PLANT REGENERATION FROM EMBRYOIDS THROUGH ISOLATED MICROSPORE CULTURE IN BRASSICA NAPUS L.

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INTRODUCTION

In recent years, many scientists abroad have reported the research on the plant regeneration from embryoids through isolated microspore culture in Brassica napus L. (Keller W. A. and K. C. Armstrong, 1983 ; P. V. Chuong and W. D. Beversdorf, 1985). The isolation and culture methods of microspore have been developed. For example, Polsoni, Kott and Beversdorf got great achievements in isolated microspore culture of B. napus and could obtain high embryoid frequency under a certain condition. All these studies have drawn attentions and emphases of many genetists and agronomists. The fact that higher microspore induction frequency, more haploids, more single cells and higher synchronization were obtained by isolated microspore culture than by anther culture have made the isolated microspore culture method can be applied to the breeding program. By taking advantage of microspores' large quantity and easy spread, people could do the research on mutation and selection as well as the mechanism of embryogenesis more convenient by isolated microspore culture than by anther culture. Hence, the breakthrough on the research of isolated microspore culture will provide effective experimental system for the genetic studies of crops on the cell and molecular levels.

According to the former reports, there were two approaches to culture the isolated microspores of B. napus. The first approach was to preculture the microspores in the anthers for several days followed by the isolation and culture of microspores. The second approach was to culture the isolated microspores directly. We adopted the second approach, that is, we directly isolated and cultured the microspores of B. napus, then, obtained pollen embryoids and got regenerated plants.

MATERIALS AND METHODS

Materials:

The hybrid progenies of winter B. napus including F1 (89-20026) and F3 (89-22015 and 89-22017) were used as the experimental materials.

METHODS:

Bud selection and harvest

After the first flower has opened, buds approximately 2.5 - 3.0 mm in size from main racemes or branches with petal/anther length ratioes approximating 1/2 and with green or transparent anthers were selected and sampled. At that time, the microspores in these buds were mostly in the late uninucleate or early binucleate stages (using cytological evidence of acetocarmine stain) and there were 8-14 buds in a raceme, then, picked 6-8 racemes with buds less than 4 mm, quickly removed all damaged buds and all buds larger than ideal determined above, wraped the selected buds with wet gauze and pretreated them in 4-6°C for 24 hours.

Bud sterilization

Buds were sterilized by placing the wet gauze containing the buds in saturated calcium hypochlorite bleach for 15 min and were rinsed throughly three times in sterile water.

Medium

B5 medium with 13% sucrose was used as the microspore extraction medium (PH 5.8) and was autoclaved. NLN medium with 13% sucrose was used as the microspore culture medium (PH 5.8) and was sterilized with 0.22 um

filter. B5 solid medium supplemented with 0.1 mg/l GA3 + 2% sucrose or modified MS medium was used as the embryoid differentiation medium (W. J. Zhong, 1987).

Isolation and collection of microspores

Sterilized buds were held in a small beaker containing B5 microspore extraction medium and were homogenized, then macerated materials were filtered through two layers of sterile nylon mesh (64 um pore size on top of 44 um) into centrifuge tubes. After centrifuging the filtrate at 500 rpm for 5 min, the pellets were resuspended and centrifuged in B5 medium three more times and in NLN medium one time. After the last wash the pellet was resuspended in fresh NLN medium. The microspores were counted using haemocytometer and were plated at a density of 5×10^5 spores/ml in 6 cm Petri plates at 3 ml suspension/plate. The plates were sealed with Parafilm.

Isolated microspore culture

The plates containing isolated microspores were incubated at 30°C in the dark and method of shallow liquid stationary culture was used.

Pollen embryoid culture

Young embryoids visible to the naked eye were transferred to B5 solid medium and were incubated at 25-27°C with a 12 hr./12 hr. day/night cycle.

Cytological observation of microspores

Microspores with optimal developing stage were determined using acetocarmine stain and the growth and development of isolated microspores in liquid culture medium were observed using inverted microscope.

RESULTS

Formation of pollen embryoids

Microspores were cultured in liquid medium. The first cell division in isolated microspores was visible five days after culture. After ten days of culture the microspores through the first cell division were increased in number and their valumes were enlarged along with the occurrence of the second cell division. Cell clusters were commonly seen 10-15 days after culture. Globular embryoids and heart-shaped embryoids occured after 20 days of culture followed by the occurrence of many types of embryoids within the next five days.

The effect of shaking culture on the growth and development of embryoids

Globular embryoids and heart-shaped embryoids were seen 20 days after culture. At this stage, some plates of these embryoids were placed on a rotating shaker (80 rpm) for shaking culture while the others were still put in 30°C incubator for stationary culture. Ten days later, extremely rapid development of embryoids which were mostly well developed, good-qualified and light yellowish but not transparent in color occured in shaking culture while very slow development of embryoids with light brown or greyish white color appeared in stationary culture.

The influence of shaking and stationary culture on the induction of cotyledonous embryoids was showed in Table 1.

Table 1. Effect of shaking culture (A) and stationary culture (B) on embryoid quality

Treatment	Mean No. of total embryoids/plate	Mean No. of cotyledonary embryoids/plate	Frequency (%)
A	62.67	30.67	48.93
B	54.33	11.00	20.62

It was revealed from Table 1 that shaking culture could speed up the

development of embryoids and the frequencies of cotyledonous embryoids were 48.93% in shaking culture and 20.62% in stationary culture respectively.

Embryoid differentiation and plant regeneration

Cotyledonous embryoids formed 25 days after culture were transferred to B5 medium supplemented with 0.1 mg/l GA3 + 2% sucrose or to modified MS solid medium. After next 7 to 10 days, like seed germination, cotyledonous embryoids turned green in color quickly and their embryonic roots full of white root hairs prolongated followed by the regeneration of normal plants with 3-4 leaves in the differentiation medium. When transferred to solid differentiation medium, the globular embryoids and abnormal embryoids, however, turned brown in color and died while some of them just developed roots but not shoots. The plant regeneration frequency from embryoids in isolated microspore culture was observed to be determined by embryoid type. Usually cotyledonous, torpedo and heart-shaped embryoids belonged to the normal embryoids while globular and abnormal embryoids had low regeneration frequencies (Table 2).

Table 2 Relationship between type of embryoids and regenerated pantlets

Type of embryoids	No. of embryoids	Regenerated plantlets	Frequency (%)
Cotyledonous	92	89	96.73
Torpedo	29	24	82.75
Heart	9	2	76.76
Globular	40	4	10.00
Abnormal	18	0	0.00

Material: 89-20026

DISCUSSION

A large quantities of pollen embryoids could be obtained in short time using microspore culture of B. napus. It took only three weeks to get embryoids from microspores and it was not necessary for microspores to be precultured in anthers for their induction before microspore isolation. This result coincided with L. Polsoni's report.

Young embryoids visible to the naked eye were placed in a rotating shaker for shaking culture so that most of the young embryoids with bipolarity could developed to cotyledonous embryoids followed by the enlargement of their cotyledons and hypocotyls. Young embryoids in stationary culture, however, could suffer nutrition shock because of lack of breath and nutrition.

Isolated microspore culture precludes the possibility of the formation of non-microspore embryoids and enhances the yield of haploidy embryoids. This method provides a way for in vitro mutation and selection as well as the application of biotechnology.

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