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The use of antimicrotubule herbicides for the production of doubled haploid plants from anther-derived maize callus

Y. Wan [1], D.R. Duncan [1], A.L. Rayburn [1], J.F. Petolino [2] and J.M. Widholm [1,*]

[1] Department of Agronomy, Turner Hall, 1102 S. Goodwin Ave., University of Illinois, Urbana, IL 61801, USA
[2] DowElanco/United AgriSeeds, P.O. Box 4011, Champaign, IL 61824, USA

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Summary. Four antimicrotubule herbicides, amiprophos-methyl (APM), pronamide, oryzalin, and trifluralin, were evaluated for their ability to induce chromosome doubling in anther-derived, haploid maize callus. Effects of various herbicide treatments on the growth and regenerative capacity of callus along with the ploidy and seed set of regenerated plants were determined. Flow cytometric analysis was also used to measure changes in ploidy levels of callus cells following treatments. More than 50% of the cells were doubled in chromosome number after the haploid callus was treated with 5 or 10 μM APM or 10 μM pronamide for 3 days. A similar proportion of plants regenerated from the treated callus produced seed upon self-pollination. APM and pronamide did not inhibit callus growth at these concentrations and the treated callus retained a high plant regeneration capacity. Oryzalin very effectively induced chromosome doubling, but severely inhibited the growth of regenerable callus and plant regeneration. Trifluralin induced chromosome doubling in a small proportion of cells at lower concentrations (0.5 and 1 μM), however, at a higher concentration (5 μM) it inhibited callus growth and plant regeneration. The results indicate that APM and pronamide may be useful agents for inducing chromosome doubling of anther-derived maize haploid callus at very low concentrations.

Key words: *Zea mays* – Chromosome doubling – Amiprophos methyl – Pronamide – Oryzalin – Trifluralin

Introduction

The rapid advance to genetic fixation which accompanies the doubling of the chromosome complement is an attractive feature of haploid breeding, especially in a species such as maize where conventional inbreeding involves several years of manual self-pollination. Recently, procedures for the production of large numbers of haploid plants from maize anther culture have been developed (reviewed by Petolino 1990). Unfortunately, the frequency of spontaneous chromosome doubling in maize haploids has been low and extremely unreliable. There is a need for an efficient, reliable, low-cost, and safe means for chromosome doubling before haploid breeding in maize becomes a viable strategy.

The most commonly used agent to induce chromosome doubling is colchicine. Colchicine disrupts mitosis by binding to tubulin, the protein subunit of microtubules, thus inhibiting the formation of microtubules and the polar migration of chromosomes, which results in a cell with a doubled chromosome number. In a previous study we used 0.025% (0.625 mM) and 0.05% (1.25 mM) colchicine to induce chromosome doubling of anther-derived, haploid maize callus (Wan et al. 1989). Doubled haploid plants were recovered at a high frequency from the colchicine-treated calli, most of which set seed following self-pollination. Several studies, however, have shown that colchicine has a much lower affinity for plant tubulins than for animal tubulins (Morejohn et al. 1984; Morejohn and Fosket 1984a; Morejohn et al. 1987b). Consequently, mM concentrations of colchicine are usually required to inhibit plant cell division and induce chromosome doubling, which compares unfavorably with the μM concentrations that affect microtubule-associated processes in animals (Hart and Sabnis 1976).

In comparison with colchicine, some antimicrotubule herbicides bind more specifically than colchicine to plant tubulin in vitro at even μM concentrations (Morejohn and Fosket 1984b; Bajer and Mole-Bajer 1986). Amiprophos-methyl (APM), a phosphoric amide herbi-

* To whom correspondence should be addressed

cide, directly poisons microtubule dynamics in plant cells (Morejohn and Fosket 1984b), which then results in the cessation of mitosis. This herbicide inhibited taxol-mediated polymerization of plant tubulin at a 10 μM concentration while 100 μM had no inhibitory effect on the assembly of brain tubulin, thus showing a higher affinity to plant tubulin than to animal tubulin (Morejohn and Fosket 1984b). In plant cell suspension cultures, μM concentrations of APM completely depolymerized microtubule arrays, resulting in a high degree of metaphase arrest and a significantly increased mitotic index (Falconer and Seagull 1987; Sree Ramulu et al. 1988; Stadler et al. 1989).

