Polyploidization of Japanese Quince (*Chaenomeles japonica*) Plants

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SUMMARY

In this paper, studies on *in vitro* polyploidization of Japanese quince (*Chaenomeles japonica*) are reported. Tetraploid and mixoploid plants were obtained both when microshoots and cotyledons were treated with colchicine or oryzalin. In general, morphogenesis seemed to be more dependent on the concentration than on time and duration of treatment. High colchicine concentrations were toxic for Japanese quince microshoots, but genotype differences were also observed. Although high oryzalin concentrations inhibited growth, intermediate and low concentrations were very efficient for induction of polyploids without severe negative effects on shoot growth. The optimal concentration for polyploidization of microshoots was 0.6–0.9% for colchicine and 30–40 μM for oryzalin. The optimal concentration for polyploidization of excised cotyledons was 0.3–0.6% for colchicine, and 20–40 μM for oryzalin. For efficient polyploidization, cotyledons should not be treated with colchicine until four days after excision, whereas oryzalin should be applied immediately.

INTRODUCTION

Japanese quince (*Chaenomeles japonica* (Thunb.) Lindl. ex Spach), Maloideae, is a minor fruit crop in Latvia and Lithuania, appreciated because of its high yield of aromatic fruits. Japanese quince orchards are currently established from open pollinated seedlings. Since Japanese quince is an out-crossing species, seed propagation results in a large variation in many important traits such as plant growth and fruit quality. The heterogeneity of the plant material makes field management difficult and hampers development of high quality food products. Consequently, there is an obvious need for crop improvement, and plant breeding programmes have recently been initiated (Rumpunen 2002). As a first step, superior seedlings have been selected in orchards, propagated and planted in comparative trials. In the long-term perspective, selection among seedlings derived from controlled crossings of superior selections would be an efficient strategy to obtain better varieties. In addition, crop improvement may be achieved by means of polyploidization.

In the past few decades, polyploidization has become increasingly successful and polyploids of several fruit plants have been developed (Roberts *et al.* 1990, Chakraborti *et al.* 1998). Through polyploidization fruit size is often increased (Brown 1975, Layne & Quamme 1975), which is one of the goals for the current Japanese quince breeding programme. A reduced number of seeds is another desirable trait, which is frequently also achieved through polyploidization. Furthermore, enzyme activity and

the production of secondary metabolites associated with fragrance and flavour could be increased (Levin 1983, Dhawan & Lavania 1996). Polyploidization can also be used to restore the fertility of interspecific hybrids. In the genera *Malus* and *Pyrus* of the subfamily Maloidae, there are many examples of polyploid varieties producing a high yield of fruits with reduced numbers of seeds (Moore & Janick 1983). This is especially characteristic of triploids, whose pollen germination is also strongly reduced (Rejman 1994). Conventional polyploidization methods often result in mixoploid plants when axillary buds are treated with mitotic disrupters (or mitotic spindle inhibitors). Non-chimeric polyploids may be obtained if the mitotic disrupters are applied during the first mitosis to a tissue from which meristematic cells are induced *de novo*.

So far, no information is available on fertility, productivity and seed set of polyploids in the genus *Chaenomeles*. Only one spontaneous tetraploid has previously been reported (Weber 1963). The objective of this study was to investigate the effects of colchicine and oryzalin treatments on morphogenesis of Japanese quince microshoots and excised cotyledons, and to develop efficient *in vitro* procedures for production of polyploids.

MATERIALS AND METHODS

Plant material and *in vitro* conditions

In the experiments, six genotypes of Japanese quince were used which had previously been propagated *in vitro*. One clone (93018) was selected in Finland (University of Helsinki), four (9221, 9218, 9226 and 9365) were selected in Lithuania (Lithuanian Institute of Horticulture), and one (C19) was selected in Sweden (Balsgård, Swedish University of Agricultural Sciences). Cotyledons were excised from seeds obtained from open pollination of clone L97, selected in Lithuania.

