Efficient Doubled Haploid Production in *Brassica napus* via Microspore Colchicine Treatment *in vitro* and Ploidy Determination by Flow Cytometry

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Abstract

An efficient microspore culture protocol was used to rapidly produce homozygous doubled haploid (DH) progeny of crosses between low erucic canola and high erucic resynthesized rapeseed (*Brassica napus* L.). Microspores of Canadian cultivars 'Excel' and 'Profit' as well as three F_1 hybrids with the resynthetic line RS239 were treated with colchicine immediately after isolation. Flow cytometry was applied for early identification of DH regenerants. The diploidization rate was subsequently verified by scoring flower morphology .
In vitro colchicine treatment had a positive effect on induced diploidization in dependence on the frequency of preliminary spontaneous diploidization which was, however, determined by the genotype. In addition, effects of colchicine treatment on embryoid formation and regeneration have been evaluated.
The method presented is feasible for commercial large-scale production of DH lines in oilseed rape since the genotype-specific diploidization can be efficiently balanced by *in vitro* colchicine treatment. In addition, the use of flow cytometry immediately after *in vitro* culture allows to efficiently select for doubled haploids thus saving labor and cost and in the laboratory and subsequent greenhouse phase.

Key words: *Brassica napus* L. – doubled haploids (DH) – flow cytometry – oilseed rape - resynthetic rapeseed

Abbreviations DAPI 4'6-diamidino-2 phenylindole, CV coefficient of variation

Introduction

Microspore culture is an established tool for the production of homozygous lines in rapeseed breeding programs. For this purpose, fertile DH progeny are required at a high and genotype independent frequency. First, Beversdorf et al. (1987) had reported an enhancement of the diploidization rate in rapeseed by a colchicine treatment *in vitro.* Usually, 70-90% of the microspore derived plants are haploid (Lichter, 1985; Chen and Beversdorf, 1992). Conventional chromosome doubling techniques are both, difficult to apply and time consuming, and are not sufficiently effective therefore. Mathias and Röbbelen (1991) proposed colchicine treatment *in vitro* with the application of low concentrations under controlled conditions to enhance the recovery of non-chimeric doubled haploids directly from microspore culture. Colchicine treatment immediately after microspore isolation causes doubling prior to the first mitosis. Thus, chimeric plants occur very rarely. Möllers et al. (1994) increased the diploidization rate to 80-90% after a 24h treatment of microspores with 50mg l^{-1} colchicine in the induction medium immediately after isolation. Correspondingly, Zamani et al. (2000) demonstrated the feasibility of colchicine treatment *in vitro* in wheat anther culture.

The efficiency of microspore culture in plant breeding is restricted by the usual occurrence of haploid plantlets which require space and need to be doubled by colchicine treatment. The determination of ploidy level in an early phase can substantially reduce the time needed (Wang et al., 1999). However, determination of ploidy by counting mitotic chromosomes is also time consuming and difficult in rapeseed due to the small chromosomes. As an alternative, ploidy may be estimated from pollen size or chloroplast number in the epidermal cells. However, these methods are not reliable, especially if the ploidy difference is small (De Laat et al. 1987). In comparison to these methods, flow cytometry offers advantages regarding simplicity, accuracy, the time required for determination and automation. In addition, flow cytometry enables the measurement of ploidy level in an early developmental stage of plantlets emerging from microspore culture. Therefore, an *in vitro* colchicine treatment coupled with an efficient determination of ploidy level by flow cytometry enhances the use of microspore culture in rapeseed breeding.

