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Full Length Research Paper

Profound improvements of isolated microspores culture techniques in winter *Brassica napus* L.

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We studied the effects of sampling stages, physical conditions (like temperature), culture conditions, embryo long-distance transportation methodology and plantlet regeneration on isolated microspores from donor plants in field. Results indicated that if microspores were sampled in bud stage instead of blooming stage to enhance the duration of microspore culture by about 9 days, embryo yield per bud reached maximum (29.61) when environmental temperature was more than 20°C. Compared with previous procedure of generating sampling buds by cutting the inflorescences, re-harvesting buds from donor plants could increase the usage efficiency of donor plants by 9 times. Use of 13% sugar solution for extracting microspores instead of B_5 solution containing 13% sugar showed the similar embryo yield per bud. This improved protocol could decrease the costs of microspore culture. Compared with normal incubating method, the improved incubating method roughly resulted in the same number of embryo and appeared markedly 4 days earlier. Liquid B_5 medium had several advantages in transporting embryos for long distance notably the number of embryo transported was about 8 times greater and the transported volume decreased by 7 times when compared with embryos transported in solid B_5 medium. Therefore, the improved procedures could undoubtedly enhance the efficiency of microspore culture in *Brassica napus.*

Key words: *Brassica napus*, microspore culture, embryogenesis, technique improvement.

INTRODUCTION

Microspores culture (MC) was widely used in *Brassica napus, Brassica rapa, Brassica juncea and Brassica oleraceae* (Cabbage and Cauliflower) for obtaining varieties and inbreed in Europe and Canada. MC in winter *B. napus* L. (Brassicaceae) assisted breeders in deriving homozygous plants in single step (Ahmad et al., 1991; Eric et al., 2004; Iva et al., 2006; Klima et al., 2004; Lionnecton et al., 2001; Laurie et al., 2004; Orr et al., 2004; Takanori et al., 2004). This technique has been earlier utilized in plant breeding and construction of double haploid (DH) population. Recently, microspore embryogenesis is used for molecular marking, genetic transformation and linkage mapping of various agronomic traits (Bin, 2008; Hafizur et al., 2007; Fan et al., 2005; Ferrieia et al., 1994; Orr et al., 2004; Pechan et al., 2004) and can be greatly simplified and speeded up using doubled haploids. Several external and internal factors notably genotype of donor plant, developmental stage of microspores, pretreatments, culture methods and physical factors (like light, temperature, etc) have been reported to influence the efficiency of microspore embryogenesis. Since Lichter (1982) successfully obtained embryos by MC, many researchers carried out extensive and intensive study from time to time to understand the role of these aforementioned factors. Although, a large body of data is available in this aspect,

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Abbreviations: DH, Doubled haploid; **B_5 medium,** Gamborg medium; **NLN medium,** Nitsch and Nitsch medium; **84 disinfectant,** 7% NaClO solution; **MC,** microspores culture.

but it is regrettable that no research group has tested all the physical and chemical parameters influencing MC. Researchers tried different culture medium to effectively isolate, culture and derive embryos from microspores (Bin, 2007; Laurie et al., 2004; Zeng et al., 2010; Yang and Seppo, 2006; Zaki and Dickinson, 1995; Zhou et al., 2002). Low temperature pre-treatment of microspores (Bin et al., 2007; Jan et al., 2004; Pechan and Keller, 1989; Zhao et al., 1996, 2004) and subsequent heat-shock for short duration before using previous cultural procedures have profound positive results on MC (Gu et al., 2004; Pechan and Smykal, 2001; Touraev et al., 1997). Previous studies also emphasized the primary role of cultural conditions in determining higher frequency of embryos (Bin et al., 2004; Bin et al., 2007; Dunwell et al., 1985; Yoshihito et al., 2005; Takahata et al., 2004).

Earlier, the donor plants of microspore culture are planted in greenhouses, where the temperature, illumination and humidity are automatically controlled resulting in providing donor plants a suitable environmental condition for easy generation of microspores derived embryos. The costs of building greenhouses and their maintenance are expensive, so researchers alternatively tried to grow the donor plants in different seasons where the environment is suitable for MC. Most of the researchers in China harvested buds from donor plants in fields and cultured isolated microspores. However, the disadvantage of harvesting buds from fields is that researchers can culture microspores only in spring, because it is the suitable time for rapeseed cultivation in many parts of China. Northwest such as Qinhai and Gansu of China can support the rapeseed in summer. But there are significant differences between MC of donor plants in field and in greenhouse with relation to plant growth, sampling stages and long distance embryo transportation.