Microshoots were grown on 3/4 MS-medium (Murashige & Skoog 1962), 30 g/l sucrose and 1 mg/l BA (benzyladenine). Cultures were put in a growth chamber in 16 h day length and a temperature of 21–25 °C. Microshoots were transferred to fresh medium every three weeks. Prior to induction of polyploids, microshoots were grown for one cycle on a medium without cytokinin to promote shoot elongation.

Polyploidization of microshoots

Sub-apical microshoots were cut into leafless two-node sections. The sections were then immediately treated, or were put on growing medium and kept cold (5 °C) in the dark for five days before being treated. For induction of polyploids, microshoots were soaked for one or two days in liquid 3/4 MS medium containing colchicine or oryzalin, 0.5–10 mg/l GA (gibberellic acid, to induce cell division) and 2% DMSO (dimethyl sulphoxide, to help chemicals penetrate cell walls). Seven concentrations of colchicine (0.01, 0.05, 0.1, 0.3, 0.6, 0.9 and 1.5% corresponding to 0.25–38 mM) and oryzalin (10, 20, 30, 40, 50, 144 and 578 µM) were tested. After induction the microshoot sections were rinsed three times in sterile distilled water and put on fresh growing medium.

A dipping method for induction of polyploids, which had previously been reported to be successful for haploid apples (Bouvier *et al.* 1994), was also tested. Two-node sections of three Japanese quince clones were dipped in a 0.2% agar solution containing 0.5 mg/l GA and 0.001% or 0.04% colchicine. After dipping (without rinsing) the sections were placed on 3/4 MS medium, containing 0.5 mg/l GA. Treatments used were a) immediate dipping after cutting the two-node sections, b) pre-treatment of two-node sections for 24 h in normal growing conditions and four days in cold and dark, and then either c) immediate dipping, or d) dipping after 24 h in normal growing conditions.

Polyploidization of isolated cotyledons

Mitotic activity can be induced *de novo* in subepidermic tissues of isolated cotyledons by use of a medium supplemented with cytokinins. For this purpose seeds from mature fruits were sterilized for 10 min in sodium hypochlorite (with 3.72% available chlorine), and cotyledons were excised. The excised coty-

ledons were grown on Nitsch-Nitsch medium (Nitsch & Nitsch 1969) with 30 g/l sucrose and 3 mg/l BA. Cultures were put in a growth chamber in 16 h day length and a temperature of 21–25 °C. Cotyledons were treated with mitotic disrupters immediately after excision or after cultivation for 4, 6, 8 or 10 days. Cotyledons were then grown for 2, 5, and 7 days on fresh medium with colchicine or immersed for one day into liquid Nitsch-Nitsch medium with oryzalin. Explants were then rinsed three times in sterile distilled water, and put on fresh shoot regeneration medium (Nitsch-Nitsch supplemented with 3 mg/l BA). Cotyledons were transferred to fresh medium every three weeks.

Mitotic activity

For successful polyploidization of cotyledons the mitotic activity of the cells must be known so that treatments can be adjusted accordingly. Excised cotyledons were kept at 20 °C, sampled each day for ten days, and fixed in a solution of ethanol and glacial acetic acid (3:1). The mitotic index was determined by microscopy in squashed sections of the basal part of a cotyledon stained with 2.5% acetocarmine solution (Pausheva 1980).

Ploidy level

The ploidy was measured by a flow cytometer (FACScan©, Becton Dickinson Immunocytometry Systems). Flow cytometer measurements were made following the procedure of Arumuganathan & Earle (1991) modified by Dikson *et al.* (1992), with double filtration according to Buckhari (1997). The samples were centrifuged for only five seconds, which was sufficient to get the chaenomeles nuclei into the pellet. The relative fluorescence intensity (FL2) was measured from at least 10 000 particles per sample. Diploid Japanese quince microshoots were used as controls. Measurements were made as soon as the microshoots had grown sufficiently to allow sampling of 25 mg from the top of the shoot. Flow cytometer measurements were confirmed by counting chromosomes in root tips (Pausheva 1980) of shoots with a presumed changed ploidy level. In addition, length of stomata was measured using a microscope.

