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# Improved microspore culture and doubled-haploid plant regeneration in the brown condiment mustard (*Brassica juncea*)

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**Abstract** The availability of doubled haploids could greatly contribute to improving seed quality in condiment mustard (*Brassica juncea*). We have developed an efficient and reliable protocol of microspore culture, modified from that of Baillie et al. (1992), based on a modification of the sucrose concentration of culture media. A comparison of microspore culture media differing in their sucrose content showed that a decrease from 17% (w/v) sucrose during the first 48 h to 10% (w/v) thereafter favoured an increase in the production of embryos whatever the responding genotype tested. Thus, out of the 13 *B. juncea* genotypes studied, 12 gave rise to embryos, and seven of these embryos could be converted into plants. Doubled-haploid plants were produced after treatment with colchicine.

**Keywords** *Brassica juncea* · Microspore culture · Doubled haploids · Sucrose

**Abbreviations** *DH* Double haploids · $GA_3$ Gibberellic acid · *MS* Murashige and Skoog (1962)

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## Introduction

The exploitation of microspore culture would clearly enhance the breeding of condiment brown mustard (*Brassica juncea*) by accelerating the process through the production of genetically stable homozygous lines.

Isolated microspore culture techniques have been reported to produce DH plants in *Brassica* (Palmer et al. 1996), but there have been few reports of this technique being applied to *B. juncea*. Among these, Thiagarajah and Stringam (1990, 1993) have described the methods they used with one oil mustard genotype, RLM 514, but gave no details on the efficiency of embryogenesis or conversion of embryos into plants. Likewise, Purnima and Rawat (1997) did not provide precise data on the embryogenesis efficiency they obtained with cv. Pusa jaikisan. In the only remaining article on *B. juncea* microspore culture, Hiramatsu et al. (1995), working with 24 commercial inbred cultivars of leaf mustard, reported obtaining seven that were embryogenic, five of which converted embryos into a total of 32 regenerated plants. Thus, the efficiency of the technique was particularly dependent on the donor plant genotype, which affected the frequency of embryogenesis, the quality of the embryos and the plant regeneration competence. On the other hand, successful results with anther cultures of *B. juncea* have been limited to a reduced number of genotypes (George and Rao 1982; Palmer et al. 1996), with the embryogenesis responses being consistently lower than those obtained from microspores (Lichter 1982; Palmer et al. 1996). Against this background, and since the protocols existing for *B. juncea* had given us a low yield of both embryos and plants with the genotypes we had tested, we decided to attempt to improve the methodology by acting on different factors and culture conditions which affect the production of embryos.

This report describes a reliable microspore culture protocol that produced several thousands of embryos and plants for condiment mustard forms of *B. juncea*.

This protocol is derived from that developed for *B. campestris* by Baillie et al. (1992) and is based on a modification of the sucrose concentration of the microspore culture media. More than 70% of the microspore-derived plants were diploid, either spontaneously or following colchicine treatment.

## Materials and methods

### Donor plants and growth conditions

Altogether, 17 genotypes from five members of the Brassicaceae were used:

- 13 *B. juncea* genotypes, selected from the *B. juncea* breeding programme of the Ecole Nationale d'Enseignement Supérieure d'Agronomie de Dijon (ENESAD); BJ-74, BJ-78 and BJ-99 were inbred lines, and BJ-1 to BJ-10 were the $F_2$, $F_3$ or $F_4$ progenies from different crosses
- *B. nigra* clone N13–95
- *B. napus* cv. Drakkar
- *B. rapa* clone R5–95
- *Sinapis alba* cv. Martigena

Except for *B. napus,* which was obtained from the Unité de Malherbologie et Agronomie, INRA Dijon, seeds of all genotypes were supplied by ENESAD.

Seeds were sown in a mixture of soil and grit; plants grew in a growth chamber under a 16 h-light photoperiod provided at an intensity of 220 $\mu E\ m^{-2}\ s^{-1}$ and a day/night temperature of 21°/18 °C. Plants were fertilised twice a week with a 15-2-9 (N-P-K) nutrient solution.

