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ORIGINAL PAPER

Doubled haploid plants following colchicine treatment of microspore-derived embryos of oilseed rape (*Brassica napus L.*)

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Abstract An efficient method for producing doubled haploid plants of oilseed rape (*Brassica napus* L.) was established using in vitro colchicine treatment of haploid embryos. Haploid embryos in the cotyledonary stage were treated with one of four colchicine concentrations (125, 250, 500 and 1,000 mg/L); for one of three treatment durations (12, 24 and 36 h) at one of the two temperatures (8 and 25°C) and were compared to control embryos (without colchicine treatment). The number of chromosomes, seed recovery, size and density of leaf stomata, and pollen grain size from regenerated plants were determined. No doubled haploid plants were regenerated from control embryos; however, the doubled haploid plants were regenerated from colchicine-treated embryos. A high doubling efficiency, 64.29 and 66.66% of regenerated plants, was obtained from 250 mg/L colchicine treatment for 24 h and 500 mg/L colchicine treatment for 36 h, respectively, at 8°C. Following 500 mg/L colchicine treatment for 36 h, a few plants regenerated (9 plants). At the higher colchicine concentration (1,000 mg/L), no plant regenerated. These results indicate that the colchicine treatment of embryos derived from microspores can induce efficient chromosome doubling for the production of doubled haploid lines of oilseed rape.

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Introduction

Microspore culture has been routinely used for doubled haploid line production in oilseed rape breeding programs because of the high frequency of embryogenesis that can be achieved in a range of genotypes (Ferrie and Caswell 2011).

Doubled haploid (DH) lines are valued for use in breeding programs because traits can be fixed without multiple generations of selfing. DH lines are also useful in hybrid breeding and in the development of mapping populations. The true magnitude of the importance of this technology in basic research was not realized until researchers had access to a highly efficient doubled haploidy system. The development of a routine, consistent, and highly efficient doubled haploidy protocol is integral to utilizing these technologies (Ferrie and Mollers 2010).

Colchicine can be applied during several stages of the microspore culture process, from isolated microspores to the regenerated plants. The usual methods for chromosome doubling involve soaking roots or whole plants in a colchicine solution (Fletcher et al. 1998), culturing plantlets in colchicine—containing medium in the greenhouse (Mathias and Robbelen 1991) or using colchicine in microspore isolation media (Zhao et al. 1996; Zhou et al. 2002a, b; Weber et al. 2005). Other alternatives are injecting colchicine into the buds or applying a colchicine-soaked cotton swab to the apex or axillary buds (Lichter et al. 1988; Gland 1981). However, the methods that involve the immersion of roots or whole plantlets in a colchicine solution are laborious, time consuming and require relatively large amounts of an

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expensive chemical. These methods often increase the percentage of chimeric plants with relatively small sectors of diploid tissue, which will produce a few selfed seeds. In addition, keeping colchicine in petri dishes is much safer than handling it with whole plants.

Using small haploid embryos derived from microspore culture, the chimeric sectors could be decreased in size or eliminated. Additionally, this method can start with manipulation of either microspores or embryos (Abdollahi et al. 2009), and the resulting doubled haploid plants can be used in mutagenesis and transformation studies (Sonntag and Rudloff 2004).

A major technical challenge in microspore culture, and a cause of inefficiency and additional cost in plant breeding programs is the separation of haploid and doubled haploid plants. Many techniques can be used for this separation, such as flow cytometry (Takahira et al. 2011), the root tip squash technique and morphological measurements. This is the first study to use the leaf stomata size and density in oilseed rape to differentiate between haploid and doubled haploid plants.

Also, the present study is the first simultaneously determine in systematic manner the effect of the colchicine concentration, the duration of the colchicine treatment and the treatment temperature on the induction of chromosome doubling and on the plant regeneration rate in comparison with untreated controls at the cotyledonary stage of embryo development in oilseed rape.

Materials and methods

Plant growth conditions

The donor plants included one F1 hybrid obtained from a SLM046 (winter) × RGS003 (spring) cross of oilseed rape (*Brassica napus* L.). Donor plants were grown in a controlled growth chamber with a 16/8 h photoperiod, a day/night temperature of 15/10°C and a light intensity of 500 $\mu molm^{-2}s^{-1}$.

