Colchicine induced embryogenesis and doubled haploid production in maize (*Zea mays* L.) anther culture

Payam Pour Mohammadi, Ahmad Moieni*, Mokhtar Jalali-Javaran

Department of Plant Breeding, Faculty of Agriculture, Tarbiat Modares University, P.O. Box 14115-336, Tehran, I.R. Iran

Abstract

This study involves *in vitro* androgenesis of *Zea mays* L. via anther culture. Combination of two embryo induction media (IMSS & YPm) in presence of different colchicine concentrations (0, 100, 200, 250, 300 and 400 mg/l) in the pretreatment medium (IML) and pretreatment duration (0, 3, 6 and 9 days) in two genotypes (DH5×DH7 and ETH-M82) were tested. After colchicine pretreatment, anthers were transferred to the induction media without colchicine to induce of embryo like structures (ELSs). The ELSs were then transferred to plant regeneration medium (YPNAS). It was found that in the genotype DH5×DH7, colchicine at a concentration of 100 mg/l significantly induced the number of ELSs (19.6). The control (without colchicine) and 400 mg/l of colchicine resulted in lower levels of ELSs (5.8, 5.7, respectively). In this genotype, colchicine pretreatment for 3 days produced highest number of ELSs (16.83) and a large increase in the ELS yield was observed in the YPm medium (14.4). In ETH-M82 genotype, 6 days of pretreatment with 300 mgl^{-1} with colchicine, produced highest frequency of ELSs (25). Also, in this genotype, a large increase in ELS yield was observed in the YPm induction medium (22.3). The frequency of spontaneous chromosome doubling in control group was very low for both genotypes (7%), but these genotypes were able to produce doubled haploid plantlets from the ELSs (63% doubled haploid) using a low concentration of colchicine in the pretreatment medium (250 mgl^{-1} for 6 days). At high concentrations of colchicine (300 and 400 mg/l), more morphological and chromosomal aberrations were observed.

Keywords**:** Maize; Anther culture; Colchicine; Embryogenesis; Doubled haploid.

Correspondence to: **Ahmad Moieni,** Ph.D.
Tel: +98 21 44196522; Fax: +98 21 44196524
E-mail: moieni_a@modares.ac.ir

INTRODUCTION

Development of inbred lines by selfing is a time consuming process in maize breeding. Therefore, many investigations on the use of doubled haploids produced by androgenesis, have been carried out (Dieu and Beckert, 1985). Anther culture capacity is characterized by embryo production and plant regeneration. Embryogenesis can be influenced by genetic and environmental factors such as anther pretreatment and embryo induction medium.

In cereal anther culture, chromosome doubling of haploid cells or tissues at certain stages is a critical step in producing doubled haploid plants (Obert and Barnabás, 2004). The frequency of spontaneous chromosome doubling occurring during androgenesis of maize is relatively low, varying between 4.5 to 22% with an average of 10% (Buter, 1997). The frequency of spontaneous chromosome doubling exceeding 50% has been reported in barley (Lyne *et al*., 1986) and rice (Rania, 1989). Several investigations investigated the effect of incorporating colchicine on anther culture derived plants in several cereals: wheat (Redha *et al*., 1998; Zamani *et al*., 2000), maize (Saisingtong *et al*., 1996; Barnabás *et al*., 1999), rice (Chen *et al*., 2001) and triticale (Arzani and Darvey, 2001). Colchicine is frequently used for chromosome doubling under both *in situ* and *in vitro* conditions, since it disrupts mitotic cell division by inhibiting the formation of spindle fibers and polar migration of chromosomes. Colchicine treatment of maize seedlings or plantlets may double the chromosome number in the tassel or the ear, but often not in both, which will make self-pollination impossible (Wan *et al*., 1989). High mortality and abnormal plant development can also be observed in colchicine-treated plant populations. This could explain the low efficiency of doubled haploid produc-

tion in maize after colchicine treatment of regenerated haploid plantlets. Genome doubling in culture media before the plantlet regeneration stage might help to circumvent the above-mentioned problems. Wan *et al*. (1989) and Wan and Widholm (1995) reported the recovery of genetically stable doubled-haploid maize plants at high frequency through the colchicine treatment of embryogenic, microspore-derived haploid calli. In other experiments, the application of colchicine treatment together with a 7-days cold shock to cultured maize anthers resulted in a considerable increase in chromosome doubling in microspore-derived plants (Saisingtong *et al*., 1996; Antoine-Michard and Beckert, 1997).