### Microspore isolation

Flower buds (2–3 mm long) were harvested from several plants of each genotype. They were surface-sterilised in 99% ethanol for 10 s, then in a 50 g/l calcium hypochlorite solution for 20 min, followed by three rinses with sterile distilled water. The buds were blended at high speed for 10 s in a Polytron Kinematica blender in B5 medium (Gamborg et al. 1968) with 13% (w/v) sucrose (B5-13). They were then filtered through a 48-μm nylon mesh and collected in 50-ml centrifuge tubes. The meshes and beakers were rinsed with 30 ml of the B5–13 medium, and the filtrates were centrifuged at 130 *g* for 10 min. The pellets were resuspended in 20 ml of B5-13 and centrifuged at 130 *g* for 10 min; this procedure was repeated twice. The last pellet was resuspended in NLN medium (Litcher 1982) with 13% (w/v) (NLN-13) or 17% (NLN-17) sucrose. The number of microspores was estimated using a Malassez cell, their density was adjusted to $5 \times 10^4$ microspores per milliliter with NLN-13, and 10 ml of microspore suspension, representing ten buds per dish, was dispensed into 9-cm petri dishes (Falcon 1005). For the induction of embryogenesis, the microspore cultures were incubated for 7 days at 32.5 °C in the dark.

### Microspore culture protocol

During the first set of experiments, microspores of BJ-3 and BJ-5 were cultured either: (1) continuously in medium NLN-13 (control), (2) during the first 48 h in media NLN-13 then transferred onto NLN-10 or (3) in NLN-17 for the first 48 h and then transferred onto NLN-10. For culture conditions (2) and (3), there was a medium change after 48 h; the microspore suspensions were pipetted into a 50-ml tube and centrifuged at 130 *g* for 10 min, then the pellet was resuspended in NLN medium with 10% (w/v) sucrose (NLN-10). A second set of experiments, based on the first one, included only continuous culture in medium NLN-13 above (control) and the media sequence NLN-17/10, but it was applied to all genotypes. There were three replicates with 1–12 dishes per replicate depending on the experiment, except for *B. nigra*, *B. napus* and *B. rapa* where only two replicates were performed.

After 7 days at 32.5 °C, the dishes were incubated at 24 °C in the dark for 1 or 2 weeks. The embryos were then counted before being transferred onto a gyratory shaker (50 rpm) and cultured at 25 °C, first under dim light (45 $\mu E\ m^{-2}\ s^{-1}$) for 1 week, then under normal light (90 $\mu E\ m^{-2}\ s^{-1}$) for 1 or 2 weeks, until well-developed cotyledons were formed.

Results were expressed as the mean ± SD of the number of embryos induced per dish, and the effect of changing the sucrose concentration was compared to the constant NLN-13 culture medium (control). The means were compared according to *F*-test at $P = 0.05$.

### Embryo culture and plant regeneration

Cotyledonary embryos were transferred onto a solid B5 medium with 2% sucrose, 1 mg/l $GA_3$ and 0.6% agar (Difco) and cultured at 21°/18 °C (day/night) under a 16-h (light) photoperiod (90 $\mu E\ m^{-2}\ s^{-1}$). Embryos which developed normal plantlets from the primary shoot apex were transferred into tubes containing a hormone-free MS medium with 0.7% agar and 2% (w/v) sucrose. Plants with developed roots and a normal shoot were transferred to soil in 7-cm pots and acclimatised in the growth chamber.

### Ploidy level measurement and colchicine doubling

After acclimatisation, the ploidy level of the plants was determined with a flow cytometer (Partec CAII). A young leaf was chopped up with a sharp razor blade in a petri dish in a ploidy buffer (0.1 *M* Tris, 2 m*M* $MgCl_2$, 0.1 *M* NaCl, 0.05% Triton X-100, pH 7; Gilissen et al. 1993). The suspension of nuclei and cellular debris was filtered through a 40-μm nylon mesh. A drop of the fluorochrome DAPI (4′,6-diamidino-2-phenylindole; Chemunex) was added, and the filtrate was mixed before it was analysed by flow cytometry.

Spontaneous diploid plants were bagged to produce selfed seeds. The haploids were treated with colchicine. The chromosome-doubling treatment used in these experiments is the one most frequently applied to *Brassica* species because of its simplicity (Palmer et al. 1996) and was initially developed for *B. napus* by Coventry et al. (1988). Thus, the roots of the nearly flowering plants were washed and immersed in a 3.4 g/l colchicine solution for 30 min, 90 min and 3 h. The roots of the treated plants were rinsed and the plants planted in soil. After 1 month, the ploidy level was confirmed by flow cytometry; the fertile inflorescences which produced seeds were noted after 2 months.

