

## Effects of colchicine on anther and microspore culture of bread wheat (*Triticum aestivum* L.)

Mercedes Soriano · Luis Cistué ·  
María P. Vallés · Ana M. Castillo

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**Abstract** The aim of this work was to study the effects of colchicine application on chromosome doubling and androgenic response in anther and microspore culture of different bread wheat genotypes. Colchicine was applied during a mannitol stress pretreatment or during the first 48 h of culture at concentrations of 0, 150 and 300 mg l<sup>-1</sup>. When colchicine was applied during stress pretreatment, the percentage of doubling depended on genotype and concentration. A significant increase in doubling was observed with 300 mg l<sup>-1</sup> in the low androgenic responding cv. Caramba. Colchicine incorporation during the first hours of culture improved percentage of doubling in all genotypes, in both anther and microspore culture. Application of 300 mg l<sup>-1</sup> colchicine improved the percentage of doubling in the two low responding genotypes, to 118% of control in DH24033, and 75% in Caramba in microspore and anther culture, respectively. Concerning the androgenic response, the effect of colchicine on embryo formation and percentage of green plants depended on the genotype and on the culture method. In cv. Pavon, a 2- and a 3-fold increase in percentage of embryogenesis and green plants, respectively, were

obtained with 300 mg l<sup>-1</sup> colchicine in microspore culture. However, no significant differences in these two variables were observed in anther culture. The number of green doubled haploid (DH) plants reflects the index of success of the procedure. Regardless of the culture method, when colchicine was incorporated during the first hours of culture, the number of green DH plants increased significantly in three of four genotypes. These results confirm the usefulness of colchicine application during the first hours of culture in wheat breeding programs.

**Keywords** Doubled haploid · Chromosome doubling · Androgenesis · Poaceae · Stress pretreatment

### Introduction

Doubled haploid (DH) plants are useful in plant breeding programmes, since homozygous plants may be obtained in a few months, thus reducing the time needed to release new cultivars. Several bread wheat cultivars selected from DH lines, obtained by microspore embryogenesis (Ouyang et al. 1983) or intersepecific crosses with maize (Laurie and Bennett 1987), have been commercialized (<http://www.scri.sari.ac.uk/assoc/COST851/Default.htm>). Microspore embryogenesis has greater potential for DH production than interspecific crosses in wheat, due to the abundance of microspores per flower compared to the

M. Soriano · L. Cistué · M. P. Vallés · A. M. Castillo (✉)  
Departamento de Genética y Producción Vegetal,  
Estación Experimental de Aula Dei, Consejo Superior de  
Investigaciones Científicas (CSIC), Avda Montañana  
1005, Zaragoza 50059, Spain  
e-mail: amcast@ead.csic.es

single ovule per floret. However, there are still several factors that hinder the application of anther and microspore culture at a commercial level, specifically the low rates of embryogenesis and regeneration, the high frequency of albinism among regenerants and the low frequency of chromosome doubling in some cultivars.

The spontaneous doubling rate in wheat depends on the genotype. Stober and Hess (1997) obtained 15–44% spontaneous doubling in German cultivars of spring wheat, whereas Barnabás (2003) reported frequencies from 25 to 68% in winter cultivars from Central and Eastern Europe. Of the different chromosome doubling agents, colchicine has been the most successful when applied to plants or callus of bread wheat (Hassawi and Liang 1991). Colchicine inhibits spindle formation during mitosis and disturbs normal polar segregation of sister chromatids resulting in doubling chromosome number (Levan 1938). Colchicine is traditionally applied to young plants at the 3–4 tillering stage by the capping technique (inverted vial) (Bell 1950) or by the immersion method (Jensen 1974). However, the application of colchicine at this stage has several disadvantages such as: (a) the high concentration and volume of colchicine solution needed, that implies dangerous handling and costly treatments, (b) a high rate of mortality in plants, and (c) production of mixoploids or chimeric plants that leads to low seed production and therefore, an additional growth cycle for seed multiplication before evaluation in the field (Chen et al. 1994). The application of colchicine during *in vitro* culture, in the induction medium (Barnabás et al. 1991) or in the regeneration medium (Mentewab and Sarrafi 1997) could minimize some of these problems.