RESULTS

Ploidy level

Flow cytometry is generally a convenient and quick technique for determining ploidy level of plants already grown *in vitro*. It is however not a very efficient technique for detecting mixoploids and aneuploids, which have only small deviations in the chromosome number. Therefore, flow cytometer results should always be confirmed by counting chromosomes in root tips. Length of stomata may also be used to estimate plant ploidy level and could be used for screening purposes. Examples of stomata length for leaves on regenerated microshoots are given in Table 1. Stomata of tetraploid shoots of the same clone were approximately one third longer than stomata of diploid shoots. For mixoploid shoots, the standard deviation for length of stomata was usually higher than for diploid and tetraploid shoots.

Table 1. Ploidy and length of stomata of leaves on shoots regenerated from microshoots of genotype 9226.

Regenerant	Ploidy	Length of 10 stomata (x 20 μm)										x±SD (x 20 μm)
0-2611-1	Diploid	13	13	14	14	14	12	12	13	14	14	13.3±0.82
0-2611-2	Diploid	15	11	14	11	10	13	11	10	11	16	12.2±2.15
0-2611-3	Diploid	15	15	14	12	14	13	13	12	12	15	13.5±1.27
0-2529-1	Diploid	12	10	11	12	10	10	8	10	11	10	10.4±1.17
0-2529-2	Mixoploid	16	21	16	16	20	21	26	15	20	18	18.9±3.38
0-2529-3	Tetraploid	18	18	21	26	21	16	20	21	22	21	20.4±2.72
0-710-1	Tetraploid	20	20	18	17	18	18	18	19	18	17	18.3±1.06
0-710-2	Tetraploid	18	15	18	17	17	17	16	16	17	19	17.0±1.15
0-710-3	Tetraploid	20	18	20	20	18	21	20	20	17	17	19.1±1.45

Polyploidization of microshoots using colchicine

When the colchicine dipping method was used according to Bouvier *et al.* (1994) no tetraploid shoots of Japanese quince were obtained (based on flow cytometer measurements), and 41% of the buds died. However, when nodes of microshoots were instead soaked in colchicine, some plants with changed chromosome numbers were obtained in almost every experiment (Table 2). Number of regenerants and the percentage of shoots with changed chromosome number depended both on colchicine concentration and on genotype. Some clones produced microshoots with a changed chromosome number already at the lowest colchicine concentration but for most clones higher colchicine concentrations were necessary to induce polyploidy. For polyploidization of Japanese quince, the optimum concentration of colchicine was approximately 0.6–0.9%.

We also tried to accumulate meristems in mitosis by keeping the microshoots at a low temperature (5 °C) before induction of polyploids. We expected to increase the number of tetraploid shoots by doing this. However, the low temperature also seemed to increase sensitivity and at 0.1% colchicine concentration over 90% of the buds died, and no tetraploid shoots were obtained.

Shoot development generally decreased at longer treatment times (Table 3) but the effect of the chemical concentration was stronger.

Table 2. Ploidy of Japanese quince (*C. japonica*) shoots obtained by treating segments of microshoots (average of genotypes) with different concentrations of colchicine for one day.

Colchicine (%)	Regenerants (*n*)	Diploids (%)	Mixoploids (%)	Tetraploids (%)
0.0	63	100.0	0.0	0.0
0.6	75	86.7	13.3	0.0
0.9	37	67.6	18.9	13.5
1.5	52	88.5	9.6	1.9

Table 3. Ploidy of Japanese quince (*C. japonica*) shoots obtained by treating segments of microshoots with 0.6% colchicine for 1 or 2 days.

