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The effect of colchicine pretreatment on isolated microspore culture of wheat (*Triticum aestivum* L.)

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Abstract

Colchicine is an alkaloid and microtubule-depolymerizing agent, disrupts microtubule formation during the cell division at metaphase stage and prevents anaphase thus resulting in an unreduced chromosome number before cell division. The main advantageous for genetic studies and plant breeding programmes using colchicine for rapid and huge production of fertile plants through anther and microspore culture. Therefore, the aim of this investigation was to find out the impact of colchicine on the response of fertile plants in wheat isolated microspore culture. In this case colchicine applied to the anthers compared with direct treatment on the isolated microspores. For first experiment 100 mg/l colchicine added seperately with three different basal media and found in all cases more or less fertile plants production compared to control (without colchicine). Colchicine application on anther culture medium showed significant increase in embryo formation and green plant regeneration. The direct treatment of colchicine to isolated microspore culture decreased three - four fold embryoids induction but improved fertile plant regeneration. Application of colchicine for both cases in anther and microspore culture increased the average chromosome doubling frequency (84.94%) compared to the control (55.26%) where colchicine was not added. For the second experiment, different concentrations of colchicine (50, 100, 150 mg/l for three days) added in AMC medium and found that reduced number of embryos and regenerated green plants, while the regeneration of albino plants was stimulated. When colchicine concentration increased the number of embryos decreased significantly, while the doubling index increased which is the main target of this study. In this case average frequency of diploid plants were increased (81.73%) compared with control (72.40%) among the three treatments. This finding has increased the knowledge about the benefit of colchicine application and optimized its concentration on isolated microspore for improving doubled haploids production in wheat microspore culture.

Key words: Chromosome doubling, Colchicine, *In vitro*, Microspore, *Triticum aestivum.*
Abbreviations: ELS- Embryo like structures; GRP- Green regenerated plants, ARP- Albino regenerated plants, DI- Doubling index, SI- Success index, LSD- Least significant difference, PC- Pre-culture medium, WM- Washing medium, AMC- Induction medium, MSR- Regeneration medium, DAPI- 4,6-diaminidino-2-phenylinodol.

Introduction

In vitro production of doubled haploid plants (DHs) has become efficient for many plant breeding programs and has increasingly become an important method for advanced research in the field of plant breeding and biotechnology. Since conventional breeding methods to derive inbred lines are difficult and time consuming, mainly anther culture has been employed to obtain homozygous lines as well as new breeding line (De Buyser et al., 1987; Pauk et al., 1995). Although anther culture method is still widely used in haploid induction. The high level of embryogenesis and regeneration of green plants by isolated microspore culture method has been elaborated by several investigators in wheat (Tuvesson and Öhlund, 1993; Puolimatka et al., 1996; Touraev et al., 1996; Kunz et al., 2000; Soriano et al., 2007; Slama-Ayed et al., 2010a). The reported advantages of isolated microspore culture are the availability of microspores, embryos for *in vitro* selection experiments (Jähne and Lörz, 1995), and as unicellular structures being targets for gene transfer (Jähne et al., 1994; Folling and Olesen, 2001).

Furthermore, in certain plant species such as *Hordium vulgare* the isolated microspore culture procedure has produced five fold green plants compared to anther culture (Hoekstra et al., 1992; Shim, et al., 2009). The protocol has been well established also last 10-15 years for other crops e.g. rice (Raina and Irfan, 1998), maize Obert et al. (2004), *Brassica napus* (Mölleres et al., 1994; Weber et al., 2005), pepper (Kim et al., 2008), *Medicago* (Ochatt et al., 2009), chickpea (Grewal et al., 2009), carrot (Górecka et al., 2010), horse chestnut (Dragosavac et al., 2010). Inspite of the improvement in wheat microspore culture (Touraev et al., 1996; Hansen and Andersen, 1998; Kunz et al., 2000), the availability of repeatable optimized *in vitro* system is needed that could be applicable to a wide range of genotypes. For success of *in vitro* culture genotype, explants, carbon source, growth regulators are important factors as well as for micropropagation (Michel et al., 2008) and androgenetic study (Islam et al., 2001). In most studies artificial manipulation in the form of physical, physolo-