Microspore culture

Buds were selected on the basis of size (2–3 mm), were placed in baskets and were surface sterilized in 5.25% sodium hypochlorite for 10 min on a shaker followed by two 5 min washes with sterile water. Up to 40 buds, the majority of which were in the late-uninucleate and early-binucleate stage, were blended with a blender in 30 ml of cold microspore isolation solution containing 13% sucrose at pH 6 (Fletcher et al. 1998). The crude suspension was filtered through a 106 μm metal mesh followed by a 53 μm mesh. Both the cups and meshes were rinsed, and a total of 50 ml was collected into two 50 ml centrifuge tubes. The microspore suspension was then centrifuged at 1,300 rpm for 4 min, the supernatant was removed, and 25 ml of microspore isolation solution was added to each tube. This step was repeated twice, and then, 4–5 ml of filter-sterilized and modified NLN-13 liquid medium (Lichter 1982) supplemented with 13% sucrose but free of potato extract and growth regulators, was added to the microspores. Then, the culture density was determined by a hemocytometer to achieve the desired density (40,000 microspores per ml), and 8 ml of microspore suspension was dispensed into each sterile Petri dish (60 × 20 mm). Cultures were incubated in the dark at 30°C for 14 days and then transferred to 25°C in the darken on a shaker (40 rpm).

Colchicine treatment

An induction medium (NLN-13 medium) containing 0.1% colchicine was prepared and filter sterilized using a 0.2 μm filter. Haploid embryos in the cotyledonary stage were treated with one of four colchicine concentrations (125, 250, 500 and 1,000 mg/L), one of three treatment durations (12, 24 and 36 h) and one of two temperatures (8 and 25°C), and then, the characteristics of the regenerated plants were compared to those of control plants. After the colchicine treatments, the embryos were transferred into colchicine-free NLN-13 medium.

Plant regeneration

After colchicine treatment the embryos were transferred to solid MS regeneration medium (2% sucrose, half-strength macro nutrients and 0.1 mg/L GA_3 (gibberellic acid), solidified with 0.7% agar–agar, pH 5.8) in plastic Petri dish (120 × 20 mm) containing 12.5 ml medium. After an initial period of 10 days at 2°C, the cultures were incubated in a controlled growth chamber at 24°C with a 16 h photoperiod with low light intensity provided by fluorescent tubes. When shoots developed, they were transferred to larger growth vessels with the same solid MS medium without GA_3. Then, the plantlets were transferred to a soil-perlite mixture (2:1) and kept for 2 weeks in a nursing room at a temperature with a 24°C with a, 16 h photoperiod length, a low light intensity and a high relative humidity.

Morphological traits measurements

The length, width, and density of the stomata were measured on the axial leaf surfaces by the observation of a thin layer of leaf. The density of the stomata was counted at 40× magnification. The length and the width of stomata were measured at 100× using a DP12 digital camera

interfaced to a BX50 Olympus microscope (Olympus Optical Co., Ltd.) for 30 leaves from plants confirmed to be doubled haploid and haploid by flow cytometry (FCM). Pollen grains were collected from flowers at anthesis, and their lengths were measured at 100× under a light microscope; for 30 samples were analyzed for both confirmed doubled haploid and haploid plants (Fig. 1).

Ploidy level evaluation

The ploidy status/DNA content of the regenerated plants was determined using flow-cytometry (PA, Partec, GmbH, Münster, Germany). Measurements were calibrated with two standards: (1) the Partec DNA standard and (2) seed-derived winter rape plants with diploid status (2n = 38) as a diploid control. A 25 mg sample of young leaf was placed in OTTO I extraction buffer (Otto 1990) for the isolation of nuclei. OTTO II (180 ml) solution containing the DNA-specific fluorochrome DAPI (4, 6-diamino-2-phenylindole) was added to the obtained suspension of isolated nuclei to visualize them (Hause et al. 1992), and then the solution was filtered through a nylon filter mesh (30 µm). After short-term incubation in the extraction solution, the filtrate was immediately analyzed by flow-cytometry. After measurements of about 2–5 thousand nuclei, the relative content of DNA was determined. At a per gain FL1 of 412–420 (relative fluorescence), a peak set at 100 and 200 FL (corresponding to the G1and, G2/M-phases, respectively) was interpreted as corresponding to diploid or doubled haploid plantlets (Fig. 2a). A peak set at 50 and 100 FL was interpreted as corresponding to haploid material (Fig. 2b) (Weber et al. 2005). Moreover, the chromosome numbers were verified using the root tip squash method (Sharma and Gupta 1982).

