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INDUCED CHROMOSOME DOUBLING IN MICROSPORES AND REGENERATED HAPLOID PLANTS OF *BRASSICA NAPUS*

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The spontaneous diploidization rates in oilseed rape (*Brassica napus* L.) via *in vitro* androgenesis are too low for practical applications. In contrast, artificial doubling of chromosomes of the microspore has proven to be more successful and allows homozygous plants to be obtained in a short time. Here, we present the efficiency of diploidization of *B. napus* haploids using three different chromosome doubling methods.
Using the *in vitro* approach in microspores, the rate of chromosome doubling in 24 populations of androgenic plants ranged from 15.8% to 94.0%. An alternative *in vivo* method for the induction of chromosome doubling involves colchicine treatment of young haploid plants, and this yielded doubling rates ranging from 47.5% to 86.4% in 10 different plant populations. Another *in vivo* method of chromosome doubling is colchicine treatment of the excised young axillary shoots of haploid plants at the early flowering stage. The high efficiency of this method was confirmed in haploid plant populations from 11 genetically distinct donors in which the frequency of occurrence of diploids ranged from 53.3% to 100%. However, in this case, the time required for seed formation from doubled haploids increased by about 3–5 months. The availability of several methods of chromosome doubling at various stages of the androgenic process – from isolated microspores through to young plants and flowering plants – allows seeds to be obtained from nearly every selected individual haploid.

Keywords: *Brassica*, chromosome doubling, colchicine, doubled haploids, *in vitro*, microspores, methods

INTRODUCTION

The use of microspore cultures to generate homozygous lines, so-called doubled haploids (DHs), has become routine for genetic research and biotechnological manipulation in oilseed rape (*Brassica napus* L.), and represents a valuable tool in modern plant breeding (Cegielska-Taras et al., 2015). Breeders are particularly interested in DH lines because, when haploid chromosomes are artificially doubled, a completely homozygous line can be obtained within one generation. In contrast, the use of conventional plant breeding methods requires at least five generations of self-fertilization to obtain acceptably pure lines (97%) from heterozygous individuals. Genetic factors are fixed in DHs and will be identical in further generations, provided they are propagated in isolation. Therefore, every DH line can potentially become a cultivar (Cegielska-Taras et al., 2015; Rahman et al., 2016).

Alleles on the homologous chromosomes of each DH line are identical, and as a result genetic traits will not segregate. This creates the possibility of an earlier examination of qualitative and quantitative traits in the breeding process. The production of DH lines in *Brassica* genotypes has three limiting factors: (1) the embryogenic potential of the microspores, (2) the capacity of the microspores to undergo artificial diploidization after treatment with an antimitotic agent, and (3) the embryo-to-plant conversion rate (Möllers and Iqbal, 2009). For all three of these factors, large variations have

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been observed between different genotypes of oilseed rape, indicating that genetics plays a major role in their control (Ferrie and Keller, 2007; Kampouridis et al., 2016).

The frequency of spontaneous genome doubling during androgenesis in microspore culture varies according to plant species and has been reported as 8.7% (Takahira et al., 2011), 10–40% (Ren et al., 2017) or 10–26% in oilseed rape (Babbar et al., 2004), 70–90% in barley, 50–60% in rice, 50–90% in rye and 25–70% in wheat (Takahira et al., 2011; Ren et al., 2017). In oilseed rape, the spontaneous chromosome doubling rate is low and insufficient for practical applications. However, there are also problems with artificial doubling of chromosomes in the isolated microspores, which might not all be at the right stage for doubling or which might have an intrinsically low capacity for duplication. Therefore, other approaches have been developed that involve doubling the number of chromosomes at a later stage of androgenic plant development.

