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Oryzalin Treatment of Potato Diploids Yields Tetraploid and Chimeric Plants from which Euploids could be Derived by Callus Induction

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Abstract Shoot apices from six diploid potato genotypes for polyploidisation were treated with oryzalin and colchicine solutions at different concentrations and incubation times. In addition to chimeric plants, diploids, one octoploid and completely ploidy doubled plants (4*x*) were regenerated in these experiments. Tetraploid plants were obtained for three of the six genotypes treated with oryzalin, and no tetraploid plants were obtained after colchicine treatments of any of the genotypes. The best result of 43% tetraploid plants was achieved using 28.8 μM oryzalin and 24 h of incubation with the L37 genotype. Moreover, we determined that doubled plants should not be selected on the basis of a single measurement and require additional ploidy checks in successive clonal generations. Some L37 plants that were initially identified as tetraploids by flow cytometry produced diploid or mixoploid plants in the next clonal generation. Morphological characters of doubled L37 plants were compared with the original genotype. The PVY resistance present in a parental clone was maintained in all regenerants and significant differences among ploidy variants were found for some quantitative characters. Molecular AFLP analyses did not show any differences among genotypes. Since polyploidizing substances may produce a large proportion of chimeric plants, we applied a method to increase the number of tetraploid plants. Callus culture of chimeric leaf explants was performed on regeneration medium and tetraploid shoots were obtained in four of the six genotypes tested. The best result of 40% tetraploid plants was achieved with explants from the L37 genotype. This method was successful in producing additional tetraploid genotypes from mixoploid plants obtained after oryzalin treatments.

Keywords callus induction · in vitro culture · ploidy · PVY · *Solanum tuberosum L.*

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Introduction

Potato breeding at the diploid level offers several advantages over classical breeding with tetraploids, such as the fast elimination of unfavorables genes, the easy monitoring of the recessive alleles after crosses, and the introgression of desirable traits from diploid wild species into cultivated potato. In order to breed at the diploid level, dihaploid potato genotypes $(2n = 2x = 24)$ may be extracted from tetraploid potatoes $(2n = 4x = 48)$ by anther culture (Veilleux 1996) or pseudogamy (Van Breukelen et al. 1977).

Polyploidization is often carried out after breeding at the diploid stage, since potato usually performs better at the tetraploid level. Dihaploids producing gametes with an unreduced chromosome number can be directly hybridised with tetraploids, but only a few genotypes have this capacity and seed production is generally low (De Maine and Fantes 1983).

Several authors have found that tetraploids can be induced by spontaneous chromosome doubling of plants regenerated through callus culture (Fleming et al. 1992; Cardi et al. 1993; Mozafari et al. 1997). A possible disadvantage of this method could be the occurrence of somaclonal variation. In addition, the success of this method is restricted to specific genotypes (Wheeler et al. 1985).

An alternative method for returning to the tetraploid level is somatic doubling using agents that disrupt mitosis. Doubled diploids obtained this way produce less heterozygous gametes, resulting in more uniform progeny genotypes. Moreover, these tetraploid clones can be easily crossed with other tetraploids of interest. If unrelated doubled genotypes with different alleles are crossed, highly heterozygous progeny, with potentially large heterotic effects, can be obtained. For these reasons, doubled diploids and their derived progenies are particularly useful for potato breeding (Golmirzaie and Mendoza 1988).

Colchicine applications in situ (Ross et al. 1967) or in vitro (De Maine and Simpson 1999) can induce somatic doubling. However, many ploidy chimeras may be obtained, or in vitro variation induced. Nevertheless, it may be applicable for polyploidisation, due to the occasionally high rates of doubled clones produced (De Maine and Fantes 1983).

Oryzalin (3,5-dinitro-N4, *N*-dipropylsulphate), a dinitroaniline herbicide derived from the toluidine chemical family (Dow AgroSciences, USA) was proposed by Bartels and Hilton (1973) as an alternative polyploidisation agent in plants. Compared to colchicine, oryzalin arrested the cells in metaphase more efficiently, induced micronucleated cells at higher frequencies and yielded a higher number of micronuclei. Flow cytometric determination of nuclear DNA content in interphase cells and analysis of chromosome numbers in mitotic cells showed that oryzalin was a highly efficient chromosome doubling agent (Doležel 1991; Ramulu et al. 1991).

