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Oryzalin-induced chromosome doubling in *Rosa* and its effect on plant morphology and pollen viability

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Abstract Shoot tips of the diploid rose Thérèse Bugnet were treated in vitro to oryzalin at concentrations of 5 and 15 μM. Tetraploid shoots were obtained in highest frequencies (40%) after exposure to 5 μM oryzalin for 14 days. Thin (1 mm) nodal sections were treated with 5 μM oryzalin and the highest frequency of tetraploids (66%) was obtained after exposure for only 1 day. The shorter exposure times required to induce chromosome doubling in thin nodal sections is attributed to the more efficient delivery of oryzalin to the meristem. Tetraploids were obtained from four diploid roses and hexaploids from two triploid roses. Chromosome doubling was accompanied by increases in thickness and a darker green colouration of the leaves and, in all diploid to tetraploid and one triploid to hexaploid conversion, the breadth/length ratio of leaflets was significantly increased. Internodes were longer in tetraploids than diploids but significantly shorter in hexaploids than triploids. The number of petals per flower in the tetraploid form of Thérèse Bugnet was double that of the diploid. Significant increases in pollen viability accompanied chromosome doubling of all four diploids and one of the two triploids.

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Introduction

Chromosome numbers in the genus *Rosa* are based on multiples of seven and range from $2n=2x=14$ to $2n=8x=56$ (Darlington and Wylie1955). Most species are diploid but most modern garden and cut-flower rose cultivars are tetraploid ($2n=4x=28$). Tetraploid cultivars have been crossed with wild diploids, often with the objective of utilising the resistance of wild roses to fungal diseases, but the resulting triploids have low fertility. Chromosome doubling of the triploid roses, which has not previously been achieved, might be expected to produce fertile hexaploids. Chromosomal doubling of diploids to obtain tetraploids that can be bred at the tetraploid level has been achieved by applying colchicine to young seedlings (Fagerlind 1958; Semeniuk and Arisumi 1968; Basye 1992) and to in vitro shoots (Ma et al. 1997). However, chromosome doubling in vegetatively propagated clones has not been achieved in vivo and only with low conversion frequencies in vitro. To achieve improved conversion rates, consideration must be given to the type of spindle inhibitor used, the manner of application and the optimal period of exposure.

Colchicine has been used for chromosome doubling in plants for several decades but causes side effects including chromosome losses, rearrangements and gene mutations (Luckett 1989). Chromosome doubling has been achieved in several genera with oryzalin (3,5-dinitro-N_4,N_4-dipropylsulfanilamide) at much lower concentrations than with colchicine, and oryzalin inhibited poly-

merisation of tubulin from 'Paul's Scarlet' rose at approximately one thousandth of the concentration of colchicine (Morejohn et al.1987). Oryzalin has a much greater affinity for plant than animal microtubules (Hugdahl and Morejohn 1993; Morejohn et al. 1987) and has the important advantage of being less hazardous to human health than colchicine, which is mutagenic.

Roberts et al. (1990) found that it took 10 h for fast-cycling cells of root meristems to complete the cell cycle in the diploid *R. wichurana* but a slightly longer exposure (12 h) to 1.25 mM colchicine was needed to maximise chromosome doubling. However, additional time may be needed for a spindle inhibitor to reach shoot meristems via stem tissues or the ensheathing leaf primordia.

The objectives of this investigation were to develop a protocol for chromosome doubling of in vitro shoot meristems of roses and to assess the fertility and morphology of the polyploids obtained. Oryzalin was used as a spindle inhibitor and its effects on cell division were first assessed in experiments on root meristems of 'New Dawn'. Ploidy levels of shoots that emerged from oryzalin-treated shoot buds were assessed by flow cytometry.

Materials and methods

Plant material

David Austin Roses Ltd, Albrighton, UK supplied plants of the diploid rose cultivars Martin Frobisher, Thérèse Bugnet, Pink Surprise, Mermaid, the triploid hybrid Alister Stella Gray×Abraham Darby and the triploid cultivar New Dawn. These cultivars are described in Cairns (2000).

