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Colchicine-mediated chromosome doubling during anther culture of maize (*Zea mays* L.)

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Abstract Efficient methods of chromosome doubling are critical for the production of microspore-derived, doubled-haploid (= DH) plants, especially if, as in maize anther culture, spontaneous chromosome doubling occurs infrequently. In the present study, colchicine (5–1000 mg/l) was added to the induction medium and maize anthers were incubated in the colchicine-containing medium for different durations (1–7 days). In order to improve overall anther culture response, the culture temperature was adjusted to 14 °C during the first 7 days. Colchicine applied at low concentration, i.e. 5 mg/l (7 days), or for short duration, i.e. 1–3 days (250 mg/l), showed beneficial effects on the formation of embryo-like structures (= ES) and thus led to increased plant production, but was comparatively ineffective regarding chromosome doubling. Optimal doubling effects were observed when anthers had been exposed to culture medium containing 250 and 1000 mg/l of colchicine (7 days); in these treatments the doubling index (= DI), defined as the quotient of the number of DH plants and the number of totally regenerated plants in a specific treatment, rose to 0.56 and 0.53, respectively, compared to 0.20 in the untreated control. However, colchicine administered at concentrations higher than 250 mg/l seemed to be detrimental to general plant production; thus, in spite of a high DI, the overall DH plant production was even lower than in the control treatment. Maximum DH plant production for three different genotypes was accomplished with culture medium containing 250 mg/l of colchicine (7 days). With the best-responding genotype (ETH-M 36) a DH plant production of 9.9 DH plants/100 anthers was accomplished, i.e. a 7-fold increase compared to the non-treated anthers. This is the first report on efficient chromosome doubling in anther culture by subjecting anthers to colchicine-containing induction medium during a post-plating cold treatment. Chromosome doubling as described here becomes an integral part of the maize anther culture protocol and thus represents a rapid and economical way to produce DH plants.

Key words Maize anther culture · Chromosome doubling · Cold treatment · Colchicine · Flow cytometer

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Introduction

Although commercial hybrids have been produced using inbred lines produced by anther culture (Wu 1986), maize remains a recalcitrant species with regard to in vitro androgenesis when compared to other plants, e.g. barley and rape seed. Frequencies of microspore-derived plants generated by anther or microspore culture that would be acceptable for breeding applications are obtained only with a few responsive genotypes (Pescitelli et al. 1989; Beckert 1994; Büter 1996). However, the aptitude for androgenic response appears to be a highly heritable trait that is determined by a limited number of genes (Cowen et al. 1992; Murigneux et al. 1994). As a consequence, significant progress has been achieved by inter-mating non- and highly responsive genotypes (Barloy et al. 1989; Büter et al. 1994).

A second restriction to be overcome in maize in vitro androgenesis consists in the comparatively low rate of spontaneous chromosome doubling. Whereas frequencies of spontaneous doubling exceeding 50% are not unusual in some cereals (Lyne et al. 1986), in maize spontaneous doubling rarely reaches frequencies above 20%; most commonly doubling rates reported for maize are 10% or lower (reviewed in Genovesi 1990). Thus, there exists an obvious need for efficient methods of artificial chromosome doubling in maize.

Unlike other monocots, maize normally does not produce tillers. Therefore, in situ doubling treatments, i.e. the application of doubling agents to regenerated

haploid plants, a commonly used and efficient method in tillering cereals, does not appear to be a promising approach in maize. So far, successful induction of chromosome doubling in maize has only been reported for treatments based on the application of colchicine or various anti-microtubule herbicides to microspore-derived, totipotent callus (Wan et al. 1989, 1991). However, this technique requires an additional culture step, i.e. callus induction. Hence, the production of DH plants will be delayed compared to an anther culture system where plants are directly regenerated, i.e. without an intermediate callus stage. Moreover, treating multicellular structures like calli carries the risk of chimeric plant formation. In contrast, the application of chromosome-doubling agents at early stages during anther culture, preferably before microspore mitosis, would allow for the rapid generation of DH plants since no additional culture step is needed. Moreover, since the doubling treatment is targeting unicellular structures (microspores), chimerism occurring during plant regeneration most probably will not become a serious problem with this approach.