A variety of molecular markers have been applied in potato to examine genetic diversity, to perform taxonomic studies or to identify cultivars (Ritter et al. 2005). Görg et al. (1992) used RFLP markers to discriminate potato cultivars on the German variety list. Ghislain et al. (1999) analyzed intraspecific variation of *S. phureja* by random amplified polymorphic DNA (RAPD) markers. Kim et al. (1998) used AFLP markers to characterize potato cultivars and McGregor et al. (2000, 2002) with the AFLP technique characterized tetraploid genotypes of potato and other wild *Solanum* species. In this work we have used the AFLP technique to detect possible differences at the DNA level between doubled and original L37 clones in the first clonal generation.

The goals of this work were to compare the efficiency of different colchicine and oryzalin treatments to induce tetraploids in six selected diploid potato genotypes, and

determine the number of clonal generations required to reach stable tetraploid genotypes. We also present a method to regenerate additional tetraploid plants from chimeric leaf callus.

Materials and Methods

Plant Material

Six diploid potato genotypes were chosen for this study based on adaptation and quality. The Max-Planck Institute für Züchtungsforschung (Cologne, Germany) provided the inter-specific diploid hybrids, L37 (which showed immunity to potato virus Y), SR260, and H84.42/4 (which showed high yield) with *S. stoloniferum* and *S. tuberosum* Tuberosum Group clones in their background. The genotypes H88GOU59, H88TAR68 and P218D8 were obtained from the local potato breeding programme at NEIKER, from crosses between wild species (*S. gourlayi and S. tarijense)* and selected cultivated diploid (*S. tuberosum* Phureja Group and *S. tuberosum* Tuberosum Group) clones.

Induction of Polyploidy

Apical shoots of about 1.5 cm length were excised from in vitro plantlets growing on modified MS (Murashige and Skoog, 1962) medium with vitamins (Duchefa; M0222), glycine (2 mg l^{-1}), *myo*-inositol (100 mg l^{-1}), and sucrose (30 g l^{-1}). The shoot apices were isolated under sterile conditions and then incubated in colchicine and oryzalin solutions. The stock oryzalin solution was prepared by dissolving 40 mg oryzalin in 1 ml DMSO (dimethyl sulfoxide) and then adding 49 ml distilled water. Concentrations of 10 mg l^{-1} (28.8 μM) and 20 mg l^{-1}oryzalin (57.6 μM) were used. Colchicine solutions were prepared by mixing colchicine with distilled water and 1 ml DMSO as a dissolvent to prepare final solutions of 100 ml. Concentrations of 5 g l^{-1} (12.5 mM) and 10 g l^{-1} (25 mM) colchicine were applied. These colchicine treatments had previously been used by De Maine and Simpson (1999). Incubations were carried out in a flow chamber under sterile conditions. Erlenmeyer flasks (250 ml) containing the polyploidisation solutions and 50 apices each were placed on a rotary shaker (180 rpm) in darkness at 18±1°C. Treatments were applied using colchicine or oryzalin at varying concentrations or incubation times (Table 1). A total of 150 apices was processed per treatment for each clone. After incubation, the shoots were washed in distilled water and transferred to glass tubes of 15 cm length and 2.2 cm diameter containing 5 ml of modified MS medium. When plants growing from shoots reached approximately 3 cm height, they were cut into single nodes to avoid chimeric plants and were transfered into new glass tubes with modified MS medium. The controls were treated similarly, except that the solutions did not contain any polyploidisation agent.

Ploidy Controls

Once the treated plants reached approximately 5 cm height, ploidy checks were carried out by flow cytometry using the PA analysis system (Partec, Germany). Leaf samples (c. 50 mg) were processed using the Partec CyStain UV precise P kit according to Brown et al. (1991). Only tetraploid plantlets were kept. They were micropropagated once. After 4 weeks, all plantlets descending from the same node were transferred to the same pot

Table 1 Distribution of ploidy levels in plantlets of six potato genotypes after various colchicine or oryzalin treatments and incubation times