Tissue culture methods

Nodal explants were introduced into culture, and shoots were multiplied and rooted in Sorbarod plugs using procedures and media similar to those of Horan et al. (1995) except that the multiplication medium contained naphthaleneacetic acid (NAA, 0.5 μM) and 6-benzylamino purine (BAP, 1.5 μM), and rooting medium contained NAA (0.25 μM) and indolebutyric acid (IBA, 0.25 μM). A medium was used to induce shoot elongation that was similar to multiplication medium but contained 1 μM BAP and no NAA. Phytagel (3 g l^{-1}; Sigma-Aldrich, Poole, UK) was added when solidified media were required. Axillary buds were cultured on the medium of Dubois and de Vries (1996). Stock solutions (50 mM) of oryzalin (British Greyhound Chromatography and Allied Chemicals, Birkenhead, UK) were dissolved in dimethyl sulfoxide and appropriate amounts were added to media after they had been autoclaved and cooled to 60°C. Cultures were maintained under high-pressure metal halide lamps (PPFD 60 μmol m^{-2} s^{-1} at the plant surface) on a 16/8 h light/dark cycle at 25±2°C. Cultures in liquid media were maintained on an orbital shaker at 50 rpm.

Effects of oryzalin on cell division in root tips

The roots of in vitro plants were immersed for 1 h in liquid rooting medium containing oryzalin. They were washed in sterile distilled water, laid on semi-solid rooting medium and root tips (10 mm) were sampled at intervals. Root tips were fixed in absolute ethanol: glacial acetic acid (3:1, v/v) for 30 min, hydrolysed in 5 M hydrochloric acid for 10 min at 23°C, then macerated and squashed in lactopropionic orcein (0.8 g l^{-1} orcein dissolved in 22.5 ml lactic acid and 22.5 ml propionic acid, then diluted with 55 ml water). In each sample, mitosis was observed in 250 cells in each of five slides prepared from different root tips and the significance of differences between mitotic indices of different samples was tested using the contingency χ^2 procedure.

Oryzalin treatment of shoot tips

The tips of in vitro multiplied shoots were cultured on shoot elongation medium for 4 weeks. Shoot tips consisting of a terminal bud plus two expanded leaves were excised and exposed to oryzalin for 14, 21 or 28 days by two methods. In the semi-solid medium treatments, 20 shoot tips per treatment were soaked in liquid medium containing oryzalin (5 or 15 μM) for 24 h to raise the concentration of the spindle inhibitor within the plant tissues as rapidly as possible. They were then transferred to semi-solid medium containing oryzalin at the same concentration for the remainder of the exposure period. In the liquid medium treatments, 20 shoot tips per treatment were exposed to oryzalin (5 or 15 μM) in liquid medium for 14 and 28 days. In both methods, treated shoots were subsequently transferred to semi-solid shoot elongation medium without oryzalin and sub-cultured every 4 weeks. Growth rates were then assessed and survival rates recorded.

Oryzalin treatment of nodal sections

In vitro shoots were cultured on solidified shoot elongation medium for six weeks. Leaves were then removed and nodal sections (approx. 1 mm thick) were prepared from the fourth, fifth and sixth nodes below the apex. The nodal sections (30 per treatment) were exposed to 5 μM oryzalin in solidified regeneration medium in Petri dishes for specified periods. The nodes were washed, transferred to fresh regeneration medium, and cultured in darkness for 1 week then under lights. Growth rates were then assessed and survival rates were recorded.