## Results and discussion

### Microspore embryogenesis

Figure 1 shows the effect of the sucrose concentration on embryo production for genotypes BJ-3 and BJ-5. In both cases, a reduction in the sucrose level in the NLN medium, from 17% to 10% (NLN-17/10), after 48 h of culture increased significantly, by six- to sevenfold, the number of embryos relative to the continuous culture in 13% sucrose (NLN-13 medium) used generally for *B. juncea* (Hiramatsu et al. 1995; Purnima and Rawat 1997). Similarly, the medium change from 13% to 10% (NLN-13/10) sucrose increased embryo production for BJ-3 and BJ-5, but on a lower scale. This result

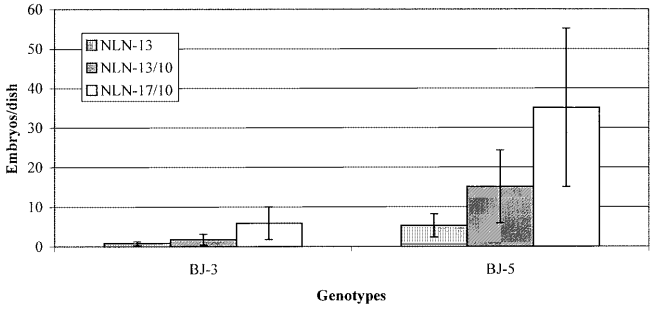


**Fig. 1** The effect of the sucrose concentration on embryo production in the BJ-3 and BJ-5 genotypes

confirms the positive effect – with respect to number of embryos – of changing the sucrose level during microspore embryogenesis, but the difference in sucrose concentration between NLN-13 and NLN-10 was not enough to induce a good rate of microspore embryogenesis as compared to the difference between NLN-17 and NLN-10, particularly for some genotypes.

The most detailed study on sucrose requirement and its effect on embryo induction and development has been published by Dunwell and Thurling (1985). They indicated that a high level of sucrose is beneficial for the induction of microspore embryogenesis of *B. napus* because a medium containing 17% sucrose generates an osmotic potential similar to that of the anther homogenate. However, a lower level of sucrose is important for a sustained level of microspore division. They suggested transferring the cultures from a high- to a low-sucrose medium to increase the rate of survival. Baillie et al. (1992), working with four genotypes of *B. campestris*, and Ferrie et al. (1999), with *B. oleracea*, demonstrated that 17% sucrose for 48 h followed by a media change to 10% sucrose increases the frequency of microspore embryogenesis. Our evaluation of the effect of a change in the sucrose level on microspore embryogenesis of *B. juncea* genotypes was in agreement with these earlier results.

By extending the protocol to different species of *Brassicaceae*, the results indicated that 17% sucrose for 48 h followed by a medium change to 10% sucrose increased significantly the production of embryos in most of the genotypes of *B. juncea* (Table 1). The yield of embryos obtained in NLN-17/10 for the responsive genotypes varied from 0.2 to 35.1 embryos per dish, which is higher than the yield obtained by Hiramatsu et al. (1995) and by Ohkawa et al. (1988). This was also 1.5- to 13-fold higher than the yield obtained from microspores cultured continuously in NLN-13 medium. The only exceptions were for genotypes BJ-99, which showed no clear tendency for one or the other strategies, and BJ-7, which always gave the lowest yields irrespective of the culture protocol applied. This change was also efficient on *B. nigra* but was not significantly different from continuous culture in NLN-13 medium for *B. napus*.

**Table 1** The effect of sucrose concentrationon embryo production in *Brassica juncea, B. nigra*, *B. napus*, *B. rapa* and *Sinapis alba*

| Genotype | NLN-13 | | NLN-17/10 | |
|---|---|---|---|---|
| | Total number of dishes | Mean number of embryos per dish[a] | Total number of dishes | Mean number of embryos per dish |
| BJ-1 | 15 | 0.6a | 15 | 1.3b |
| BJ-2 | 13 | 0.3a | 13 | 0.5[b] |
| BJ-3 | 9 | 0.8a | 14 | 5.9b |
| BJ-4 | 12 | 2.2a | 13 | 3.8b |
| BJ-5 | 7 | 5.3a | 11 | 35.1b |
| BJ-6 | 17 | 0.5a | 15 | 4.7b |
| BJ-7 | 12 | 0.1a | 14 | 0b |
| BJ-8 | 13 | 0a | 14 | 0.3b |
| BJ-9 | 12 | 0 | 13 | 0 |
| BJ-10 | 13 | 0.8a | 9 | 10.7b |
| BJ-J74 | 16 | 0a | 12 | 0.2b |
| BJ-J78 | 10 | 0a | 7 | 3.4b |
| BJ-J99 | 14 | 17.6a | 15 | 16.6a |
| *B. nigra* | 14 | 0.1a | 12 | 14.4b |
| *B. napus* | 13 | 410.1a | 7 | 477.1a |
| *B. rapa* | 17 | 1.4a | 10 | 0.5b |
| *S. alba* | 13 | 0.2a | 12 | 0b |

[a] Means followed by the same letter, for each genotype, do not differ significantly according to the *F*-test ($P = 0.05$)

A strong genotype effect was observed with respect to embryo production (Table 1). Thus, genotypes BJ-3, BJ-5, BJ-10, BJ-99 were highly responsive, regenerating embryos and plants easily, while BJ-9 gave no embryos. All the remaining genotypes gave a low yield of embryos, and both embryo development (BJ-2, BJ-7, BJ-8, BJ-74, BJ-78) and the formation of normal plantlets (BJ-1, BJ-4, BJ-6) were difficult.