The present paper reports the effects of colchicine pretreatment of maize anthers on embryogenesis and chromosome doubling.

MATERIALS AND METHODS

Plant material: Two maize genotypes DH5×DH7, and ETH-M82 (provided kindly by Dr. M. Beckert, INRH, Clermont-Ferrand, France and Dr. IE. Aulinger, Swiss Federal Institute of Technology, Zurich, Switzerland, respectively) were used as anther donor plants. They were grown in a growth chamber at 25°C (day) and 15°C (night), with a photoperiod of 16 h and a light intensity of 500 $\mu molm^{-2}s^{-1}$.

Anther culture and colchicine pretreatment: Tassels were collected prior to the emergence of the main leaf blade and tested for microspore development. As a cold pretreatment, the tassels were covered with an aluminum foil and then kept at 7°C for 10 days (Jumpatong *et al*., 1996). Tassel fragments containing anthers in the late-uninuclear microspore developmental stage (determined by acetocarmine squash) were surface sterilized with 2% w/v sodium hypochlorite for 10 min and then washed three times with sterile distilled water. The anthers were then dissected under sterile conditions and were placed in 55×15 mm plastic petri-dishes containing 8 ml of filter-sterilized liquid pretreatment medium (IML); Saisingtong *et al*., 1996) and different concentrations of colchicine, in order to double the chromosome number of the embryogenic microspores. Colchicine was dissolved in distilled water and then added to medium. The cultures were then incubated for 3, 6 and 9 days at 7°C in the dark. After colchicine pretreatments, the anthers were transferred to colchicine-free semi-solid induction media (IMSS); (Saisingtong *et al*., 1996) and YPm) medium (Genovesi and Collins, 1981) and then incubated at 28°C in the dark for one month. The number of responding anthers and the frequency of microspore-derived structures (embryo-like structures; ELSs) were then calculated as a percentage of the cultured anthers.

The experiment was carried out in a 4-factorial experiment (based on a completely randomized design) with 5 replications. An external analysis of sum of squares (SS) was employed, because of the significance of most of the interactions. Therefore, in each genotype an independent analysis was carried out based on a three-factorial experiment. Each replication consisted of one petri-dish containing 25 anthers. Three studied factors consisted of colchicine concentration in the pretreatment medium (0, 100, 200, 250, 300 and 400 mg/l), incubation duration in the colchicine pretreatment medium (3, 6 and 9 days) and embryo induction medium (IMSS and YPm media). Analysis of variance (ANOVA) was carried out using the SPSS statistical software (version 10.0).

Plant regeneration: The produced ELSs were removed from the one-month cultured anthers and transferred directly to the 10 cm plastic petri-dishes containing 15 ml of plant regeneration medium (YPNAS; Genovesi and Collins, 1981). They were incubated under a 16 h illumination period (50 µmol $m^{-2}s^{-1}$ light intensity) at 25°C. The regenerated plantlets were then transferred into the 250 ml glass containers containing 30 ml of basal MS medium for further growth. Healthy green plantlets were then transplanted into 10-cm pots containing vermiculite and peat (1:1). Finally, the plantlets were transferred to soil and grown to maturity in a growth chamber. Plants which produced normal male and female inflorescences were self-pollinated and the seed set was recorded.