Most in situ and in vitro studies on chromosome doubling have used colchicine as a doubling agent. Colchicine inhibits spindle formation, disturbs normal polar migration of the chromosomes and thus impedes regular cell division (Jensen 1974). In addition, colchicine has been reported to have further attributes on plant cell development which are not necessarily related to cell division (Pickett-Heaps 1967).

In other cereals, e.g. wheat (Barnabas et al. 1991), Tritordeum (= *Hordeum chilense* × *Triticum turgidum* cv *durum* (Barcelo et al. 1994) and rice anther culture (Alemanno and Guiderdoni 1994), the production of fertile, DH plants has been improved by adding colchicine to the induction medium and culturing anthers on this medium for 1–3 days at a normal culture temperature (26–29 °C). Here, we report on the effects of colchicine in maize anther culture when anthers are subjected to colchicine-containing induction medium during a post-plating cold treatment (14 °C), i.e. during the first 1–7 days of in vitro culture.

Materials and methods

The following genotypes were used as donor plants: ETH-M 36 (Exp. A), ETH-DT 34 (Exp. B) and ETH-M 24 (Exp. C). ETH-M 36 and ETH-M 24 were both produced at the experimental station of ETH Zürich in Lindau, Switzerland. Genetically these two genotypes are closely related, since they were obtained by fertilizing two anther culture-derived plants of the Chinese hybrid DAN SAN 91 with pollen from a single anther culture-derived plant of the US hybrid PA91 × FR16. Due to an insufficient quantity of seeds, in Exp. C ETH-M 24 was employed instead of ETH-M 36. ETH-DT 34 was produced at the National Corn and Sorghum Research Center, Nakornratchasima, Thailand, by inter-mating an anther culture-derived plant of DAN SAN 91 (female) with the Thai inbred line KI 3 (male).

The donor plants of ETH-M 36 and ETH-DT 34 were grown in the greenhouse at Lindau during the period November 1993 – February 1994 (25/20 °C ± 3 °C; 16 h light). ETH-M 24 donors were grown during the period October – December 1994. Tassel harvest, cold pretreatment, stage determination and anther inoculation followed previously described procedures (Büter et al. 1993).

A liquid induction medium (= IML) was used for colchicine application; it consisted of YP basal salts (Ku et al. 1978) supplemented by 0.1 mg/l triiodobenzoic acid, 0.25 mg/l thiamine-HCl, 1.3 mg/l nicotinic acid, 125 mg/l L-proline, 125 mg/l L-glutamine, 15 mg/l L-aspagarine, 5 g/l activated charcoal (= AC) and 90 g/l sucrose. All media were adjusted to pH 5.8 and autoclaved; 24 h after autoclaving AC was removed by filtering (Millipore Sterivex GS filter, 0.22 µM, 1.0 bar). For preparation of colchicine-containing induction media, appropriate amounts of colchicine were dissolved in 50 ml of IML (after AC removal) and added back to the medium by filter sterilization.

Without pre-culture anthers containing microspores, preferentially at the late-uninucleate stage, were directly inoculated on colchicine-containing induction media. Anthers were randomly distributed to the culture dishes (60 × 15 mm; 10 ml medium and 24 anthers per dish); each culture dish was considered as one replicate. After the relevant duration, anthers were transferred to a colchicine-free, semi-solid induction medium (IMSS) of similar composition, but supplemented by phytagel (1.5 g/l) and AC (5 g/l). Anthers in control treatments, i.e. without colchicine application, were inoculated on IML and transferred to IMSS 7 days after inoculation.

Immediately after inoculation culture dishes were moved to 14 °C for 7 days. Subsequently, all culture steps were conducted at 27 °C. Twenty one days after culture initiation anthers were transferred to regeneration medium (= RM) consisting of YP basal salts, 0.5 mg/l thiamine-HCl, 1.5 mg/l nicotinic acid, 2.5 mg/l kinetin, 25 mg/l succinic acid, 100 mg/l myo-inositol, 250 mg/l l-glutamine, 2 g/l phytagel and 30 g/l sucrose adjusted to pH 5.8. Every 2 weeks after anther transfer to RM, embryo-like structures (= ES) and regenerated plants (= RP) were counted and transferred to fresh RM.