Assessment of the effects of oryzalin on ploidy

Ploidy was investigated in shoots arising from the meristems of axillary or terminal buds that had been exposed to oryzalin. It was established, by micro-dissection, that both in vitro shoot tips and axillary buds of Thérèse Bugnet contained 5–6 leaf primordia. It was expected that ploidy changes in these preformed leaves would be independent of the axillary buds they subtended, whereas in subsequently initiated nodes, the ploidy of a leaf and its axillary bud would be the same because they arose, after treatment, from the same meristem initials. Therefore, treated shoot tips and axillary buds were cultured on shoot elongation medium and only nodes distal to the first six leaves to emerge were investigated. Three adjacent leaves were selected per shoot, the leaves were assessed for ploidy by flow cytometry and the axillary buds they subtended were re-cultured as nodal sections.

Ploidy was assessed by flow cytometry using the same procedures, reagents and cytometer as Yokoya et al. (2000). *Petroselinum crispum* 'Champion Moss Curled' ($2n=2x=22$; 2C DNA amount=4.46 pg) was used as an internal calibration standard and 4′,6-diamidino-2-phenylindole (DAPI) was used as the fluorochrome. *P. crispum* was chosen as the internal calibration standard because its DNA amount was similar to, but distinct from, those of the roses under investigation (Yokoya et al.2000). Nuclei of rose leaves are mostly at the G_1 (pre-replicative) stage of cell division and few are at the G_2 (post-replicative) stage, so that mixoploids can be identified in flow cytometry histograms by the presence of two large peaks.

Assessment of morphology and pollen viability after transfer to soil

The morphology of five plants per genotype was assessed. Measurements of leaves were based on the means of five mid-stem leaves and breadth/width ratios were measured on the terminal leaflets. The significance of differences between means was assessed by the Student's *t*-test.

The viability of pollen was recorded as the proportion of grains in a 9.6×10^{-6} M solution of fluorescein diacetate (Sigma-Aldrich) in an aqueous solution of sucrose (0.4 M) that fluoresced in UV light (Ueda 1994). The proportion of viable pollen was based on 100 pollen grains per plant in five plants per genotype, except that for the hexaploids only 100 grains per genotype was assessed because of a shortage of flowers. Contingency χ^2 tests were used to test the significance of differences between chromosomally doubled and undoubled genotypes.

Results

Effects of oryzalin on cell division in roots of 'New Dawn'

The effects of oryzalin were first assessed in root meristems because it was easier to study cell division in this tissue than in shoot meristems. Cells at anaphase or telophase were absent from all treatments 4 h after the start of the 1 h exposure, indicating complete spindle arrest, but were seen again after 8 h in the treatment with 0.3 μM oryzalin and after 96 h in the treatments with 5 and 15 μM oryzalin (Fig. 1). In the treatments with 5 and 15 μM oryzalin, the mitotic indices rose to levels of more than 7 times the control after 8 h ($P<0.001$) and were maintained at similar levels over the 96 h period of observation. The raised mitotic indices in treated cells indicated that mitosis took longer to complete in the absence of a spindle and that the viability of cells was undiminished by exposure to oryzalin. As treatments with 5 and 15 μM oryzalin gave prolonged mitotic arrest without apparent loss of cell viability, these concentrations were used to induce chromosome doubling in shoots.

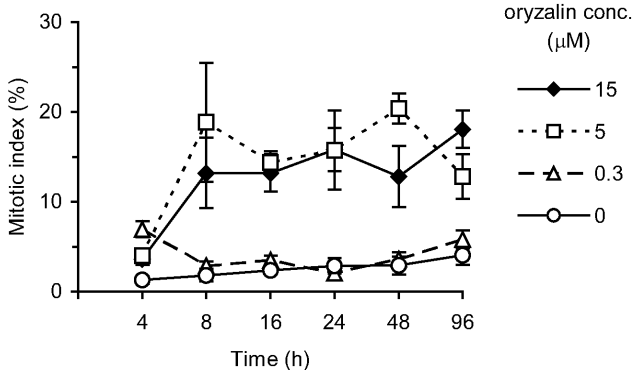

Fig. 1 Mitotic indices of root tips in relation to time from the start of 1 h exposures to oryzalin at concentrations of 0 (control), 0.3, 5, 15 μM. *Bars*=standard errors