Trifluralin and oryzalin, which are dinitroaniline herbicides, also disrupt mitosis by inhibiting the formation of microtubules (Lignowski and Scott 1972; Bartels and Hilton 1973; Strachan and Hess 1983; Morejohn et al. 1987a; Cleary and Hardham 1988). Both herbicides also specifically inhibit plant tubulin assembly and have no inhibitory effect on the assembly of animal tubulin to form microtubules at the concentrations inhibiting plant tubulin assembly (Bartels and Hilton 1973; Strachan and Hess 1983; Morejohn et al. 1987a). The plant response to μM concentrations of these herbicides are similar to the responses of plants treated with mM concentrations of colchicine (Upadhyaya and Nooden 1977; Quader and Filner 1980).

Pronamide, a benzamide herbicide, disrupts mitosis by a similar mechanism (Bartels and Hilton 1973; Carlson et al. 1975). This herbicide probably does not cause complete microtubule loss, but apparently causes shortening of the microtubules (Vaughan and Vaughn 1987).

Although these four herbicides are known to have an effect similar to that of colchicine on microtubules, they have not been used to induce chromosome doubling for doubled haploid plant production. The present study demonstrates that some of these herbicides could be viable alternatives to colchicine for chromosome doubling in vitro.

Materials and methods

Anther culture

Field-grown F1 plants of the cross, H99 × Pa91, were used as donor plants for anther culture. The anther culture procedure and the establishment of callus lines were the same as in a previous study (Wan et al. 1989). One highly regenerable callus line initiated from a single embryo-like structure was used throughout the present study.

Herbicide treatment

Amiprophos-methyl (APM) was obtained from the Mobay Corp. Kansas City, Mo., pronamide from Rohm and Haas Co., Philadelphia, Pa., and oryzalin and trifluralin from Eli Lilly and Co. Indianapolis, Ind. Herbicide-containing media were prepared by adding the 1 mM stock solutions in acetone to petri dishes (100 × 25 mm), each containing 20 ml liquid D medium (Duncan et al. 1985). For each treatment, 0.5 g of regenerable calli was cut into fine pieces and incubated for 2 or 3 days in the herbicide-containing medium and then rinsed as described for the colchicine treatments (Wan et al. 1989).

Determination of callus growth

Following treatment, calli were transferred to agar-containing D medium, and maintained at 28 °C in the dark for 25 days. The regenerable calli and non-regenerable calli were then separated, and the fresh weights were determined. The regenerable calli were transferred to regeneration media for plant regeneration as described below.

Flow cytometric analysis

Ten to twenty days after treatment, nuclei from about 50 mg of randomly chosen regenerable callus tissue were isolated, stained, and analyzed by flow cytometry as described by Rayburn et al. (1989) with the following modifications. The callus tissue was crushed slightly in a small amount of nuclear isolation buffer using a mortar and pestle before being ground with a small homogenizer. After the concentration of the nuclei in the suspension was determined with a hemacytometer, a fluorochrome, DAPI (4′-6-diamidino-2-phenylindole), was used to stain the nuclei at a concentration of 1 µg DAPI per 1 million nuclei. Haploid nuclei from control cultures were analyzed each day to adjust for day to day variability. The cell ploidy compositions in each population can be estimated by comparing the peaks in the DNA histogram for the treated cells with that of the control, assuming that the GO + G1 to G2 peak ratio is constant for each ploidy level in the population.

Plant regeneration, growth, and determination of ploidy level

Plant regeneration procedures were as described before (Wan et al. 1989). The total number of plantlets regenerated in each treatment was recorded when the plantlets were transferred from petri dishes to culture tubes. Plants were transplanted in the field or greenhouse following 10–30 days in peat pots (5.7 × 5.7 × 10.0 cm) containing a 1:1 (v/v) mixture of peatmoss and vermiculite in the greenhouse. Since flow cytometric analysis showed that oryzalin treatments induced many tetraploid cells in the calli, one or two root tips of each plant regenerated from oryzalin-treated calli were collected before transplanting to the field, and the chromosome numbers were determined microscopically after staining (Wan et al. 1989). The plants from the other herbicide treatments were classified as haploid or diploid-like according to observed vigor, morphology, and pollen shed. The haploid plants were small, less vigorous and did not have well-developed pollen grains. The plants which grew vigorously and shed mature pollen grains were defined as diploid-like (double haploid-like) plants. Plants which shed pollen and had silks were self-pollinated.