The following parameters were recorded in order to describe the colchicine effects: ES productin (ES/100 anthers), plant regeneration capacity (RP/100 ES), plant production (RP/100 anthers), doubling index (number of DH plants divided by total number of plants), and DH plant production (DH/100 anthers).

Determinations of ploidy status were conducted by flow cytometry (CA II; Partec GmbH, Münster, Germany). Young leaves of 3-week-old regenerated plants were chopped with a sharp razor-blade in 2 ml of Partec DNA staining solution. The solution was then passed through a 30-µm nylon gauze filter. On average, 50 regenerated plants were analyzed for each treatment. Nuclei isolated from the leaf blades of 2-week-old, diploid ETH-M 36 (Exp. A), ETH-DT 34 (Exp. B) and ETH-M 24 (Exp. C) seedlings were used as controls; 2000–5000 nuclei were analyzed for each sample (Fig. 1).

Experiment A was designed to investigate the optimal duration of colchicine application with regard to the production of DH plants. Anthers of ETH-M 36 were plated on colchicine-containing media (250 mg/l of colchicine) for 1, 2, 3, 4, 5, 6, and 7 days. Nineteen replicates, resulting in a total of 456 anthers, were inoculated for each treatment.

Similarly, in Experiment B different durations (3, 5 and 7 days) of colchicine application were tested for the genotype ETH-DT 34. The colchicine concentration was 250 mg/l. For each treatment 23 replicates (552 anthers) were inoculated.

The effects of different colchicine concentrations on DH plant production were analyzed in Experiment C. Anthers of ETH-M 24 were plated on induction media containing 0, 5, 10, 100, 250, 500 and 1000 mg/l of colchicine. Seven days after inoculation on colchicine-containing media all anthers were transferred to IMSS (without colchicine). Twenty five replicates (600 anthers) were cultured for each treatment.

Results

The data obtained in Exp. A revealed that short-term colchicine (250 mg/l) applications, i.e. for 1–3 days after inoculation, led to increased ES formation in maize

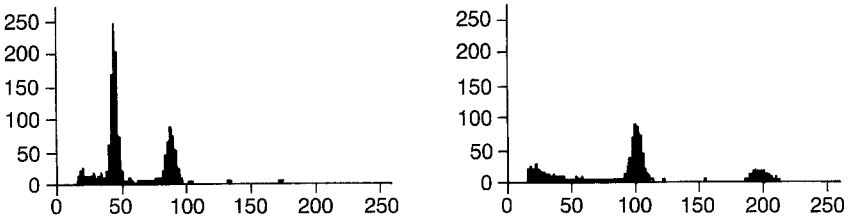

Fig. 1 Flow cytometer histogram of a haploid (left) and a doubled-haploid maize plant (genotype: ETH-M24). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI); x-axis: histogram channels revealing the DNA content in relative fluorescence units; y-axis: number of nuclei counted per histogram channel

anther culture (Fig. 2 a). Using the genotype ETH-M 36 a maximum ES formation of 199.0 ES/100 anthers was accomplished by a 1-day application of colchicine compared to 167.2 ES/100 anthers in the corresponding control treatment. Analogously, colchicine applied at low concentration (5 mg/l), but for longer duration (7 days), also increased ES formation (217.9 vs 173.6 ES/100 anthers) (Exp. C; Fig. 2 a). In both experiments, the frequencies of plant regeneration in the ES-promoting colchicine treatments were found to be similar to the control (Exp. A: 20.4 plants/100 ES after a 1-day application of 250 mg/l of colchicine vs 18.9 in the control; Exp. C: 10.9 plants/100 ES after a 7-day application of 5 mg/l colchicine vs 11.4 in the control) (Tables 1 and 2). Consequently, plant production in these colchicine treatments was also higher than in the control treatments (Exp. A:40.6 vs 31.7; exp. C: 23.8 vs 19.8 RP/100 anthers) (Fig. 2 b).