Cytological observations: The ploidy analyzer I (Partec GmbH, Germany) was used to determine the ploidy level of regenerated plants. Using a sharp razor blade, 1 cm^2 leaf segment was cut into small pieces in 2 ml of an 8°C, DAPI (4,6-diamidino-2-phenylindole) staining solution (5 µg/ml, Partec GmbH) and passed through a 50 µm sized nylon mesh (Aulinger, 2002). The filtrate was then used for flow cytometric analysis; at a per gain FL_1 of 412 to 420 (relative fluorescence), a peak set at 100 and 200 FL (corresponding to the G1, G2 or M-phases, respectively) was interpreted as cor-

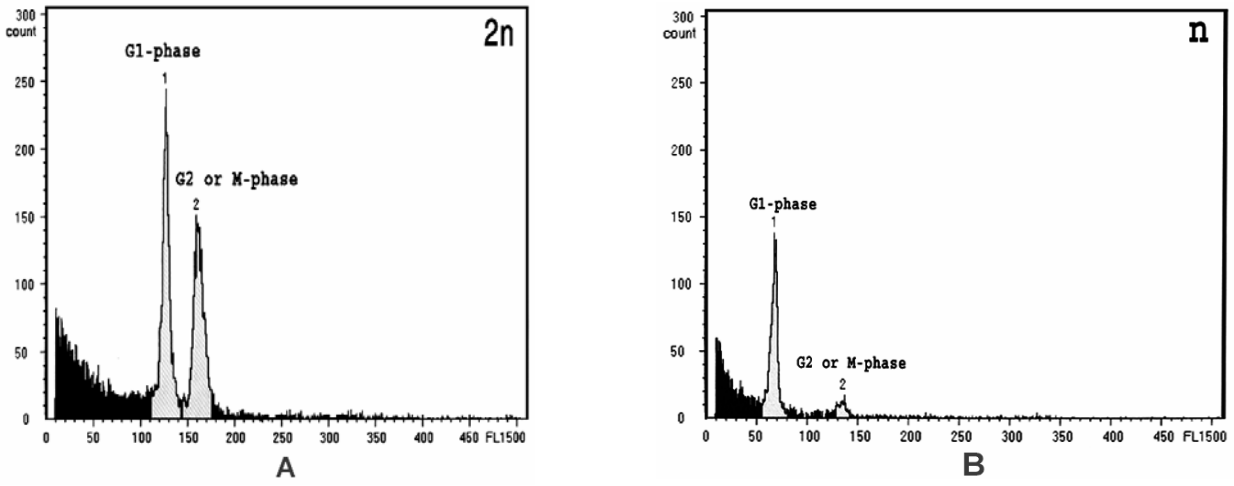

Figure 1. Flow cytometric analysis of the ploidy level. The x-axis of the histogram represents the intensity of DNA fluorescence in relative units; the y-axis represents the number of nuclei counted per histogram channel. **(A)** A representative peak set for diploid or doubled haploid material. **(B)** Peaks corresponding to a typical haploid individual.

responding to diploid or doubled haploid plantlets (Figure 1A). A peak set at 50 and 100 FL was interpreted as corresponding to haploid material (Figure 1B). Moreover, the chromosome numbers were checked using the root tips squash method (Figure 2).

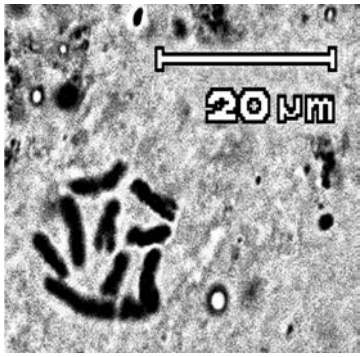

Figure 2. A somatic cell showing 10 chromosomes in regenerated haploid maize plants.