Data analysis

The experiment was carried out in a 3-factorial manner (based on a completely randomized design) with 5 replications. Each replication consisted of one Petri dish containing 25 embryos. The three studied factors were colchicine concentration, treatment duration and temperature.

Analysis of variance (ANOVA) and multiple comparisons with Duncan's test were carried out using SPSS statistical software (version 13.0).

Results

Plant regeneration

Colchicine treatment (125, 250, 500 and 1,000 mg/L) was applied to cotyledonary embryos of F1 *Brassica napus* for 12, 24 and 36 h in embryo induction medium (NLN-13). The embryos had a normal slightly elongated root/shoot axis with two very conspicuous cotyledons surrounding the shoot apex (Fig. 1a). Most of these healthy embryos could directly regenerate vigorous shoots and successfully developed into normal plants. After treatment with 1,000 mg/L of colchicines, there were no regenerated plants. Similarly, a longer duration of colchicine treatment (36 h) had negative effects on plant regeneration.

In addition, the highest plant regeneration frequency was achieved using a low concentration of colchicine (125 and 250 mg/L) for 12 and 24 h (Table 1). The temperature of 8°C was better than 24°C for plant regeneration at all colchicine concentrations (Table 2).

Morphological investigation

Some morphological characteristics such as the size and density of leaf stomata and the size of pollen grains were used to identify haploid and doubled haploid plants. Colchicine treatment delayed shoot growth in the first month after treatment, but afterward, both treated and untreated embryos grew similarly. The haploid plants did not produce any seeds, and siliques were depleted.

Leaf stomata length, width, and density were significantly different between the doubled haploid and haploid plants. The length and width of the stomata in doubled

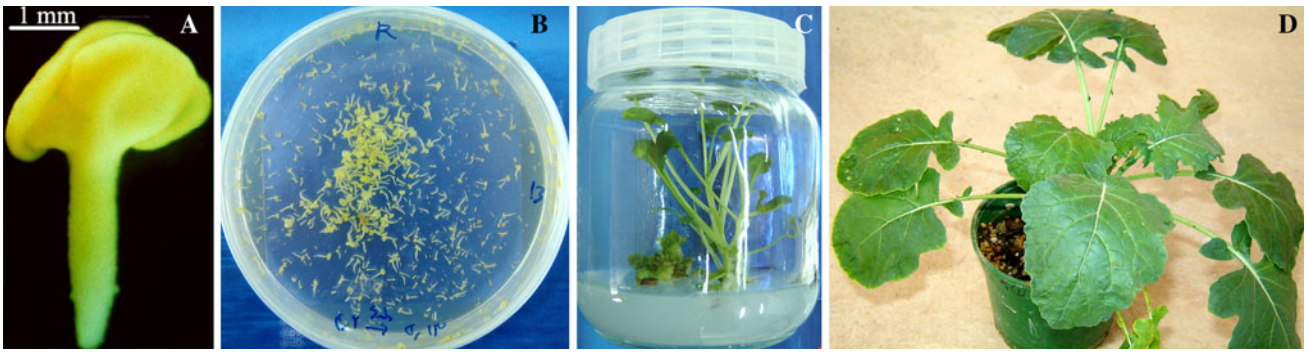

Fig. 1 Haploid embryos in cotyledonary stage (**a** and **b**) normal regenerated plant (**c**) normal doubled haploid plant (**d**)