The process of induced doubling of the genome *in vitro* and *in vivo* depends on a large number of variables: plant species, application methods, media, explant type, antimitotic agents and their concentration, as well as exposure times. The interaction between these parameters is high. Various antimitotic agents such as colchicine, antimicrotubule herbicides like oryzalin, trifluralin and amiprophos-methyl, and dinitroanilines, among others, are used to promote chromosome doubling to obtain homozygous, fertile DH plants (Hansen and Andersen, 1996; Rahman et al., 2016). Colchicine is still the most widely applied antimitotic agent both *in vitro* and *in vivo*, especially for agricultural plant species (Dhooghe et al., 2011; Rahman et al., 2016). In *Brassica* spp., colchicine can be applied at several stages of the androgenesis process, from isolated microspores (Cegielska-Taras et al., 2002) and microspore-derived embryos (Mohammadi et al., 2012) to the regenerated plants (Fletcher et al., 1998). In this paper we show various methods of chromosome doubling at different stages of haploid development in *B. napus*.

MATERIALS AND METHODS

PLANT MATERIAL

Plant material consisted of 41 genetically distinct microspore donors of winter oilseed rape (*Brassica napus* L.). Donor plants marked as D3-D27 and S × T, as well as 12r, 17r and 29r, were F1 hybrids obtained by crossing resynthesized lines with natural oilseed rape. The other donors were F1 hybrids of natural *B. napus*. After seven weeks of vernalization, the plants were grown in a phytotron under controlled conditions (16 h photoperiod at 11/7°C day/night). From each donor plant, flower buds were selected and used immediately for microspore isolation (Cegielska-Taras et al., 2002).

IN VITRO COLCHICINE TREATMENT

After three washes in NLN13 culture medium, microspores were placed in medium with the addition of 0.05% colchicine (Fig. 1). After 22 h in the dark at 30°C or 32°C, the microspores were centrifuged and washed twice with fresh NLN13 medium. Microspores in NLN13 medium were kept in the dark at 30°C for 10 days or 32°C for 2 days and then incubated at 24°C still in the darkness

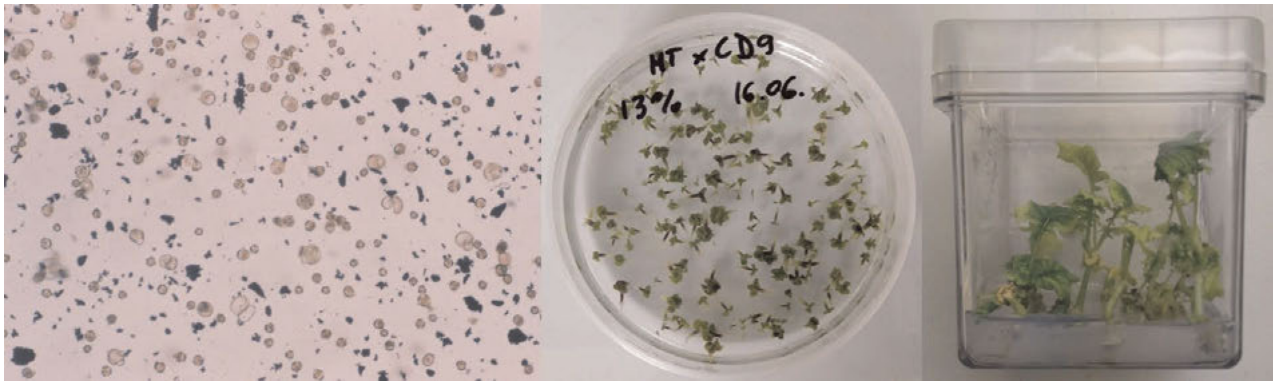

Fig. 1. Development of *Brassica napus* androgenic plant from microspores treated with *in vitro* colchicine.

Fig. 2. Development of doubled haploids from *Brassica napus* haploid plantlets after treatment with *in vivo* colchicine.

until the embryos developed sufficiently to be visible to the naked eye (Cegielska-Taras et al., 2002). To assess the effect of spontaneous doubling of the number of chromosomes, isolated microspores of the winter oilseed rape cultivar Monolit were cultured without the addition of colchicine.

IN VIVO COLCHICINE TREATMENT: YOUNG PLANTS

The roots of haploid plants after vernalization, when several leaves had developed, were washed free of soil and immersed in 0.05% colchicine solution with 1.5% DMSO for 22 h in the dark. After this time and repeated rinsing of roots, young plants were again transplanted into soil. At first the plants were grown in a growth chamber with 16 h of light at 18–20°C and then in a greenhouse (Fig. 2).