gical and/or chemical treatment are needed to switch on the signal for the sporophytic pathway of the microspores (Zheng, 2003). A variety of pretreatments have been used such as applying stress to the developing microspores at a critical stage, causing a block or delay in their development (Jähne and Lörz, 1995; Touraev et al., 1996). These stresses include cold, heat, starvation, osmotic shock or microtubule disruption agents as colchicine, pronamide, oryzalin and amiprophosmethyl (AMP). Out of that microtubule-depolymerizing agent colchicine still prove to be a useful chemical for production of doubled haploids (Soriano et al., 2007). The pretreatment effect of colchicine in addition to the induction medium investigated for anther culture using different concentrations and durations, causing an increase in the frequency of fertile plants up to 76% (Barnbás et al., 1991; Navarro-Alvarez et al., 1994; Redha et al., 1998; Zamani et al., 2000; Obert and Barnbás, 2004). Hansen and Andersen (1998) applied colchicine to microspore culture, which caused an increase in the frequency of fertile plants up to 53%. Albino plants are very often produced during the regeneration of microspore-derived plants in cereals (Olmedilla, 2010). For androgenetic study in cereal crops still albinism has a great problem. Talebi et al. (2007) reported that the frequency of albinos may vary from 5 -100% in rice anther culture. Different stress application for shorter periods (3 - 4 days) may be cause to microspore survival problem and stimulated albinism (Torp and Andersen, 2009; Slama Ayed, 2010). Therefore, the present investigation has been undertaken for better understanding of the impact of certain stress factor such as colchicine and optimizing its concentration for increasing the fertile plant production and reducing albinism in wheat microspore culture.

Materials and methods

Plant material

The spring wheat genotype of DH83Z118.32 was used as donor plant material for this study. It was selected for its agronomic traits by the breeding department of the Swiss Federal Research Station for Agronomy (FAL), Zürich-Reckenholz and for its high androgenetic response by ETH-Zürich, Switzerland (Schmid et al., 1994). Its androgenetic response was confirmed for anther as well as for isolated microspore culture (Kunz et al., 2000). Donor plants were grown in the greenhouse with approximately 25/18ºC day/night temperature and 16/8 h light/dark.

Microspore isolation

Flowering spikes with microspores at late uninucleate to early binucleate stage were removed from donor plants, and subjected to a cold pretreatment for 3-14 days, at 4ºC, in the dark. After surface sterilized with 70% ethanol, excised anthers were squeezing with a sterile glass slide in washing medium. The suspension was further diluted with 10 ml washing medium, filtered through a sieve with 100 µm mesh and centrifuge for three minutes at 750 rpm. The sediment was carefully re-suspended in 2 ml induction medium and transferred to a Corning, surface treated 35 × 10 mm Petri dishes. Around six to eight ovaries isolated form the same spikes were added to each culture dish (Puolimatka et al., 1996). Each dish contained approximately 8×10^4 microspores

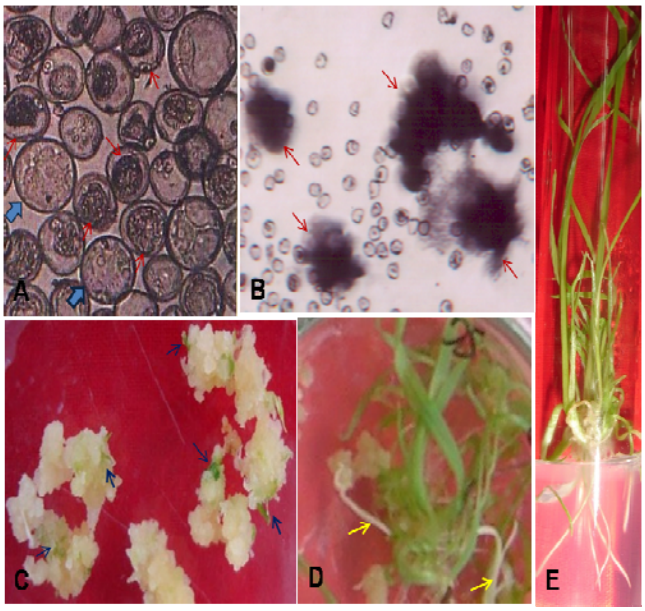

Fig 1. Microspore cell division, embryo/callus formation and green plant regeneration from colchicine pre-treated microspore culture of wheat.

A: Maximum colchicine pre-treatment microspores are showing non-viability (narrow arrow) and few are showing viability (thick arrow) after five days of culture initiation.

B: Only viable microspores are showing embryos formation after two - three weeks of culture initiation.

C: Callus with re-generable green structures.

D: Green and albino (yellow arrow) regenerated plantlets.