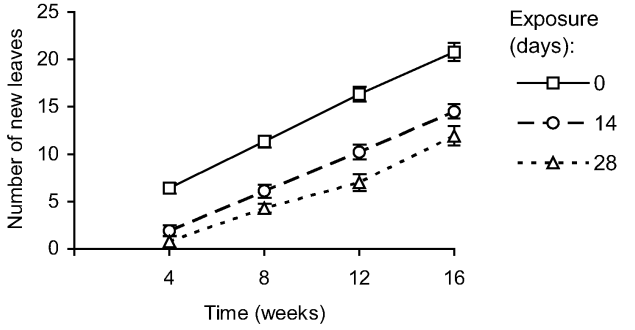

Fig. 2 The cumulative number of new leaves formed per shoot in relation to time after exposure of shoot tips to oryzalin (5 μM) in semi-solid medium for 0 (control), 14 and 28 days. *Bars*=standard errors

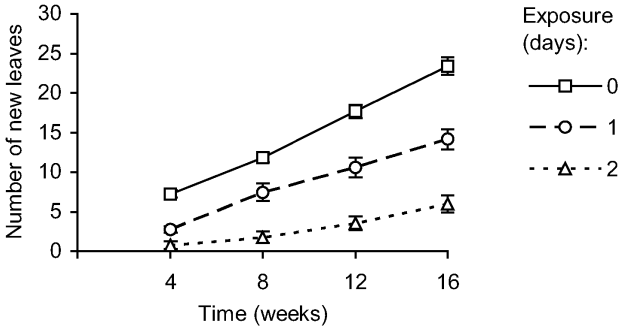

Fig. 3 The cumulative number of new leaves formed per shoot in relation to time after nodes on semi-solid medium were exposed to oryzalin (5 μM) for 0 (control), 1, and 2 days. *Bars*=standard errors

Oryzalin treatment of shoots tips of Thérèse Bugnet

In preliminary experiments, shoot tips exposed to 5 and 15 μM oryzalin for 7 days produced no tetraploids and 48-day exposures were lethal. Thus, in the experiments described below, shoot tips were exposed to oryzalin at these concentrations for 14, 21 and 28 days.

In the semi-solid medium treatments of shoot tips, survival rates decreased from 100% in the control, to 50%, 35% and 20%, respectively, after exposure to 5 μM oryzalin for 14, 21 and 28 days (Table 1). No shoot tips survived treatments with 15 μM oryzalin. The growth of shoots treated with 5 μM oryzalin was initially retarded (Fig. 2) but resumed after 4 weeks. Mixoploid and/or tetraploid leaves were found after exposures for 14, 21 and 28 days and the highest frequency of tetraploid leaves (40%) was observed after a 14-day exposure (Table 1).

In liquid medium treatments of shoot tips, survival rates were higher (Table 1) and growth was not delayed after exposures to 5 μM oryzalin. Mixoploid and/or tetraploid leaves were found after exposures for 14 and 28 days (Table 1) and the frequencies of unchanged (diploid) shoots were similar to those for corresponding exposures in semi-solid medium. The highest frequency of tetraploid leaves (18.8%) was recorded following the 14-day treatment, (Table 1). Unlike the treatments on solid

Table 1 Survival and ploidy of shoot-tips of Thérèse Bugnet (20 per treatment) after exposure to oryzalin in liquid and semi-solid media

Concentration of oryzalin (μM)	Exposure (days)	Number of shoots surviving	Ploidy of leaves (three tested per shoot) (%)		
			2*x*	2*x*/4*x*	4*x*
Semi-solid medium					
5	14	10	40	20	40
	21	7	71.4	28.6	0
	28	10	53.4	26.6	20
Liquid medium					
5	14	16	43.8	37.5	18.8
	28	11	54.5	36.4	9.1
15	14	5	20	60	20
	28	5	100	0	0