This study described our studies on the earlier mentioned factors and developing improved protocol to efficiently and speedily carry out MC in field condition. We improve these techniques for generating doubled haploid plants from isolated microspores embryogenesis in winter *B. napus*.

MATERIALS AND METHODS

Plant materials

The F_1 combination H155×Qva was sown in the fields in the second week of May at Xining city, Qinhai province in 2006, 2007 and 2009. The line and plant distance were 40 and 13 cm, respectively. Nitrogen, phosphorus and potash fertilizers were applied in the soil before tilling. Nitrogen fertilizer was also added in seedling stage. Irrigation and spraying of pesticide were conducted if necessary.

Isolation of microspore

The previous cultural methods (PCM) of isolating microspores were followed (Zeng et al., 2010). We developed the improved cultural protocol (ICP) and details are as follow. 20 to 40 buds (2.8 to 3.5 mm in length) from mid-uninucleate to early bicellular stage from each single donor plant at bud stage (mid-august) were harvested and stored in 50 ml centrifuge tube with ice. The centrifuge tubes were stored in an ice box and taken to laboratory. The buds were disinfected with 84 disinfectants for 10 min, subsequent rinsing for 5 min with sterile distilled water poured in a 100 ml triangular flask. The buds were put into another 50 ml centrifuge tube and mashed in 13% (w per v, pH 5.8, 4°C) sugar solution and filtered through a 40 µm nylon filter into a 50 centrifuge tube. The crude microspore suspension was centrifuged at 800 rpm for 5 min. Microspores were then re-suspended with 13% (w per v, pH 5.8, 4°C) sugar solution and centrifuged at 800 rpm for 5 min. Microspores were then re-suspended with 50 ml NLN (16% (w per v sugar, pH5.8) medium (Lichter, 1982; Nitsch and Nitsch, 1967) and distributed into 60 × 15 mm dishes and were resealed with a double layer of Para film, and incubated in 32°C in dark. As soon as embryos were visible to naked eyes (about 10 days after culture), the dishes were transferred to a rotary shaker at 50 rpm and incubated in dark at 25°C. After the embryos grew up to cotyledon stage, they were moved to refrigerator to verbalize for 10 days at 4°C. Finally, all embryos were transplanted to B_5 solid medium without any plant growth regulators.

Sampling stages

The influence of sampling stages on embryo yields was studied. Buds of suitable stages were sampled and MC was conducted every three days from the 9th day before blooming to the 15th day after blooming. Three replications were done to check the sampling stage (three days after blooming). Additionally, the influence of sampling times on embryo yields was studied by sampling and MC at 7, 10, 14 and 17 h in the same day. Three replications were observed to check conventional sampling time at 7 h.

Sampling method

Two sampling methods were carried out to study the efficiency of donor plants. One was the PCM: suitable-sized buds were selected in laboratory from cut inflorescence (2.8 to 3.5 mm in length). Another was our ICP: 20 to 40 buds (2.8 to 3.5 mm in length) were selected from mid-uninucleate to early bicellular stage from one donor plant at bud appearance stage (almost in mid-August) in the field. The buds were sampled leaving the inappropriate buds.

Extract solution

13% sugar solution was used as extraction solution instead of B_5-13 (13% sugar) liquid medium and their influences on embryo yields was studied.

Two kinds of microspore incubating procedures

Two types of microspore incubating temperatures were set. One was the PCM, in which incubation of microspores were set at 32°C for 2 days in dark and then transfer to 25°C in dark. The ICP directly incubate microspores at 32°C in dark. The embryo yields of the two methods and the days before embryo was visible in plates were compared.