Colchicine has been successfully applied during the first hours of anther and microspore culture, resulting in an increase of chromosome doubling in different species such as wheat (Barnabás et al. 1991, Hansen and Andersen 1998), tritordeum (Barcelo et al. 1994), maize (Saisingtong et al. 1996), rice (Alemanno and Guiderdoni 1994), *Miscanthus* (Peterson et al. 2002), *Brassica* (Möllers et al. 1994) and tobacco (Takashima et al. 1995). Colchicine can affect not only the percentage of doubling but also the whole androgenetic process. In *Brassica*, the application of colchicine to isolated microspore cultures resulted in a high embryo induction rate and almost synchronized embryogenesis (Möllers et al.

1994; Zaki and Dickinson 1995; Zhao et al. 1996a, b; Zhou et al. 2002a, b). Studies performed in tobacco and maize indicated that the optimal concentration for embryogenesis had no effect on chromosome doubling while optimal concentration for doubling had negative effects on plant production (Eady et al. 1995; Saisingtong et al. 1996). In bread wheat, application of colchicine during the first hours of anther culture produced contradictory results regarding embryogenesis and percentages of green plants (Barnabás et al. 1991; Navarro-Alvarez et al. 1994; Redha et al. 1998a, b; Zamani et al. 2000, 2003). Only the study from Hansen and Andersen (1998) reported the effect of colchicine on isolated microspore culture in wheat.

The application of a stress pretreatment is necessary for efficient induction of microspore embryogenesis in cereals. Sugar starvation by placing anthers on a medium with mannitol as the carbohydrate source is one the most commonly used stress pretreatments. This pretreatment has provided consistently high chromosome doubling rates in barley (Kasha et al. 2001; Shim et al. 2006) and wheat (Hu and Kasha 1997). In wheat, a stress pretreatment that delayed nuclear division and retained microspores at the uninucleate stage produced more green plants than pretreatments in which microspore division occurred (Hu and Kasha 1999). There is only one report in maize in which colchicine was applied during a cold stress pretreatment (Antoine-Michard and Beckert 1997). In that study, colchicine had no effect on percentage of doubling but significantly increased the number of fertile DH plants.

The aim of this work was to study the effect of colchicine on chromosome doubling and on the androgenic response when applied not only during the first hours of anther and microspore culture, but also during mannitol stress pretreatment in bread wheat.

## Materials and methods

### Plant material

Spring wheat cvs. Chris, Pavon, Caramba and the doubled haploid line DH24033 were used as donor plants for isolated microspore and anther culture. Chris is a model cultivar for microspore embryogenesis, Pavon is a medium-high responding cultivar, whereas Caramba and DH24033 are low responding.

The DH line was chosen because of its low percentage of spontaneous chromosome doubling.

Seeds of donor plants were sown in a paper-pot of 3 cm in diameter with a mixture of peat, vermiculite and sand (1:1:1). Plants were vernalized for 5 weeks in a growth chamber at 4°C, under an 8 h photoperiod. Plants were transplanted to 15 cm diameter pots with the above soil mixture and cultivated in a growth chamber at 12°C with a 12 h photoperiod. After 5 weeks, the temperature was increased to 21/18°C and the photoperiod lengthened to 16 h. Relative humidity was kept at 70–80%. Fertilization of the plants was performed as described by Cistué et al. (2006).

#### Anther and microspore culture

##### *Microspore developmental stage assessment*

For every genotype and batch of plants, four spikes were chosen in order to associate their morphology to the developmental stage of the microspores. One or two anthers were taken from the central flower and squashed in 127.5 g l<sup>-1</sup> mannitol. A drop of 4', 6'-diamidino-2-phenylindole (DAPI) stock solution (4 µg µl<sup>-1</sup> with 0.01% Tween 20) was added (Vergne et al. 1987) and incubation was performed until nuclei were visible under a UV lamp in an epifluorescent microscope (Nikon Eclipse T300).