It is currently acknowledged that the donor plant genotype is one of the most important factors affecting microspore culture in *Brassica* (Palmer et al. 1996). In the present study, 12 of the 13 *B. juncea* genotypes studied produced embryos; in seven of these embryos were converted into plants and only one remained unresponsive to androgenesis. This compares very favourably with the results of other authors for *B. juncea*, where the ratio of responsive genotypes was around 30% for microspore cultures (Hiramatsu et al. 1995) and less than 7% for anther cultures (George and Rao 1982).

*B. napus* cv. Drakkar was highly responsive to microspore embryogenesis, but the embryos produced in the NLN-17/10 media sequence never regenerated plants as opposed to those produced in NLN-13 medium (results not shown). Conversely, the *B. rapa* genotype studied was a low-responsive one – few embryos were produced and the yield was better in the standard medium, NLN-13. This is in line with results showing that *B. rapa* is a relatively recalcitrant species in cell culture, particularly with respect to microspore culture (Baillie et al. 1992; Guo and Pulli 1996). *Sinapis*

*alba* microspores showed a very low response under our experimental conditions; this contrasts with data by Leelavathi et al (1984), but they studied anther culture of this species.

## Regenerated plants

Globular embryos were visible after 15 days of culture. After 2 or 3 weeks of culture, the cotyledonary embryos were transferred under light conditions to a solid medium, and plants could be regenerated after 2 months. We observed that none of the globular embryos produced after 3 weeks of culture developed normally or converted into a plant. Only embryos transferred at the cotyledonary stage gave a high plant regeneration frequency. As an example, the data in Table 2 summarise the results obtained in an independent experiment, where up to 29% of the microspore-derived embryos produced normal plants in BJ-1, BJ-3, BJ-4, BJ-5, BJ-6, BJ-10 and BJ-99 genotypes; however, the yield of regenerated plants was always better from embryos derived in NLN-17/10 media than with those derived in NLN-13 medium. In this context, it is worthwhile remarking that data on the actual efficiency of embryo conversion into plants in the literature are scant. Hiramatsu et al. (1995) state that they obtained 21% of embryos at the torpedo to cotyledonary stage (the only ones from which plants could be regenerated).

## Ploidy level and colchicine doubling

The ploidy level was determined in all regenerated plants from the BJ-1, BJ-3, BJ-4, BJ-5, BJ-10 and BJ-99 genotypes obtained in independent experiments (Table 3). For these *B. juncea* genotypes, 0 (BJ-1) to 22% (BJ-5) of the microspore-derived plants were spontaneous diploids. Spontaneous doubling of chromosomes in vitro is an inherent tendency in several species (Rao and Suprasanna 1996). In *Brassica*, the frequency of spontaneous doubling depends on the genotype used, the microspore stage and the culture conditions, particularly the low temperature treatments (Keller and Armstrong 1978; Charne et al. 1988; Chen and Beversdorf 1992). Thus, in microspore cultures of *B. juncea*, Hiramatsu et al. (1995) regenerated 19% of fertile plants, considered to be diploids, without any chromosome doubling treatment. In an earlier study with anther cultures of *B. juncea*, George and Rao (1982) obtained embryos that never developed into normal plants, but plants were eventually regenerated from hypocotyl explants derived from such abnormal embryos, whereby 57% of such regenerants proved to be diploid. Given the intervening phase of regeneration from hypocotyls, and coupled with the fact that this was preceded by callusing, there is no evidence that such diploids were actually derived from the original microspores. Indeed, the genetic instability of callus tissues is commonplace, and several mechanisms, i.e. endomitosis, endoreduplication or nuclear fusion, could explain the spontaneous doubling of chromosomes (Narayanaswamy and Chandry 1971). Such spontaneous diploids can be useful to breeders for a rapid production of lines on the condition that they are homozygous.