Two types of long-distance embryo transportation

Two methods for studying the long-distance transportation of embryos and their differences in transportation efficiency were

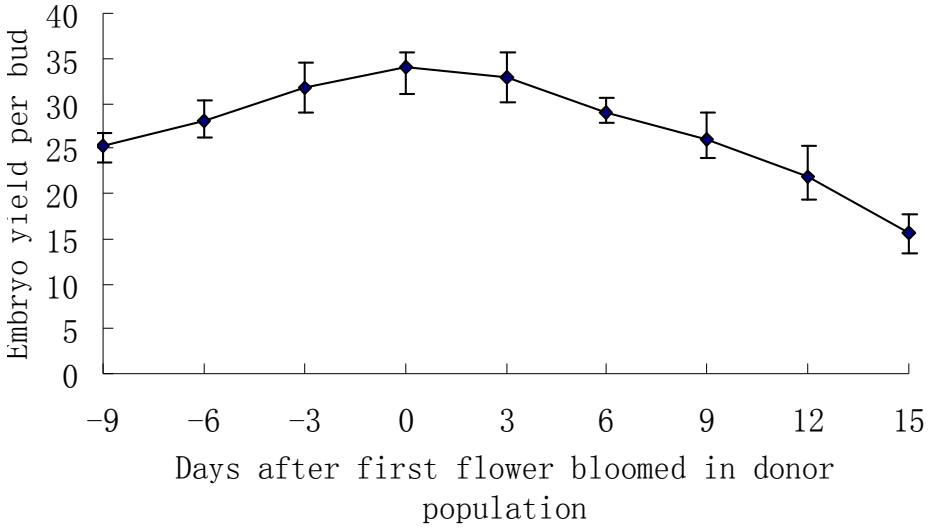

Figure 1. Effects of sampling stages on embryo yield in microspore culture.

studied. In one method, 30 embryos were transported in 80 mm × 110 mm triangular flasks with B_5 solid medium and in another method, 300 embryos were transported in 50 ml centrifuge tubes (30 mm × 110 mm) with B_5 liquid medium.

Data analysis

Experimental data were analyzed by software of statistic analysis system (SAS Institute, 1999) and Microsoft excel. Student's t-test was also used.

RESULTS

Influences of sampling stages on embryo yields

Embryos were obtained by sampling and MC from the 9th day before blossoming to the 15th day after blossoming and the yield reached to 15.60 to 34.08 embryos per bud (Figure 1). However, the highest embryo yield (31.71 to 34.08 embryos per bud) occurred on the 3rd and 4th day before and after blossoming. The embryo yield (27.99 to 28.93 embryos per bud) was still higher when sampled and cultured on the 3rd and 6th day after blossoming, which was not different when compared with that on the 3rd and 4th day before and after blossoming. The embryo yield (21.87 to 25.94 embryos per bud) decreased when sampled on the 9th and 12th day after blossoming. The embryo yield (15.60 embryos per bud) was the lowest when sampled on the 15th day after blossoming. Therefore, the most suitable sampling stage was on the 3rd and 4th day before and after blossoming. Embryo yield was also higher if sampled on the 9th day before blossoming. However, the buds of suitable sizes were not enough at that time. Compared with previous sampling method (PSM) (sampling after blooming), the improved sampling method (ISM) was earlier by 9 days.

Influences of sampling times on embryo yields

MC showed that embryogenesis efficiency among different sampling times in one day (Table 1) was different. In 2006, 2007 and 2009, no embryos were obtained by sampling at 7 h. Embryos could be obtained by sampling at 10 and 17 h, but embryo yield was low (18.53 embryos per bud and 20.74 embryos per bud, respectively), which was not different. The embryo yield (29.61 embryos per bud) reached the highest by sampling at the 14 h. The embryogenesis efficiency among different sampling times was different. In mid-august at Xining, Qinhai Province, the temperature at 7 h was usually about 10℃, at 10 and 17 h, the temperature was below 20℃. At 14 h, temperature was above 20℃. Consequently, when temperature in the field was above 20℃, it was suitable for sampling the buds and this showed maximum embryo yield.

Influences of sampling methods on embryo yields

PSM used to cut inflorescences in field and select suitable-sized buds (2.8 to 3.5 mm in length) in the laboratory. ICP directly select suitable-sized buds (2.8 to 3.5 mm in length) from donor plants in the field. Results showed that there was only a little difference in the embryo yields between these two methods. The average embryo yield in three years for ISM was 29.75 embryos per bud and that of ISM was 28.30 embryos per bud (Table 2). In PSM, there were only 4 to 5 suitable-sized buds in one inflorescence and about 40 suitable-sized buds were needed in single isolated microspore culture. In one season, about 40 to 50 donor plants were sampled. In some cases, it was difficult to get enough donor plants. In ISM, only suitable-sized buds were

Table 1. Effects of sampling time on embryo yield in microspore culture.