RESULTS

The results of ANOVA (Table 1) for ELS production in the genotype DH5×DH7 showed significant differences among colchicine concentrations in pretreatment medium (A), the durations of colchicine pretreatment (B) and embryo induction media (C). In the genotype ETH-M82; the main effect of the embryo induction medium (C) and the interaction between colchicine concentration and its duration in the pretreatment medium (A×B) were significant. In the genotype DH5×DH7 (Figure 3), colchicine at a concentration of 100 mg/l produced significantly, the highest number of ELSs (19.6), while the control (colchicine-free pretreatment medium) and 400 mg/l of colchicine produced the least ELSs (5.8 and 5.7, respectively). Three days of colchicine pretreatment produced the highest frequency of ELSs (16.83; Figure 4). Also, a signifi-

Table 1. Mean squares (M.S.) values for embryo like structure (ELS) production in the anther culture of *Zea mays* L. (genotypes DH7×DH5 & ETH-M82).

Source of variation (S.O.V)	d.f.	M.S.	
		DH5×DH7	ETH-M82
Colchicine concentration in pretreatment medium (A)	5	520.42**	387.44*
Duration of colchicine pretreatment (B)	2	769.07**	170.60^{ns}
Embryo induction medium (C)	1	275.11*	392.14*
A × B	10	178.53^{ns}	695.28**
A × C	5	35.79^{ns}	86.79^{ns}
B × C	2	205.14^{ns}	112.96^{ns}
A × B × C	10	140.18^{ns}	197.21^{ns}

**, * Significant difference at 1 & 5% probability level, respectively
ns: Non Significant at 5% probability level. d.f: degree of freedom

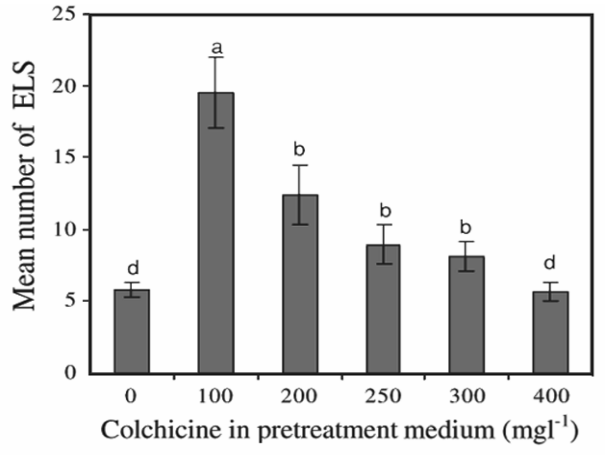

Figure 3. Influence of colchicine in the pretreatment medium on ELS production in the *DH5×DH7* genotype. Means with the same lettar are not significantly different at p=0.05.

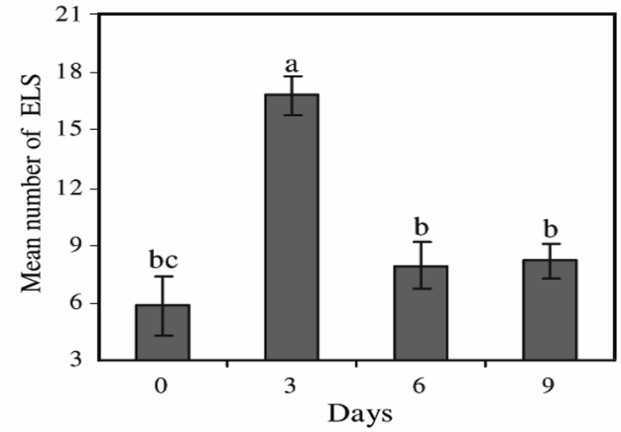

Figure 4. Effect of the duration of colchicine pretreatment of anthers on ELS production in the *DH5×DH7* genotype. Means with the same lettar are not significantly different at p= 0.05.

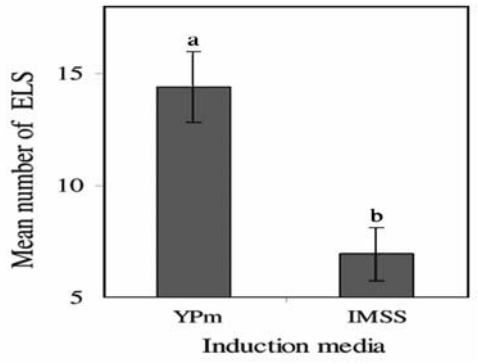

Figure 5. Effect of induction medium on ELS production in the *DH5×DH7* genotype. Means with the same lettar are not significantly different at p=0.05.

cant increase in ELS yield was observed in the YPm induction medium (14.4; Figure 5) in comparison with the IMSS medium.