Materials and Methods

Plant material and microspore culture

Three low erucic (<0.5% erucic acid) Canadian cultivars, i.e. 'Excel', 'Profit' and 'Tristar', the resynthesized rapeseed line 'RS 239' (Lühs and Friedt, 1994) and three hybrids (F_1), i.e. 'Excel' x RS 239 (ExR), 'Profit' x RS 239 (PxR) and 'Tristar' x RS 239 (TxR) were used in the present experiment. Flower buds 2-7mm in size were selected, and isolation and microspore culture was carried out according to Coventry *et al*. (1988). Plating density was 105 microspores ml^{-1} in a modified liquid NLN medium (Lichter, 1985; Gland et al., 1988) in petridishes (94 x 16 mm) containing 15ml microspore suspension. For colchicine treatment microspores were suspended immediately after isolation in the same medium containing 50mg l^{-1} colchicine. Cultures were then incubated in the dark at 30°C for 24h as described by Möllers et al. (1994). After this period, medium was exchanged to remove colchicine and cultures incubated under the conditions mentioned above up to 14 days. After that embryoids were visible and were transferred to a shaker (60 rpm) in the light (16h photoperiod, 25°C). Well developed embryoids were transferred to solid MS medium (Murashige and Skoog, 1962) containing 3% sucrose. Shoots emerging from these embryoids were cut, transferred to soil and adapted to greenhouse conditions in a mist chamber.

Determination of ploidy level

Samples were prepared and subsequently stained according to Otto (1988). The composition of DAPI-stock solution was modified as indicated below. Fully expanded leaves of *in vitro* plantlets at the 3-4 leaf stage regenerated from microspore-derived embryoids were selected for the preparation. Leaves were placed in a petri dish (∅ 5 cm) and chopped with a sharp razor blade. Afterwards, 5ml citric acid/Tween 20 solution (21g l^{-1} citric acid H_2O, 5ml l^{-1} Tween 20) were added and incubated for 20min at room temperature while slightly shaking. After incubation, the slurry was filtered through a nylon mesh (42.5µm) and the resulting suspension centrifuged at 100*g* for 10min. The pellets were either fixed in 5ml 70% ethanol or directly resuspended in 1ml citric acid/Tween 20 solution. Ethanol was removed by centrifuging (200*g,* 10min) and resulting pellets resuspended in 1ml citric acid/ Tween 20 solution as well. After incubation for 10min, 5ml DAPI-stock solution [56.6g l^{-1} Na_2HPO_4, 0.025mg l^{-1} DAPI (4'6-diamidino-2 phenylindole), 0.03g l^{-1} PIPES] was added for fluorescent staining.
The samples were measured using a Partec flow cytometer with a mercury lamp (HBO100 W/2). This procedure is based on the measurement of nuclear DNA content. The measurement was carried out after staining with the DAPI solution at a flow rate of 100 to 300 nuclei

per second. High voltage was adjusted to 320 V and the discriminator to 50 V. The suspension of cells was absorbed through a tube and focused by a covered stream.
The results were displayed as one-parameter DNA-histograms with the x-axis showing the number of the channels which is proportional to DNA content. The y-axis displays the frequency of single cells per channel, i.e. the number of cells of a certain ploidy level as recorded. The peak positions of DNA-histograms were compared to standard peaks derived from either microspores or somatic cell suspensions from leaf tissue of a control plant.
The regenerated plants were again scored for ploidy during flowering. Haploid plants showed degenerated flowers or anthers, whereas diploid individuals were characterized by normal flower and pollen development.