IN VIVO COLCHICINE TREATMENT: AXILLARY SHOOTS OF HAPLOID PLANTS

Excised axillary shoots of haploid plants (e.g., after vernalization) were immersed in 0.05% aqueous colchicine solution with the addition of 1.5% DMSO for 22 h. Shoots were then rinsed several times in tap water and placed in soil for rooting and further growth. The shoots were rooted in the conditions of a growth chamber and then, the newly developed plants were moved to a greenhouse. Such developed plants did not require re-vernalization. From meristem angular leaves, new shoots grew up and developed into proper flowering shoots (Fig. 3). Shoots from untreated haploid plants were assumed to be haploid, while colchicine-treated angular shoots with at least one fertile flower were assumed to be diploid.

Fig. 3. Development of doubled haploids from *Brassica napus* haploid auxiliary shoots after treatment with *in vivo* colchicine

CONFIRMATION OF PLOIDY

The ploidy level of the populations of androgenic plants was determined from leaf fragments using a Ploidy Analyser flow cytometer.

STATISTICAL ANALYSIS

Analysis of variance (ANOVA) was carried out to test the hypotheses of no differences between the average rate of chromosome doubling after colchicine treatment of microspores, haploid plantlets, haploid axillary shoots and spontaneous chromosome doubling of isolated microspores. The method of contrasts was used to compare the average value for the rate of chromosome doubling between these three methods of chromosome doubling at different stages of haploid development and spontaneous chromosome doubling of isolated microspores.

RESULTS

COLCHICINE TREATMENT OF MICROSPORES IMMEDIATELY AFTER ISOLATION

The method of doubling chromosomes in microspores has proven to be particularly beneficial because it allows homozygous plants to be obtained in a short time. This was confirmed by the results from 24 genetically distinct donor plant populations, each population consisting of 55 to 205 androgenic plants (Table 1). Androgenic plants, obtained from colchicine-treated microspores, grew normally (Fig. 1). The rate of chromosome doubling ranged from 15.8% to 94.0%, depending on the donor genotype (Table 1). As a control, 162 androgenic plants of cv. Monolit were developed from colchicine untreated microspores. The rate of spontaneous chromosome doubling was 14.2%.

TABLE 1. Ploidy level of androgenic winter oilseed rape plants developed from *in vitro* colchicine-treated microspores.

Population	Number of plants in population	Ploidy level			Rate of chromosome doubling (%)
		Haploid	Diploid	Others	
BK5793	60	10	47	3	78.3
BK26	133	83	47	3	35.3
BK1	205	162	43	0	21.0
BK4	141	42	99	0	70.2
D3	73	53	16	4	21.9
D5	76	50	18	8	23.7
D10	153	65	72	16	47.1
D12	89	59	29	1	32.6
D14	55	24	26	5	47.3
D18	58	15	38	5	65.5
D22	141	34	104	3	73.8
D23	136	56	76	4	55.9
D25	120	98	19	3	**15.8**
D27	64	6	54	4	84.4
KP10/14	62	7	45	10	72.6

Population	Number of plants in population	Ploidy level			Rate of chromosome doubling (%)
		Haploid	Diploid	Others	
K82/op	69	27	40	2	58.0
KS17/14	134	2	126	6	**94.0**
SxT	102	30	65	7	63.7
12r	124	60	61	3	49.2
17r	165	74	86	3	52.1
29r	75	36	38	1	50.7
DT1	73	16	48	9	65.8
DT2	168	15	129	17	76.8
DT4	103	29	63	11	61.2
Mean					54.9
Control – without colchicine treatment					
cv. Monolit	162	133	23	6	14.2

COLCHICINE TREATMENT OF YOUNG HAPLOID PLANTS

Colchicine treatment of young haploid plants, at the several-leaf stage just after the end of vernalization period, was used after determination of their ploidy level by cytometry. Colchicine is toxic and consequently the young plants lost some of their leaves (Fig. 2). However, the efficiency of chromosome doubling by this method was satisfactory: in 10 different androgenic plant populations, the doubling rate ranged from 47.5% to 86.4% (Table 2). However, it took 2–3 months longer to obtain seeds from such plants compared to DH plants obtained by colchicine treatment of microspores; seed harvesting is delayed because plants need to regenerate the root system and repair the damage caused by colchicine. Nevertheless, this approach results in a better chromosome doubling rate than when the flowering stage of the plant is used, and it is less labor-intensive and time-consuming.