##### *Stress pretreatment*

Anthers containing the majority of microspores at the mid- to late-uninucleate stage for anther culture or at late-uninucleate to early-binucleate stages for microspore culture were excised from the flower and plated in 127.5 g l<sup>-1</sup> mannitol, 5.9 g l<sup>-1</sup> CaCl<sub>2</sub> plus FHG macronutrients (Hunter 1988) solidified with 0.8 g l<sup>-1</sup> Sea Plaque agarose.

##### *Ovary preconditioned medium*

Ovaries from flowers containing microspores at the late binucleate or trinucleate stages were excised and used for preconditioning MMS3 medium (MS medium modified by Hu and Kasha 1997), containing 62 g l<sup>-1</sup> maltose, 1 mg l<sup>-1</sup> 2,4-dichlorophenoxyacetic

acid (2,4-D) and 1 mg l<sup>-1</sup> benzyladenine (BA) (MS3M). This medium was supplemented with 200 g l<sup>-1</sup> (MS3MF200) or 300 g l<sup>-1</sup> Ficoll 400 (MS3MF300) for anther and microspore culture, respectively. Medium was preconditioned for 5 days at 25°C with 10 ovaries per 1.5 ml of culture media in 3 cm Petri plates.

##### *Microspore isolation and culture*

After 5 days of pretreatment, anthers were harvested in 54.7 g l<sup>-1</sup> mannitol and microspores were released using a glass rod homogenizer. The isolation procedure was performed including viable microspore enrichment by centrifugation on a maltose band, as described by Castillo et al. (2000). Around 50 spikes were used for each isolation. The total number of microspores was estimated using a haemocytometer (Neubauer) and the percentage of viable microspores was determined by the fluorescein diacetate technique (Widholm 1972). After counting, we centrifuged the microspore suspension (5 min, 110 g), removed the mannitol solution with a Pasteur pipet and resuspended the pellet in MS3M medium. Microspores were inoculated at a final density of 80–100 × 10<sup>3</sup> microspores/ml in 3 cm Petri plates containing ovary preconditioned MS3MF300. After 8–10 days of culture, an equal amount of similar fresh medium was added to each plate.

##### *Anther culture*

After mannitol pretreatment, anthers were inoculated in 3 cm plates containing 1.5 ml ovary preconditioned MS3MF200 medium. Around 40 anthers were cultured per plate. Ten to 12 days after culture, an equal amount of MS3MF300 was added to each Petri dish.

##### *Plant regeneration and soil transfer*

Embryos that developed after 30–60 days of culture were transferred to J25-8 medium (Jensen 1983) for regeneration. Embryos were kept in the dark at 25°C for 2 days and transferred to the light. After 20–30 days, plants were counted and transferred to

Magenta boxes containing similar medium plus NAA ( $2 \text{ mg l}^{-1}$ ) for root development. Plants were vernalized for 5 weeks at  $4^\circ\text{C}$  and 8 h photoperiod before transfer to soil. Afterwards, plants were transferred to the greenhouse for seed production. Plants with seed set greater than 90% were considered doubled haploids.

## Colchicine application

### *Colchicine application during mannitol pretreatment*

The three anthers of each flower were randomly distributed to three pretreatment media containing different concentrations of colchicine: 0 (Control), 150 (Col 150) and  $300 \text{ mg l}^{-1}$  (Col 300), so that each pretreatment medium contained one anther from every flower. After 5 days of pretreatment, microspores were isolated and cultivated as previously described.

### *Colchicine application during the first hours of microspore and anther culture*

In microspore culture, four treatments were used: one untreated control in which microspores were cultivated as previously described; two treatments in liquid MS3M with colchicine ( $150$  and  $300 \text{ mg l}^{-1}$ ); and one control treatment in liquid MS3M without colchicine. In anther culture, three treatments were applied: 0, 150 and  $300 \text{ mg l}^{-1}$  colchicine in liquid MS3M medium. In both types of culture, colchicine treatments were conducted in 3 cm plates containing 1.5 ml medium for 48 h. After this time, treated microspores were transferred to a 15 ml centrifuge tube and washed in liquid MS3M by centrifugation (5 min, 110 g). Treated anthers were washed with 2 ml liquid MS3M. Afterwards, microspore and anther cultures were performed as previously described.