An extended duration of application, i.e. 4–7 days, and elevated concentrations of colchicine, i.e. 50–1000 mg/l, led to a decline in ES formation and plant production (Fig. 2 a, b). Reductions in ES and plant production were particularly severe in the treatment with 1000 mg/l of colchicine (2.9 plants/100 anthers compared to 19.8 for untreated anthers). Again, no distinct positive or negative effects on the plant regeneration capacity were observed (Tables 1 and 2).

Concerning the effects on chromosome doubling, colchicine treatments with extended durations or elevated concentrations proved to be most suitable. Opti-

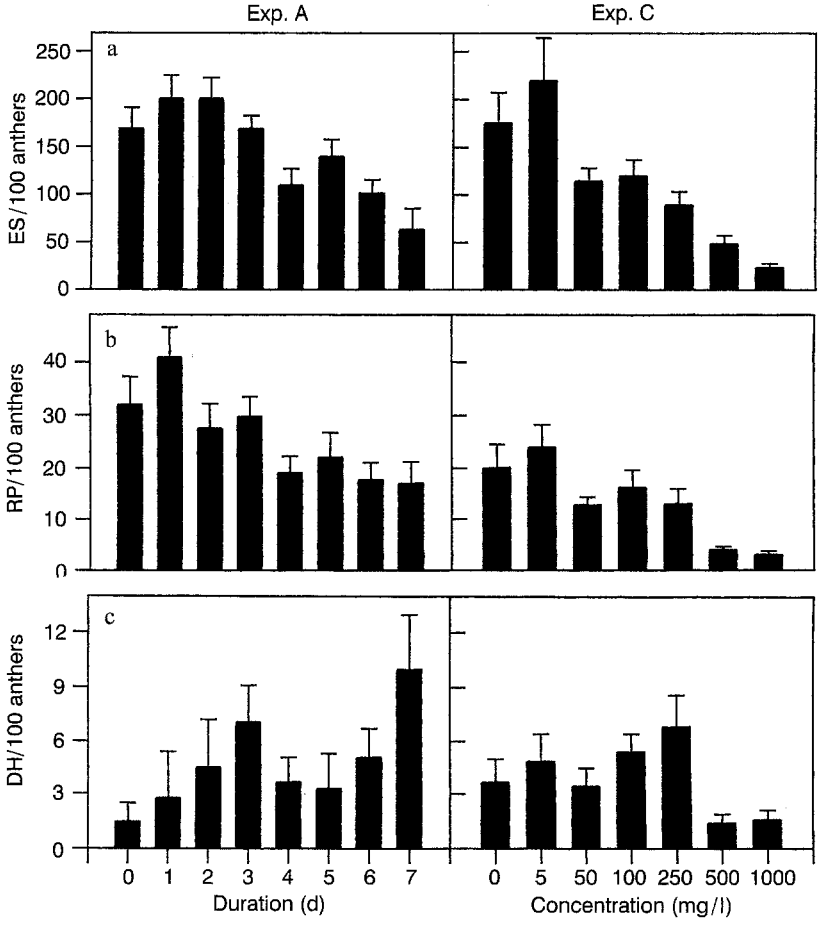

Fig. 2a–c Colchicine treatment during maize anther culture: effects of different treatment durations and colchicine concentrations on the production of (a) embryo-like structures (= ES); (**b**) regenerated plants (= RP); (**c**) doubled haploid (= DH) plants. Genotypes: ETH-M 36 (Exp. A) and ETH-M 24 (Exp. C)

Table 1 Different duration of colchicine application in maize anther culture: effects on plant regeneration and chromosome doubling[a]

Duration of colchicine application (days)		1	2	3	4	5	6	7	0
Plant regeneration (plants/100 ES)	Exp. A[b]	20.4	12.9	17.7	17.3	14.7	16.9	20.5	18.9
	Exp. B[c]	nt[e]	nt	8.1	nt	10.3	nt	14.4	14.1
Doubling index[d]	Exp. A[b]	0.09	0.20	0.17	0.16	0.18	0.30	0.49	0.08
	Exp. B[c]	nt	nt	0.17	nt	0.33	nt	0.50	0.18