Genotype[a]	Agent	Concentration (mgl^{-1})	Time (h)	Number of dead shoots after treatment	Number of regenerated plantlets analyzed	Tetraploid plants	Mixoploid plants	Diploid plants	Octoploid plants
L37	Oryzalin	10	24	10	93	39	28	25	1
L37	Oryzalin	20	24	42	60	3	4	53	0
L37	Oryzalin	10	48	48	76	2	3	71	0
L37	Colchicine	5×10^3	24	38	65	0	1	64	0
L37	Colchicine	10×10^3	24	40	87	0	3	84	0
L37	Colchicine	5×10^3	48	48	53	0	4	49	0
SR260	Oryzalin	10	24	20	73	0	3	70	0
SR260	Oryzalin	20	24	64	21	0	0	21	0
SR260	Oryzalin	10	48	80	4	0	0	4	0
SR260	Colchicine	5×10^3	24	30	60	0	0	60	0
SR260	Colchicine	10×10^3	24	36	50	0	1	49	0
SR260	Colchicine	5×10^3	48	50	48	0	0	48	0
H84.42/4	Oryzalin	10	24	14	30	0	2	28	0
H84.42/4	Oryzalin	20	24	60	11	0	0	11	0
H84.42/4	Oryzalin	10	48	70	5	0	0	5	0
H84.42/4	Colchicine	5×10^3	24	40	55	0	0	55	0
H84.42/4	Colchicine	10×10^3	24	50	61	0	0	61	0
H84.42/4	Colchicine	5×10^3	48	58	50	0	1	49	0

P218D8	Oryzalin	10	24	16	109	0	5	104	0
P218D8	Oryzalin	20	24	44	73	0	0	73	0
P218D8	Oryzalin	10	48	74	30	0	0	30	0
P218D8	Colchicine	5×10^3	24	26	55	0	0	55	0
P218D8	Colchicine	10×10^3	24	38	50	0	1	49	0
P218D8	Colchicine	5×10^3	48	52	50	0	1	49	0
H88TAR68	Oryzalin	10	24	10	133	2	11	120	0
H88TAR68	Oryzalin	20	24	52	65	0	0	65	0
H88TAR68	Oryzalin	10	48	68	41	0	0	41	0
H88TAR68	Colchicine	5×10^3	24	50	39	0	0	39	0
H88TAR68	Colchicine	10×10^3	24	48	67	0	2	65	0
H88TAR68	Colchicine	5×10^3	48	78	23	0	0	23	0
H88GOU59	Oryzalin	10	24	14	95	5	3	87	0
H88GOU59	Oryzalin	20	24	54	54	0	0	54	0
H88GOU59	Oryzalin	10	48	62	47	0	0	47	0
H88GOU59	Colchicine	5×10^3	24	44	25	0	0	25	0
H88GOU59	Colchicine	10×10^3	24	66	50	0	1	49	0
H88GOU59	Colchicine	5×10^3	48	80	24	0	0	24	0

[a] 150 shoots were used in all treatments

Table 2 Ploidy levels of ten plants raised from first generation tubers of 15 selected doubled L37 genotypes. The mother plants were previously indicated as tetraploid by flow cytometry

Clone code of doubled L37	Mixoploid plants	Diploid plants	Tetraploid plants
290	3	2	5
312	0	0	10
197	0	0	10
186	10	0	0
192	9	1	0
288	2	1	7
161	0	0	10
243	0	0	10
198	0	10	0
203	6	0	4
285	2	3	5
282	3	7	0
686	2	0	8
472	0	0	10
685	0	0	10
L37 diploid control	0	10	0

(6.4 l), in the greenhouse for first generation tuber production. Tubers were harvested and stored at room temperature until dormancy was broken. Then, 10 tubers per clone were planted in pots. Ploidy levels were analysed again in the emerging plants. Again, only tetraploid plants were kept for the production of second generation tubers in the greenhouse. They were planted in the field and the ploidy levels checked again. Finally, a fourth round of ploidy checks was performed in the following clonal generation in the field. In all generations, ploidy checks were carried out by flow cytometry.

Characterisation of Diploids

In the first clonal generation (plants grown from first generation tubers) of tetraploids derived from L37, mechanical inoculations with Potato Virus Y, strain N (PVY^N), were performed in the greenhouse to compare the resistance to the original L37 genotype. A total of 89 tetraploid plants belonging to 11 clones were inoculated, using carborundum (600 mesh) as an abrasive agent. The PVY^N inoculum (isolate D613) was prepared from leaves of artificially infected *Nicotiana tabacum*. Sap was diluted 1:10 (v/v) in phosphate–PVP buffer. Two leaves per plant were inoculated and then rinsed with water to remove excess abrasive and inoculum. ELISA–DAS (Clark and Adams 1977) was used to evaluate PVY^N infections 6 weeks after inoculation using commercial antibodies (Bioreba, Switzerland). Spectrophotometric readings at 405 nm were performed with air blanking after various times of substrate reaction, until the highest values were about $A_{405} = 1.5 - 2.0$ (after 0.5 to 2 h of reaction). The exact positive/negative threshold was set for each batch of microplates with the aid of histograms of the log-transformed data (Crofts et al. 1988). All plants were checked again in the next clonal propagation cycle for PVY^N infection using ELISA–DAS.