### *Ploidy analysis*

Ploidy was estimated by flow cytometry after transferring plants to soil. Young leaves were chopped in 2 ml Cystain UV ploidy solution (Partec) and filtered through a  $30 \mu\text{m}$  nylon filter. Samples were analyzed

in PA or PAS cytometer (Partec). Leaves from young seedlings were used as control. At least 50 plants from each treatment were analyzed, unless the number of green plants was lower.

## Data analysis

For microspore cultures, each microspore isolation was considered a replicate. Four replicates from different batches of plants were used for studying the effect of colchicine application during pretreatment or culture. Anther culture experiments consisted of ten replicates of approx. 40 anthers per treatment and genotype. The following variables were recorded: number of embryos (nEmb), number of green plants (nGP), and total plants, all per 100 anthers, and percentage of doubling expressed as number of doubled haploids per 100 analyzed plants (pDH). Percentage of regeneration (number of regenerated plants per 100 embryos; pReg), percentage of green plants (number of green plants per 100 total plants; pGP) and number of green DH plants per 100 anthers (nGDH) were calculated.

All experiments were established in a completely randomized design. Standard SAS/STAT procedures were used for statistical analysis (SAS 1988). ANOVA was conducted for each genotype separately, using the GLM (Generalized Linear Model) procedure for all the variables, except for pDH which was analyzed using the  $\chi^2$ -test. Mean separation was tested by the Duncan method ( $\alpha \leq 0.05$ ).

## Results

### Colchicine application during mannitol pretreatment

In the model cv. Chris, either of the two colchicine concentrations used did not significantly affect any of the variables related to plant production: nEmb, nGP, pReg or pGP (Table 1). Among these variables, only nGP was significantly increased in the medium-high responding cv. Pavon, from 7 in control to 11 with  $300 \text{ mg l}^{-1}$  colchicine. In Caramba, similar percentages of regeneration and green plants were obtained in control and  $300 \text{ mg l}^{-1}$  colchicine cultures, although a detrimental effect was observed with  $150 \text{ mg l}^{-1}$ . Similar numbers of embryos and green plants were obtained in control and colchicine treated cultures of Caramba.

**Table 1** Effect of the incorporation of colchicine during the mannitol stress pretreatment applied to anthers, on microspore culture response of three cultivars of bread wheat

Cultivar	Treatment (mg l <sup>-1</sup> )	nEmb*	pReg	pGP	nGP*	pDH	nGDH*
Chris	Col 0	90 a <sup>+</sup>	69 a	98 a	61 a	70 a	42 a
	Col 150	123 a	65 a	99 a	79 a	60 a	50 a
	Col 300	96 a	70 a	98 a	66 a	67 a	44 a
Pavon	Col 0	34 a	54 a	37 a	7 b	66 a	4 b
	Col 150	46 a	49 a	37 a	8 ab	72 a	5 ab
	Col 300	55 a	49 a	43 a	11 a	56 b	6 a
Caramba	Col 0	21 a	56 a	50 a	6 a	55 b	4 a
	Col 150	27 a	49 b	28 b	4 a	60 ab	3 a
	Col 300	21 a	52 ab	45 a	5 a	69 a	3 a

\* Values are based on 100 anthers

<sup>+</sup> Values followed by the same letter within cultivars and columns are not significantly different ( $P < 0.05$ )

Regarding percentage of chromosome doubling (pDH), the 150 mg l<sup>-1</sup> colchicine treatment did not differ significantly from controls of any cultivar (Table 1). However, the 300 mg l<sup>-1</sup> treatment was detrimental in cv. Pavon, stimulatory in cv. Caramba and ineffective in cv. Chris.

The number of green DH plants, which expresses the index of success of the procedure, was unaffected by colchicine in Chris and Caramba. However, this variable was significantly improved in Pavon, from 4 in control to 6 with 300 mg l<sup>-1</sup> colchicine, mainly due to the greater number of green plants produced.