Sampling time	Number of inoculated bud	Number of obtained embryo	Embryo yield (embryo number per bud)*
7am	40	0	0[c]
10am	40	741	18.53[b]
14pm	40	1184	29.61[a]
17pm	40	829	20.74[b]

*Within columns, the same letter means are not significantly different at the 0.05 level of probability (t-test).

Table 2. Effects of sampling methods on embryo yield in isolated microspore culture.

Sampling method	Number of sampled plant	Sampling time per plant	Number of Inoculated bud	Yield of embryo	Embryo number per bud
Cutting inflorescence	10	1	40	1190	29.75[a]
Harvesting buds from donor plant	1	10	40	1132	28.30[a]

*Within columns, the same letter means are not significantly different at the 0.05 level of probability (t-test).

removed from donor plants in the field and other small buds were left to develop further. Therefore, every donor plant could be used for several times. In this study, every donor plant could be sampled for about 10 times. In one season, 5 to 10 donor plants were appropriate to get the bud. In addition, re-sampling from the same donor plant could increase the chances to derive embryos with the same genetic background. Compared with TSM, the ISM could sharply decrease the number of donor plants and size of experimental field.

Influences of different extraction solutions

The previous extraction solution method (PESM) in isolated microspore culture was B_5 medium with 13% sugar (w per v, pH 5.8). The improved extraction solution method (IESM) was 13% sugar solution (w per v, pH5.8). Comparatively, embryo yield of PESM and IESM were 31.72 embryos per bud and 27.41 embryos per bud, respectively in 2006. However, the embryo yield of IESM was slightly higher in both 2007 and 2009. 2006, 2007 and 2009 results in this respect clearly showed that there was no difference between the PESM and IESM in terms of embryogenesis efficiency. The average embryo yield in three years was 30.55 embryos per bud in 13% sugar solution and it was 29.79 embryos per bud in B_5 medium containing 13% sugar (Figure 2).

Comparison of two kinds of microspore incubating procedures

Previous incubating method (PIM) was used to incubate isolated microspores at 32°C for 2 days in the dark and then transfer them to 25°C in the dark till the embryos were visible with the naked eyes. In this study, the microspores were directly incubated at 32°C in the dark till the embryos were visible with the naked eyes. We observed the embryos were visible on the 10th day, which decreased the time for embryo visibility by four days (Table 3). The embryo yield was almost the same in PIM and developed incubating protocol.

The long-distance transportation of embryos without injection

Solid B_5 medium had several advantages in transporting embryos notably stability of solid medium; embryos could be separated from each other and no injection occurred during transportation (Table 4). However, there were also disadvantages in this kind of transportation. The efficiency of solid B_5 medium was low. Only 50 embryos could be transferred in a 150 ml triangular flask and each embryo occupied a space of 11.06 cm^3. If transported by liquid B_5 medium, more than 400 embryos could be placed in one centrifuge tube which occupied 78 cm^3 and each embryo occupied a space of 0.195 cm^3 resulting in 54 times space efficiency. However, liquid medium in tubes frequently shook during transportation and ratio of injection tubes was about 27.42%. Therefore, these two kinds of transportations had their advantages and disadvantages.