E: Well develop plantlets with root and shoot ready to transfer to soil.

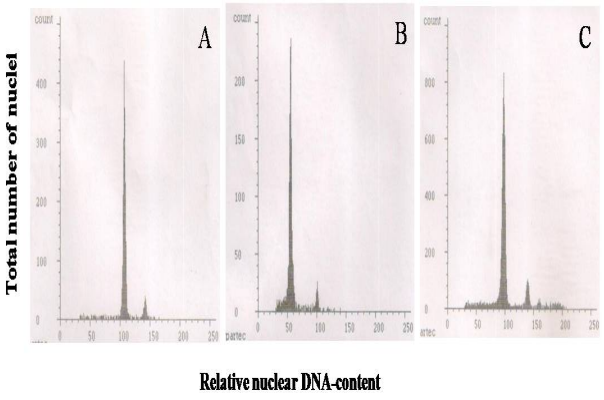

Fig. 2. Flow cytometry histograms presenting ploidy levels of colchicine pre-treated microspore derived plants. Nuclei stained with DAPI solution.

A. Control (diploid, 2n)

B. Haploid (n)

C. Doubled haploids/Fertile plants (2n).

isolated from 80 anthers. The cultures were incubated for three - four weeks in the dark at 27ºC for embryo induction.

Culture media

Three media as PC, WM and AMC was considered for this study. The pre-culture medium was composed of D-manitol 72.87 g/l, L-ascorbic acid 50 mg/l and L-proline 125 mg/l. The WM and AMC modified by AM (Schmid, 1990). For all three media 100 mg/l colchicine was added along with other composition.

Embryogenesis, plant regeneration and ploidy level evaluation

Microspore development stages observed after 5 days of culture initiation under colchicines application and continued up to three - four weeks for embryoids development (Fig. 1. A, B). Good qualities of embryoids were transferred to semi-solid regeneration medium (MSR, Henry and De Buyser, 1990) and culture plates were kept at 27ºC with 16h light in growth chamber. Normally embryoids become greenish after 3 - 4 days of transferring to regeneration medium (Fig. 1. C). The number of green and albino plants were recorded after 2 - 3 weeks (Fig. 1. D). Well developed shoot and rooted plants (Fig. 2. E) are raised in the greenhouse at 25/18ºC day/night, till maturity. Genomic variation in regenerated plants, which have been regenerated from callus or microspore cultures treated with colchicines, has been detected by flow cytometry (CAII; Partec GmbH, Münster, Germany). Flow cytometry analysis was the based on the use of DNA-specific fluorochromes of the relative fluorescence intensity of stained nuclei. The number of nuclei counted per sample was 2-3 thousand. The ploidy level of each sample was estimated by comparing the peak of histogram. The peak of histogram made from the analysis of isolated nuclei of 10 - 12 days old seedlings leaf blades of its standard genotype (2n) was used as control (Fig. 2. A) with the comparison of regenerated treated plants. Colchicines treated microspore-derived plants was analyzed for confirming haploid (Fig. 2. B) and doubled haploid levels (Fig. 2. C).

Effect of colchicine pre-treatment for both anther and microspore culture of wheat

For Part-I (anther culture) excised anther were incubated in PC (T_1), WM (T_2) and AMC (T_3) medium and all medium was supplemented with 100 mg/l colchicines. For control inoculated anthers were incubated in colchicine free pre-culture medium (PC). After the pre-treatment duration anthers were rinsed 2 - 3 times with colchicine free washing medium. For Part-II, isolated microspores were incubated directly in PC (T_1), WM (T_2) and AMC (T_3) medium. Then microspores were rinsed two to three times by centrifugation with colchicine free washing medium. For both cases microspore isolation and other procedure are same as described earlier.

Effect of different concentrations of colchicine on isolated microspore culture

The different concentrations of colchicine (50, 100 and 150 mg/l) were added to AMC (induction) medium and incubated at 27ºC for three days. After colchicine treatment microspores were re-suspended in fresh induction medium, and kept in the dark at 27ºC around three to four weeks for embryos formation.