Colchicine application during the first hours of microspore and anther culture

#### *Effect of the application method in microspore culture*

To study the effect of the application method itself, we compared untreated microspores (Control) and microspores treated with 0 mg l<sup>-1</sup> colchicine (Col 0) (Table 2). There was a negative effect of the treatment procedure in cvs. Chris and Pavon. The percentage of green plants in cv. Pavon was reduced significantly from 66% to 18%. This led to a decrease

**Table 2** Effect of the incorporation of colchicine during the first 48 h of microspore culture of three genotypes of bread wheat

Genotype	Treatment (mg l <sup>-1</sup> )	nEmb*	pReg	pGP	nGP*	pDH	nGDH*
Chris	Control	58 a <sup>+</sup>	41 a	98 a	23 a	62 b	14.3 a
	Col 0	44 a	29 a	99 a	13 ab	53 b	6.7 a
	Col 150	33 a	37 a	100 a	12 ab	55 b	6.6 a
	Col 300	37 a	26 a	99 a	9 b	77 a	7.2 a
Pavon	Control	13 ab	47 a	66 a	4 a	35 c	1.4 a
	Col 0	9 b	37 a	18 b	1 a	50 b	0.3 b
	Col 150	21 a	55 a	30 ab	3 a	57 b	1.9 a
	Col 300	21 a	36 a	38 a	3 a	88 a	2.5 a
DH24033	Control	9 a	17 a	87 a	1 a	22 c	0.3 b
	Col 0	28 a	14 a	93 a	4 a	34 b	1.2 ab
	Col 150	34 a	32 a	94 a	10 a	60 a	6.2 a
	Col 300	32 a	27 a	90 a	8 a	73 a	5.6 a

\* Values are based on 100 anthers

<sup>+</sup> Values followed by the same letter within genotypes and columns are not significantly different ( $P < 0.05$ )

in the nGP, with a two-fold and a four-fold decrease in Chris and Pavon, respectively. However, in the DH24033 line, the method of application enhanced nEmb, without affecting pReg or pGP, and thus resulted in a four-fold increase in the number of green plants.

The percentage of spontaneous doubling was significantly increased by the application method in cv. Pavon (from 35% to 50%) and DH24033 (from 22% to 34%) (Table 2). The number of green DH plants was also affected by the application method. A two-fold and a five-fold decrease in this variable were observed in cvs. Chris and Pavon, respectively, mainly due to the lower number of green plants produced in the treated control. Conversely, the number of green DH plants from DH24033 was increased from 0.3 in control cultures to 1.2 in treated control, because of the greater number of embryos in this treatment.

#### *Effect of the colchicine application during the first hours of microspore culture*

The effect of colchicine at 0 (Col 0), 150 (Col 150) and 300 mg l<sup>-1</sup> (Col 300) in the induction medium on green plant production varied with the genotype (Table 2). In Pavon, a 2-fold increase in nEmb was observed with both concentrations compared with 0 mg l<sup>-1</sup>. Percentage of green plants was also significantly increased up to 2-fold with 300 mg l<sup>-1</sup> in Pavon. As a consequence, the number of green plants increased from 1 with 0 mg l<sup>-1</sup> to 3 with 150 mg l<sup>-1</sup>. No significant differences were observed in nEmb, pReg, nGP and pGP in cv. Chris and DH24033.

The percentage of chromosome doubling was significantly increased with 300 mg l<sup>-1</sup> colchicine

compared with 0 mg l<sup>-1</sup> in the three genotypes used. The greatest effect was observed for DH24033 and cv. Pavon with a 118% and 76% increase in the percentage of doubling, respectively.

Similar numbers of green DH plants in cv. Chris were obtained with 0 mg l<sup>-1</sup> and the two colchicine concentrations used. In Pavon, the number of green DH plants increased up to 8 times with 300 mg l<sup>-1</sup>, due not only to the greater percentage of chromosome doubling but also to the greater number of embryos and percentage of green plants obtained compared with 0 mg l<sup>-1</sup>. In DH24033 this variable increased, although not significantly, from 1.2 with 0 mg l<sup>-1</sup> to 5.6 with 300 mg l<sup>-1</sup> colchicine.

#### *Effect of the colchicine application during the first hours of anther culture*

Colchicine did not significantly affect the number of responding anthers, embryos and green plants, or percentages of regeneration and green plants in either of the two cultivars used (Table 3), although a slight decrease in the number of embryos occurred in Caramba with 150 mg l<sup>-1</sup>.

