Production of triploid *Sandersonia aurantiaca* plants

ER Morgan[1]*, JF Seelye[1], BL Hofmann[1] and JE Grant[2]

[1] *New Zealand Institute for Crop & Food Research Limited, Private Bag 11600, Palmerston North, New Zealand*
[2] *New Zealand Institute for Crop & Food Research Limited, Private Bag 4704, Christchurch, New Zealand*
* Corresponding author, email: morgane@crop.cri.nz

Triploid plants of *Sandersonia aurantiaca* were produced by crossing diploid and tetraploid forms of *S. aurantiaca*. Enlarged ovules were transferred to *in vitro* culture 14–30 days after pollination. The triploid nature of the embryo derived plants was determined by flow cytometry and chromosome counts both of which showed that the triploid plants had features that were midway between those of the two parents. The mean nuclear DNA contents of 2C nuclei from diploid, triploid and tetraploid forms of *S. aurantiaca* were 6.86pg, 10.04pg and 13.55pg, respectively. The nuclear DNA content of 1C nuclei of sperm cells from pollen grains was 2.94pg. Mitotic chromosome counts from the three plants gave 2n = 24, 36 and 48 chromosomes for the diploid, triploid and tetraploid forms, respectively. Meiotic chromosome counts for the diploid and tetraploid plants were n = 12 and n = 24, respectively. The triploid showed mainly bivalents, but lagging chromosomes led to micronuclei and infertility in gametes. The morphological features of the various plants corroborated other evidence indicating that the triploid plants were the result of a cross between diploid and tetraploid plants.

Introduction

Sandersonia aurantiaca is a tuberous plant from the KwaZulu-Natal and Cape Provinces of South Africa. *Sandersonia* is a member of the Colchicaceae family and is related to *Littonia* and *Gloriosa*. In New Zealand it is grown for cut flowers and tubers both of which are exported. *Sandersonia* is a monospecific genus in which little variation has been observed. New forms may have significant commercial value, e.g. a short stemmed form could have potential as a potted plant and a strong stemmed variety would permit winter production of this crop in New Zealand.

Embryo rescue or ovule culture techniques have been used to introduce new traits into a number of flower crop species (e.g. Van Tuyl *et al*. 1990, Kishi *et al*. 1994, Morgan *et al*. 1995, 1998, 2001), by rescuing abortive embryos from wide crosses. Embryo rescue or ovule culture techniques are also used in the production of triploid plants from embryos generated by crossing diploid and tetraploid parents (e.g. Yao and Cohen 1996). Tetraploid *Sandersonia* plants have been investigated for their potential as cut flower crops (Morgan *et al*. 1999, 2002). Embryo rescue techniques were used to develop an inter-generic hybrid between *Sandersonia* and *Littonia modesta* (Morgan *et al*. 2001), which was released as the variety Santonia 'Golden Lights'.

The aims of this work were to produce a triploid form of *Sandersonia aurantiaca* and to compare it to diploid and tetraploid forms. Preliminary studies indicated seeds did not form when diploid and tetraploid plants were crossed, suggesting that ovule culture would be necessary to produce triploid plants.

Materials and Methods

Production of triploid plants

Tubers of diploid and tetraploid forms of *S. aurantiaca* were planted so as to give synchronous flowering in both sets of plants. The potted plants were grown in a glasshouse with a minimum temperature of 15°C and a ventilation temperature of 24°C.

Flowers on the diploid and tetraploid plants used as female parents were emasculated before anther dehiscence. Anthers from the male plants were collected soon after dehiscence and used to pollinate flowers on the female parents with care taken to leave as much pollen as possible on each stigma.

The procedures described by Morgan *et al*. (2001) were used to rescue abortive embryos with flowers being collected for ovule culture 14–30 days after pollination. All cultures were maintained at 24 ± 2°C with a 16h photoperiod. The light intensity of 35–45μmol m^{-2} s^{-1} was provided by cool white fluorescent tubes.

After the plants had been established in the greenhouse they were propagated by tuber division and grown using current commercial techniques established for diploid plants.

Characterisation of the ovule derived plants

Various features of the diploid, triploid and tetraploid plants, including plant form, flower colour, and guard cell and pollen grain lengths of greenhouse-grown plants were compared. Comparative measurements were made of nuclear DNA contents determined using mitotic and meiotic chromosome counts, and flow cytometry. Observations of plant form and size were made over several years to ensure that there were no effects related to tuber size.

Flower colour was measured with a Chroma Meter (Minolta CR-200) using the procedure described by Nielsen *et al*. (2003). Five mature flowers (at anther dehiscence) were measured from diploid, triploid and tetraploid plants. These data were analysed using the Kruskal-Wallis one way analysis of variance (Conover 1971).

The lengths of guard cells on the lower surfaces of fully expanded mature leaves were measured. Epidermal peels were prepared and the lengths of guard cells measured directly. Pollen grains were prepared by tapping dehiscent anthers on to a microscope slide and then adding Alexander stain (Alexander 1969). Observations were made of pollen grain length and staining. Tetraploid plants were self pollinated to check seed production as no enlarged ovules were observed after pollination with pollen from a diploid parent.

