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Determination by flow cytometry of the chromosome doubling capacity of colchicine and oryzalin in gynogenetic haploids of Gerbera

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Summary. *In vitro* plants of the gynogenetic haploid line 86122/560 of gerbera were treated with colchicine or oryzalin dissolved in dimethylsulfoxide, to compare the antimitotic efficiency of these substances. The ploidy level was evaluated by flow cytometry two months after the treatment. Decrement of the multiplication rate was taken into account for the evaluation of the toxic effect of the antimitotic substances. Controls both with and without dimethylsulfoxide maintained the haploid status. At comparable doses, oryzalin proved to be as efficient as colchicine, but slightly less phytotoxic. Longer oryzalin treatments could probably induce the diploidization of a larger number of cells and reduce the problem of chimaeric plants.

Abbreviations: DAPI, 4', 6 diamidino-2-phenylindole; DMSO, dimethylsulfoxide; MS, Murashige and Skoog; TRIS, tris(hydroxymethyl)aminomethane; UV, ultraviolet

Introduction

Homozygous doubled-haploid genotypes, obtained through gynogenesis, constitute the most suitable material for synthesis of true F1 hybrids in *Gerbera jamesonii* H. Bolus (Tosca *et al.* 1990). In this species about 80-90% of plants regenerated via gynogenesis are haploid, the remainder being spontaneous diploid (Tosca *et al.* 1990 cit; Honkanen *et al.* 1991).

A key step in the production of isogenic lines is the diploidization of haploid plants, commonly achieved by means of antimitotic substances.

An ideal chromosome doubling agent should be simple and rapid to apply, efficient, reliable and suitable for a wide range of plant species. It should also avoid physiological problems and genetic alterations, such as intrasomatic selection, punctiform or genomic mutations and aneuploidy.

Colchicine, an antimitotic substance, is commonly used in diploidization of haploids of gerbera (Honkanen *et al.* 1991), but in many species it is reported to cause side effects, such as sterility, abnormal growth and morphology and to induce chimaeric plants, due to asynchrony of cell divisions (Wan *et al.* 1989). Chromosome losses or rearrangements and gene mutations caused by colchicine are reported in flax, sunflower, barley and cotton (Luckett 1989).

Chimaeras of gerbera after colchicine treatments and the disturbed development of pollen/stamen or male sterility of many doubled haploids were reported by Honkanen *et al.* (1991, cit.).

The herbicides oryzalin and amiprophos-methyl have strong binding affinity to plant tubulins (Morejohn and Fosket 1986; Morejohn *et al.* 1987; Falconer and Seagull 1987; Falconer *et al.* 1988). Oryzalin proved to be a more efficient chromosome-doubling agent than amiprophos-methyl and colchicine in potato cell suspension culture (Sree Ramulu *et al.* 1991).

To verify an alternative to colchicine, we tested the diploidization provided by oryzalin by flow cytometry.

Materials and Methods

Plant material. The haploid line 86122/560 of *Gerbera jamesonii* was obtained by gynogenesis from genotype 86122 and its *in vitro* multiplied shoots were used for the antimitotic evaluation by means of flow cytometric analysis.

Media and antimitotic treatment. A basal medium containing half-strength MS macrosalts (Murashige and Skoog 1962), microsalts of Heller (1953), MS vitamins, 0.217 mM adenine sulphate, 29.2 mM sucrose was used.

Antimitotic treatments were carried out by transferring haploid plantlets into test tubes containing Sorbarod plugs (Baumgartner Papier SA), soaked with 12 ml of liquid basal medium supplemented with 4.44 μM benzyladenine and 0.57 μM indoleacetic acid.

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Two independent trials were carried out. In the first, colchicine (Sigma Co.) dissolved in DMSO was tested at 0, 30, 60, 120, 180, 240, 360, 480, 720 and 960 μM. In the second, oryzalin (Dr. Ehrenstorfer GmbH, Augsburg - D) dissolved in DMSO was tested at 0, 30, 60, 120, 240, 480 μM. The DMSO concentration was adjusted to 1% (v/v) in each treatment. Controls with and without DMSO were carried out.

The shoots remained in the presence of the antimitotic agent for 48 hours and were then placed into test tubes containing 20 ml of the same medium solidified with 0.7% agar. A month later the newly proliferated shoots were transferred to a medium with 23.2 μM kinetin instead of benzyladenine.

