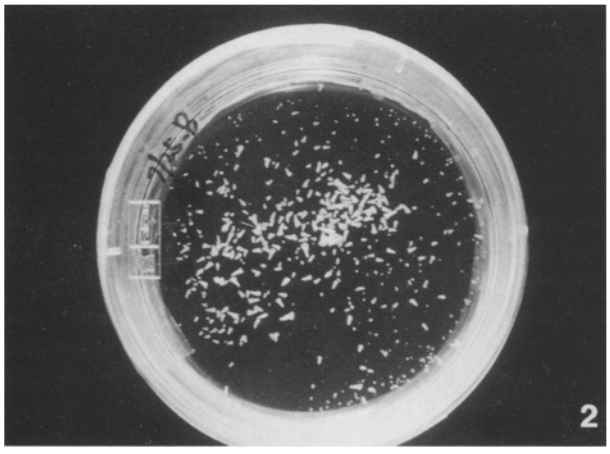

Fig.1. Embryos developed from microspores of Chinese cabbage after 8 days in culture. Bar=100μm.
Fig.2. Embryos derived from microspores of Chinese cabbage after 14 days in culture. Petri dish is 60 mm in diameter.
Fig.3. A haploid plant regenerated from a microspore.

weeks of culture, an equal volume of culture medium supplemented with 3% sucrose and without plant growth regulators was added. The embryos were transferred onto B5 medium with 3% sucrose and 0.8% agar after 3 to 4 weeks of culture and kept under fluorescent light (ca. 3,000 lx) at 25°C.

Cytological analysis of root tips of microspore-derived regenerates was done according to the procedure of Kuroiwa *et al.* (1982) and Nishibayashi *et al.* (1986).

RESULTS AND DISCUSSION

After 2 to 3 days of culture, the microspores increased in volume, and some cell division was observed. Small embryos consisting of compact cells were formed after 7 days of culture (Fig.1). The embryos grew to globular and heart stages after 7 to 10 days of culture, and to torpedo and cotyledonary stages after 14 days (Fig.2). More than 500 embryos were obtained in a 60 mm petri dish after 14 days of culture. Isolated microspore culture was repeated more than ten times, and efficiency of embryogenesis from isolated microspores was estimated to be 0.1% to 0.25%. After 14 days of culture, approximately 10% of embryos were at cotyledonary stage, about 40% at torpedo stage, 30% at heart stage and 20% remained at the globular stage.

Addition of the same volume of culture medium supplemented with 3% sucrose and without plant growth regulators after 2 to 3 weeks of culture promoted the growth of embryos under fluorescent light (ca. 3,000 lx). The embryos at heart stage were less lobated at the top than normal zygotic embryos. Most of the embryos at heart stage grew to form trumpet-shaped cotyledons at the torpedo stage. After transfer onto B5 agar medium, some of these trumpet-shaped embryos grew into plants; others, lacking on apical meristem, could not develop leaves. The regenerated plants were transplanted to pots with sterilized soil and grown under light at 20°C. The efficiency of plant regeneration from cultured embryos was ca. 5% to 10%. One haploid plant was found (Fig.3), but seven plants were doubled haploids with 20 chromosomes.

At the beginning of our study, the procedure for microspore culture of *B. napus* (Ohkawa *et al.* 1988) was applied to the Chinese cabbage line having high

embryogenic ability. Less than 50 embryos were obtained in each petri dish. Several factors influencing embryo yields were examined in our effort to improve the culture procedure (details of the examination will be published elsewhere.). Using half strength macronutrients of NN gave more embryos than the original concentration. The medium without auxin and cytokinin gave as many embryos as the medium containing 0.5 mg/l NAA and 0.05 mg/l BA. The best concentration of sucrose for embryo yield was 10%. Lower concentrations of sucrose gave higher percentages of normally developed embryos, but lower embryo yields. Of the constituents of the medium tested so far, the concentration of macronutrients had the greatest effect on embryo yields.

The efficiency of embryogenesis of Chinese cabbage obtained here was still much less than those of *B.napus*, where embryos were generated from about 7% of isolated microspores (Kott *et al.* 1988). To increase the efficiency of microspore cultures of Chinese cabbage, other potential factors affecting embryogenesis from isolated microspores, such as growth conditions of the donor plants, bud size and high temperature treatment after plating, should be examined extensively. A large proportion of embryos were abnormal, had no shoot meristem, and could not grow into plants. Embryo culture procedures should also be improved to increase the efficiency of plant regeneration.

In the genus *Brassica*, *B.campestris* is one of the most recalcitrant species in cell and tissue culture. Shoot formation on hypocotyl derived calli was less frequent in *B.campestris* than *B.oleracea* or *B.napus* (Murata and Orton 1987). Regenerated plants were successfully obtained from mesophyll protoplasts by Yamagishi *et al.* (1988), but efficiency of plant regeneration was low (29% of protoplast-derived calli and 0.02% of cultured protoplasts). The frequency of plant regeneration from isolated microspores reported here is remarkably higher than frequencies observed with somatic cells and tissue of *B.campestris*. This isolated microspore culture technique can be used not only for the production of haploids and doubled-haploid plants for breeding programs but also as a cell culture system for gene transfer in *B.campestris*.

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