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Oryzalin combined with adventitious regeneration for an efficient chromosome doubling of trihaploid kiwifruit

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Summary. Chromosome doubling of one parthenogenetic trihaploid from cultivar Hayward of *Actinidia deliciosa* was investigated. Two antimitotic agents, colchicine and oryzalin, applied *in vitro* on shoots and leaves at different concentrations were compared with regard to their efficiency. Survival and regeneration rates were determined and ploidy level of regenerated plantlets was evaluated by flow cytometry. Differences were observed between the two antimitotic agents depending on whether shoots or leaves were treated. Hexaploid plantlets were obtained with highest efficiency by adventitious regeneration from leaves treated by oryzalin at 5 µM, constituting an original and promising result which was corroborated for another trihaploid clone. Dodecaploid plantlets were also induced but only from oryzalin treated leaves. On the other hand, colchicine applied to leaves was very phytotoxic. This study demonstrates that oryzalin combined with adventitious regeneration is particularly efficient to induce chromosome doubling of trihaploid kiwifruit.

Key words: Trihaploid kiwifruit - *In vitro* culture - Colchicine - Oryzalin - Flow cytometry

Abbreviations: MS, Murashige and Skoog; IBA, indole-3-butyric acid; DMSO, dimethylsulfoxide; PBS, phosphate buffer salin; DTT, dithiothreitol.

Introduction

In a polyploid species such as *Actinidia deliciosa* (6x = 174), highly heterozygous and mainly dioecious, isogenic lines may not be obtained by conventional breeding. Therefore chromosome doubling of trihaploid clones constitutes the only efficient mean for inducing homozygous plants for a large number of loci. Procedures for the production of trihaploid plants (3x) from the main kiwifruit female, cultivar Hayward, have been developed by Pandey et al. (1990) and recently by Chalak and Legave (submitted).

Before the use of kiwifruit trihaploids in breeding programmes can become a viable strategy, there is a need for efficient and reliable techniques for chromosome doubling.

Colchicine has been used since 1937 for producing polyploid plants (Blakeslee 1937), and is commonly used for metaphase arrest (Hadlaczky et al. 1983). In many species, colchicine is known to cause side effects, such as sterility or abnormal growth, and to induce chimeric plants, due to asynchrony of cell divisions (Wan et al. 1989). In kiwifruit, Fraser et al. (1991) mentioned a first investigation of doubling chromosome number from trihaploid petiole explants treated with colchicine at a high concentration (2.5 mM), but no doubled trihaploid has been obtained. In haploid apple, Bouvier et al. (1994) observed a better efficiency with lower concentration of colchicine (0.025 mM) applied to *in vitro* shoots. Furthermore, immunocytochemical studies in various species suggest that the herbicide oryzalin has a strong binding affinity to plant tubulin and hence a high microtubule depolymerizing activity with a low concentration of a few micromolar (Morejohn et al. 1987). Sree Ramulu et al. (1991), Wan et al. (1991) and Bouvier et al. (1994) showed that oryzalin was more efficient than colchicine for chromosome doubling in their material.

In this study, *in vitro* antimitotic effects of colchicine and oryzalin were tested and compared in regard to their efficiency in inducing chromosome doubling of a trihaploid kiwifruit.

Materials and methods

Plant material. A parthenogenetic trihaploid named H1 was mainly used. Another one, H11, was also used to confirm

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the efficiency of the result obtained for H1. They were obtained by pollination of the cultivar Hayward with lethally irradiated pollen of the male Tomuri and were introduced *in vitro* by adventitious regeneration from petiole explants. All regenerated shoots were found to be trihaploid by means of flow cytometric analysis. They were multiplied on MS medium (Murashige and Skoog 1962) supplemented with 1 $mg.l^{-1}$ zeatin (Z1 medium) and the trihaploid level was checked after 12 months of culture before using the antimitotic treatments.