In the second clonal generation a morphological and physiological characterization of the 11 doubled L37 clones compared to the original L37 genotype was performed in the field. The field trial consisted of completely randomized blocks with two replicates of five tubers of each of the 11 clones and L37. The plots were planted with intra- and inter-row distances of 35 cm and 75 cm, respectively. Check cultivars to assess haulm maturity were

Table 3 Means and standard errors of morphological traits in plants from 11 doubled L37 clones and from the original L37 genotype in the second clonal generation

Genotype	Plant height (cm)	Leaf length (cm)	Leaf width (cm)	Pollen viability (%)	Dry matter (%)	Reducing sugars (%)
L37 (2*x*)	30.1±2.1b	4.2±0.2a	2.0±0.1b	50±4.2a	22.4±4.5a	0.20±0.01a
Doubled L37 (4*x*)	35.7±2.8a	4.4±0.3a	3.6±0.2a	50±3.5a	17.0±3.6b	0.22±0.02a

Means in a column followed by the same letter did not differ significantly at $P < 0.05$ according to the Duncan test.

cv. Jaerla (very early), cv. Kennebec (mid-early) and cv. Désirée (mid-late). All plants were evaluated for height, terminal leaf length and width, flower colour, pollen viability and vegetative cycle length. Pollen viability was measured by observing the percentage of pollen grains that stained uniformly with acetocarmine. After harvest, skin colour, flesh colour and eye depth were recorded and analyses of dry matter (Simmonds 1977) and reducing sugar contents (Miller 1959) were performed. SAS Software (SAS 2000) and particularly the Duncan test, was used for the separation of means.

The AFLP technique was applied to detect possible differences at the DNA level between doubled and original L37 clones in the first clonal generation. DNA extraction was carried out using the DNAeasy Plant Mini kit from Qiagen (Valencia, USA). AFLP analysis with EcoRI/MseI adapters was performed according to Vos et al. (1995). Four primer combinations, E63 (5′-GAC TGC GTA CCA ATT CGA-A-3′) / M64 (5′-GAT GAG TCC TGA GTA AGA C-3′), E78 (5′-GAC TGC GTA CCA ATT CGT-T-3′) / M73 (5′-GAT GAG TCC TGA GTA AGG G-3′), E75 (5′-GAC TGC GTA CCA ATT CGT-A-3′) / M74 (5′-GAT GAG TCC TGA GTA AGG T-3′), and E75 (5′-GAC TGC GTA CCA ATT CGT-A-3′) / M92(5′-GAT GAG TCC TGA GTA ATT C-3′) were evaluated. Amplification products were separated on 6% denaturing polyacrylamide gels. AFLP fragments were detected on a LI-COR 4200-S1 DNA Sequencer. Primers were labeled with the fluorescent infrared dye IRD800 (LI-COR, Lincoln, Nebraska, USA) and fragment analysis recorded by a laser system as described by the manufacturer (LI-COR 1997).

Callus Induction

Six of the chimeric plants were used for callus induction. Small leaf pieces (c. 1 cm^2) were cut and placed in Petri dishes (9 cm Ø) containing 25 ml of CM medium (Barandalla et al.

Table 4 Distribution of ploidy levels in plantlets of six potato genotypes regenerated through callus induction of mixoploid plants obtained by oryzalin treatments

Genotype	Clone	Number of shoots	Plantlets analyzed	Tetraploid plants	Diploid plants	Mixoploid plants
L37	88	38	20	8 (40)[a]	12 (60)	0
SR260	785	27	20	0	20 (100)	0
H84.42/4	629	21	20	0	20 (100)	0
P218D8	19	20	20	2 (10)	5 (25)	13 (65)
H88TAR68	25	34	20	6 (30)	13 (65)	1 (5)
H88GOU59	9	39	20	3 (15)	16 (80)	1 (5)

[a] Percentage is indicated in parentheses

2003). Five leaf pieces of each genotype were incubated at 22 °C and 16 h light for 4–6 weeks. Calli were subcultured every 3 weeks. Shoots were excised and transferred to glass tubes containing modified MS medium. When plants reached a height of c. 3 cm, single nodes were transferred to new glass tubes with MS medium. Once plants were c. 5 cm tall ploidy checks were performed by flow cytometry as described above (20 plants per genotype). Tetraploid clones were evaluated in the same way as the other tetraploids obtained with oryzalin.