Genotype	Exposure (days)	Regenerants (*n*)	Diploids (%)	Mixoploids (%)	Tetraploids (%)
9218	1	23	73.9	17.4	8.7
	2	6	83.3	16.7	0.0
9221	1	16	81.2	18.8	0.0
	2	23	82.6	17.7	0.0
9226	1	38	89.4	5.3	5.3
	2	21	76.2	14.3	9.5
9365	1	14	100.0	0.0	0.0
	2	6	66.6	33.4	0.0
Totals	1	91	85.7	9.9	4.4
Totals	2	56	78.5	17.8	3.3

Polyploidization of microshoots using oryzalin

When oryzalin concentrations of 10–50 μM were used polyploids were successfully induced from microshoots and regeneration of shoots was stimulated (Table 4). This was true for all genotypes, even for genotypes that were adversely affected by high colchicine concentrations. The number of compact shoots increased with increasing oryzalin concentration. For polyploidization of Japanese quince, the most efficient concentration of oryzalin was 30–40 μM. A concentration of 144 μM and higher increased mortality of treated sections, and no polyploid shoots were obtained.

A summary of the procedures for induction of polyploids using colchicine and oryzalin on nodal sections of microshoots is shown in Figure 1.

Polyploidization of cotyledons using colchicine

In excised cotyledons mitotic cells were detected on the third-fourth day after isolation, and on the sixth day dividing cells were observed in all excised cotyledons. Shoot regeneration depended on time and duration of colchicine treatment. Colchicine concentrations between 0.3–0.6% did not differ in efficiency for polyploidization (Table 5). Higher colchicine concentrations inhibited shoot regeneration and therefore the percentage of shoots with a changed chromosome number was considerably reduced.

Table 4. Ploidy of regenerated Japanese quince (*C. japonica*) microshoots. For polyploidization 25 sections of microshoots per treatment were exposed to oryzalin for 1 day in liquid MS medium.

Genotype	Oryzalin (μM)	Regenerants (*n*)	Diploids (%)	Mixoploids (%)	Tetraploids (%)
9218	0	28	100.0	0.0	0.0
	10	72	90.3	5.5	4.2
	20	65	90.8	6.2	3.0
	30	48	83.3	8.3	8.3
	40	51	84.3	13.7	2.0
	50	28	89.3	0.0	10.7
9221	0	32	100.0	0.0	0.0
	10	26	92.3	7.7	0.0
	20	16	87.5	6.2	6.3
	30	38	97.4	2.6	0.0
	40	53	83.0	7.5	9.5
	50	53	88.7	5.6	5.7
9226	0	71	100.0	0.0	0.0
	10	117	93.2	5.1	1.7
	20	102	95.1	3.9	1.0
	30	65	90.7	7.7	1.6
	40	171	95.3	2.3	2.4
	50	58	100.0	0.0	0.0
9365	0	60	100.0	0.0	0.0
	10	130	92.3	3.8	3.9
	20	110	96.4	1.8	1.8
	30	100	89.0	7.0	4.0
	40	118	86.4	10.2	3.4
	50	85	94.1	4.7	1.2