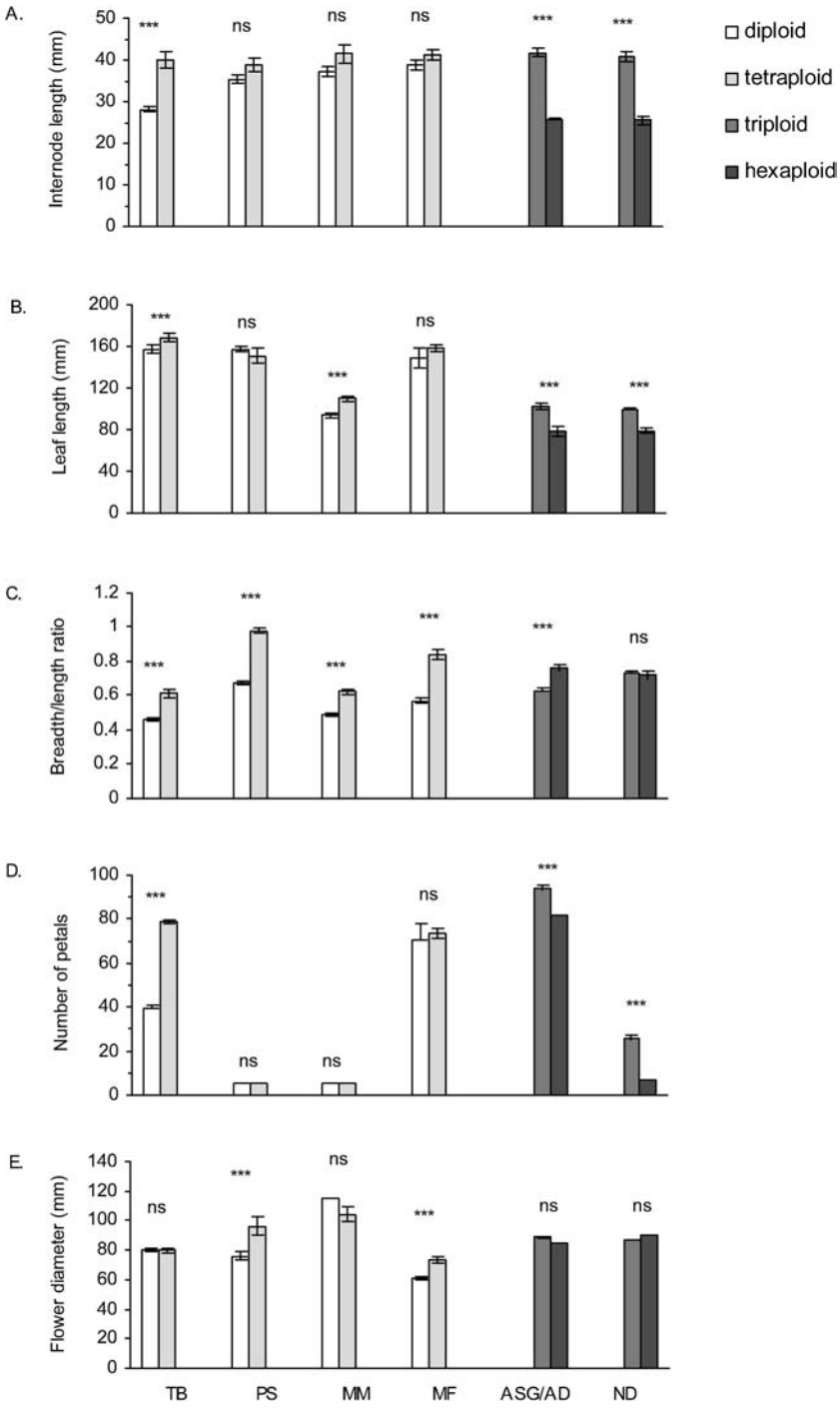

Fig. 4A–E Morphological characters of plants of the original ploidy level and their chromosome-doubled derivatives. **A** Intermode length. **B** Leaf length. **C** Breadth/length ratio. **D** Number of petals. **E** Flower diameter. Abbreviations: *TB* Thérèse Bugnet; *PS* Pink Surprise; *MM* Mermaid; *MF* Martin Frobisher; *ASG/AD* Alister Stella Gray×Abraham Darby; *ND* New Dawn. Abbreviations: *ns* indicates $P>0.05$; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$. *Bars*=standard errors