[a] Colchicine concentration: 250 mg/l
[b] Experiment A: genotype ETH-M 36
[c] Experiment B: genotype ETH-DT 34
[d] Doubling index: number of DH plants divided by total number of regenerated plants
[e] nt = not tested

Table 2 Impact of different colchicine concentrations on the frequency of plant regeneration (RP/100 ES) and chromosome doubling in maize anther culture (Genotype: ETH-M 24 [Exp. C])[a]

Concentratin of colchicine (mg/l)	5	50	100	250	500	1000	0 (control)
Plant regeneration (plants/100 ES)	10.9	10.9	13.4	14.4	7.6	13.0	11.4
Doubling index[b]	0.23	0.29	0.41	0.56	0.35	0.53	0.20

[a] Duration of colchicine application: 7 days
[b] Doubling index: number of DH plants divided by total number of regenerated plants

mal effects were obtained with a 7-day application of either 250 or 1000 mg/l of colchicine (Exp. A and C; Tables 1 and 2). In these treatments doubling indices of more than 0.5 were obtained, i.e. more than 50% of the totally regenerated plants were DH. Spontaneous chromosome doubling in the control treatments varied with the genotype and ranged from 8 to 20%.

In spite of high doubling indices, treatments with colchicine concentrations above 250 mg/l produced only low numbers of DH plants (Exp. C; Fig. 2c). As mentioned above, ES formation and plant production were severely inhibited by the presence of highly concentrated colchicine in the induction medium. Maximum DH production occurred on induction medium containing 250 mg/l of colchicine; in this treatment the decline in ES and plant production was less striking (12.7 plants/100 anthers compared to 19.8 in the control treatment; Exp. C) and was counterbalanced by the increase of the DI. Best results were obtained with the genotype ETH-M 36 (Exp. A) reaching a DH plant production of 9.9 DH/100 anthers, i.e. an increase by a factor of seven in comparison to the control (Fig. 2c). Smaller, but still significant increases were also observed for the genotype ETH-M 24 (6.7 vs 3.6 DH plants/100 anthers in the control; Exp. C; Fig. 2c).

Data in Exp. B which was meant to re-examine the duration effects observed in Exp. A with another genotype, principally confirmed the previously obtained results, although culture response was generally lower than in Exp. A (Fig. 3). As in Exp. A, maximum DH plant production was accomplished by subjecting anthers to induction medium with 250 mg/l of colchicine for 7 days (5.3 DH plants/100 anthers compared to 1.9 in the control).

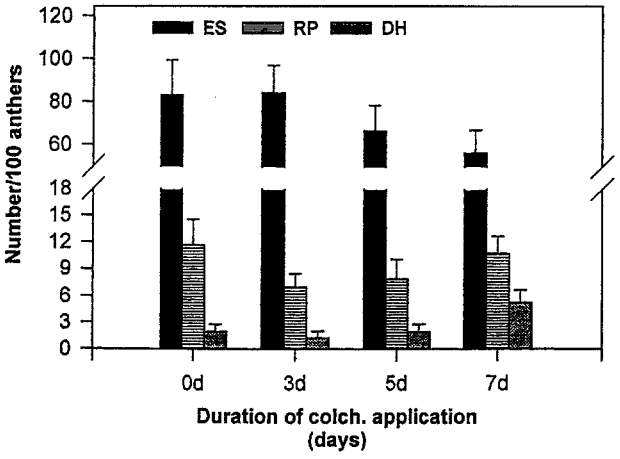

Fig. 3 Colchicine tratment during maize anther culture: effects of different treatment durations on the production of embryo-like structures (= *ES*), regenerated plants (= *RP*) and doubled-haploid (= *DH*) plants. Genotype: DT 34 (Exp. B)

Discussion

Beneficial effects of colchicine as a component of the induction medium in anther or microspore culture have been reported for several species, e.g. *Brassica napus* L. (Chen et al. 1994; Iqbal et al. 1994; Möllers et al. 1994), *Triticum aestivum* L. (Barnabas et al. 1991), Tritordeum (Barcelo et al. 1994) and *Oryza sativa* L. (Hu and Liang 1979; Alemanno and Guiderdoni 1994).