Results

Effect of herbicides on callus growth

Control calli, without herbicide or acetone treatment, grew to about 10–13 g fresh weight in 25 days and consisted of about 80% regenerable tissue. Calli which were treated with 2% acetone for 3 days produced somewhat less fresh weight when compared to the control (8 g), but did maintain a high proportion of regenerable

Table 1. Effect of herbicide treatment on the number of regenerated plants, and the ploidy levels of those which survived in the field or greenhouse

Treatment			Total no. of plants	No. of plants which survived			
Agent	μM	Day		Total	% 1N	% 2N	% 4N
	0	2	14	8	100.0	0	0
	0	3	101	67	100.0	0	0
Acetone	2% [a]	3	26	18	100.0	0	0
APM	1	3	150	41	95.1	4.9	0
	5	3	149	75	36.0	64.0	0
	10	3	61	28	35.7	64.3	0
	15	3	168	91	33.0	67.0	0
	20	3	189	88	49.9	51.1	0
Pronamide	1	2	73	21	47.6	52.4	0
	5	3	299	151	26.5	73.5	0
	10	3	152	64	31.3	68.8	0
Oryzalin	5	3	46	24	16.7	75.0	8.3
	10	2	21	9	11.1	77.8	11.1
	10	3	28	14	7.1	57.1	35.7
	20	3	0	0	0	0	0
Trifluralin	0.5	3	124	42	76.2	23.8	0
	1	2	419	177	80.2	19.8	0
	5	3	14	8	0	100.0	0

[a] Concentration of acetone in percentage, not μM

callus (85.3%). Calli treated with from 1 to 20 μM APM or 1 to 10 μM pronamide displayed increased growth (11–20 g fresh weight) due to the proliferation of non-regenerable calli; they thus consisted of less regenerable calli when compared to the controls (20 to 54%), although the proportion of regenerable calli in each APM and pronamide treatment decreased, a large number of regenerated plants were obtained (Table 1). Treatments with oryzalin (5–20 μM) greatly decreased the proportion of regenerable calli (5%–14%) such that fewer plants were obtained (Table 1). At 20 μM oryzalin, callus growth was inhibited (7.8 g fresh weight) and less than 5% of the callus was visually estimated to be regenerable. No plants were obtained from this treatment. Trifluralin at 0.5 μM did not affect the proportion of regenerable calli, but as the concentration increased to 1 and 5 μM, the amount of regenerable calli decreased dramatically to 29% and 3.5%, respectively.

Effect of herbicide treatments on calli ploidy levels

Calli ploidy levels were determined by flow cytometric analysis 10–20 days after treatment. The DNA histograms of representative treatments are given in Fig. 1. In the haploid control (Fig. 1a), the first peak in the histogram represents those nuclei with 1C DNA content, which is the DNA content of haploid cells in the G0 or G1 stage of the cell cycle. The second peak, which is located at twice the channel number of the first peak, represents the nuclei with 2C DNA content (G2 stage or prophase of mitosis), with S-stage nuclei between these two peaks. There are no detectable peaks where the 4C nuclei would be, indicating that essentially all of the cells were haploid. In these experiments the second peak with the haploid cells usually represented about 15% of the total nuclei. In Fig. 1a 75.6% of the nuclei are in the first peak and 16.3% are in the second peak. Nuclei isolated from calli treated with the herbicides displayed a change in the DNA histogram. For example, treatment with 1 μM trifluralin caused an increase in the size of the second peak as well as the appearance of a very small peak where 4C nuclei would be (Fig. 1b). When 10 μM APM or 10 μM oryzalin-treated calli were analyzed, even greater increases in the higher ploidy peaks were obtained (Fig. 1c, d), including a clear peak representing 8C nuclei for the 10 μM oryzalin treatment.