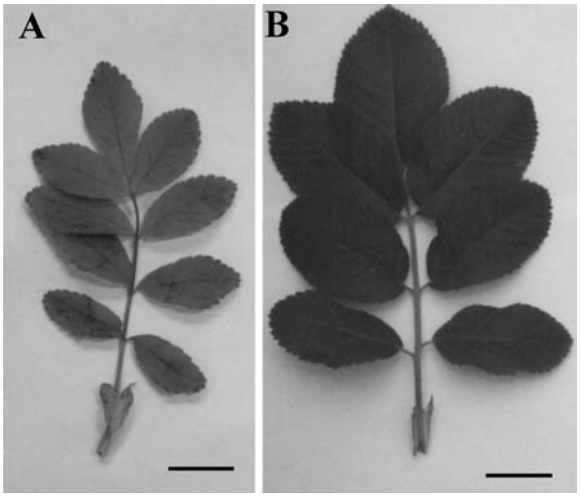

Fig. 5 Leaves of diploid **A** and tetraploid **B** forms of Thérèse Bugnet. *Bars*=40 mm. Note the greater breadth to length ratio and the darker colour of leaflets in the tetraploid form

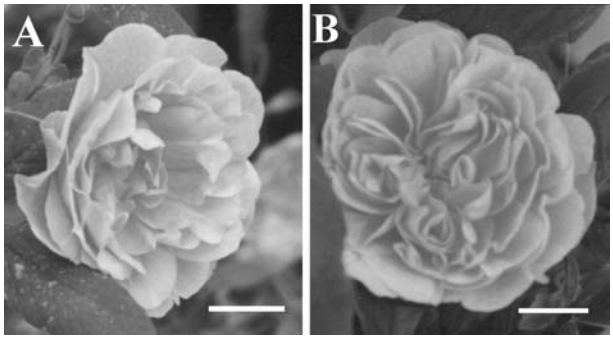

Fig. 6 Flowers of diploid **A** and tetraploid **B** forms of Thérèse Bugnet. *Bars*=20 mm. Note the greater number of petals in the tetraploid form

medium, some plants survived exposure to 15 μM oryzalin in liquid medium and tetraploid leaves were recorded in the 14-day treatment.

After treatments in both semi-solid and liquid media, the nodes subtended by leaves identified as tetraploid were re-cultured. Three adjacent leaves on the resulting shoots were tested for ploidy and were found, without exception, to be tetraploid.

Oryzalin treatment of nodal sections of Thérèse Bugnet

Nodal sections were exposed to 5 μM oryzalin for 0–3 days. The survival rates were 80% in the control (0-day), 20% in the 1-day treatment, 10% in the 2-day treatment and 0% in the 3-day treatment. The growth of shoots from nodes that survived treatments with 5 μM oryzalin was initially retarded but resumed after 4 weeks (Fig. 3). Treatments for 1 and 2 days both resulted in mixoploid and tetraploid leaves. The highest frequency of tetraploid leaves (66.6%) was observed in shoots exposed to oryzalin for 1 day. The nodes of leaves identified as tetraploid were re-cultured. On each of the resulting shoots, three adjacent leaves were tested for ploidy and all were found to be tetraploid.

Morphology and pollen viability of plants after transfer to soil

In addition to Thérèse Bugnet, chromosome doubled plants were obtained from three diploids (‘Martin Frobisher’, ‘Mermaid’ and ‘Pink Surprise’) and two triploids (‘Alister Stella Gray’×‘Abraham Darby’ and ‘New Dawn’) by exposure of shoot tips to 5 μM oryzalin in semi-solid medium for 14–28 days.