In the ETH-M82 genotype, the interaction between colchicine concentration in the pretreatment medium and the incubation duration of anthers in this medium was highly significant (Figure 6). Anthers pretreated with 300 mgl^{-1} of colchicine for 6 days showed the highest frequency of ELSs (25). Moreover, in this genotype, a large increase in ELS yield was observed in the YPm medium (18; Figure 7).

The frequency of spontaneous chromosome doubling in the colchicine-free pretreatment medium was very low in both genotypes (7%), and according to the phonological observations, most of the plants

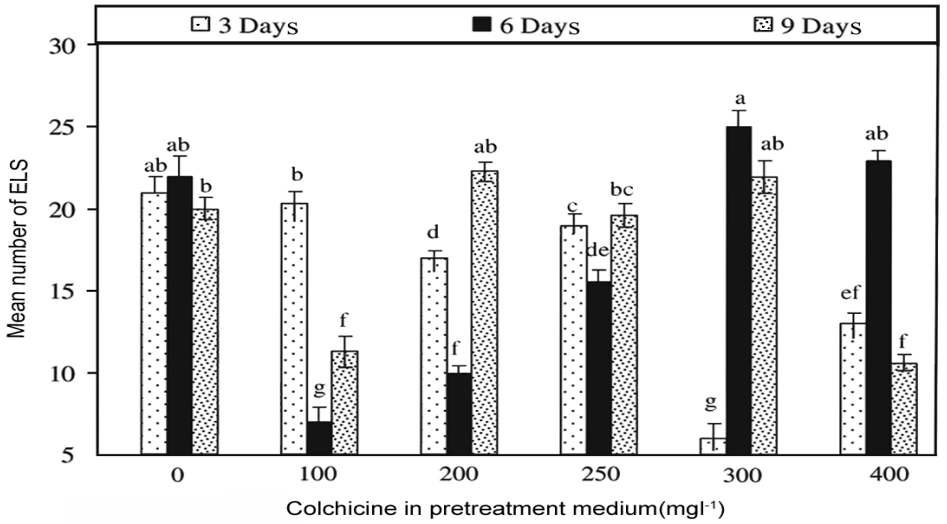

Figure 6. The interaction between the colchicine concentration in the pretreatment medium and the duration of colchicine pretreatment on the ELSs production in the ETH-M82 genotype. Means with the same lettar are not significantly different at p=0.05.

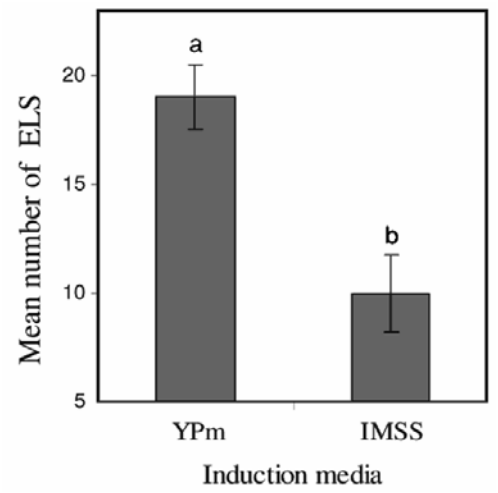

Figure 7. Effect of induction medium on ELS production in the *ETH-M82* genotype. Means with the same lettar are not significantly different at p= 0.05.

were chimeric, having sectorial fertility in the tassels. On the other hand, both the genotypes were able to produce doubled haploid plantlets from ELSs (63% doubled haploids) using a low concentration of colchicine (250 mg/l for 6 days). By increasing the concentration of colchicine (300 and 400 mg/l), more morphological and chromosomal aberrations were observed. Both haploids and doubled haploids showed variation in agronomic characters including plant height, ear height, days to anthesis and days to silking (data not presented). Abnormal morphological characters were also observed in both haploids and doubled haploids. Generally, they were short in plant stature with some narrow leaves and had abnormal reproductive organs such as tassel seeds with varying degree of pistillate and staminate flowers, very small ears and tassels, prolific ears, terminal ears, no tassel and/or ear formation, non-shedding tassels and non-silking ears. However, the degree of abnormality was higher in haploids than doubled haploids.