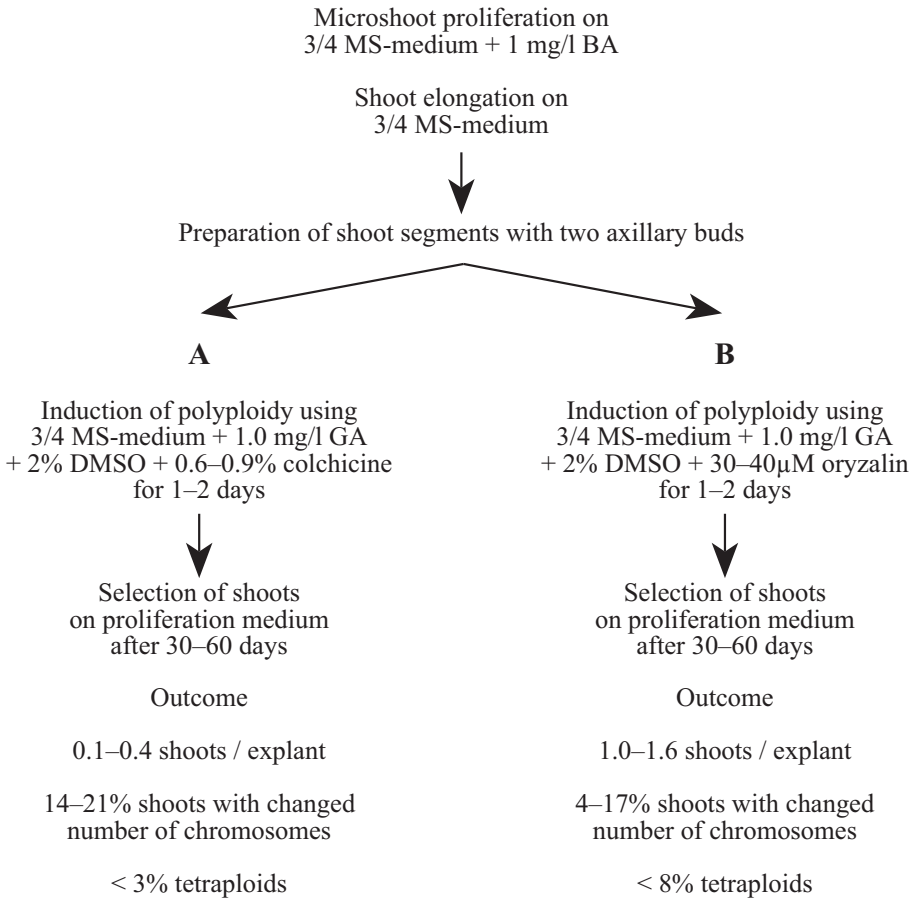

Figure 1. Optimal procedures for induction of polyploids in Japanese quince (*C. japonica*) microshoots using colchicine (A) or oryzalin (B).

The most notable depression in shoot regeneration was observed when cotyledons were treated with colchicine immediately after excision (Table 6). Treating cotyledons between day 4–10 after excision resulted in higher regeneration percentages. Non-chimeric tetraploid shoots were obtained by treating excised cotyledons during the start of the first mitoses. Later, chimeric shoots were obtained. Duration of treatment had little effect on the percentage of regenerated shoots.

Polyploidization of cotyledons using oryzalin

All oryzalin concentrations tested induced shoots with changed chromosome numbers (Table 7). The highest number of tetraploid and mixoploid shoots was obtained using 20–40 µM oryzalin. When the cotyledons were treated with 10 µM oryzalin immediately after excision, the number of regenerants increased compared to the control, and more callus was formed. At oryzalin concentrations of 20–40 µM the number of regenerants and callus development decreased, but the percentage of polyploid plants increased. When oryzalin was applied on the fourth day after excision of cotyledons no shoots could be regenerated, and the rate of callus formation decreased further, except in the 10 µM treatment (result not shown).

The overall number of shoots with changed chromosome numbers obtained from the oryzalin treatments was nearly double in comparison with the colchicine treatments, and more shoots were regenerated in total. A summary of the outcome and efficient procedures for induction of polyploids using colchicine and oryzalin on excised cotyledons is shown in Figure 2.

Table 5. Ploidy of Japanese quince (*C. japonica*) shoots regenerated from excised cotyledons treated with colchicine.

Colchicine (%)	Regenerants (*n*)	Diploids (%)	Mixoploids (%)	Tetraploids (%)
0.0	21	100.0	0.0	0.0
0.3	83	89.2	9.6	1.2
0.6	33	87.8	12.1	0.0

Table 6. Regeneration percentages of Japanese quince (*C. japonica*) shoots obtained from cotyledons treated with 0.6% colchicine at different times after excision (0, 4, 6, 8 and 10 days) and for different periods of exposure (2, 5 and 7 days).

Time (days)	Exp. (days)	Segments (*n*)	Regenerants (%)	Exp. (days)	Segments (*n*)	Regenerants (%)	Exp. (days)	Segments (*n*)	Regenerants (%)
Control	0	20	70.0a	0	20	70.0a	0	20	70.0a
0	2	200	32.0e	5	100	26.0c	7	141	2.8d
4	2	200	42.5e	5	100	61.0b	7	180	34.4c
6	2	199	62.3b	5	100	66.0ab	7	180	32.2c
8	2	195	56.9bc	5	100	59.0b	7	180	36.7bc
10	2	200	55.5c	5	95	67.4a	7	180	41.7b

Table 7. Ploidy level of Japanese quince (*C. japonica*) shoots regenerated from cotyledons treated with oryzalin for 1 day in liquid Nitsch-Nitsch medium immediately after excision.