The data presented here show that in maize anther culture the exposure of anthers to a colchicine-containing induction medium at a reduced culture temperature also greatly improves the production of DH plants. Both treatment duration and colchicine concentration

were found to be relevant for the anther culture response: even colchicine applications limited to only 1 day (250 mg/l) and colchicine concentrations as low as 5 mg/l (7 days) revealed clear effects on culture response.

Generally, beneficial colchicine effects on final DH plant production may be related to:

(1) enhanced ES formation
(2) improved plant regenertion
(3) increased doubling indices, i.e. frequencies of chromosome doubling, or to
(4) the combined effects of 1, 2 and 3

Enhanced ES formation by colchicine application has been reported for several anther culture systems. In rice, treating anthers with colchicine (500 mg/l) for 1–2 days significantly increased both the number of responding anthers and total ES production ("calli/100 anthers") (Alemanno and Guiderdoni 1994). For wheat, a colchicine-related increase of pollen embryos was observed (Barnabas et al. 1991) in spite of a decreased frequency of responding anthers and total ES production ("haploid structures/100 anthers"). Beneficial colchicine efffects on ES formation were also reported for both anther and microspore culture in rape seed (Iqbal et al. 1994; Möllers et al. 1994; Zaki and Dickinson 1995). In our experiments we also observed colchicine-related increases in ES production (Figs. 2 and 3); however these increases were moderate and not significant statistically (data not shown). The reasons for the colchicine effects on ES formation are not clearly known. It has been shown in *B. napus* that colchicine may induce elevated levels of symmetrical microspore division which is thought to be associated with microspore embryogenesis and it has been assumed that these symmetrical divisions are a consequence of the disruption of cytoskeletal components at or before the first pollen mitosis (Zaki and Dickinson 1991; Iqbal et al. 1994). However, for maize the significance of symmetrical divisions during first pollen mitosis for subsequent microspore embryogenesis so far remains questionable. Studies on this subject would require the exact timing of the colchicine treatment, i.e. colchicine needs to be applied before mitosis. The anther culture method does not allow for a such precise determination of the microspore stage, since microspores in different stages are usually present in the same anther. In addition, the anther wall represents a barrier with unknown permeability. Therefore, cultures of isolated microspores facilitating comparisons of homogeneous microspore populations (Gaillard et al. 1991) and permitting direct access of colchicine seem to be more suitable for investigations into this subject.

Interestingly, in our experiments enhanced ES formation was only obtained with colchicine treatments that were not, or else were less, effective with respect to chromosome doubling. In fact, our results indicated the existence of two different optima for colchicine application in maize anther culture:

(1) Treatments optimal with regard to ES formation and plant production, e.g. anther treatment with 250 mg/l of colchicine for 1–3 days (Exp. A) or with 5 mg/l of colchicine for 7 days (Exp. C) (Fig. 2 a, b)
(2) Treatments optimal with regard to chromosome doubling, e.g. 250 or 1000 mg/l of colchicine applied for 7 days (Tables 1, 2).

Similarly, in wheat, colchicine concentrations that were most effective regarding chromosome doubling caused a decreased proportion of responding anthers and, as a consequence, in the quantity of "haploid structures". However, the quality of the "haploid structures" obtained from colchicine-treated anthers seemed to be improved (Barnabas et al. 1991). In contrast, Alemanno and Guiderdoni (1994) reported that colchicine (500 mg/l; 1 or 2 days) applied to rice anthers increased the doubling frequency and at the same time improved callus formation. Similar results were reported for rape seed (Iqbal et al. 1994).

Concerning the plant regeneration capacity, our data indicated a genotype-dependent variation, but no consistent positive or negative colchicine effects (Tables 1 and 2): ES from colchicine-treated anthers seemed to regenerate at the same frequencies as those from non-treated anthers. Similar observations were made in wheat and rice anther culture (Barnabas et al. 1991; Alemanno and Guiderdoni 1994); thus, for plant regeneration, the ploidy status of the original tissue, i.e. haploid or doubled haploid, appears to be of minor importance.

