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Efficient production of doubled haploid plants through colchicine treatment of anther-derived maize callus

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Summary. A chromosome doubling technique, involving colchicine treatment of an embryogenic, haploid callus line of maize (*Zea mays* L., derived through anther culture), was evaluated. Two colchicine levels (0.025% and 0.05%) and three treatment durations (24, 48, and 72 h) were used and compared to untreated controls. Chromosome counts and seed recovery from regenerated plants were determined. No doubled haploid plants were regenerated from calli without colchicine treatment. After treatment with colchicine for 24 h, the callus tissue regenerated about 50% doubled haploid plants. All of the plants regenerated from the calli treated with colchicine for 72 h were doubled haploids, except for a few tetraploid plants. No significant difference in chromosome doubling was observed between the two colchicine levels. Most of the doubled haploid plants produced viable pollen and a total of 107 of 136 doubled haploid plants produced from 1 to 256 seeds. Less extensive studies with two other genotypes gave similar results. These results demonstrate that colchicine treatment of haploid callus tissue can be a very effective and relatively easy method of obtaining a high frequency of doubled haploid plants through anther culture.

Key words: *Zea mays* – Anther culture – Embryogenic haploid callus – Chromosome doubling

Introduction

The success of producing haploid plants in maize through anther culture makes it possible to generate inbred lines through chromosome doubling (Kuo et al. 1986). However, the application of anther culture to plant breeding is largely dependent on the production of large numbers of haploid plants and the high frequency of induction of chromosome doubling. In maize, anther-derived lines have been developed and used commercially (Wu et al. 1983). However, the frequency of chromosome doubling of anther-derived haploid plants either spontaneously or through colchicine treatment has been undesirably low (Ku et al. 1978; Nitsch et al. 1982; Miao et al. 1978). Ku et al. (1978) and Nitsch et al. (1982) observed only 6.3% and 4.5% spontaneously doubled haploids among plants regenerated from cultured maize anthers, respectively. Miao et al. (1978) treated anther-derived plantlets and obtained only one plant which set seeds from the 24 plants that survived.

With many plant species, chromosome doubling can be achieved by the use of an antimitotic agent treatment of anther-derived, haploid plantlets. Since antimitotic agents such as colchicine usually induce chromosome doubling in only some cells due to the asynchrony of cell divisions, chimeric plants are common after colchicine treatment. For plant species which produce bisexual flowers and tillers or branchs, chimeras are acceptable since some tillers or branchs may develop from the chromosome-doubled cells. In contrast, maize plants usually do not produce tillers and cell lines which give rise to the tassel and ear are already determined in the mature seed (Coe and Neuffer 1978). Colchicine treatment of maize seedlings or plantlets may double the chromosome number in the tassel or ear, but often not in both, which will make self-pollination impossible. These reasons may explain why the efficiency of inducing doubled haploid plants in maize is very low by colchicine treatment of regenerated haploid plantlets (Miao et al. 1978).

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Since somatic embryos from tissue cultures may develop from one or a few cells, it may be possible to induce chromosome doubling in embryogenic haploid callus and then induce plant regeneration from this tissue (Genovesi and Collins 1982). The use of a long term haploid culture system capable of plant regeneration may make the chromosome doubling technique effective as proposed by Tsay et al. (1986). This paper reports the recovery of doubled haploid plants with high frequency through colchicine treatment of embryogenic haploid callus initiated from maize anther culture.

Materials and methods

Establishment of callus cultures. F1 plants of a maize hybrid, H99 × Fr16, were grown in the field in 1987. Tassel collection to anther plating were carried out by previously described methods (Petolino and Thompson 1987). Petri dishes containing anthers were placed in plastic boxes covered with aluminum foil at 28 °C. About 1 month later, embryo-like structures began to appear from responding anthers. The embryo-like structures were removed from the anthers and were transferred to a callus induction medium. The callus induction medium consisted of macronutrients and vitamins of N6 medium (Chu et al. 1975), micronutrients of B5 medium (Gamborg et al. 1968) with 2,4-D (0.45 μ*M*), dicamba (11.3 μ*M*), myo-inositol (0.55 m*M*), L-proline (25.0 m*M*), enzymatic casein hydrolysate (0.1 g/l), sucrose (87.6 m*M*), Na_2EDTA (110.55 μ*M*) and $FeSO_4 \cdot 7\,H_2O$ (100.2 μ*M*). Callus lines, each of which was derived from a single embryo-like structure, were maintained in the callus induction medium through subcultures by selective transfer of the embryogenic calli at 4-week intervals. One highly regenerable callus line was used 6 months after culture initiation.

Colchicine treatment. Colchicine was dissolved in water to make a stock solution which was filter-sterilized and then added to liquid D medium (Duncan et al. 1985) to the required final concentrations and stored in the dark. About 20 ml of the medium was placed in Petri dishes (100 × 25 mm) and a filter paper disc supported by a stainless steel screen, which were autoclaved previously, was saturated with the liquid medium. Embryogenic calli, 20 days after subculture, were cut into 0.5–1.0 mm pieces and were plated on the moist filter paper and incubated in the dark at 28 °C. Following treatment, the calli were placed on a stainless teel screen and were rinsed twice in liquid D medium without colchicine. Two colchicine levels (0.025% and 0.05%) and three treatment durations were used and compared with untreated control.