Oryzalin (µM)	Regenerants (*n*)	Diploids (%)	Mixoploids (%)	Tetraploids (%)
0	21	100.0	0.0	0.0
10	57	96.5	3.5	0.0
20	49	77.6	18.4	4.0
30	46	80.0	15.7	4.3
40	63	74.6	17.5	7.9

DISCUSSION

For Japanese quince tetraploid (and mixoploid) shoots were obtained when excised cotyledons and microshoots were used in the different polyploidization experiments. Both colchicine and oryzalin were successful in inducing a change in the chromosome number. However, colchicine strongly inhibited shoot growth, whereas low concentrations of oryzalin did not apparently affect development of shoots although high concentrations made shoots more compact. For microshoots, the number of regenerants with a changed number of chromosomes was similar using colchicine and oryzalin at optimal concentrations. For excised cotyledons the number of regenerants with a changed number of chromosomes was almost doubled when oryzalin was used compared to when colchicine was used. We presume that this result was due to a higher toxicity of colchicine in combination with the different mechanisms of plant regeneration from microshoots and cotyledons. In experiments with rhododendrons (Väinölä 2000) and haploid apples (Bouvier *et al.* 1994), oryzalin was also more efficient in chromosome doubling than colchicine. In our trials, oryzalin was more efficient for excised cotyledons but not for microshoots. The

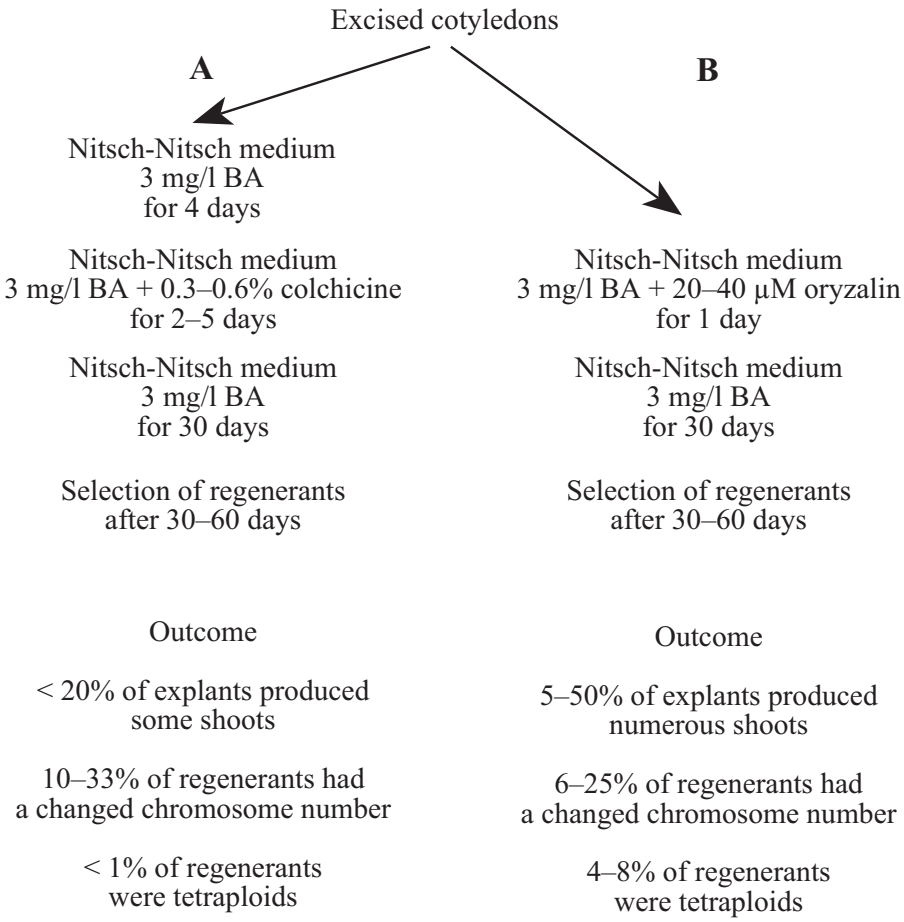

Figure 2. Optimal procedures for induction of polyploids in Japanese quince (*C. japonica*) cotyledons using colchicine (A) and oryzalin (B).