Self-pollination of each doubled haploid plant was attempted in order to produce selfed seeds for inbred line formation. Only a few doubled haploids (13 plants in ETH-M82 and 19 plants in DH5×DH7) could produce selfed seeds due to the abnormality of reproductive organs and the non-synchronization of pollen shedding and ear silking.

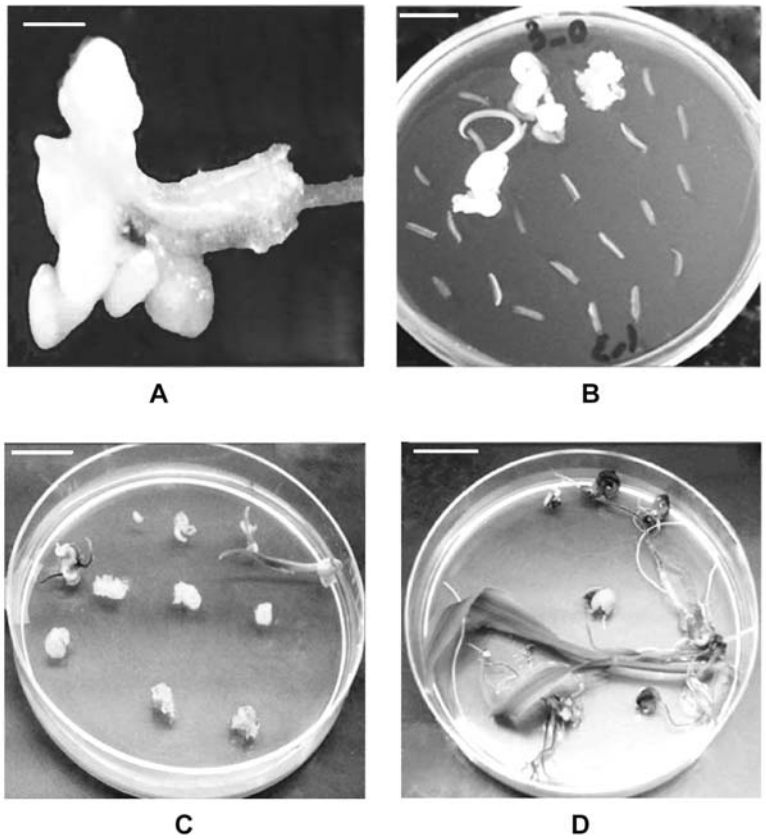

Figure 8. Embryogenesis and plantlet regeneration in maize anther culture. A & B: ELSs in the induction medium. C & D: Regenerated plantlets. (Bar A = 1 mm, Bar B, C, D = 1cm).

DISCUSSION

In this study, the pretreatment medium containing colchicine at a concentration of 100 mg/l increased significantly the number of ELSs in the genotype DH5×DH7 (Figure 8), while in genotype ETH-M82 the concentration of 300 mg/l produced the highest frequency of ELS. The negative effect of 400 mg/l of colchicine on ELSs formation in genotype DH5×DH7 might be due to the toxic effect of colchicine on microspores. In fact, two main physiological factors are important for the achievement of a successful androgenic response in maize: the developmental stage of the microspores and the exogenous stimulus inducing an androgenic response (Reynolds, 1997). In maize anther culture, the first asymmetric division of the androgenic microspore increases embryogenesis (Szakács and Barnabás, 1995). The signal responsible for the switch in the genetic programme of the microspores from gametophytic to the sporophytic developmental pathways is stress. Stress acts as a launching mechanism for redirection to embryogenesis and stops the development of the fertile pollen grain (Touraev *et al*., 1997). Anther and microspore culture could be affected by colchicine treatment. Increase in the embryo frequency due to colchicine treatment has already been reported in maize genotypes (Barnabás *et al*., 1999).