Analysis of plantlet tissues. Five replications per treatment were carried out in the colchicine trial, and 4 to 7 in the oryzalin trial.

To verify the phytotoxicity of the antimitotic agents tested, the inhibitory effect on the multiplication rate was used as a control parameter. The number of proliferated shoots, obtained from each treated one, was determined one month after the treatment. Data are presented as the ratio between the number of shoots after the treatments and the average number of shoots of the control (hereafter called multiplication rate).

Data on ploidy level were collected 2 months after the treatments as percent of haploid, diploid and tetraploid nuclei.

Flow cytometry. Tissues cut into small pieces were fixed with 4% (w/v) formaldehyde in TRIS buffer as reported by Sgorbati *et al.* (1986). After washing with buffer, 1 ml of a solution of TRIS + 0.1% (v/v) Triton X-100 was added and a slight pressure was applied to extract nuclei. The suspension of nuclei was passed through two nylon filters with pore size of 60 and 15 μm. The nuclei (2.10^5 ml^{-1}) were stained with 4', 6'-diamidino-2-phenylindole (DAPI, Sigma Co.) at 14 μM.

Nuclei were analysed with the flow cytometer PAS II (Partec, Münster), provided with a mercury arc lamp (HBO 100/2) and a 40 X, 0.8 NA objective.

To select the UV band (350 nm) KG1, BG38 and UG1 filters coupled with a dichroic lamina TK420 were used. The blue emission was collected through the barrier filter GG435.

Root apices of imbibed pea seeds were chosen as an internal biological standard.

As the DNA content of *Pisum sativum* (9.4 pg), is about double that of gerbera (5.2 pg), each sample was analysed both with and without the standard to detect tetraploid nuclei, if any.

For ploidy evaluation, a ratio between the average channels of the tissue analyzed and that of the 2C peak of the standard was determined.

Results

Multiplication rate

As shown in Fig. 1, the multiplication ratio decrement caused by oryzalin stopped at 60 μM; at this concentration it was about 40% of the control. The colchicine treatment reduced the multiplication ratio to 20% of the control, starting from the concentration of 480 μM.

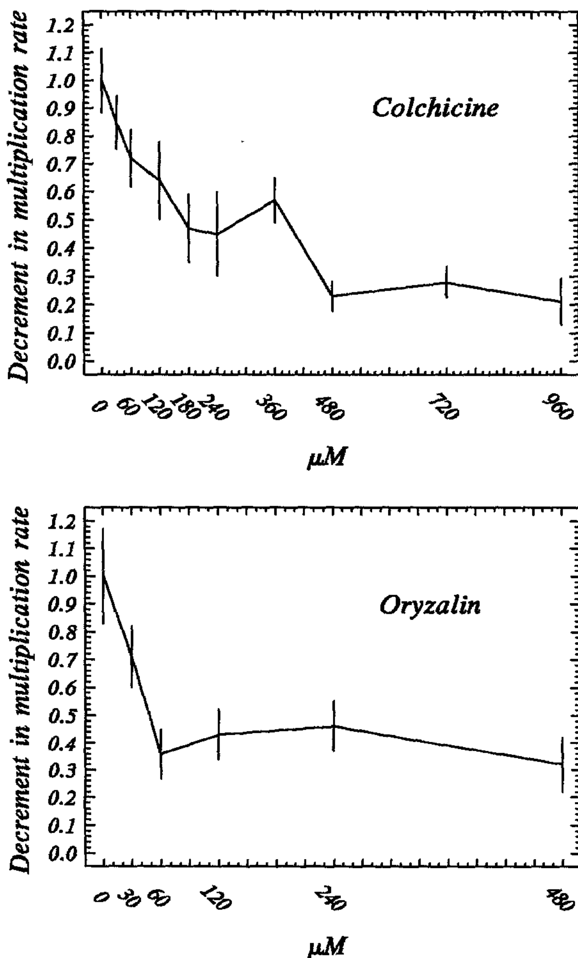

Fig. 1. Effect of different concentrations of colchicine and oryzalin on the multiplication rate.
Bars represent ± SE (n=5).