Plant regeneration from treated calli. Colchicine treated calli were subcultured two times with an interval of 10 days on agar-solidified D medium. For plant regeneration, calli were transferred to H medium (Duncan et al. 1985) with 3.5 mg/l 6-benzyladenine for 3 days. The calli were then cultured in H medium until some regenerated plantlets grew to 3–4 cm long, which occurred within about 20 days. The regenerated plantlets were transferred to H medium minus RT vitamins and glucose in culture tubes for further growth. After 7–10 days, they were transplanted to soil in 11.5-cm pots and grown for another 10–15 days (or even longer depending on the growth of each plant). Finally, the plants were transplanted to 27.5-cm pots in the greenhouse and at least two root tips were collected from each plant for mitotic examination.

Plants with pollen and silks were self-pollinated on successive days. The dates of the first day of pollen shed and the first day of silk emergence were recorded for 33 representative doubled haploid plants. Seeds were harvested 40–45 days after pollination.

Determination of ploidy level. The root tips were cold-treated in ice water for 24 h and fixed in 3:1, 95% ethanol:glacial acetic acid for 24 h and then stored in 70% ethanol. For mitotic examiantion, the root tips from each plant were placed in a small vial with 1% acetocarmine and heated to the boiling point several times. The meristematic region was excised and squashed in one drop of 45% glacial acetic acid on a slide. At least two root tips from each regenerated plant were examined to determine the ploidy level.

Results

All 24 plants regenerated from the untreated calli contained the haploid number of ten chromosomes (Table 1, Fig. 1 a). Of 96 plants regenerated from calli treated for 24 h with either 0.025% or 0.05% colchicine, 49 were diploid with 20 chromosomes (Fig. 1 b), and the other 47 were haploid with ten chromosomes. Of 53 plants from the calli treated with colchicine for 48 h, 29 were diploid plants. Calli treated for 72 h did not regenerate any haploid plants, with most being diploid plants except for one and four tetraploid plants with 40 chromosomes obtained from the two 72-h treatments of 0.025% and 0.05% colchicine, respectively. No significant difference in chromosome doubling was observed between these two colchicine levels (Table 1).

The haploid plants regenerated in this study all displayed a characteristic morphology (short, narrow leaves, reduced vigor, and no pollen shed). Under the same growing conditions, the doubled haploid plants

Table 1. Ploidy of plants regenerated from colchicine-treated haploid calli as determined from root tip squashes

Colchicine treatment		No. of plants regenerated			
Hours	Concentration	Total	Haploid	Diploid	Tetraploid
–	–	24	24	0	0
24	0.025%	48	23	25	0
	0.05%	48	24	24	0
Total		96	47	49	0
48	0.025%	22	8	14	0
	0.05%	31	16	15	0
Total		53	24	29	0
72	0.025%	31	0	30	1
	0.05%	32	0	28	4
Total		63	0	58	5

were generally more vigorous in appearance and grew more rapidly when compared with the haploid plants (Fig. 2). The doubled haploid plants from different treatments exhibited similar morphology. Most of them produced abundant, viable pollen. A common feature of many of the doubled haploid plants was the appearance of tassels with some female flowers. The ears of these plants could, however, still be self-pollinated if the silks emerged in time.

Most of the doubled haploid plants, 107 of 136, produced from 1 to 256 seed per ear after self-pollination. A few ears had almost normal seed set (Fig. 3). Among 29 doubled haploid plants which did not produce seed, 21 of the plants could not be pollinated due to asynchronous pollen shed and silk emergence, the lack of ear development, or to stunted growth. Eight other plants produced no seed even after being pollinated one or two times on successive days. The synchrony of pollen shed and silk emergence were the main factors which affected the seed production by the doubled haploid plants. As shown in Table 2, if the silks emerged for pollination 1–3 days later than the first pollen was shed, an average of more than 87 seeds per ear were set. If the pollination was started 4 days later than the first pollen was shed, the seed set was dramatically decreased to 39 seeds per ear. Most plants would not set seed if silk emergence was delayed 5 days or more after pollen shed began.

Five tetraploid plants were found among the plants regenerated after the two 72-h colchicine treatments. Of these five plants, two plants had terminal ears and three plants had good pollen shed, but due to late silk emergence, only two of the plants produced one seed each after self-pollination.