DISCUSSION

B. napus is one of the most important oilseed crops in the world and studies on breeding, genomics, metabolomics,

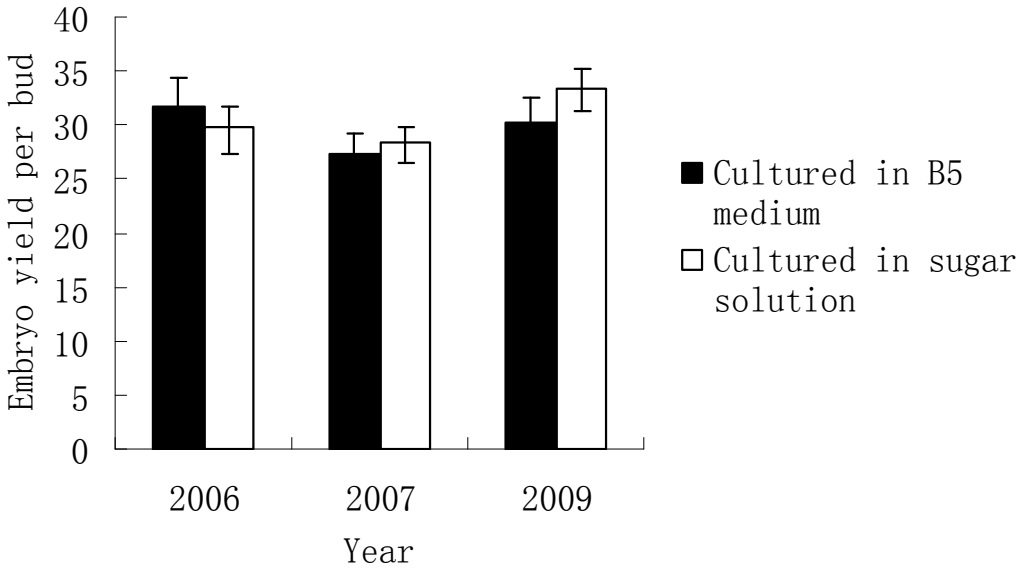

Figure 2. Effects of extraction solutions on embryo yield in isolated microspore culture.

Table 3. Comparison of two kinds of microspore incubating protocols.

Incubating procedure	Day before embryo were visible	Embryo yield	Embryo number per bud
Incubate in 32°C for 2 days in dark and then transfer to 25°C in dark	14	1208	30.20[a]
Directly incubate in 32°C in dark	10	1235	30.86[a]

*Within columns, the same letter means are not significantly different at the 0.05 level of probability (t-test).

Table 4. Comparison of embryo transportation methods without injection.

Transporting method	Container	Space (cm^3)	Number of Inoculated embryo	Space efficiency (cm^3/embryo)	Ratio of injected tube (%)
Inoculating in solid B_5 medium	Triangular flask (150 ml)	553	50	11.06	0
Inoculating in liquid B_5 medium	Centrifuge tube (50 ml)	78	400	0.195	27.42

germplasm, cytogenesis and molecular genetics have greatest impact in characterizing the qualitative and quantitative traits. Isolated microspore culture of *B. napus* is extensively used to study earlier mentioned research areas (Chery et al., 2005; David et al., 2004; Eric et al., 2004, Zhang and Takahata, 2001). Lichter (1982) studied microspore culture in greenhouse grown *B. napus* where the temperatures, humidity and illumination were controlled and can be used for round the year microspore embryo generation. Some researchers tried to improve these techniques for higher embryo efficiency. Breakthrough work was carried out by developing NLN medium having iron content reduced by half for initial microspore culture (Laurie et al., 2004). Decrease of sucrose in the first 48 h from 17% (w/v) to 10% (w/v) thereafter supports an increase in the production of embryos (Lionneton et al., 2001). Embryo yield increased when 2, 4-D and 6-BA were added in NLN medium

(Ferrieia et al., 1994). Isolation of microspores pretreated with NLN and colchicines showed higher ratio of embryos (Zhou et al., 2002; Zaki et al., 1995).

Sampling stage is one of the most important influencing factors on embryo yields. Yoshihito et al. (2005) found that optimal bud size for microspore culture differed between genotypes. Both Takahata et al. (2004) and Yang et al. (2006) concluded that microspore isolated from buds of older plants had a higher embryo yield than those of younger plants. Although, the stage at which the ratio of uninucleate cells reached maximum differ among genotypes. The microspores from 2.8 to 3.5 mm buds had the highest ratio of uninucleate cells. In this study, we found that each donor plants could be a potential source of buds for MC at different developing stages. Sampling was done before and after blossoming to select bud size between 2.8 and 3.5 mm having higher ratio of uninucleate cells and embryo yield. In addition, the culture period was extended by 9 days in ICP, which increased the culture time by about 30% in the total culture time and it was the cause of limited resource available at sampling stage.