Chromosome doubling significantly increased in colchicine treatments in both cultivars (Table 3). Similar percentages of spontaneous doubling were obtained in the control treatment for both cultivars (33%), and it improved significantly up to 50% in Pavon and 58% in Caramba, after treatment with 300 mg l<sup>-1</sup> of colchicine. As a consequence, the number of green DH plants also increased significantly (about 50%) in both cultivars when 300 mg l<sup>-1</sup> colchicine was incorporated during the first hours in the induction medium.

**Table 3** Effect of the incorporation of colchicine during the first 48 h of anther culture of two cultivars of bread wheat

Cultivar	Treatment (mg l <sup>-1</sup> )	Resp. Ant (%)	nEmb*	pReg	pGP	nGP*	pDH	nGDH*
Pavon	Col 0	44 a <sup>+</sup>	419 a	50 a	62 a	117 a	33 b	38 b
	Col 150	43 a	361 a	51 a	66 a	144 a	35 b	46 b
	Col 300	43 a	376 a	48 a	60 a	129 a	50 a	60 a
Caramba	Col 0	23 a	178 a	49 a	61 a	68 a	33 b	23 b
	Col 150	21 a	103 a	46 a	61 a	32 a	54 a	15 b
	Col 300	22 a	159 a	58 a	64 a	70 a	58 a	35 a

\* Values are based on 100 anthers

<sup>+</sup> Values followed by the same letter within cultivar and columns are not significantly different ( $P < 0.05$ )

Comparison of ploidy and plant fertility measured by percent seed set was performed. The correlation between ploidy determined by flow cytometry and fertility of plants was high ( $r^2 = 87.8$ ,  $P = 0.01$ ). The percentage of aneuploid plants that produced few seeds was 0.1%. Seed set from DH plants from colchicine treated cultures was similar to that of control plants.

## Discussion

This is the first report of the influence of colchicine incorporated into the mannitol stress pretreatment on the androgenetic process. Mannitol pretreatments at 28°C to wheat uninucleate microspores have delayed nuclear division and produced more green plants (Hu and Kasha 1999). Since colchicine inhibits spindle formation, and thus could have accumulated microspores at mitosis, an increase in the number of green plants might have been expected by the application of colchicine during the pretreatment. However, in this study, a significant increase in the number of green plants was observed with 300 mg l<sup>-1</sup> only for cv. Pavon. Incorporation of colchicine during cold pretreatment in maize improved anther response and embryogenesis, without affecting percentage of doubling (Antoine-Michard and Beckert 1997). On the contrary, in this study, a significant increase in percentage of doubling was observed in cv. Caramba with 300 mg l<sup>-1</sup> whereas this concentration produced a significant decrease in cv. Pavon. These results show that the colchicine effect on green plant production and percentage of doubling was genotype dependent.

When colchicine was applied during the first hours of culture, its effects on number of embryos and on percentage of green plants depended on the genotype and on the culture method. In microspore culture, colchicine improved these two variables only in cultivar Pavon, but had no influence in either of the two cultivars used in anther culture. An increase in embryogenesis was also reported in microspore culture of *Brassica* (Zhou et al. 2002a, b), whereas no effect was observed in wheat microspore culture (Hansen and Andersen 1998). In wheat anther culture, enhancement of embryogenesis (Barnabás et al. 1991) and toxic effects (Navarro-Alvaréz et al. 1994; Redha et al. 1998a, b) have been described.

The different genotypes used in these studies could explain these contradictory results. Also, toxic effects have been reported in F1 durum × wheat hybrids (Tersi et al. 2006). Stimulation of embryogenesis has also been reported in anther culture of some genotypes of maize (Obert and Barnabás 2004) and rice (Alemanno and Guiderdoni 1994). Different actions of colchicine have been proposed that could explain the positive effects on embryogenesis, as an increase of symmetrical divisions (Szakacs and Barnabás 1995), the depression in the synthesis of pollen specific tubulins (Cleveland et al. 1983; Carpenter et al. 1992), and cytoskeletal restructuration (Shariatpanahi et al. 2006).