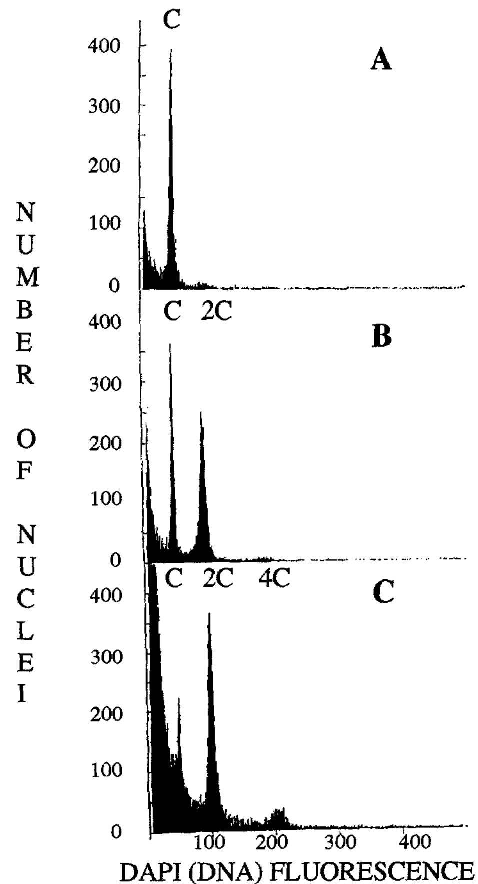

Fig. 2. Flow cytometric histograms of control (A), 120 μM oryzalin (B) and 120 μM colchicine (C) treated plantlets. After DAPI staining, nuclei released from plantlets revealed C, 2C and 4C DNA content, depending on their relative fluorescence intensity.