Results

Table 1 shows the distribution of ploidy levels in plantlets derived from the six diploids treated with colchicine or oryzalin. No tetraploid plants were obtained after colchicine treatment. However, the frequences of mixoploid plants increased with increasing colchicine concentration or incubation time. With oryzalin, tetraploid plants were obtained for three of the six genotypes. The best result of 43% tetraploids was obtained with 10 mg l^{-1} oryzalin and 24 h incubation of the L37 genotype. The only octoploid plant occurred in this treatment. For genotypes SR260, H84.42/4 and P218D8, mixoploids plants were obtained at the lowest oryzalin concentration and no tetraploid plants were produced in any treatment. In two genotypes (H88TAR68 and H88GOU59) a few tetraploids and mixoploids plants were obtained with 10 mg l^{-1} oryzalin.

Some of the mixoploid plantlets were micropropagated and the propagules were analysed again. All mixoploid plantlets maintained their chimeric character through the next vegetative cycle. Table 2 shows the distribution of ploidy levels in 15 polyploidized L37 clones of the first clonal generation. They had previously been determined to be tetraploids based on flow cytometry. The propagules of 6 out of 15 selected tetraploid plants were completely tetraploid, and one plant each were completely diploid and mixoploid. The other seven polyploidized L37 clones showed a varying proportion of mixo-ploid, diploid and tetraploid plants (Table 2). Ploidy checks were also performed on all plants in the following clonal generations as described above. In the second clonal generation only one diploid and one mixoploid (2*x*/4*x*) plant was detected, while in the third clonal generation all plants were tetraploid.

The results of the ELISA–DAS analyses of 11 doubled L37 clones after mechanical inoculations with PVY^{N} indicated that all doubled plants retained their resistance to this virus strain. After multiplication in the field, the plants showed no PVY virus symptoms, and ELISA tests were again negative.

A morphological characterization of the 11 doubled L37 clones compared to the original L37 genotype was performed in the second clonal generation in the field (Table 3). Plant height and leaf width were significantly higher in doubled L37 clones than in the original genotype. Flower, tuber skin and tuber flesh colours were maintained in the doubled clones. Pollen viability, maturation and eye depth were similar in both groups. Tubers of doubled clones had significantly lower dry matter content than L37 but reducing sugar content was similar in both groups.

AFLP analyses generated a total of 199 bands with the four primer combinations and no differences in the banding patterns were observed between original and doubled L37 (4*x*) clones.

Callus was induced from leaf pieces of chimeric plants of six genotypes and a variable number of shoots subsequently regenerated (Table 4). One clone per genotype was used for this experiment. It was possible to induce calli in each genotype and to regenerate a variable number of shoots. Table 4 shows the results of the ploidy checks in the regenerated plants.

Tetraploids were obtained in four of the six genotypes. Of particular interest is the success with P218D8, since with this genotype no tetraploids were obtained from the oryzalin treatment. Multiplication and evaluation continued as described for the other doubled genotypes and stable tetraploids were obtained after two generations. The 686, 472, and 685 tetraploid clones came from callus induction of chimeric plants.

Discussion

Ross et al. (1967) performed polyploidisation experiments in vivo using 0.25, 0.375 and 0.5% colchicine concentrations in various combinations with two treatment times (24 and 48 h) in 54 diploid potato clones and obtained doubled genotypes in 33 of them (61% success). The best treatment was 0.5% colchicine for 24 h. However, these high percentages might be overestimated since the authors evaluated ploidy levels by counting chromosomes in root tips and considered therefore only layer LIII. Completely doubled plants are expected to be tetraploid in all three layers. Ross et al. (1967) also observed the induction of large numbers of chimeric plants and, as in our work they observed that the number of living meristems in all the genotypes was lower after increased incubation time or concentration of the polyploidisation agent.