One factor, which is limiting in the application of doubled haploid plants to maize breeding, is low induction of spontaneous chromosome doubling. A low rate of spontaneous chromosome doubling yields only a small number of doubled haploid plants useful for breeding purposes. In the present study, the frequency of spontaneous chromosome doubling in the control treatment was very low for the two studied genotypes (7%). In a review by Buter (1997) the frequency of spontaneous chromosome doubling in maize was reported to range from 4.5 to 22%, with an average frequency of approximately10%. While, Antoine-Michard and Beckert (1997) reported that spontaneous chromosome doubling occurred at the rate of 26.92% during direct embryogenesis of maize genotypes F1937×DH229 and DH147×L1. Both endoreduplication and nuclear fusion of the microspores occurring during anther culture are presumed to be the cause of spontaneous chromosome doubling (Chen and Wu, 1983).

Use of antimitotic drugs at the beginning of cultivation of the anthers or microspores, for the direct doubling of chromosomes in the genome of haploid microspores, seems to be very effective for stable dihaploid offspring production in cereals (Barnabás *et al*., 1991 and 1999; Saisingtong *et al*., 1996; Antoine-Michard and Beckert, 1997). In the present research, the studied genotypeds produced doubled haploid plantlets when their anthers were pretreated with 250 mgl^{-1} of colchicine for 6 days, at 7°C. Saisingtong *et al.,* (1996) observed that the maximum frequency of chromosome doubling (49%) was accomplished when anthers of the maize genotype ETH-M36 were treated with 250 mg/l of colchicine for 7 days at 14°C. In the present research, many of the produced plants from pretreatment media containing high concentrations of colchicine (300 and 400 mg/l) showed morphological abnormality and chromosomal aberrations. Wan *et al.* (1989) faced the problem that some doubled haploid plants derived from colchicine-treated anthers could not set seed after self-pollination which was mainly caused by delayed silk emergence or the lack of ear formation. The abnormality of reproductive organ and the non-synchronization of pollen shedding and ear silking are common phenomena among tissue culture-derived maize plants (Miao *et al.,* 1978; Petolino and Jones, 1986; Wan *et al.,* 1989). Under the experimental conditions used in the present study, some seeds were set on the plants showing normal morphological feathers to allow the further cultivation of doubled haploid plants in the field.

The results presented here indicate that the best embryo induction can be achieved by combination of all four factors (genotype, colchicine concentration in the pretreatment medium, duration of colchicine pretreatment and the embryo induction medium) and that the colchicine pretreatment of maize anthers can be used to induce *in vitro* chromosome doubling.

References

Antoine-Michard S, Beckert M (1997). Spontaneous versus colchicine-induced chromosome doubling in maize anther culture. Plant Cell Tiss Org. 48: 203-207.

Arzani A, Darvey NL (2001). The effect of colchicine on triticale anther-derived plants: Microspore pre-treatment and haploid plant treatment using a hydroponics recovery system. *Euphytica* 122: 235-241.

Aulinger IE (2002). Combination of *in vitro* androgenesis and biolistic transformation: an approach for breeding transgenic maize (*Zea mays* L.) lines. *Ph.D. Thesis*, Swiss Federal Institute of Technology (ETH), Zurich, Switzerland.

Barnabás B, Pfahler P, Kovacs G (1991). Direct effect of colchicine on the microspore embryogenesis to produce dihaploid plants in wheat (*Triticum aestivum* L.). *Theor Appl Genet*. 81: 675-678.

Barnabás B, Obert B, Kovacs G (1999). Colchicine, an efficient genome-doubling agent for maize *(Zea mays* L) microspore cultured *in vitro*. *Plant Cell Rep*. 18: 858-862.