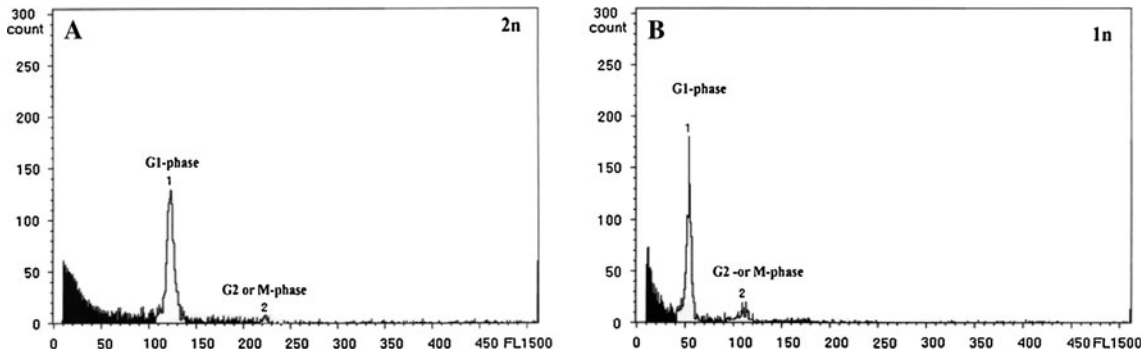

Fig. 2 Flow cytometric analysis of the ploidy level. The x-axis of the histogram represents the intensity of DNA fluorescence in relative units; the y-axis represents the number of nuclei counted per histogram channel. **a** A representative peak set for doubled haploid material. **b** Peaks corresponding to a typical haploid individual

Table 1 Effects of colchicine concentration and duration treatment on regeneration and recovery of doubled haploid plants of oilseed rape

Colchicine treatment concentration (mg/L)	Colchicine treatment duration (h)	Number of regenerated plants	Number of doubled haploid plants
0 (control)	0	53 ± 2.4^{a}	0^{f}
125	12	49 ± 6.1^{ab}	10 ± 1.8^{bc}
	24	37 ± 3.3^{bc}	9 ± 0.9^{bc}
	36	18 ± 1.2^{d}	8 ± 0.5^{c}
250	12	41 ± 3.1^{b}	13 ± 2.9^{b}
	24	42 ± 2.8^{b}	27 ± 1.3^{a}
	36	15 ± 3^{d}	9 ± 0.9^{bc}
500	12	17 ± 1.1^{d}	7 ± 0.8^{cd}
	24	14 ± 4.6^{d}	8 ± 0.6^{c}
	36	9 ± 1^{e}	6 ± 0.6^{d}
1,000	12	0^{f}	0^{e}
	24	0^{f}	0^{e}
	36	0^{f}	0^{e}

A total of 500 embryos were used per replication and this was repeated three times. Mean with different letters within columns are significantly ($P < 0.01$) different following Duncan's multiple range test

Table 2 Effects of temperature during colchicine treatment on regeneration and recovery of doubled haploid plants of oilseed rape

Colchicine treatment temperature (°C)	Number of regenerated plants	Number of doubled haploid plants
8	167 ± 8.8^{a}	62 ± 7.7^{a}
24	75 ± 5.9^{b}	35 ± 4.1^{b}

A total of 500 embryos were used per replication and this was repeated three times. Mean with different letters within columns are significantly ($P < 0.01$) different following student's *T*-test

haploid leaves were larger than those of haploid leaves (23.05 μm length and 18.26 μm width in doubled haploid leaves and 17.32 μm length and 14.11 μm width in haploid leaves; Fig. 3). However, the density of stomata was reduced to about half in doubled haploid plants compared with haploid plants (8.5 and 16, respectively, in an area of 312 × 234 μm; Fig. 3).

Larger anthers and pollen grains were observed for the doubled haploids than for the haploids (29.09 and 17.95 μm, respectively; Fig. 4). Additionally, the number pollen grains in the haploid plants was reduced considerably.

Chromosome doubling efficiency

Colchicine treatment during the microspore culture at the cotyledonary stage caused an increase in doubling efficiency. The highest doubling efficiencies, 64.29 and 66.66%, were obtained using treatment with 250 mg/L colchicine for 24 h and 500 mg/L colchicine for 36 h, respectively. Treatment with 500 mg/L colchicine for 36 h significantly decreased the frequency of regenerated plants, and at the colchicine concentration of 1,000 mg/L, no plant regenerated. The best treatment for chromosome doubling was 250 mg/L colchicine for 24 h. This treatment produced the highest frequencies of doubled haploids and regenerated plants (Table 1). In addition, the temperature of 8°C was better than 24°C for inducing doubled haploid plants (Table 2).