Root tips and flower buds for chromosome counts were harvested from glasshouse-grown plants as described by Morgan *et al*. (2001).

The protocol for flow cytometric determination of nuclear DNA contents of diploid, tetraploid and putative triploid plants used here was that described by Morgan *et al*. (2001). Greenhouse-grown *Hordeum vulgare* cv. Sultan was used as an internal standard and the nuclear DNA content of these plants was taken to be 11.12pg DNA per 2C nucleus (Bennett and Smith 1991).

It was difficult to obtain consistent meiotic chromosome counts for the putative triploid plants and flow cytometry was used in an attempt to verify the spread of chromosome numbers observed in the manual counts of the triploid plants. Pollen was collected from anthers of diploid, tetraploid and putative triploid plants for germination at 22°C in a liquid medium comprising sucrose (10% w/v) and boric acid (0.1% w/v). Ten dehiscent anthers were added to 10ml of pollen germination medium to allow the pollen to dissociate from the dehiscent anthers. The pollen was incubated on an orbital shaker (90rpm) overnight to allow time for pollen tube growth. Pollen tubes were lysed by adding excess ice cold Galbraith's buffer (Galbraith *et al.* 1983) to the germination medium followed by rapid syringing through a pipette and filtration through 32µm steel mesh. Nuclei were recovered by centrifugation (400x*g*, 7min) and treated as described by Morgan *et al.* (2001) except that nuclei from the internal standard were chopped and then added to the pollen nuclei at the time of staining.

Flow cytometry measurements were repeated three times for each plant and for the diploid and triploid pollen types.

Results and Discussion

Three enlarged ovules were transferred into *in vitro* culture from the diploid female parent. No enlarged ovules or seed were found on the tetraploid female parent after either self pollination or pollination with a diploid male parent. Many embryos produced by interspecific or inter-ploidy crossing are thought to perish because the endosperm fails to develop. Histological studies of seed development in *S. aurantiaca* revealed that about 28 days after pollination a clear area appeared in the endosperm adjacent to the embryo, which was interpreted as the remains of digested endosperm (Zou *et al.* 2000). It was therefore likely that embryos from the cross between the diploid and tetraploid plants would not develop beyond 28 to 30 days after pollination, and embryo culture before this time would be necessary to produce triploid plants.

The ovules grew a smooth callus-like growth from which shoots eventually developed. The callus could take two or three months to develop. Tissue taken from this callus and analysed using the flow cytometer provided the first evidence for the triploid nature of the ovule culture derived plant.

The time for shoot production from the callus was variable and it could take 8–10 months. A shoot usually produced three to five leaves before becoming senescent, leaving a small tuber at its base. These tubers were quiescent or dormant and would sprout after transfer to fresh media but the time for this was also quite variable. The behaviour of the ovule culture derived plants was very similar to that described by Morgan *et al*. (2001) for *Sandersonia* x *Littonia* hybrids.

The triploid plants were very vigorous and almost always grew taller than the diploid plants when grown from similar sized tubers. The tetraploid plants were smaller than the diploid and triploid plants (Figure 1) and were much less vigorous. They produced fewer flowers and these flowers were often distorted and less attractive than those of the diploid and triploid plants.

Flowers of the polyploid plants appeared to be the same colour, though brighter, than those of the diploid plants. No significant differences were detected in L values between the diploid, triploid or tetraploid flowers. A significant difference was detected in the chroma values between the diploid and tetraploid flowers but not between the diploid and triploid, or triploid and tetraploid flowers (P = 0.031). There was no significant difference in hue angle measurements between the triploid (67.12) and tetraploid (67.08) flowers which were significantly different from the diploid (70.96) flowers (P = 0.028).

Chromosome numbers of 2n = 24, 2n = 36 and 2n = 48 were found for the diploid, triploid and tetraploid plants, respectively, with mitotic chromosome counts from the root tips. For the meiotic pairing analysis, the diploid *Sandersonia* gave the expected 12 bivalents, and the tetraploid gave 0.25IV, 23.25II and 0.5I. However the triploid was 17.36II and 0.68I. Cells were seen that had less than the expected 36 chromosomes and these had either 16 or 17 bivalents with no univalents. At anaphase I there was clearly unequal separation of the chromosomes (Figure 2). Figure 2c shows anaphase I where there are cells showing variation in the number of lagging chromosomes. The resulting gametes would lead to infertile pollen grains and the lag-

Figure 1: Left to right, diploid, triploid and tetraploid plants of *Sandersonia aurantiaca*

Figure 2: Chromosomes from pollen mother cell meiosis of diploid and triploid *Sandersonia aurantiaca.* (a) metaphase I in the diploid showing 12 bivalents; (b) metaphase I in the triploid showing 18 bivalents; (c) anaphase I in the triploid showing cells with different numbers of lagging chromosomes

ging chromosomes could possibly form micronuclei. At anaphase II, gametes with chromosome numbers between 15–19 were observed, confirming the unequal division in meiosis.