Antimitotic treatments and media. Concentrated solutions of colchicine (Sigma) and oryzalin (Duchefa) were prepared freshly at 100 $mg.l^{-1}$ in 10% DMSO and then sterilized by filtration (22 µm). Colchicine was used at 0.025 and 1.25 mM, and oryzalin at 5, 15 and 30 µM, mixed with an autoclaved agarose solution at 0.6%. For each treatment, a sample of twenty *in vitro* shoots was taken 30 days after subculture onto Z1 medium. After the leaves and apex were taken off, the shoots were coated with the agar solution containing colchicine or oryzalin and then placed into culture tubes containing half strength MS medium without hormones (MS0 medium). Furthermore, the leaves removed were scarified and also coated with the mixture before transfer to Petri dishes containing Z1 medium. Eight weeks after the antimitotic treatment, young shoots arising from axillary buds were removed from treated shoots and placed onto MS0 medium for rhizogenesis. For treated leaves, adventitious shoots were removed from the callus and transferred for rhizogenesis on MS0 medium after soaking overnight in a sterile aqueous solution of IBA (2 $mg.l^{-1}$). At the same time, untreated apices and leaves were cultured as respective controls of trihaploid level for the two treatments. At the different stages and subcultures, 100 $mg.l^{-1}$ of cefotaxime was added into the culture medium, in order to avoid bacterial infection.

Analysis of antimitotic effects. To assess the phytotoxicity of the antimitotic agents, survival of treated explants and shoot regeneration were regarded as physiological parameters. The number of regenerated shoots was recorded for treated shoots after 2 months, and for treated leaves, when they were transferred from Petri dishes to culture tubes. The ploidy level of each newly developed plantlet was assessed at least twice by flow cytometry 3 to 12 months after the antimitotic treatments.

Flow cytometry. Analyses were carried out from samples of young leaves and roots. For each sample, nuclei were released from a small and fresh portion of tissue chopped with a razor blade in 1 ml of PBS buffer (BioMerieux) added with 0.3% Triton 100 and $1g.l^{-1}$ DTT. After filtering (30µm), the nuclei were stained on ice with 100 $mg.l^{-1}$ propidium iodide solution for 10 mn. A relative fluorescence of total DNA was measured for each stained nucleus with a FACS Becton Dickinson cytometer equipped with an argon laser tuned to 488 nm and operating at 15 mW. Red fluorescence emission was collected by a data processing system (Lysis II) and displayed in histograms of nuclei number according to one fluorescence intensity which was proportional to DNA content. For each histogram, peaks of fluorescence corresponding to G0/G1 cells and sometimes G2 cells were clearly discernable from a number of 2000 nuclei analysed. During these analyses, control samples of cv. Hayward and clones H1 or H11 were regularly run in the cytometer in order to monitor the stability of the peak position corresponding to the hexaploid (6x) and trihaploid (3x) levels, respectively. Also, a few µl of fish blood were added to some plant filtrates as an internal biological standard.

Results

Survival and shoot regeneration

As listed in Table 1, the treatment of H1 shoots by colchicine and oryzalin did not induce obvious physiological effects concerning shoot development from axillary buds, by comparison with the corresponding control sample. The range of survival rates was very high (85-100%). Only a delay of 1 week was noted for shoots treated with colchicine at 1.25 mM.

The treatment of leaves with antimitotic agents induced phytotoxic effects, particularly with colchicine (Table 1). When leaves were treated with 1.25 mM colchicine, necrosis appeared very quickly and all the explants were killed after 1 or 2 days. With 0.025 mM colchicine, only 13.3% of the leaves developped calli 10 weeks after treatment; but these calli did not survive. Concerning oryzalin treatment, the number of regenerated shoots decreased with the increase of oryzalin concentration, especially from 30 µM. Indeed at 5 µM, 15 µM and 30 µM, the survival rate was 81.6, 71.6 and 16.6% respectively.

Table 1. Effects of antimitotic treatments on survival and shoot development estimated with clone H1.

Treatment	Treated shoots			Treated leaves		
	No. of treated shoots	No. of survi-ving shoots	No. of develo-ping shoots	No. of treated leaves	No. of survi-ving leaves	No. of regene-rated shoots
Control	20	20	24[a]	60	54	80
Colchicine						
0.025 mM	20	18	21[a]	60	8	0
1.25 mM	20	17	17	60	0	-
Oryzalin						
5 µM	20	18	24[a]	60	49	37
15 µM	16	16	24[a]	120	86	50
30 µM	20	18	18	60	10	3

[a] Some shoots carried more than one axillary bud

Table 2. Ploidy levels achieved with antimitotic treatments applied to clone H1.