De Maine and Fantes (1983) used three colchicine treatments: 0.5% aqueous colchicine solution for 24 h, 0.5% for 48 h, and 1% for 24 h, in subaxilary meristems of dihaploid stems grafted onto tomato roots. They were able to double 9 of 11 genotypes in all layers. There were no significant differences among the three colchicine treatments. They also indicated that this method usually produces high numbers of mixoploid plants. However, De Maine and Simpson (1999) obtained a maximum of 37% doubled plants in only one of three genotypes using different colchicine treatments of 0.01, 0.05, 0.07 and 0.5% and 1, 5, 10, 24 and 48 h incubation times. In our polyploidisation experiments with colchicine, only some chimeric plants and no tetraploid plants were obtained. Chauvin et al. (2003) indicated that the success of the oryzalin treatments depends on the genotypes. Success with colchicine treatment is likely to be genotype specific as well. However, in our work, three of six genotypes produced some tetraploids after oryzalin treatments. Despite some oryzalin precipitation in the flasks, also observed by Chauvin et al. (2003) for concentrations above 10 mg l^{-1}, the best result was obtained with the L37 genotype with a concentration of 10 mg l^{-1} oryzalin and a 24 h incubation time, leading to 43% tetraploid plants.

Considering these results, it is evident that strong interactions among treatments and genotypes exist. Therefore, it is difficult to establish a general procedure for doubling diploids, because a specific protocol is required for each genotype. Moreover, doubled plants should not be selected on the basis of a single measurement and additional ploidy checks in the next clonal generations are required.

Dolezël et al. (1992) checked ploidy levels using different fluorochromes (DAPI, PI, and MI) for flow cytometry and found that this method is reliable to check ploidy levels using any of the fluorochromes. Therefore, we also applied this technique in our experiments using DAPI fluorochrome. Nonetheless, some plants that were initially identified as tetraploids by flow cytometry produced diploid or mixoploid plants in the next generation. This was also observed by Chauvin et al. (2003), who also obtained completely diploid descendents. It could be because some selected tetraploids were not completely doubled clones in the three layers of the shoot (L1 or epidermis, L2 or hypodermis and L3 or internal tissues). So, a selection procedure in the progeny was necessary to obtain stable

doubled clones. Chauvin et al. (2003), observed that the only way to obtain stable 4*x* plants was to try to select in the vegetative progeny stable 4*x* plants, and they observed that nearly all selected 4*x* plants displayed a stable ploidy level after 2 years. Doubled L37 plants maintained resistance to PVY^N as confirmed by ELISA tests after artificial inoculation and field retesting in the next generation. With respect to other qualitative characters, the same tuber and flesh color as in the original diploid was observed in the doubled clones. Considering quantitative characters, no differences were observed for leaf length and reducing sugar contents. Leaf width was higher in the doubled clones There were no pollen viability differences between doubled clones and the original diploid, and androsterile plants were not found, in contrast to De Maine and Stewart's (1988) studies. Dry matter content was lower in tetraploid clones. Finally, plant height was higher in tetraploid clones. This was also observed by Uijtewaal et al. (1987).

De Maine and Stewart (1988) observed that doubled clones had the same protein bands as the original dihaploids. Our molecular AFLP analysis in the L37 genotype revealed the same banding pattern in both doubled and original clones, which at least suggests the absence of major gene losses or chromosomal rearrangements during chromosome doubling by mitotic polyploidisation.

We regenerated shoots from calli of mixoploid plants in an attempt to increase the number of doubled plants. In addition to diploid and mixoploid shoots, we were able to obtain tetraploids with this technique in four of the tested genotypes and particularly in one genotype, where no tetraploids had been obtained with colchicine or oryzalin treatments. So, we conclude that this method is useful when oryzalin or colchicine treatments produce only mixoploids. Hulme et al. (1992) applied a method to regenerate plants from leaf explants and stated that the regeneration capacity depends on the genotype and other external factors.

There are previous studies about spontaneous doubling using dihaploid leaves (Rousselle and Rousselle-Bourgeois 1989; Chauvin et al. 2003). However to our knowledge this is the first report about the production of tetraploids following callus regeneration of mixoploid clones. Some authors (Wheeler et al. 1985; Karp et al. 1989) indicated that tissue culture which includes a callus phase could result in phenotypical or somaclonal variability, but somaclonal variation was not found either phenotypically or at the molecular level in this study. Therefore we conclude that callus induction from chimeric explants could be a promising method in some genotypes when polyploidisation produces only mixoploids.

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