The temperatures at which both the donor plants grew and the isolated microspores were incubated affects embryogenesis. Jan et al. (2004) studied that 32.5°C temperature control the development of *B. napus* microspores resulting in sporophytic development and the formation of embryos. Dunwell et al. (1985) thought that in majority of genotypes grown at 15°C produced more productive anthers than grown at 20°C. Dunwell et al. (1985) and Laurie et al. (2004) found that embryo yield could be increased after the buds were incubated at 33 to 35°C for 1 to 2 days followed by maintenance at 25°C. Seiki et al. (2002) found that low temperature pretreatment of buds or inflorescence could increase the embryo yield in *B. rapa*. According to the authors' experiments of this study, embryo yield was very low or no embryo when the buds were sampled in low temperature in field. The temperature was above 20°C in the field when buds were sampled was suitable for microspore culture. In Xining city of Qinhai province, no embryos were obtained when buds were sampled in early morning. Usually, the temperature in noon and afternoon was above 20°C in mid-august in Xining, by sampling at this time; higher embryo yield could be obtained.

The inflorescences of *B. napus* are large and the buds are at different development stages. The PSM cut inflorescences in the field and select suitable buds in laboratory for microspore culture. Usually only 4 to 5 suitable buds are accepted from more than 50 buds in one inflorescence. Therefore, most of buds from donor plants are wasted and the large population of donor plants is required. In improved sampling methods, only suitable-sized buds were harvested from donor plants in the field and smaller buds were left to grow on the inflorescences, so it was the efficient way to use buds of same donor plants for isolation of microspores from the same genetic background. Moreover, this sampling method required a smaller population of donor plants.

B_5 medium with 13% sugar is extensively used as extraction solution for isolation of microspores. Extraction solution (ES) plays an important role in supplying osmotic pressure and nutrition for isolated microspores. The treatment of microspore with ES is carried out for limited time and ES is discarded immediately after extraction, so the nutrition absorbed from the solution by microspore is very limited. In our study, we substituted B_5 medium with 13% sugar solution for treating microspore. Results indicated that 13% sugar solution had the similar function of supplying osmotic pressure and got the same embryo yield as B_5 medium. This improvement not only saved the costs of many organic and inorganic compounds, but also decreased the labor for B_5 medium preparation.

The traditional procedure of isolating microspore culture was used to incubate isolated microspores at 32°C for 2 days in the dark and then transfer them to 25°C in the dark till the embryos were visible for naked eyes. Compared with traditional procedures, the improved procedures not only produced the similar yield of embryos, but also decreased the time of embryo visibility by 4 days. In addition, it could save the labors of transferring embryos from 32°C incubators to 25°C incubators and 25°C incubators were not necessary.

In China, many laboratories prefer to culture isolated microspores for more than one time in a year. The first culture is usually conducted in local laboratory and another culture is carried out in other places in another suitable seasons. Therefore, embryos must be transported long-distance from remote sites to local laboratories and many laboratories are daunted by embryo injection during transportation. We studied two types of transportation methods. In one transportation method, embryos were transferred in sealed sterile triangular flasks with solid B_5 medium and in another it was transferred in sealed sterile centrifuge tubes with liquid B_5 medium. Each of these transfer method had its advantage and disadvantages. Since solid B_5 medium was stable, embryos were separated from each other and no injection occurred during transportation. However, the efficiency of this type of transportation was low. Compared with the transportation with solid B_5 medium, the space efficiency of transportation with liquid B_5 medium was 54 times higher. However, in liquid medium tubes frequently shook during transportation and ratio of injection tubes was also higher. Depending on the requirements researchers could choose suitable embryo transfer method.

In studying vernalization, embryos were stored at 4°C for about 10 days before they were transplanted to B_5 solid medium. Although, there was no plant growth regulator present in B_5 medium, most of the embryos develop into normal plantlets and subculture was not necessary. Therefore, the improved techniques of isolated microspore culture have wide range of practical applications.

Conclusion

In this study, we modified previous cultural techniques of isolated microspores. The sampling stage was carried out by about 9 days before the first flower in population bloomed and required more than 20°C. Suitable-sized buds were selected (2.8 to 3.5 μm) from the inflorescences in the field and leave small buds. The selected buds were disinfected with 84 disinfectants. The microspores were extracted with 13% sugar solution instead of B_5 medium. And the isolated microspores were incubated in NLN medium with 16% of sugar at 32°C in dark until the embryos became visible to naked eyes. Then the embryos were transferred to shakers in 25°C in dark. We established that for long distance transport of embryo, solid B_5 medium could be used. However, for transporting large number of embryos to a short distance, liquid B_5 medium could be a better choice.

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