The most significant positive effect of colchicine with regard to final DH plant production in our experiments consisted in the enhancement of the doubling indices, i.e. a relatively high proportion of plants with a doubled chromosome set was obtained in the optimal colchicine treatment (250 mg/l of colchicine; 7 days; Tables 1 and 2). Some 49–53% of the plants obtained in this treatment were DH, i.e., an increase by a factor of 3–6 (depending on the genotype) when compared to the control. Consequently, even considerable reductions in the total number plants obtained, e.g. up to 50% (Exp. A; Fig. 1), were compensated for by the high DI. Similar colchicine effects were reported for rice anther culture where, in the optimal treatment, 65% of the regenerated plants were DH (control: 31%; Alemanno and Guiderdoni 1994). For wheat anther culture no ploidy analyses are available; however, from colchicine-treated anthers significantly higher rates of fertile plants were obtained than from non-treated anthers (Barnabas et al. 1991).

It is noticeable though that in our study the optimal treatment duration was considerably longer than in most other studies mentioned above. The requirement for a longer application is obviously associated with the different culture temperatures at which colchicine was applied. In our experiments the colchicine application was combined with a post-plating cold treatment at 14 °C, a culture step that previously had been shown to considerably improve ES and RP production (Büter

et al. 1991); usually colchicine is applied at culture temperatures of 26–29 °C. It is conceivable that due to reduced metabolic activity at low temperatures the colchicine action is delayed and thus a longer exposure is required to obtain results similar to those produced at a higher temperature. It is feasible that there exist specific combination effects of the cold and the colchicine treatment, e.g. that toxic colchicine effects on the microspore cells are reduced at low temperature without impairing the doubling effect. Experiments with direct comparisons of colchicine applications at a normal (27 °C) and a reduced culture temperature (14 °C) are in progress to address this issue.

For practical reasons only a few combinations of colchicine concentration and treatment duration were tested in this study. Further experiments should focus especially on prolonged exposure of anthers to colchicine, because maximum DH plant production was achieved with the longest duration (7 days) tested in our study. Preliminary studies with a continuous application of colchicine at low concentration, i.e. colchicine application during the entire induction period (21 days), gave promising results (data not shown). This approach may represent a potential technique for future routine application, since it does not require an anther transfer from colchicine-containing to colchicine-free culture medium.

In conclusion, colchicine application to maize anthers during a post-plating cold treatment, i.e. during the first 7 days of anther culture, led to considerable increases of DH production. The cold treatment allows for high frequencies of ES formation and plant production, whereas the colchicine increases the percentage of plants with a doubled-haploid chromosome set. Thus, these two treatments seem to supplement each other resulting in a high DH plant production. Efficient chromosome doubling in maize antherculture may also be implemented by the induction of microspore-derived totipotent calli and a subsequent callus treatment with colchicine or other drugs suitable for chromosome doubling (Wan et al. 1989, 1991). The technique described in the present paper avoids a callus stage; instead, plants carrying a doubled chromosome set are produced by direct regeneration at high frequencies. As a consequence time can be saved, DH plants can be obtained from more individual microspores, and the risk of chimeric plant formation is reduced.

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References

Alemanno L, Guiderdoni E (1994) Increased doubled haploid plant regeneration from rice (*Oryza sativa* L.) anthers cultured on colchicine-supplemented media. Plant Cell Rep 13:432–436

Barcelo P, Cabrera A, Hagle C, Lörz H (1994) Production of doubled-haploid plants from tridordeum anther culture. Theor Appl Genet 87:741–745

Barloy D, Denis L, Beckert M (1989) Comparison of the aptitude of anther culture in some androgenetic doubled haploid maize lines. Maydica 34: 303–308

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

Beckert M (1994) Advantages and disadvantages of the use of in vitro/in situ-produced DH maize lines. In: Bajaj YPS (ed) Biotechnology in agriculture and forestry, vol. 25. Maize. Springer-Verlag, Berlin Heidelberg, pp 201–213

Büter B (1996) Maize. In: Jain (ed) In vitro haploid production of higher plants. Kluwer Academic Publishers, (in press)

Büter B (1996) Maize. In: Jain M, Sopory SK, Veilleux RE (eds) In vitro haploid production of higher plants. Kluwer Academic Publishers, (in press)