Treatment	Treated shoots						Treated leaves					
	No. of plantlets analysed	No. of 3x plantlets	No. of 6x plantlets	No. of 12x plantlets	No. of mixoploid plantlets 3/6x	6/12x	No. of plantlets analysed	No. of 3x plantlets	No. of 6x plantlets	No. of 12x plantlets	No. of mixoploid plantlets 3/6x	6/12x
Control	24	24	0	0	0	0	60	60	0	0	0	0
Colchicine												
0.025 mM	21	21	0	0	0	0	0	-	-	-	-	-
1.25 mM	17	13	3	0	1	0	0	-	-	-	-	-
Oryzalin												
5 µM	24	22	0	0	2	0	35	5	16	0	14	0
15 µM	24	8	0	0	16	0	46	3	19	12	9	3
30 µM	18	12	1	0	5	0	2	0	0	2	0	0
Total	104	76	4	0	24	0	83	8	35	14	23	3

Ploidy evaluation

According to the size and development of the plantlets, flow cytometric analyses were carried out on one or two leaves and one root when possible. In each round, the ploidy of a given sample was deduced by comparison with Hayward and H1 control samples. Fig. 1 illustrates representative data on flow cytometric determination of DNA content of nuclei for controls and shoots issued from antimitotic treatment.

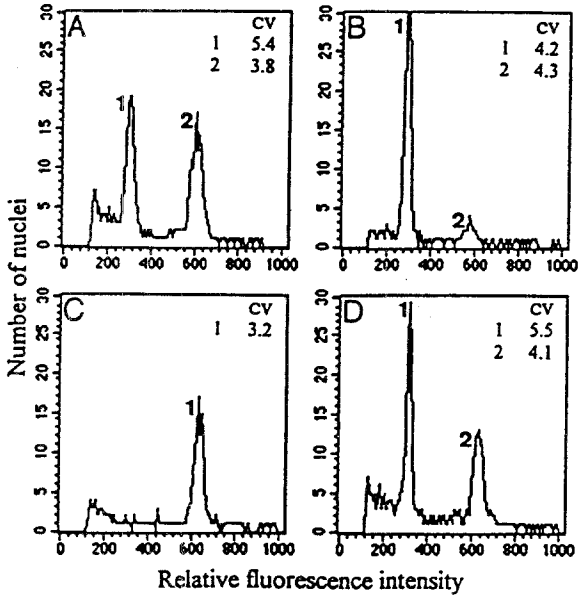

Fig. 1. Flow cytometric determination of relative DNA content of nuclei released from: **A**, leaf mixture of controls H1 and Hayward, the peak 1 corresponding to G0/G1 cells of H1 (3C) and peak 2 mainly to G0/G1 cells of Hayward (6C). **B, C** and **D**, leaves of shoots issued from antimitotic treatments showing respectively a DNA content on **B** of 3C (peak 1), on **C** of 6C (peak 1) and on **D** a mixture of 3C (peak 1) and 6C (peak 2). Peak 2 on **B** may be considered as a G2 peak. The coefficient of variation (CV) is given for each peak. DNA contents of dodecaploid (12C) and mixoploid 6/12 C are not illustrated because their fluorescence intensity was detected outside this scale.

Interphase nuclei released from control shoots of H1 and Hayward appeared to contain only 3C and 6C DNA respectively, while those issued from the different treatments appeared to contain 3C, 6C, 12C, 3/6C or 6/12C DNA. Such shoots were then considered as trihaploid (3x), hexaploid (6x), dodecaploid (12x) or mixoploid (3/6x, 6/12x), respectively.

Evaluation of antimitotic effects on ploidy

As shown in Table 2, no spontaneous doubling was screened in plantlets issued from axillary or adventitious regeneration of H1 control explants. The antimitotic effect on ploidy appeared to vary greatly depending on the agent, the concentration, and particularly, the explant treated.

In the case of treated shoots, 73% of developing plantlets were trihaploids, 23% mixoploids (3/6x) and only 3.8% hexaploids. Most of the hexaploids were obtained with 1.25 mM colchicine, while mixoploids (3/6x) were essentially screened among plantlets issued from 15 µM oryzalin.

When leaves were treated, no shoots derived from colchicine treatment were analysed since no regeneration was possible, while 83 plantlets issued from oryzalin treatment were analysed by flow cytometry: 9.6% of them were trihaploids, 42% hexaploids, 16.8% dodecaploids and 31% mixoploids (3/6x, 6/12x). For a given antimitotic treatment, various ploidy levels were induced. Nevertheless, dodecaploid plantlets were obtained only at the highest concentrations of oryzalin (15 and 30 µM).

On the other hand, hexaploid and dodecaploid plantlets were acclimated in the greenhouse. Flow cytometry from leaves and roots confirmed their ploidy level in these growth conditions.

The treatment of leaves with 5 µM oryzalin appeared as the more efficient for clone H1, by inducing the highest rate of hexaploids and only a moderate phytotoxic effect. Applied to clone H11, the same treatment induced very similar results (Table 3).