Haploid and spontaneous diploid plants of BJ-1, BJ-3, BJ-4, BJ-5, BJ-10, BJ-99 were acclimatised in soil and, thereafter, haploid plants were diploidised with a 3.4 g/l colchicine solution. In a first experiment with BJ-3 and BJ-5, various durations of colchicine treatment were assessed (Table 4). The chromosome-doubling effect was dependent on the duration of the treatment. Doubling was usually incomplete and, after 2 months, produced chimaeric plants with both haploid and doubled-haploid flowers. The highest diploidisation rate was reached by the 3-h treatment, but others factors like the percentage of mortality and fertile plants have to be considered in the determination of the optimal treatment. Indeed, the 90-min treatment appeared to be the optimum one under our experimental conditions, as all treated plants were fertile and

**Table 2** Microspore-derived normal plants for seven responsive genotypes of *B. juncea*

| Genotype | Medium | Embryos per dish[a] | Total no. of embryos | Regenerated plants |
|---|---|---|---|---|
| BJ-1 | NLN-13 | 0.6a | 9 | 0 |
| | NLN-17/10 | 1.3b | 20 | 1 |
| BJ-3 | NLN-13 | 0.8a | 6 | 0 |
| | NLN-17/10 | 5.9b | 77 | 13 |
| BJ-4 | NLN-13 | 2.2a | 26 | 0 |
| | NLN-17/10 | 3.8b | 49 | 8 |
| BJ-5 | NLN-13 | 5.3a | 41 | 5 |
| | NLN-17/10 | 35.1b | 364 | 106 |
| BJ-6 | NLN-13 | 0.5a | 9 | 0 |
| | NLN-17/10 | 4.7b | 71 | 12 |
| BJ-10 | NLN-13 | 0.8a | 10 | 0 |
| | NLN-17/10 | 10.7b | 118 | 33 |
| BJ-99 | NLN-13 | 17.6a | 247 | 24 |
| | NLN-17/10 | 16.6a | 249 | 68 |

[a] Means followed by the same letter, for each genotype, do not differ significantly at $P=0.05$ according to the $F$-test

**Table 3** Ploidy level of microspore-derived plants for six genotypes of *B. juncea*

| Genotype | Total of plants analysed | Percentage of haploids | Percentage of diploids |
|---|---|---|---|
| BJ-1 | 10 | 100 | 0 |
| BJ-3 | 16 | 87.5 | 12.5 |
| BJ-4 | 9 | 89 | 11 |
| BJ-5 | 125 | 75 | 22 |
| BJ-10 | 14 | 86 | 14 |
| BJ-99 | 40 | 77.5 | 20 |

**Table 4** Doubled-haploid production with colchicine treatment of microspore-derived haploids of *B. juncea* (BJ-3 and BJ-5)

| Time of treatment | Number of treated plants | Percentage of chromosome doubling | Percentage of mortality | Percentage of fertile plants |
|---|---|---|---|---|
| Control | 10 | 0 | 0 | 0 |
| 45 min | 10 | 70 | 0 | 80 |
| 90 min | 10 | 90 | 0 | 100 |
| 180 min | 10 | 100 | 10 | 90 |

**Table 5** Doubled-haploid production following a 3.4 g/l colchicine solution treatment of microspore-derived haploids of *B. juncea* for 90 min

| Genotypes | Number of treated plants | Percentage of fertile plants | Percentage of mortality |
|---|---|---|---|
| BJ-1 | 10 | 50 | 10 |
| BJ-3 | 4 | 100 | 0 |
| BJ-4 | 6 | 100 | 0 |
| BJ-5 | 16 | 81 | 0 |
| BJ-10 | 12 | 100 | 0 |

produced seeds without any mortality. The results obtained are consistent with those described by Coventry et al. (1988) for *B. napus* and by M.R. Thiagarajah for *B. juncea* (personal communication). Therefore, a doubling protocol of a 3.4 g/l colchicine solution for 90 min was then applied to the BJ-1, BJ-3, BJ-4, BJ-5 and BJ-10 haploid plants; the results are reported in detail in Table 5.

Despite the fact that we obtained an improvement in the microspore culture of condiment mustard, some of the genotypes remained recalcitrant to in vitro microspore embryogenesis. Nevertheless, the protocol optimised here enabled the production of several hundred DH plants from different genotypes of *B. juncea,* which can be used further in selection, either directly as lines or as parents for crossing.

A DH population derived from one $F_1$ plant from the cross of two interesting lines was produced by this protocol. More than 5,000 embryos were induced with a mean of 38 embryos per dish, and 714 plants were regenerated. Of the 381 acclimatised plants, 59% were haploids, 22% were diploids and more than 72% of the colchicine-treated haploid plants were fertile.

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