Büter B, Pescitelli SM, Berger K, Schmid JE, Stamp P (1993) Autoclaved and filter-sterilized liquid media in maize anther culture: significance of activated charcoal. Plant Cell Rep 13:79–82

Büter B, Jumpatong C, Berger K, Saisingtong S, Schmid JE, Thiraporn R, Stamp P (1994) Application of the anther culture technique in a corn breeding project in Thailand. Abstracts VIIIth Int Congr Plant Tissue and Cell Culture, Firenze, June 12–17, p 85

Chen ZZ, Snyder S, Fan ZG, Loh WH (1994) Efficient production of doubled haploid plants through chromosome doubling of isolated microspores in *Brassica napus*. Plant Breed 113:217–221

Cowen NM, Johnson CD, Armstrong K, Miller M, Woosley A, Pescitelli S, Skokut M, Belmar S, Petolino JF (1992) Mapping genes conditioning in vitro androgenesis in maize using RFLP analysis. Theor Appl Genet 84:720–724

Gaillard A, Vergne P, Beckert M (1991) Optimization of maize microspore isolation and culture conditions for reliable plant regeneration. Plant Cell Rep 10:55–58

Genovesi AD (1990) Maize (*Zea mays* L.): In vitro production of haploids. In: Bajaj YPS (ed) Biotechnology in agriculture and forestry vol 12. Haploids in crop improvement I. Springer-Verlag, Berlin, pp 176–203

Hu Z, Liang HC (1979) Ways of improving the method of rice anther culture. Acta Phytophysiol Sin 5:131–139

Iqbal MCM, Möllers C, Röbbelen G (1994) Increased embryogenesis after colchicine treatment of microspore cultures of *Brassica napus* L. J Plant Physiol 143:222–226

Jensen CJ (1994) Chromosome dounling techniques in haploids. In: Kasha KJ (ed) Haploids in higher plants – advances and potential. Proc 1st Int Symp Guelph, Ontario, Canada, June 10–14, pp 153–190

Ku MK, Chen GWC, Ju LC, Kuan YL, An HP, Huang CH (1981) Induction factors and morpho-cytological characteristics of pollen-derived plants in maize (*Zea mays* L.). In: Proc Symp Plant Tissue Culture 1978. Science Press, Peking, pp 35–42

Lyne RL, Ribennet CP, Hunter C (1986) Embryoid and plant production from cultured barley anthers. In: Withers LA, Anderson PG (eds) Plant tissue culture and its agricultural application. Butterworths, Guildford, pp 405–411

Möllers C, Iqbal MCM, Röbbelen G (1994) Efficient production of doubled haploid *Brassica napus* plants by colchicine treatment of microspores. Euphytica 75:95–104

Murigneux A, Bentolila S, Hardy T, Baud S, Guitton C, Jullien H, Ben Tahar S, Freyssinet G, Beckert M (1994) Genotypic variation of quantitative trait loci controlling in vitro androgenesis in maize. Genome 37:970–976

Pescitelli SM, Mitchell JC, Jones AM, Pareddy DR, Petolino JF (1989) High-frequency androgenesis from isolated microspores of maize. Plant Cell Rep 7:673–676

Pickett-Heaps JD (1967) The effect of colchicine on the ultrastructure of dividing plant cells, xylem wall differentiation and the distribution of cytoplasmatic microtubules. Dev Biol 15:206–236

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

Wan Y, Duncan DR, Rayburn AL, Petolino JF, Widholm JM (1991) The use of anti-microtubule herbicides for the production of doubled haploid plants from anther-derived maize callus. Theor Appl Genet 81:205–211

Wu J (1986) Breeding haploid corn by anther culture. In: Hu H, Yang HY (eds) Haploids in higher plants. Springer-Verlag, Berlin Heidelberg New York Tokyo, pp 149–161

Zaki M, Dickinson H (1991) Microspore-derived embryos in *Brassica*: the significance of division symmetry in pollen mitosis to embryogenic development. Sex Plant Rep 4:48–55

Zaki M, Dickinson H (1995) Modification of cell development in vitro: the effect of colchicine on anther and isolated microspore culture in *Brassica napus*. Platn Cell Tissue Org Cult 40: 255–270