The estimated cell ploidy compositions in each treatment are listed in Table 2. As the concentration of APM and pronamide increased from 1 μM to 10 μM, the percentage of diploid cells increased to nearly 60%. However at 20 μM concentrations of both herbicides, the percentage of diploid cells decreased somewhat. The oryzalin treatments were even more efficient in inducing chromosome doubling. The 5 μM oryzalin treatment resulted in the appearance of 44.1% diploid cells and 2% tetraploid cells. As the concentration increased to 10 μM, the percentage of tetraploid cells increased to 32% for the 2-day treatment and 41.2% for the 3-day treatment. The treatments with 0.5 μM or 1 μM trifluralin caused chromosome doubling in 12.8% and 22.0% of the cells,

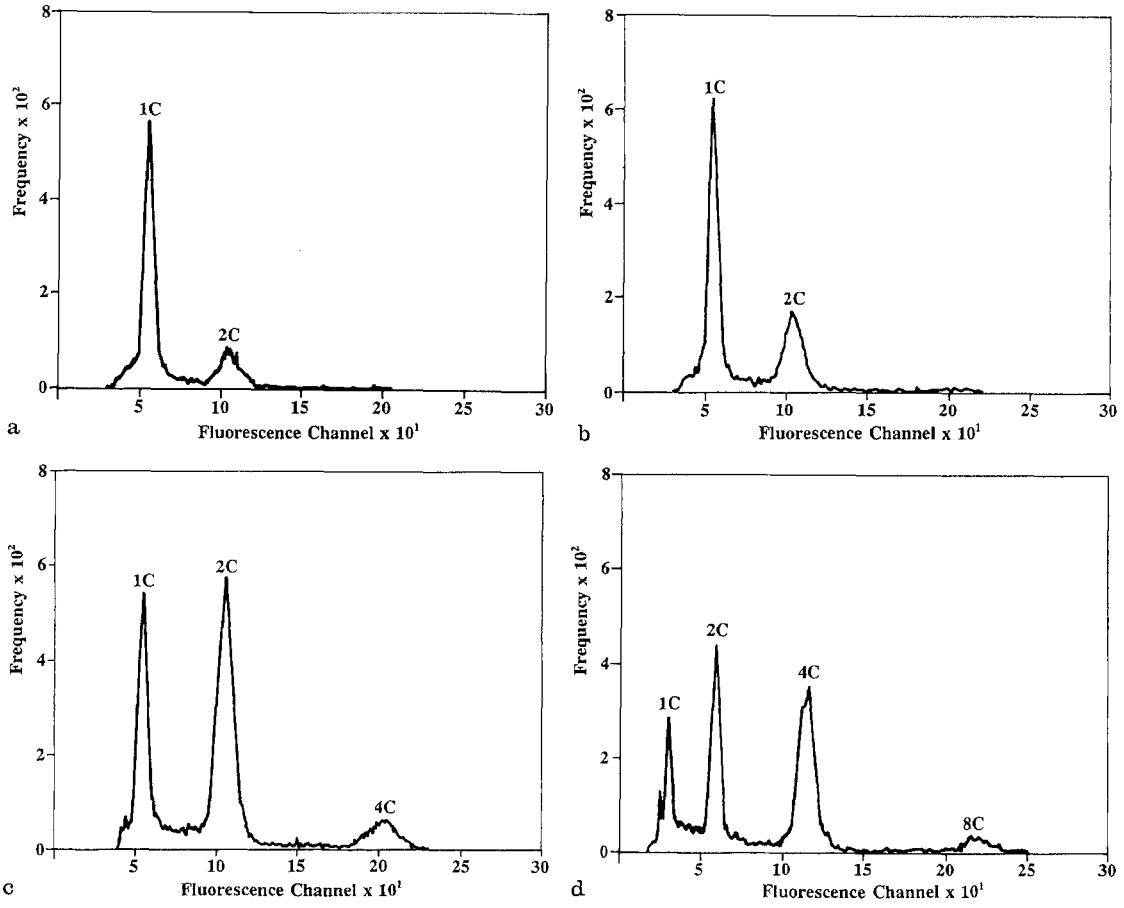

Fig. 1. Flow cytometric analysis of DAPI-stained nuclei from maize anther culture-derived callus tissue without treatment (*a*), with 1 μ*M* trifluralin (*b*), 10 μ*M* APM (*c*), and 10 μ*M* oryzalin (*d*) for 3 days. The DNA content of each peak is indicated on the top. The photomultiplier tube voltage has been lowered in *d* to accommodate the wide range of fluorescence intensity of the various peaks

Table 2. Ploidy of callus tissue treated with herbicides as determined by flow cytometry

Treatment			% of callus cells		
Agent	μ*M*	Day	1N	2N	4N
	0	2	100.0	0	0
	0	3	100.0	0	0
APM	1	3	93.8	6.2	0
	5	3	42.2	57.8	0
	10	3	42.2	56.8	1.0
	20	3	62.0	38.0	0
Pronamide	1	2	84.8	15.2	0
	5	3	53.7	42.1	4.2
	10	3	37.9	62.1	0
	20	3	59.9	40.1	0
Oryzalin	5	3	53.9	44.1	2.0
	10	2	26.0	42.0	32.0
	10	3	24.6	34.2	41.2
Trifluralin	0.5	3	87.2	12.8	0
	1	2	78.0	22.0	0

respectively. The data from 5 μ*M* trifluralin were not obtained due to microbial contamination of this callus sample.