Anther-derived callus lines from two other hybrids, H99 × Pa91 and Pa91 × Fr16, were also treated with colchicine. Due to lower regenerability of the callus line from Pa91 × Fr16 and incomplete experiment design for the callus line from H99 × Pa91, the data from these two lines are not included. However, these experiments also showed that the longer the callus cultures were incubated

Table 2. The relationship between the delay in silk emergence after the beginning of pollen shed and the average number of seed produced per ear from 33 randomly selected doubled haploid plants

Silk emergence delay (d)	No. of plants pollinated*	Average seeds per ear	No. of ears without seed
1	4	100.3	0
2	8	91.0	0
3	8	87.1	0
4	5	39.0	0
>5	8	2.5	5

* One ear per plant was self-pollinated

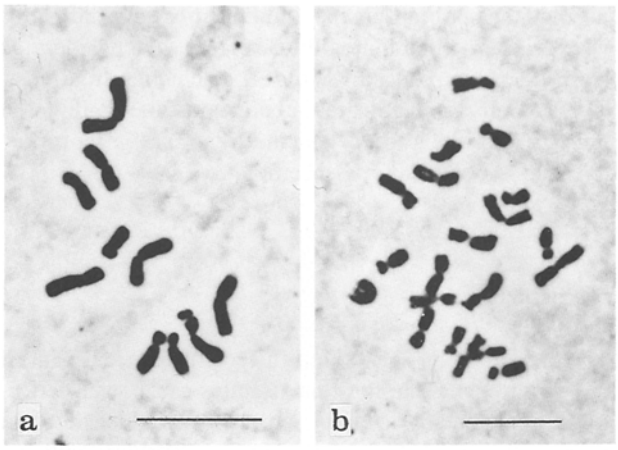

Fig. 1 a and b. Root tip chromosomes from **a** a haploid cell with 10 chromosomes from a plant regenerated from untreated callus and **b** a diploid cell with 20 chromosomes from a plant regenerated from colchicine treated callus. *Bar* represents 10 μm

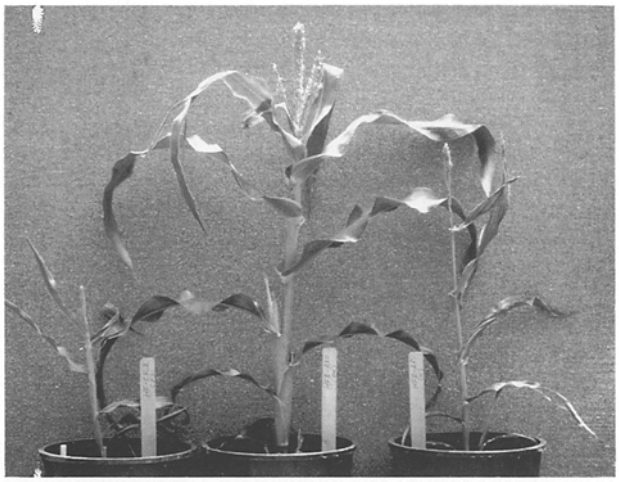

Fig. 2. Typical appearance of a doubled haploid plant (middle) from colchicine treated haploid callus, haploid plant (right) from colchicine-treated haploid callus, and haploid plant (left) from untreated callus. The pot diameters are 27.5 cm

Fig. 3. Mature ears resulting from self-pollination of some doubled haploid plants

in colchicine-containing medium, the more diploid plants were regenerated, and no diploid plants were regenerated from the control calli without colchicine treatment. These results then indicate that chromosome doubling of maize callus tissue by colchicine treatment is reproducible and is not genotype-specific.

Discussion

The present study shows that the colchicine treatment of the embryogenic haploid maize callus can be very effective for producing a large number of doubled haploid plants. By incubating embryogenic haploid calli on colchicine-containing medium, doubled haploid plants were produced at high frequencies. Since all the plants from untreated calli were haploids, the occurrence of doubled haploid plants must be due to the effect of colchicine. The method is rapid since it only required 6 months from colchicine treatment of calli to the harvest of seeds from the regenerated doubled haploid plant.

The results of this study suggest that the duration of colchicine treatment is important. The treatment of more than 48 h is necessary in order to get higher frequency of doubled haploids among the regenerated plants. If the treatment is 72 h, tetraploid plants could be produced, which may not be desirable. The two concentrations of colchicine used, 0.025% and 0.05%, did not show significant differences in their chromosome doubling efficiency.

There was no indication that ploidy chimeras were regenerated, since most of the doubled haploid plants produced seeds after self-pollination. The problem which caused the doubled haploid plants to not set seeds was mainly delayed silk emergence or the lack of ear formation, which are common phenomena among tissue culture-derived maize plants (Miao et al. 1978; Petolino and Jones 1986). The abnormal plants found among the regenerates were probably due to the tissue culture conditions rather than the colchicine treatment since the same abnormalities (stunted growth, terminal ear, the lack of normal ear) existed among the plants regenerated from untreated control calli. In practice, only vigorous plantlets should be selected before transplanting to greenhouse or field. This should reduce the frequency of abnormal plants.

The results show that colchicine treatment of embryogenic haploid callus can result in the production of entire doubled haploid plants with high frequency, which produce fertile maize inbred lines within a short time at a high frequency, thus making the anther culture technique more useful to the plant breeder.

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