Data recording and statistical analysis

Data were recorded on the basis of embryogenesis, regeneration and diploidization of microspores particularly on the following traits, i) embryos per 10^5 microspores (Embryo like structures = ELS/100 anthers), ii) green regenerated plants (GPR) per 100 embryos (GPR/100 ELS), iii) albino regenerated plants (ARP) per 100 embryos (ARP/100 ELS), iv) doubling index (DI) = doubled haploids or fertile plants / total green regenerated plants × 100, and v) success index (SI) = doubled haploids / total number of anthers × 100. Analysis of variance (ANOVA) was performed on the following traits, e.g. embryos per 10^5 microspores, TRP, GRP, ARP, DI and SI per 100 anthers. In order to get normal distribution, all data were transformed by the ArcSin√P value before ANOVA. List significant difference (LSD) was estimated at 0.05 and 0.01 level of probability. The analysis was computed following the working schedule of Gomez and Gomez (1976).

Results

Effect of colchicine pre-treatment for both anther and microspore culture of wheat

Colchicine treatment of the microspores while still inside the anthers compared with direct treatment of the isolated microspores were studied with three different basal media containing the drug (Table 1). Chromosome doubling is an important step to obtain doubled haploid plants in wheat anther and microspore culture. Results indicated that colchicine treatment on anthers (microspores inside) showed not so clear effect of colchicines of the response of variables. In contrast, direct treatment of colchicine into the isolated microspores differed significantly from the untreated control for all response variables.

The direct treatment of microspores with colchicine, decreased the frequency of embryo formation (47.56%) compared with untreated control embryos (207.83%) per 10^5 microspores (Table 1). The direct treatment had a positive effect on the frequency of green plants regenerated from 9.38% per 100 embryos in the control to an average 33.78% per 100 embryos for all three media in microspore culture system (Table 1). The direct treatment also induced regeneration of more albino plants (44.15%) compared to the control (8.74%) per 100 embryos (Table 1). Finally, the direct microspore treatment increased chromosome doubling frequency of green regenerated plants (DI = doubling index) to produce 84.94% diploids on average among green plants regenerated from the three different treatment media (Table 1) compared with only 55.26% diploids from the untreated control.

Differences among the three media holding colchicine during treatments were only significant in a few cases. When anthers were treated with colchicine in three different basal media, only significant effects was observed for embryo formation. This effect of the media for anther treatment apparently was caused by a much higher embryo formation from anthers cultured on the induction medium compared with pre-culture and washing medium (325.50 vs 207.83 in Table 1). For the direct treatment of colchicine into microspore culture media showed significant effects on green plant formation and for the number of diploid plants per 100 anthers (SI). The significant media effect on green plant regeneration from embryos was apparently due to a much lower (13.33% green plants per 100 embryos) in T_3

Table 1. Effect of colchicine (100 mg/l for 3 d) with three basic media for both anther and isolated microspore culture of wheat.

Treatment (Media)	ELS	GRP (%)	ARP (%)	TGRP	DI (%)	SI (%)
Part- I: Anthers						
Control	207.83	9.38	8.74	76	55.26	7.00
T_1	178.00	21.25	16.29	83	66.27	9.17
T_2	186.40	15.24	8.91	68	48.53	6.60
T_3	325.50**	27.09	10.04	64	37.50	4.00
Average	229.97	21.19	11.75	71.67	50.77	6.59
Part- II: Isolated microspores						
T_1	54.00	34.57	40.12	49	91.84*	15.00**
T_2	38.67	53.45**	39.66	52	94.23*	16.33**
T_3	50.00	13.33	52.67**	16	68.75	3.67
Average	47.56	33.78	44.15	39	84.94	11.67
Mean	148.63	24.90	25.20	-	66.05	8.82
± S. E.	40.19	5.76	6.96	-	8.03	1.91

ELS: Embryo like structures per 10^5 microspores. Part I: Colchicine treatment of excised anther before microspore isolation. Part II: Direct treatment of colchicines to isolated microspores.
T_1 = Pre-culture (PC), T_2 = Washing (WM) and T_3 = Microspore culture (AMC) induction media.
*and ** indicating significant at 0.05 and 0.01 probability level by t-test.

Table 2. Effect of different concentrations of colchicine (50, 100, 150 mg/l for 3 d) on isolated microspore culture of wheat.

Treatment (Col. conc.)	ELS per 10^5 microspores	GRP/100 ELS	ARP/100 ELS	Fertile plants	DI (%)
Control	560.8	40.27	15.47	127	72.40
T_1	359.39	30.72	22.03	82	78.10
T_2	326.06	35.59	24.81	105	80.90
T_3	314.75	32.70	25.26	94	86.20
Average	333.40	33.00	24.03	93.67	81.73
LSD 0.05	-	14.46	8.43	-	19.21
LSD 0.01	-	21.04	12.26	-	27.95

Control = without colchicine. T_1 = 50 mg/l, T_2 = 100 mg/l and T_3 = 150 mg/l colchicine supplemented in AMC medium.