Numbers and ploidy levels of the regenerated plants

As listed in Table 1, more plants were regenerated from all of the APM treatments (except for 10 μ*M*) than with the untreated control with the highest number of regenerated plants, although the amount of regenerable calli in each APM treatment was found to be less than that observed with the controls. The 5 and 10 μ*M* pronamide treatments also produced more plants than the controls. All of the oryzalin treatments resulted in the regeneration of fewer plants, and no plants were obtained from the 20 μ*M* treatment. Calli treated with 0.5 and 1 μ*M* trifluralin regenerated 124 and 419 plants, respectively, but the number of plants from the 5 μ*M* trifluralin treatment was only 14, largely because of the decreased amount of regenerable calli.

Table 3. Number of regenerated doubled haploid and tetraploid plants which survived and produced seed after self-pollination

Treatment			No. of 2N plants			No. of 4N plants		
Agent	μM	Day	Total	w/seeds	(%)	Total	w/seeds	(%)
	0	2	0	0	0	0	0	0
	0	3	0	0	0	0	0	0
Acetone	2%[a]	3	0	0	0	0	0	0
APM	1	3	2	2	100.0	0	0	0
	5	3	48	40	83.3	0	0	0
	10	3	18	10	55.6	0	0	0
	15	3	61	48	78.7	0	0	0
	20	3	45	40	88.9	0	0	0
Pronamide	1	2	11	8	72.7	0	0	0
	5	3	111	83	74.8	0	0	0
	10	3	44	34	77.3	0	0	0
Oryzalin	5	3	18	10	55.6	2	1	50.0
	10	2	7	4	57.1	1	1	100.0
	10	3	8	3	37.5	5	0	0
Trifluralin	0.5	3	10	8	80.0	0	0	0
	1	2	35	25	71.4	0	0	0
	5	3	8	4	50.0	0	0	0

[a] Concentration of acetone in percentage, not μM

The ploidy level of each surviving plant was determined and the data is summarized in Table 1. In the case of the oryzalin treatments, the ploidy level of each regenerated plant was determined by counting the root-tip chromosomes. The ploidy levels of the plants from the other herbicide treatments were classified morphologically in the later stages of development. The chromosome counting and morphological observations correlated exactly for the 47 plants from the three oryzalin treatments, which survived in the field, although the tetraploid plants could only be determined by chromosome counting.

As shown in Table 1, all 93 plants which regenerated from the control calli incubated in liquid medium without herbicide or with 2% acetone had typical haploid morphology, and no diploid-like plants were observed. A few of the plants extruded anthers from a few spikelets, but no seed was produced after selfing. In the APM treatments, as the concentration increased from 1 to 5 μM, the percentage of diploid-like plants increased from 4.9 to 64.0. The percentage of diploid-like plants did not change as the concentration of APM increased to 15 μM. At 20 μM, the proportion of diploid-like plants decreased slightly. The pronamide treatments produced more than 50% diploid-like plants even at the 1 μM concentration. At 5 and 10 μM, about 70% of the plants were diploid-like. Among the plants regenerated from calli treated with 0.5 μM or 1 μM trifluralin there was a lower proportion of diploid-like plants – 23.8% for the 0.5 μM treatment and 19.8% for the 1 μM treatment. As the concentration was increased to 5 μM, all the plants in the field were diploid-like, but there were only 14 plants regenerated, 8 of which survived (Table 1). Since the ploidy level of each plant from the oryzalin treatments was known from chromosome counting, the tetraploid plants could be identified. In the 5 μM concentration, 75.0% of the plants were doubled haploid, 8.3% tetraploid, and only 16.7% haploid. In the case of the 10 μM oryzalin treatments the proportion of tetraploid plants increased to 11.1% for the 2-day treatment and to 35.7% for the 3-day treatment; the percentage of doubled haploid plants were 77.8 and 57.1, respectively (Table 1).