Table 3. Efficiency of oryzalin application to leaves at 5 μM for chromosome doubling in two trihaploid clones.

Clone	No. of treated leaves	% of regeneration [a]	% of 6x plantlets [b]
H1	60	61	45.7
H11	60	66	50.0

[a] No. of regenerated shoots divided per No. of treated leaves
[b] No. of hexaploids divided per No. of regenerated shoots

Discussion

This study supports the interest in oryzalin associated with adventitious regeneration for chromosome doubling in a trihaploid clone of kiwifruit. The efficiency of the two antimitotic agents was very low when they were applied to shoots, though a few hexaploids were induced especially by colchicine. These results are not in agreement with those of Bouvier et al. (1994) who observed in haploid apple shoots a higher efficiency of both oryzalin and colchicine in chromosome doubling.

Toxicity of colchicine was reported by Fraser et al. (1991) who found that treatment of haploid kiwifruit petioles with 2.5 mM for 4-6 h severely decreased callus growth and regeneration ability. In this study, a similar observation was made on leaves even with the lowest colchicine concentration (0.025 mM).

The induction of different ploidy levels within the same treatment could be due to the stage of the cells targeted. High variability was also obtained in the response of haploid gerbera shoots to oryzalin (Tosca et al. 1995) and in the response of maize callus to the antimitotic herbicides amiprophosmethyl and oryzalin (Wan et al. 1991), and pronamide (Beaumont and Widholm 1993). On the other hand the screening of mixoploid shoots among the adventitious regenerants suggests that adventitious buds may be derived from pluricellular origin.

The screening of dodecaploid shoots from adventitious regenerations was unexpected, though Boase and Hopping (1995) mentioned two dodecaploid plants identified by flow cytometry in a population derived from adventitious regeneration through extended callus culture. Thus the relative contributions of the oryzalin treatment and the adventive regeneration process in inducing ploidy changes must be further investigated, even if the length of time in culture before regeneration is probably important (Fraser et al. 1991).

On the basis of the high number of hexaploid regenerated shoots, the absence of dodecaploid shoots, the lower phytotoxicity on leaves and the better regeneration rate, 5 μM of oryzalin applied to leaves seems to be the best treatment to induce chromosome doubling. The efficiency of this treament has proved to be reproducible for two trihaploid clones. Similar observations were made by Tosca et al. (1995) who tested a wide range of useful doses of oryzalin in haploid gerbera and suggested that the lower dose was the best one to avoid genetic disorders. Additional research should be carried out to verify their hypothesis.

Flow cytometric analysis has proved to be a rapid, accurate and sensitive method to evaluate the ploidy level in *Actinidia*, as previously reported (Ollitrault-Sammarcelli et al. 1994, Hopping 1994, Boase and Hopping 1995). In this work, this technique was of unquestionable utility to investigate the ability of antimitotic agents for chromosome doubling from *in vitro* trihaploid explants and to select the desirable treatment.

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References

Beaumont VH, Widholm JM (1993) Plant Cell Reports 12: 648-651
Blakeslee AF (1937) C R Acad Sciences 205: 476-479
Boase MR, Hopping, ME (1995) Plant Cell Reports 14: 319-323
Bouvier L, Fillon FR, Lespinasse Y (1994) Plant Breeding 113: 343-346
Fraser LG, Harvey CF, Kent J (1991) Acta Hort 297: 109-114
Hadlaczky G, Bistrary G, Parznovszky T, Dudits D (1983) Planta 157: 278-285
Hopping ME (1994) N Z J Bot 32: 85-93
Morejohn LC, Bureau TE, Bajer J, Bajer AS, Fosket DE (1987) Planta 172: 252-264
Murashige T, Skoog F (1962) Physiol Plant 15: 473-497
Ollitrault-Sammarcelli F, Legave JM, Michaux-Ferriere N, Hirsch AM (1994). Sci Hort 57: 303-313
Pandey KK, Przywara L, Sanders PM (1990) Euphytica 51: 1-9
Sree Ramulu K, Verhoeven HA, Dijkhuis P (1991) Protoplasma 160: 65-71
Tosca A, Pandolfi R, Citterio S, Fasoli A, Sgorbati S (1995) Plant Cell Reports 14: 455-458
Wan Y, Petolino JF, Widholm JM (1989) Theor Appl Genet 77: 889-892
Wan Y, Duncan DR, Rayburn AL, Petolino JF, Widholm JM (1991) Theor Appl Genet 81: 205-211