Results and Discussion

Microspore culture and regeneration

The microspore embryoid yield achieved varied from 0.9 to 31.2 embryoids ml^{-1}. Lower yields may be due to the high density used (10^5 microspores ml^{-1}) and the wide range of bud size. However, the frequency of embryoid formation was clearly determined by rapeseed genotype. The highest level of embryoid production was observed for 'Excel', a Canadian spring cultivar which originates from a cross between a conventional variety and a DH breeding line (Sernyk, 1994). The lowest frequency of embryoid production was observed for cv. 'Tristar' and no embryoid formation has been observed in RS 239. All three hybrids delivered lower yields than 'Excel'. The colchicine treatment had no significant effect on embryoid yield. A slight reduction was observed for 'Excel' and the hybrid TxR while beneficial colchicine effect was detected for the hybrids ExR and PxR.
Plantlet regeneration mainly occurred directly and ranged from 18 to 40% of subcultured embryoids. It was clearly determined by genotype and, in contrast to embryoid yield, the highest frequencies were observed for the hybrids. Colchicine treatment had a significant positive effect on the regeneration frequency. With this treatment organogenesis still occurred at the same level as for embryoids emerging from the non-treated control, but direct regeneration was stimulated when microspores had been colchicine-treated.
Due to the genotypic variation regarding both, frequency of embryoid development and regeneration from microspore derived embryoids, the genotypes used in the present study can be distinguished by these parameters according to Weber et al. (1995). The positive effect of colchicine treatment especially on direct regeneration may be related to a higher number of already doubled embryoids at the time of induction of regeneration.

Determination of ploidy level

DNA-content was identified by fluorescence intensity using somatic cells from an ordinary rapeseed plant (2n=38) as a check at channel 86 which showed the highest degree of synchrony. Standard samples from microspores, however, were less effective because peaks were more difficult to interpret. However, this may be explained by the presence of tetrads and microspores of later developmental stages in the suspensions examined. The samples from regenerants were therefore compared to standard peaks of amphidiploid checks; this way their ploidy could be identified unequivocally.
The coefficient of variation (CV) of cytometry values, as a criterion for the reliability of measurement, varied mainly between 3 and 6% (sometimes <1%). This is in agreement with observations of Galbraith (1990) who obtained CV values ranging from 3 to 6 % for *Nicotiana tabacum* protoplasts. Ulrich and Ulrich (1991) reported lower CV values (<1%) using protoplasts from different species, while nuclei obtained from chopped material showed only

slightly higher CV values (1.0-1.5%). In the present study, the physiological state and age of samples were identified as determining parameters for the interpretation of the distributions (cf. De Laat et al., 1987). The clearest results were obtained using tissue from plants at the 3-4 leaf stage. The intensity of chopping was an additional factor for the interpretation of the distributions. Rapeseed material has to be chopped very intensively to obtain satisfactory results while for example cereals require less intensity (M. Jäger-Gussen, pers. comm.).

A major problem for routine application of flow cytometry remains the preparation of appropriate samples for measurement. Because of the compact plant cell wall it is difficult to release cell nuclei. This can be done either by chopping of leaf tissue (Möllers *et al*. 1994) or by enzymatic isolation of protoplasts (De Laat et al., 1987; Ulrich and Ulrich, 1991). In the former case, cellular debris can not be prevented. An optical separation of cells by DNA content is therefore not always possible. Depending on the preparation technique, fluorescence from the cellular debris may be classified as a certain cellular development stage and therefore can lead to false interpretation (P. Rase, pers. comm.). As we obtained CV values from chopped rapeseed leaf material comparable to protoplast isolations (Galbraith, 1990), time-consuming isolation of protoplasts is not required for determination of ploidy level by flow cytometry in this species. However, very intensive chopping had to be carried out to obtain these results. An alternative strategy to achieve comparable results may be chromatin isolation (Kleine et al., 1995) which can be done under non-aseptic conditions in a short time as well.

Fixation of the nuclei by ethanol (70%) prior to DNA analysis has been discussed as well. According to Galbraith (1990) the fixation of nuclei could be one reason for the large variation observed. In the present study no difference was observed between fixed and non-fixed samples regarding the exactness of measurement.