COLCHICINE TREATMENT OF AXILLARY SHOOTS OF HAPLOID PLANTS

Haploid shoots after colchicine treatment were rooted in 10–14 days, but new shoots developed from axillary buds for over three weeks. The time required for production of viable pollen grains from regenerated plants, i.e., DHs, was on average more than three months (Fig. 3). From one haploid plant, several lateral shoots can be taken. Although usually the new plant, which has developed from colchicine-treated shoots, produces both haploid and diploid shoots, fertile shoots produce enough seeds for further research. However, the time required for seed formation by DH plants deveoped from shoots increased by about 3–5 months, despite the fact that plants regenerated from the shoots did not require re-vernalization. The high efficiency of this method was confirmed in haploid plant populations from eleven genetically distinct donors in which the frequency of occurrence of diploids ranged from 53.3% to 100% (Table 3).

STATISTICAL ANALYSIS

Preliminary ANOVA indicated highly significant differences between the studied methods of chromosome doubling and spontaneous chromosome doubling of isolated microspores (Table 4). The highest average rate of chromosome doubling was recorded for haploid axillary shoots treated with colchicine. It was significantly higher than the average rate of chromosome doubling after colchi-

TABLE 2. Ploidy level of winter oilseed rape haploids plantlets after colchicine treatment.

Population	Number of plants in population	Ploidy level			Rate of chromosome doubling (%)
		Haploid	Diploid	Others	
A1	90	11	74	5	82.2
A2	22	3	19	0	**86.4**
A3	100	19	79	2	79.0
BK	100	19	79	2	79.0
DC	22	3	19	0	**86.4**
D10	60	8	49	3	81.7
D24	21	6	15	0	71.4
D23	54	20	34	0	63.0
D-25	103	54	49	0	**47.6**
HZxCD	28	9	19	0	67.8
Mean					74.5

TABLE 3. Ploidy level of androgenic winter rape plants developed from colchicine-treated haploid axillary shoots.

Population	Number of plants in population	Ploidy level		Rate of chromosome doubling (%)
		Haploid	Diploid	
12x12A	53	16	38	69.8
12x12B	53	11	43	79.2
12x12C	53	21	33	60.3
12x17A	45	21	35	**53.3**
12x17B	45	4	42	91.1
12x17C	45	7	39	84.4
MTxCD	24	3	21	87.5
BK4	21	0	21	**100.0**
BK1	120	8	112	93.3
EG	51	15	36	70.6
Gr	75	35	55	73.3
Mean				81.6

TABLE 4. Analysis of variance for average rate of chromosome doubling after colchicine treatment of microspores, haploid plantlets, haploid axillary shoots and isolated microspores without colchicine treatment.

Source of variation	Degree of freedom	Sum of squares	Mean square	F-statistic
Group	3	0.897	0.30	9.528**
Error	42	0.132	0.31	
Critical value				
$F_{00.5}$				2.83
$F_{00.1}$				4.29

cine treatment of microspores. The spontaneous chromosome doubling of isolated microspores was significantly lower than the average rate of chromosome doubling at the three studied stages of haploid development of oilseed rape (Table 5).

DISCUSSION

Haploid and DH plants obtained via microspore culture are routinely used in oilseed rape breeding and genetic research (Xu et al., 2007; Cegielska-Taras et al., 2015). The low percentage of haploid microspores from *B. napus* that undergo spontaneous chromosome doubling, which ranges from 8.7% to 40% (Takahira et al., 2011; Ren et al., 2017), depends on the genotype of the microspore donor. In our study, the spontaneous rate of development of DHs, i.e., without colchicine treatment of microspores, was 14.2%.