fact that oryzalin affected shoot regeneration *in vitro* less adversely than colchicine makes it useful *e.g.* for polyploidization of Japanese quince genotypes supersensitive to colchicine. In our experiments, as in polyploidization experiments on cocoyam (Tambong *et al.* 1998), even the lowest concentrations of mitotic disrupters were sufficient to induce polyploids. Apparently, any sublethal concentration could result in chromosome doubling. The effect of mitotic disrupters is strongly dependent on the physiological state of the treated tissue and the phase of cell cycle during the treatment. Pre-treatment of microshoots in the cold and dark predisposed the tissue to colchicine, and the mortality of buds increased. The high mortality in combination with high colchicine concentrations can, however, also be considered as an advantage provided that polyploidization is really achieved. A treatment with colchicine decreases the number of regenerants and thus reduces the number of ploidy measurements needed. In experiments with haploid apples (Bouvier *et al.* 1994) and beet (Hansen *et al.* 1998), low concentrations of colchicine and oryzalin have been found to be more efficient in producing tetraploids than high concentrations. According to Roberts *et al.* (1990) this is probably due to the toxic effects of colchicine on mitosis and the decrease in the mitotic index. Colchicine significantly reduced the length and number of regenerated Japanese quince microshoots. This clearly proves the toxic properties of the chemical. For Japanese quince microshoots, colchicine concentrations between 0.6–0.9% and oryzalin concentrations between 20–40µM were the most efficient for polyploidization.

According to Väinölä (2000) polyploid rhododendron shoots emerged more slowly than diploid shoots, and the first vigorously growing shoots were mostly diploids. This also proved to be the case for Japanese quince regenerants and the proportion of tetraploid shoots in flow cytometer measurements increased with time (results not shown). More mixoploids than tetraploids were obtained in every treatment.

Mixoploid regenerants were also obtained from cotyledons that were treated during the beginning of the first mitosis. Thus, already in the very beginning of the cell divisions, the morphogenetic tip of a cotyledon consists of several cells, which because of asynchronic division become differently affected by the mitotic disrupters.

The colchicine dipping method did not result in tetraploid shoots, although Bouvier *et al.* (1994) successfully used the same method for diploidizing haploid apple microshoots. The dipping method did not work with diploid Japanese quince shoot sections, probably because no DMSO was used. Therefore colchicine may not have been able to penetrate the inner cell layers of the buds, and only part of the cells may have become affected and possibly tetraploid. According to Sanford (1983) polyploid cells are eventually displaced by diploid cells if they are present in the same tissue, because diploid cells grow and divide faster than polyploid cells. This is in agreement with our finding that when mixoploid shoots were propagated *in vitro* most shoots remained mixoploid but also tetraploid and an increasing number of diploid shoots were obtained (results not shown).

Tetraploid genotypes with a large genetic variation may in the future be obtained from seedlings derived from controlled crossings among the tetraploid plants developed. By crossing tetraploids and diploids it will also be possible to create triploids that will further expand the range of genetic variation and hopefully result in plant material useful in applied breeding.

CONCLUSION

Procedures were successfully optimized for *in vitro* polyploidization of Japanese quince (*Chaenomeles japonica*) plants. Polyploids of Japanese quince were developed both from microshoots and from excised cotyledons using colchicine and oryzalin. The regenerated tetraploid plants were viable, although slower in growth and development than their diploid counterparts. The use of cotyledons did not eliminate the production of mixoploid and chimeric shoots. A treatment with 0.6–0.9% colchicine or 30–40 μM oryzalin is recommended for induction of polyploids using Japanese quince microshoots, whereas a treatment with 0.3–0.6% colchicine or 20–40 μM oryzalin is recommended for induction of polyploids in excised cotyledons.

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