Buter B (1997). *In vitro* haploid production in maize. In: S.M. Jain, S.K. Sapory and R.E. Veilleux (eds). *In vitro Haploid Production in Higher Plants*. Vol. 4. Kluwer Academic Publishers, Dordrecht, PP. 37-71.

Chen CC, Wu YH (1983). Segmentations in microspores of rice during anther culture. *Proc Natl Sci Counc*. 78: 151-157.

Chen QF, Wang CL, Lu YM, Shen M, Afza R, Duren MY,Brunner H (2001). Anther culture in connection with induced mutations for rice improvement. *Euphytica* 120: 401-408.

Dieu P, Beckert M (1985). Further studies of androgenetic embryo production and plant regeneration from *in vitro* cultured anthers in maize *(Zea mays* L.). *Maydica* 31: 245-259.

Genovesi AD, Collins GB (1981). *In vitro* production of haploid plants of corn via anther culture. *Crop Sci*. 22: 1137-1144.

Jumpatong C, Boonyai P, Sangduen N, Thiraporn R, Saisingtong S, Buter B (1996). Anther culture, a new tool for generation of doubled haploid, homozygous maize in Thailand. *Thai J Agric Sci*. 29: 469- 487.

Lyne Rl, Benette RI, Hunter CP (1986). Embryoid and plant production from cultured barley anthers, In: L.A. Withers and P.G. Anderson (eds). *Plant Tissue Culture and Its Agricultural Application*. Butterworths, Guildford, pp. 405-411.

Miao SH, Kuo CS, Kwei YL, Sun AT, Ku SY, Lu WL, Wang YY, Chen ML, Wu MK, Hang L (1978). Induction of pollen plants of maize and observations on their progeny. In: *Proceedings of symposium plant tissue culture*. Science Press, Peking, PP. 23-33.

Obert B, Barnabás B (2004). Colchicine induced embryogenesis in maize. Plant Cell Tissue Organ Cult. 77: 283-285.

Petolino IF, Jones AM (1986). Anther culture of elite genotypes of maize. *Crop Sci*. 26: 1072-1074.

Raina SK (1989). Tissue culture in rice improvement: Status and potential. *Adv Agron*. 42: 339-398.

Redha A, Attia T, Buter B, Saisingtong S, Stamp P, Schmid lE (1998). Improved production of doubled haploids by colchicine application to wheat *(Triticum aestivum* L.) anther culture. *Plant Cell Rep*. 17: 974-979.

Reynolds TL (1997). Pollen embryogenesis. *Plant Mol Biol*. 33: 1-10.

Szakács E, Barnabás B (1988). Cytological aspects of *in vitro* androgenesis in wheat *(Triticum aestivum* L.) using fluorescent microscopy. *Sex Plant Report* 1: 217-222.

Szakács E, Barnabás B (1995). The effect of colchicine treatment on microspore division and microspore-derived embryo differentiation in wheat *(Triticum aestivum* L.) anther culture. *Euphytica* 83: 209-213.

Saisingtong S, Schmid IE, Stamp P, Buter B (1996). Colchicine mediated chromosome doubling during anther culture of maize *(Zea mays* L.). *Theor.Appl.Genet*. 92: 1017-1023.

Touraev A, Vicente O, HeberleBors E (1997). Initiation of microspore embryogenesis by stress. *Trends Plant Sci*. 2: 297-302.

Wan Y, Petolino IF, Widholm lM. (1989). Efficient production of doubled haploid plants through colchicine treatment of anther-derived maize callus. *Theor Appl Genet*. 77: 889-892.

Wan Y, Widholm JM. (1995). Effect of chromosome-doubling agents on somaclonal variation in the progeny of doubled-haploids in maize. *Plant Breed*. 114: 253-255.

Zamani I, Kovacs G, Gouli-Vavdinoudi E, Roupakjas DG, Barnabás B (2000). Regeneration of fertile doubled haploid plants from colchicine-supplemented media in wheat anther culture. *Plant Breed*. 119: 461-465.