(AMC), compared with other two media (T_1, PC) and (T_2, WM). This low regeneration of green plants also led to a very low SI score for the AMC treatment media (T_3), which caused the significant media effects for this response variable.

Effect of different concentrations of colchicine on isolated microspore culture

Direct treatment of isolated microspores with different concentrations of colchicine was performed in an attempt to estimate an optimal concentration for the compound during treatment of the microspore (Table 2). Analysis of variance showed clear difference between the untreated control and average of all treatments, for embryo formation, green plant regeneration and albino plant frequency. Also in this experiment direct treatment of microspores with colchicine had a negative effect on the subsequent formation of embryos. Frequencies of green plants regenerated were affected negatively of the treatment while formation of albinos was stimulated (Table 2). Effects of the three different concentrations of the colchicine treatment, however, were significant only for embryo formation, which was the result of reduced embryo regeneration with higher concentration of the drug during microspore treatment. The spontaneous frequency of chromosome doubling in this experiment was high, 72.4 percent diploid regenerates from the untreated control. Direct treatment of the microspores with colchicine in this experiment also increased the frequency of diploid plants from 72.4% in the control to an average of 81.73 percent among the three treatments. However, due to the high spontaneous level of chromosome doubling in this experiment the effect of the three different concentrations of colchicine were not significant, although there was a tendency to higher diploid frequencies with increased concentration (150 mg/l) of the colchicines in T_3 (86.20%).

Discussion

The level of spontaneous chromosome doubling in anther culture of wheat is in general low 15-25% (Hansen and Andersen, 1998; Navarro-Alvarez et al., 1994) which has lead to studies of anther pretreatment with colchicine to increase the frequency of diploid regenerated plants. In anther culture these pretreatments have often been shown even to increase frequency of green regenerates by reducing the albino percentage (Navarro-Alvarez et al., 1994; Redha et al., 1998). Hassawi and Liang (1991) used three antimitotic agents *viz.* colchicines, trifluralin and oryzalin for different durations (48 and 72 h) in wheat. They found that out of three, colchicine is the most effective compound for chromosome doubling in wheat anther culture systems. In this study only colchicine used and found interesting results on spontaneous chromosome doubling in control (55.26%) and in average of three media were 50.77%. Barnabás et al. (1996) mentioned that the frequency of symmetrical divisions of microspores was significantly increased after *in vitro* colchicine treatment. In a

report Ouyang et al. (1994) mentioned that the best concentration of colchicine for wheat anther is 250 mg/l and the optimum duration for the treatment of calli in colchicine-containing medium is 24 h. However, in this case isolated microspore was incubated with colchicines containing medium, so the concentration was differed. In most of the cases for wheat isolated microspore culture the spontaneous chromosome doubling frequencies are only about 15 - 25 percent, which has also inspired to studies of *in vitro* treatment of microspores with chromosome doubling chemicals before culture (Hansen et al., 1988; Hansen and Andersen, 1998). Soriano et al. (2007) applied colchicines in wheat during mannitol stress pretreatment or during the first 48 h of culture at concentrations of 0, 150 and 300 mg/l. They observed that significant increase in doubling with 300 mg/l but low androgenetic response found. In the present study when colchicines concentration was increased (150 mg/l) then found very similar type of results on chromosome doubling efficiency and reduced ELS. For applied purposes, it is important that a high frequency of regenerated plants are diploid because it is very resource consuming to chromosome double a high number of plants after establishment in soil. This results support with the findings of above mentioned reports. In the present investigation, albino plants were significantly increased when colchicine (100 mg/l) was directly applied to the microspore culture medium. Navarro-Alvarez et al. (1994) reported that lower concentrations of colchicine are better than higher concentrations because of the positive effects of the critical steps of embryoid formation and albinisms. Barnabás et al. (1991) reported that colchicine may selectively eliminate microspores carrying abnormalities that cause the development of albino plants. Hansen and Andersen (1988) also reported that albino plant production was increased when the higher concentration of colchicine was used in wheat microspore culture. The reported findings are very consistent with the present results. In this study it was observed that when increased colchicine concentration the number of embryos decreased significantly, while the doubling index increased. This finding has increased the knowledge about the benefit of colchicine application and optimization of its concentration for improvement of doubled haploid plant production in wheat microspore culture.

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