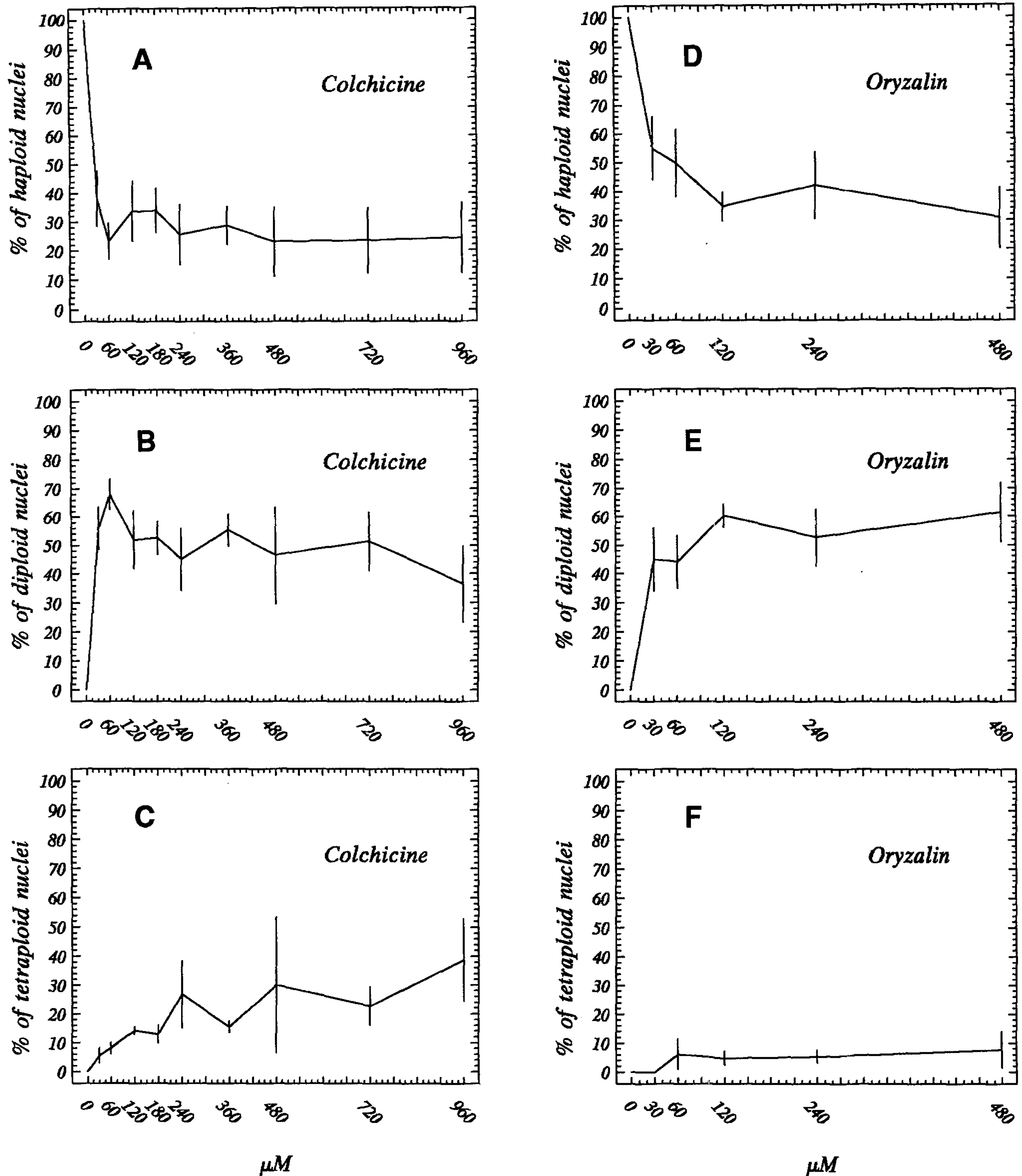

Fig. 3. Effect of different concentrations of colchicine and oryzalin on ploidy level of *Gerbera jamesonii* hybr. (A, B, C respectively % of haploid, diploid and tetraploid nuclei for colchicine; D, E, F respectively % of haploid, diploid and tetraploid nuclei for oryzalin).
Bars represent ± SE (n=5).

Flow cytometric analysis

Fig. 2 shows an example of flow cytometric analysis of the control (A), and of 120 µM oryzalin (B) and 120 µM colchicine (C) treated plantlets. Nuclei released from control plantlets (A) appeared to contain only C DNA, while those released from treated plantlets (B, C) appeared to contain C, 2C and 4C DNA, depending on the treatment.

Evaluation of antimitotic effect in haploid plantlets

Spontaneous diploidization never occurred in the controls, with or without DMSO.

Haploid and diplo-haploid percentages did not decrease significantly with the increase of colchicine concentrations (Fig. 3, A and B), while tetraploid nuclei increased constantly, varying between 5.8% and 40% at the highest concentration (960 μM) of colchicine (Fig.3, C). The best result (68.2% of diplo-haploid nuclei) was achieved at 60 μM colchicine.

Oryzalin from 120 to 480 μM induced the maximum number of diplo-haploid nuclei (59-61%, Fig. 3, E), without significant differences between these concentrations. At the same doses haploid cells reached the minimum level (Fig. 3, D). Tetraploids increased slightly with increase of the dose, never exceeding 7.5% (Fig. 3, F).

Concentrations of oryzalin below 30 μM did not significantly affect the ploidy level (data not reported).

Discussion

Flow cytometry has proved to be of unquestionable utility as a rapid and precise tool to detect the ploidy level of a plant, as previously reported (Sgorbati *et al.* 1986; Dickson *et al.* 1992; Le Gall *et al.* 1993). In this work we used this technique to monitor the capability of antimitotic drugs to double the chromosomes of *in vitro* plants without major side effects.

As colchicine treatments induced a higher tetraploidy percentage than oryzalin, a long term effect of colchicine in our material can be hypothesized. In *Luzula purpurea*, Nelson (1972) pointed out that after even short treatments of colchicine (10^{-4} - 10^{-3} M), dividing cells continue at higher ploidy levels and do not return to normal mitosis, even after 72 hours of recovery. Sakharov *et al.* (1969) observed the same phenomenon in *Crepis capillaris*, a member, like gerbera, of the Asteraceae family.

On the basis of the low tetraploidy percentages scored and the lower phytotoxicity revealed by the multiplication rate, oryzalin seems to have much fewer side effects than colchicine.

To establish an optimal protocol for diploidization of haploids of gerbera, the duration of treatments should be more carefully investigated. In fact Wan *et al.* (1989) reported a higher efficiency of haploid maize diploidization when the duration of treatment was 72 hours instead of 48. The doses of the antimitotic substances seem to be irrelevant in the range tested, except in the case of the tetraploidy increase when colchicine was used.

The variability of the ploidy level within the same treatment could be due to the stage of the cells targeted. Beaumont and Widholm (1993) reported high variabilty among replicates in the response of maize callus to the antimitotic herbicide pronamide.

We can state, on the basis of this preliminary investigation, that the antimitotic substances tested could have a wide range of useful doses, the lower being the best to avoid genetic disorders. Oryzalin may be considered superior to colchicine because of its lower phytotoxicity and the absence of long term effects. Longer times of treatment could help to obtain complete diploid plants. Additional research to verify this hypothesis should be carried out.

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