The choice of the technique used to double the chromosome number is strongly influenced by the stage of development of the object (microspore, plantlets or axillary shoots from flowering haploid plants). The diploid number (2n) is 38 chromosomes in *B. napus* and polyploid or aneuploid forms resulting from an artificial duplication of chromosomes are rare.

The fastest and most convenient way to achieve chromosome doubling is by colchicine treatment of the haploid microspores just after isolation. In our research, from 15.8% to 94% DHs were obtained using this approach. A similar broad range of diploidization rates, from 37% to 94%, has been reported in the literature (Kampouridis et al., 2016). Artificial doubling of chromosomes is the most popular method used for production of DHs on a large scale, but the sensitivity to doubling is very dependent on the genotype. Marked differences in diploidization rates were observed between different genotypes in this study (Tables 1, 2 and 3).

Although production of DHs by treatment of microspores with antimitotic agents is advantageous and has been used for mass production of

TABLE 5. Estimates and testing contrasts between groups for average rate of chromosome doubling.

Contrast between groups	Difference	LSD, α=0.05	LSD, α=0.01
1 – 4	40.66*	36.48	48.78
1 – 2	- 19.59**	13.45	17.99
1 – 3	- 26.74**	13.02	17.40
2 – 3	-7.16	36.48	48.78

Groups: 1 – isolated microspores, 2 - haploid plantlets, 3 - haploid axillary secondary shoots, 4 – isolated microspores without colchicine treatment

oilseed rape DHs, there are also disadvantages. Thus, the stage of development of microspores is particularly important: due to non-synchronous development of the microspores, when the antimitotic substance is administered not all microspores will have doubled chromosomes. Where low chromosome doubling rates occur with this method, alternative approaches can be used. Due to different methods for doubling the number of chromosomes at different stages of haploid development, seeds can be obtained from almost every androgenic plant. Androgenic plants from populations D23 and D25 are examples of this phenomenon, where there was a relatively low efficiency of doubling by the *in vitro* method (Table 1), while the *in vivo* approach (Table 2) was more successful. The influence of the genotype, however, could be seen in the population of androgenic plants from donor D10, in which low chromosome doubling rates were obtained by both *in vitro* (Table 1) and *in vivo* methods (Table 2). Indeed, in the case of BK4 donor populations, doubling was high using both *in vitro* (Table 1) and *in vivo* methods (Table 3). In the androgenic populations of plants from donor BK1, the effectiveness of colchicine *in vitro* was low, with only a 21% doubling rate (Table 1), while colchicine treatment of excised shoots yielded 94% developmentally fertile new shoots. There were no differences in the chromosome doubling potential between androgenic populations obtained from hybrids that derived from crosses of natural and resynthesized oilseed rape.

Determination of the number of chromosomes in young androgenic plants (4–6 leaf stage) on the basis of the amount of nuclear DNA facilitated the selection of plants for *in vivo* application of colchicine (Doležel et al., 2007). When the rate of microspore chromosome doubling is very low, the method of colchicine treatment of young haploid plants is particularly convenient. This method is mostly used in the creation of resynthesized oilseed rape lines from ancestral genotypes (Sosnowska and Cegielska-Taras, 2014) or when haploid explants are used for genetic manipulation, e.g., genetic transformation (Cegielska-Taras et al., 2008). Another method of *in vivo* chromosome doubling is by colchicine treatment of excised young axillary shoots of haploid plants at the stage of budding and flowering. This method is used when the ploidy of androgenic plants has not been determined and haploids are recognized based on the morphology of buds or flowers. It is also used when the yield of androgenic plants is very low and chromosome doubling is unsatisfactory, but the androgenic plants have important features.

CONCLUSION

The various methods available for doubling the number of chromosomes at various stages of the androgenic process (from isolated microspores, through young plants to flowering plants) allow seeds to be obtained from nearly every selected individual plant.

AUTHORS' CONRTIBUTIONS

LS: research concept, regeneration of plants, data analysis, writing the article – review and editing. KS: regeneration of plants, visualization, review and editing the article. TCT: research concept, data analysis, writing the article.

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