Negative effects on percentage of green plants in microspore culture (Hansen and Andersen 1998) and negative and positive effects in anther culture (Barnabás et al. 2001; Ouyang et al. 1994; Redha et al. 1998a, b; Zamani et al. 2000) have been previously described in wheat. It has been suggested that colchicine could cause a reduction of chloroplast DNA abnormalities (Aubry et al. 1989) or a selective elimination of microspores having abnormalities (Barnabás et al. 1991), increasing the percentage of green plants.

In this study, colchicine application during the first hours of microspore or anther culture resulted in higher rates of chromosome doubling in all the genotypes. These results are in accordance with those previously reported in anther culture (Barnabás et al. 1991; Navarro-Alvarez et al. 1994; Redha et al. 1998a, b; Zamani et al. 2000) and microspore culture of wheat (Hansen and Andersen 1998), as in other species, such as maize (Saisintong et al. 1996; Barnabás et al. 1999), triticale (Arzani and Darvey 2001) and *Brassica* (Möllers et al. 1994; Hansen and Andersen 1996; Zhou et al. 2002a, b). The doubling rate increased more significantly in cv. Pavon and DH24033 than in cv. Chris. Genotypic dependence has also been described previously in wheat (Barnabás 2003; Zamani et al. 2003) and *Brassica* (Möllers et al. 1994; Weber et al. 2005). The differences among genotypes in their responses to colchicine might be related to the kinetics of mitotic division in culture (Zaki and Dickinson 1991).

A compromise should be adopted for the selection of concentration and treatment duration. Colchicine concentrations above 120 mg/l for 48 h in microspore culture (Hansen and Andersen 1998) and

100–200 mg l<sup>-1</sup> for 72 h in anther culture (Navarro-Alvarez et al. 1994; Redha et al. 1998a) have been reported to increase percentage of doubling but also reduced embryogenesis and/or green plant regeneration in wheat. However, the concentrations of colchicine applied in the present study allowed us to increase percentage of doubling and still no deleterious effects, or even positive effects on androgenic response were produced. This suggests that higher concentration of colchicine could further improve DH plant production.

We observed a lower increase in the percentage of chromosome doubling in anther culture than in microspore culture of cv. Pavon with 300 mg l<sup>-1</sup> colchicine (51% and 76%, respectively). Also, positive effects produced by colchicine on embryogenesis and percentage of green plants in microspore culture of Pavon were not observed in anther culture. This variation of colchicine effects between microspore and anther culture could be due to the fact that different microspore stages were used. Also, the anther wall could be acting as a filter (Pulido et al. 2005), avoiding complete absorption of colchicine. If so, longer treatments or higher concentrations of colchicine would be necessary in anther than in microspore culture to produce the same effect.

The negative effect of the application method itself in cvs. Chris and Pavon could be due to the initial culture of microspores in liquid medium and subsequent manipulation of microspores during washing. Similar effects have been described in wheat (Hansen and Andersen 1998) and in *Brassica* (Hansen and Andersen 1996; Zhao et al. 1996b). However, in this study around a 3-fold increase in the number of embryos was produced by the application method in the DH line. This positive effect could be explained by the high percentage of mortality of the microspores during the first 2 days of culture in this cultivar. Washing microspores could be helpful for the elimination of toxic compounds released by dead or deteriorating microspores into the culture medium, thus increasing the number of embryos. These results agree with those reported in *Brassica* by Fletcher et al. (1988) and Zhou et al. (2002b) who found that a change of the induction medium 15–24 h after microspore isolation could be essential to allow normal embryo initiation and development.

The incorporation of colchicine during the first hours of anther and microspore culture increased the

number of green DH plants compared with treated controls in two cultivars and the DH24033 whereas colchicine application during the stress pretreatment only increased the number of green DH plants in one of the two cultivars used. From these results we conclude that colchicine application during the first hours of culture is more effective than during the stress pretreatment. Colchicine was successfully used in both anther and microspore culture, in cultivars with medium or low rates of doubling. These results encourage the introduction of this methodology into wheat breeding programs. Further improvements could be achieved by optimization of the application method in microspore culture and by the application of longer treatments in anther culture.

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