After transfer to soil, significant changes associated with chromosome doubling were observed in two or more morphological characters in each of the plants investigated (Fig. 4). The leaves of all chromosome doubled plants were thicker and darker green than those of the original undoubled plants. No other character was significantly changed in all plants although all tetraploids had significantly greater leaf breadth/length ratios than their diploid progenitors (Figs. 4, 5). Some tendencies for morphological change associated with the triploid to hexaploid conversion were the reverse of those in the diploid to tetraploid conversion. For example, internode length was increased in all diploid to tetraploid conversions (although the increase was significant only in Thérèse Bugnet) whereas it was significantly decreased in both of the

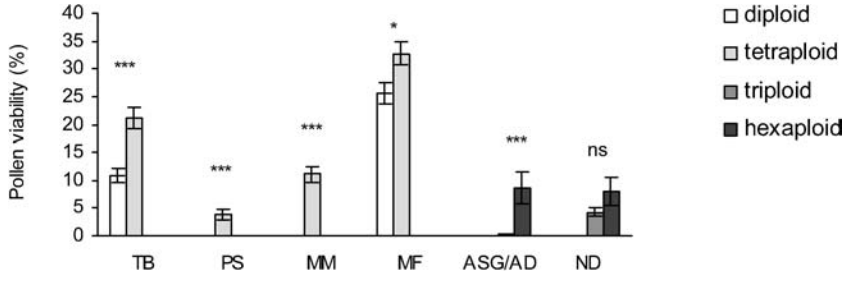

Fig. 7 Pollen viability of plants of the original ploidy level and their chromosome-doubled derivatives. Abbreviations: *TB* Thérèse Bugnet; *PS* Pink Surprise; *MM* Mermaid; *MF* Martin Frobisher; *ASG/AD* Alister Stella Gray×Abraham Darby; *ND* New Dawn. Abbreviations: *ns* indicates $P>0.05$; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$. *Bars*=standard errors

triploid to hexaploid conversions. The number of petals in tetraploid Thérèse Bugnet was nearly double that of its diploid progenitor (Figs. 4, 6) but did not vary significantly with ploidy in the other plants. The hexaploid plants produced flowers in early summer but, in later months and in the next flowering season, very few flowers were produced.

Diploid forms of 'Pink Surprise' and 'Mermaid' produced no viable pollen but the tetraploid forms produced 3.8% and 11.2% viable pollen, respectively (Fig. 7). These tetraploids have been successfully used as pollen parents by David Austin Roses Ltd (personal communication), who raised 1,998 seedlings using pollen of the tetraploid 'Mermaid' and 20 seedlings using pollen of the tetraploid 'Pink Surprise'. The tetraploid form of Thérèse Bugnet and the hexaploid form of 'Alister Stella Gray'×'Abraham Darby' had significantly ($P<0.001$) more viable pollen than the original forms (Fig. 7).

Discussion

Exposure of root meristems to oryzalin at concentrations of 5 and 15 μM for 1 h inhibited mitosis for 48 h or more, apparently without adverse viability effects. However, the survival rates of shoot tips and nodes, which were exposed to oryzalin for longer periods, were inversely related to the period of exposure. Thus the toxicity of oryzalin, whether or not it is a direct consequence of spindle inhibition, is an important consideration when seeking to maximise the efficiency of chromosome doubling protocols.