In order to verify the results of cytological analysis, we have also scored the regenerants for ploidy by flower characteristics, as the size of petals and pollen production of the anthers differ significantly between haploid and diploid (amphidiploid) individuals. The results of the two assessments were clearly related irrespective of the treatment (R^2=0.97, Fig. 1), however, the correlation was even stronger in colchicine treated material (R^2=0.98). Only 10 plants which had initially been identified as haploid by flow cytometry were later classified as amphidiploid on the basis of flower morphology. However, no regenerant which showed the amphidiploid DNA content in the flow cytometer was later scored haploid according to flower characteristics. The escapes described might be related to the occurrence of diploidization at a developmental stage later than 3-4 leaf stage, probably restricted to flower organs. Therefore, flow cytometry can be considered a quick and reliable method to determine ploidy level at an early growth stage.

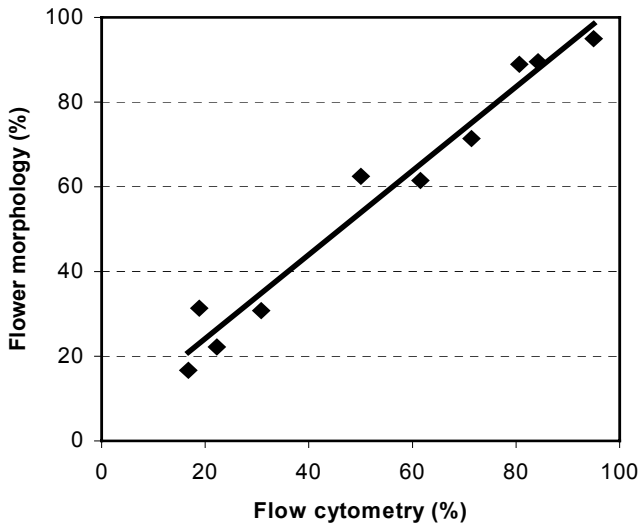

Fig. 1: Correlation between diploidization rates (%) as determined by flow cytometry and flower morphology.

Effect of colchicine treatment

Examination by flower morphology revealed an average

spontaneous diploidization rate of 44.9%. Furthermore, spontaneous diploidization varied largely depending on genotype (Tab. 1); it ranged from 16.7% for TxR ('Tristar' x RS 239) to 71.4% ('Profit') and was generally lower for the hybrids. This corresponds to findings of Möllers et al. (1994) who observed variation of diploidization between genotypes as well. The latter authors consider the different stages of microspores at the start of culture as a reason for spontaneous diploidization. In our view, this might be due to the results of embryoid yield and spontaneous diploidization which showed coincidence in the controls (data not shown). Embryoid yield reflects the distribution pattern of developmental stages in the microspore suspension at the time of isolation, and yield is clearly related to this pattern. Therefore, suspensions with a high amount of microspores in the bi-nucleate stage yield fewer embryoids and spontaneous diploids as well. This aspect may be improved by a more careful selection of donor material.

Table 1: Frequency of diploidization depending on genotype and colchicine treatment as determined by flower morphology

Genotype	Diploidization frequency (%)	
	Control	colchicine
Excel	62.5	88.9
Profit	71.4	89.5
Excel x RS239	22.2	61.5
Profit x RS239	31.3	95.0
Tristar x RS239	16.7	30.8
Average	44.9	79.2

In general, colchicine treatment was very effective for all genotypes as the frequency of DHs was almost doubled as compared to the non-treated controls (Tab. 1). The highest number of colchicine induced diploids (95.0%) was observed for the hybrid PxR ('Profit' x 'RS239'), while spontaneous diploidization of this genotype was low (18.8%). Thus, *in vitro* colchicine treatment balanced the genotypic effect on diploidization which has been considered a major obstacle for the application of this technology in practical breeding (Rudolf et al., 1999). Moreover, the simplicity of the protocol allows combinations with other microspore pretreatments, i.e. induced mutagenesis (Barro et al., 2001)

The frequency of doubled haploids obtained in the present study allows to avoid labor intensive and time-consuming colchicine treatment of established plantlets in a later development stage.

In addition, *in vitro* colchicine treatment considerably enhances the efficiency of the system regarding plant regeneration from embryoids. This effect allows saving work and resources during the plant regeneration phase.

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