Discussion

The present results show that an efficient chromosome doubling of haploid microspores of *Brassica napus* can be achieved by treating microspore derived embryos with colchicine. A high chromosome doubling efficiency of 64.29% was obtained using 250 mg/L of colchicine for

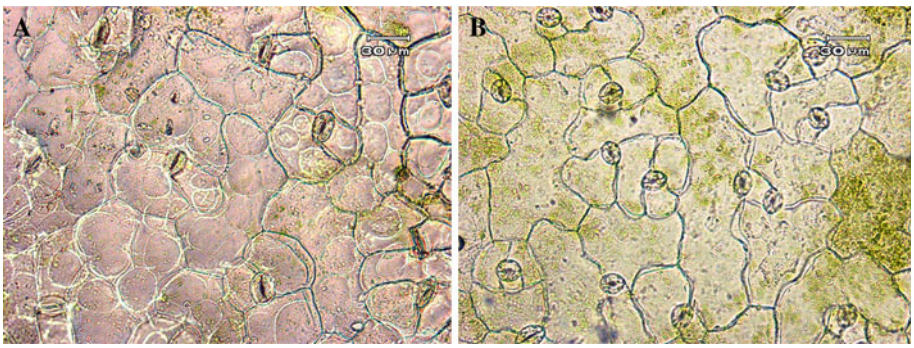

Fig. 3 Density and size of stomata in doubled haploid (**a**) and haploid plant (**b**)

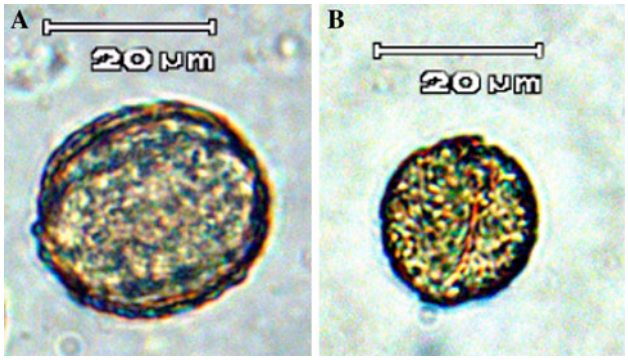

Fig. 4 Size of pollen grain in doubled haploid (**a**) and haploid plant (**b**)

24 h at 8°C. These results are in good agreement with previous studies on the colchicine treatment of plants or plantlets.

The duration of colchicine treatment is a critical parameter for chromosome doubling. The duration of colchicine exposure had a considerable effect on chromosome doubling induction and the survival rate. The present experiment showed that a treatment duration of 12 h had fewer positive effects on the doubling efficiency, especially at the 125 mg/L colchicine concentration.

Plants regeneration directly and rapidly from the shoot apex of microspore-derived embryos is an important step in doubled haploid production. Direct and rapid regeneration ensures minimal occurrence of cytogenetic abnormalities, which is extremely important in a breeding program (Fletcher et al. 1998). Large and healthy-looking embryos were obtained after colchicine treatments (except 1,000 mg/L colchicine), and after being transferred to solid plant regeneration medium and an initial period of low temperature (2°C) for 10 days, these embryos germinated well at 24°C and easily regenerated vigorous plants. These plants were complete with roots, stems, leaves and shoot apexes and were ready to be transferred to soil. The time period from microspore isolation to the transfer of regenerated plants to soil was only 8–9 weeks, and more importantly, most of these plants were already doubled haploid plants. Compared to colchicine treatment of microspore derived plants, immediate colchicine treatment of microspore-derived embryos results in a high chromosome doubling rate and a low chimeric percentage in *Brassica napus* (Chen et al. 1994; Zhou et al. 2002a, b).

The enlarged stomata and pollen grains of induced doubled haploid plants reported in our study have alson bee observed in other plants (Takamura and Miyajima 1996; Thao et al. 2003; Majdi et al. 2010). In the present study, an increased ploidy level was correlated with a larger size of the guard cells and a lower density of leaf stomata. The reduced stomatal density in doubled haploid plants was similar to that observed in other studies (Chakraborti et al. 1998; Khazaei et al. 2009). A positive correlation between the amount of genomic DNA and the seed, pollen, and leaf sizes has been reported previously (Chung et al. 1998). A positive correlation between cell and organ size and nuclear DNA content has also been reported (Leitch and Bennett 2003). Increased cell size resulting from doubled haploidy induction may be one of the important reasons for the enlargement of plant organs, e.g., the leaves and flowers. The positive effect of ploidy on cell size was also reported recently in tetraploid Arabidopsis in which increasing DNA content led to further cell growth (Breuer et al. 2007).

It seems that leaf stomata size measurements are the simplest parameters for identifying doubled haploids at early growth stages. The length and width of stomata of doubled haploids were twofold larger than those of haploid plants. Such alterations in leaf stomata size have also been reported for other plants (e.g., Chakraborti et al. 1998; Sikdar and Jolly 1994).

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