When shoot tissues of Thérèse Bugnet were exposed to 5 μM oryzalin, tetraploid shoots were obtained in the highest frequency from shoot tips after a 14-day exposure (40%) and in the highest frequency from thin nodal sections after a 1-day exposure (66%). The period of exposure needed for nodal sections is much closer to the optimal time of 12 h needed to induce chromosome doubling with colchicine in root meristems of *R. wichurana* (Roberts et al. 1990) and its short duration indicates that the delivery of oryzalin to the meristem was more efficient than in shoot tips. Likewise, Ma et al. (1997) obtained tetraploids from nodal sections (unspecified length) of diploid roses after exposing them to colchicine for 2 days whereas shoot tips required exposures of at least 5 days and most tetraploids were obtained after exposures of 8–10 days. Access of oryzalin to the meristem via the intact surfaces of the terminal and axillary buds may be prevented by epidermal and cuticular layers that cover the ensheathing leaf primordia and adjacent stem, and the path of least resistance may be via the cut surface of the stem. In the case of the nodal sections (1 mm thick in the present investigation), oryzalin could enter the stem tissues via the cuts above and below the node, and the diffusion path was shorter than the 10-mm path between the single cut at the base of the stem and the apical meristem in shoot tips. The highest frequency of tetraploids obtained in the present study (66% when nodal sections were exposed to 5 μM oryzalin for 1 day), exceeded the best obtained by Ma et al. (1997) in the diploid hybrid *R. wichurana*×*roxburghii* (13% of nodal sections exposed to 2.5 mM colchicine for 2 days). Factors that may have contributed to this improvement include the use of oryzalin rather than colchicine and the use of thinner nodal sections.

Some morphological changes associated with chromosome doubling, including the greater number of petals observed in Thérèse Bugnet, suggest that chromosome doubling could be used to modify plant morphology. However, the main interest lies in the greater fertility of chromosome-doubled hybrids, which will facilitate introgression of genes from wild roses into cultivated roses.

We believe that the hexaploid forms of 'Alister Stella Gray'×'Abraham Darby' and 'New Dawn' represent the first recurrent-flowering cultivars at this ploidy level. Leitch and Bennett (1997) attributed the success of polyploid plants as wild and cultivated species to the combination of genetically different genomes. The hexaploid level of ploidy, in which three genomes are combined, may provide interesting opportunities in rose breeding.

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References

Basye R (1992) The future of the rose. Am Rose Annu 31:62–63
Cairns T (ed) (2000) Modern roses XI. Academic Press, London
Darlington CD, Wylie AP (1955) Chromosome atlas of flowering plants, 2nd edn Allen and Unwin, London
Dubois LAM, de Vries DP (1996) The direct regeneration of adventitious buds on leaf explants of glasshouse-grown cut rose cultivars. Acta Hortic 424:327–329
Fagerlind F (1958) Hip and seed formation in newly formed *Rosa* polyploids. Acta Hortic Berg 17:229–256
Horan I, Walker S, Roberts AV, Mottley J, Simpkins I (1995) Micropropagation of roses: the benefits of pruned mother-plantlets at Stage II and a greenhouse environment at Stage III. J Hortic Sci 70:799–806
Hugdahl JD, Morejohn LC (1993) Rapid and reversible high-affinity binding of the dinitroaniline herbicide oryzalin to tubulin from *Zea mays* L. Plant Physiol 102:725–740
Leitch IJ, Bennett MD, (1997) Polyploidy in angiosperms. Trends Plant Sci 2:470–476
Luckett DJ (1989) Colchicine mutagenesis is associated with substantial heritable variation in cotton. Euphytica 42:177–182
Ma Y, Byrne DH, Chen J (1997) Amphidiploid induction from diploid rose interspecific hybrids. Hortscience 32:292–295
Morejohn LC, Bureau TE, Molè-Bajer J, Bajer AS, Fosket DE (1987) Oryzalin, a dinitroaniline herbicide, binds to plant tubulin and inhibits microtubule polymerization in vitro. Planta 172:252–264
Roberts AV, Lloyd D, Short KC (1990) In vitro procedures for the induction of tetraploidy in a diploid rose. Euphytica 49:33–38
Semeniuk P, Arisumi T (1968) Colchicine-induced tetraploid and cytochimeral roses. Bot Gaz 129:190–193
Ueda Y (1994) Systematic studies in the genus *Rosa*. Techn Bull Fac Hortic, Chiba University, Japan 48:241–328
Yokoya K, Roberts AV, Lewis R, Mottley J, Brandham PE (2000) Nuclear DNA amounts in roses. Ann Bot 85:557–561