Seed set by the doubled haploid plants

Most of the regenerated doubled haploid plants (ploidy actually determined by chromosome counting or assumed based on morphological observation) set seed after self-pollination (Table 3). Some plants, however, did not set any seed after self-pollination or could not be self-pollinated. More than 72% of the doubled haploid plants regenerated from APM- or pronamide-treated calli set seed, although only 55.6% from the 10 μM APM treatment. However, among the 8 plants (44.4%) which did not produce seed in the 10 μM APM treatment, 5 were transplanted to the field too late to reach maturity. Fewer of the doubled haploid plants regenerated from the oryzalin-treated calli produced seed; 55.6% for 5 μM, 57.1% and 37.5% with 10 μM for 2 and 3 days, respectively. In the treatments with lower concentrations (0.5 and 1 μM) of trifluralin, a higher proportion of the doubled haploid plants produced seed, 80.0% for 0.5 μM and 71.4% for 1 μM, respectively. As the concentration increased to 5 μM only 50% of the plants produced seed.

Discussion

The present study demonstrated that some herbicidals can produce effects similar to those of colchicine, but much lower concentrations (<20 μM) than required by colchicine. APM had little effect on callus growth and plant regeneration ability. This observation is consistent with results of Stadler et al. (1989) in which 50 μM APM did not have any growth-inhibiting effects on a maize "Black Mexican Sweet" suspension culture treated for 21–28 h. Similarly, pronamide did not severely inhibit callus growth or decrease the regeneration ability of treated calli at concentrations of 5 or 10 μM. More than 50% of the cells were doubled in chromosome number after treatment with 5 or 10 μM APM or 10 μM pronamide. Most of the doubled haploid plants from APM and pronamide treatment produced seed normally after self-pollination, which may indicate that these two herbicides do not have severe side effects at these concentrations.

Oryzalin and trifluralin have similar chemical structures and similar structural and ultrastructural effects on plant cells (Bartels and Hilton 1973). The present study suggests that oryzalin is a very effective antimicrotubule agent. Compared with APM and pronamide, oryzalin treatment resulted in the appearance of a high proportion of tetraploids under the same conditions. This may have resulted from the rapid uptake and accumulation of high doses by the callus tissue (Upadhyaya and Nooden 1980). However, the results also indicate that oryzalin treatments (>5 μM) severely decrease the proportion of regenerable callus so that fewer regenerants were obtained. This confirmed our previous observations that oryzalin inhibits the growth of regenerable callus (Duncan and Widholm 1989). Trifluralin at lower concentrations (0.5 μM and 1 μM) did not affect callus growth or callus regeneration ability, and also did not efficiently induce chromosome doubling. At 5 μM trifluralin severely decreased callus regeneration ability and caused browning of the treated callus tissue, as was also observed with tobacco callus treated with the same concentration of trifluralin (Young and Camper 1979). Trifluralin at 5 μM was also shown to suppress RNA, DNA, and protein synthesis in tobacco callus tissue (Young and Camper 1979). Whether the loss of regeneration ability of treated callus tissue is related to these inhibitory effects is unknown.

Flow cytometric analysis has proven to be a rapid, accurate, convenient and sensitive method to determine the cell cycle status and the ploidy levels of cells by determining the DNA contents (reviewed by Brown et al. 1986). Our study suggests that changes in the ploidy levels of cells in callus tissue after herbicide treatment can be easily detected by flow cytometric analysis, so the effect of the treatment can be evaluated rapidly, which enables one to select the desirable treatments and eliminate the unpromising ones.

A comparison of the frequency of diploid cells from flow cytometric analysis and the frequency of regenerated diploid plants which survived, show that the latter frequency is somewhat higher (Tables 1, 2). Thus, the diploid cells can regenerate plants at a somewhat higher frequency and/or the diploid plants survive somewhat better during transplanting.

In conclusion, APM and pronamide effectively induced chromsome doubling of maize anther culture-derived haploid callus and could be used as alternatives to colchicine. Little negative effect on callus growth, plant regeneration, or subsequent development of the regenerants was detected in APM or pronamide treatments. Oryzalin and trifluralin, however, are not as desirable. Oryzalin severely inhibited the growth of regenerable callus, although this herbicide was the most efficient in inducing chromosome doubling. Trifluralin was not effective in inducing chromosome doubling at lower concentrations and severely decreased callus regeneration ability at higher concentrations.

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