The average length of guard cells from the diploid plant was (mean ± SE) 0.49 ± 0.003µm (n = 62), for the triploid plant it was 0.57 ± 0.004µm (n = 62), and for the tetraploid it was 0.7 ± 0.005µm (n = 62). These results are similar to reported comparisons between diploid and tetraploid plants of other genera, e.g. *Zantedeschia* (Cohen and Yao 1996) or *Gentiana* (Morgan *et al.* 2003). Pollen grain length of a diploid plant averaged 0.3µm (n = 40) with an average staining percentage of 97% (n = 464). Pollen grain length for the tetraploid plant was 0.4µm (n = 40) with an average staining percentage of 62% (n = 670). For the triploid plant, pollen grain length averaged 0.35µm (n = 40) with 55% of pollen grains stained (n = 398). When Griesbach and Bhat (1990) compared pollen grain diameters between diploid and tetraploid *Eustoma grandiflorum* plants they observed the pollen grains were larger in tetraploid plants and that pollen viability was reduced by 80%. No seed production was observed on self pollinated tetraploid *Sandersonia* plants compared with 2% seed set in tetraploid *Eustoma grandiflorum* (Griesbach and Bhat 1990).

Sufficient numbers of nuclei for flow cytometry were readily extracted using the techniques described by Morgan *et al.* (2001). Outlines of histograms from these measurements are presented in Figure 3. The mean 2C nuclear DNA contents of nuclei from diploid (Figure 3b), triploid (Figure 3c) and tetraploid *Sandersonia* (Figure 3d) plants were 6.86pg, 10.04pg and 13.55pg DNA per 2C nucleus, respectively. There was little difficulty resolving the peak of the standard (Sultan barley at 11.12pg DNA per 2C nucleus) from that of the triploid *Sandersonia* at 10.04pg DNA per 2C nucleus (Figure 3c). Obtaining measurements of the nuclear DNA content of vegetative cell and sperm cell nuclei from the disrupted pollen tubes of the diploid plants was also straightforward (Figure 3a). The nuclear DNA content of the sperm
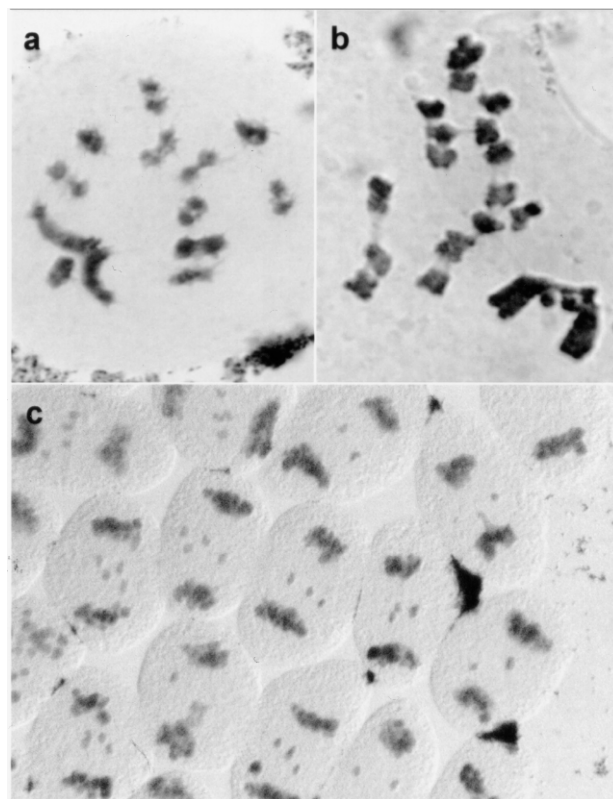
cell nuclei was 2.9pg DNA per 1C nucleus and that of the vegetative nuclei was measured at 6.53pg DNA per 2C nucleus, which is comparable with the 6.86pg recorded above for the diploid leaf tissue. The diploid nuclear DNA content recorded for the vegetative nucleus is attributed to either a large proportion of these cells undergoing a mitotic division in the pollen tube or to the meiotic division of these nuclei occurring as the nuclei passed down the pollen tube. This was not investigated further. There was not enough pollen from the triploid plant to provide sufficient numbers of nuclei for any conclusions about the nuclear DNA contents of these pollen grains to be reached. It would be interesting to repeat this experiment attempting to verify the irregular meiotic chromosome count results using flow cytometry. However, the analysis should be carried out using intact anthers at the time the pollen mother cells would be undergoing meiosis since the nuclei may not have broken down as they appear to when the pollen grains are allowed to mature.

That the plants obtained from ovule culture are embryo derived is confirmed by the triploid nature of these plants. Had they been derived from endosperm they would have been tetraploid (a diploid sperm nucleus fusing with two haploid polar nuclei to give a tetraploid chromosome complement) and if derived from maternal tissue they would have been diploid.

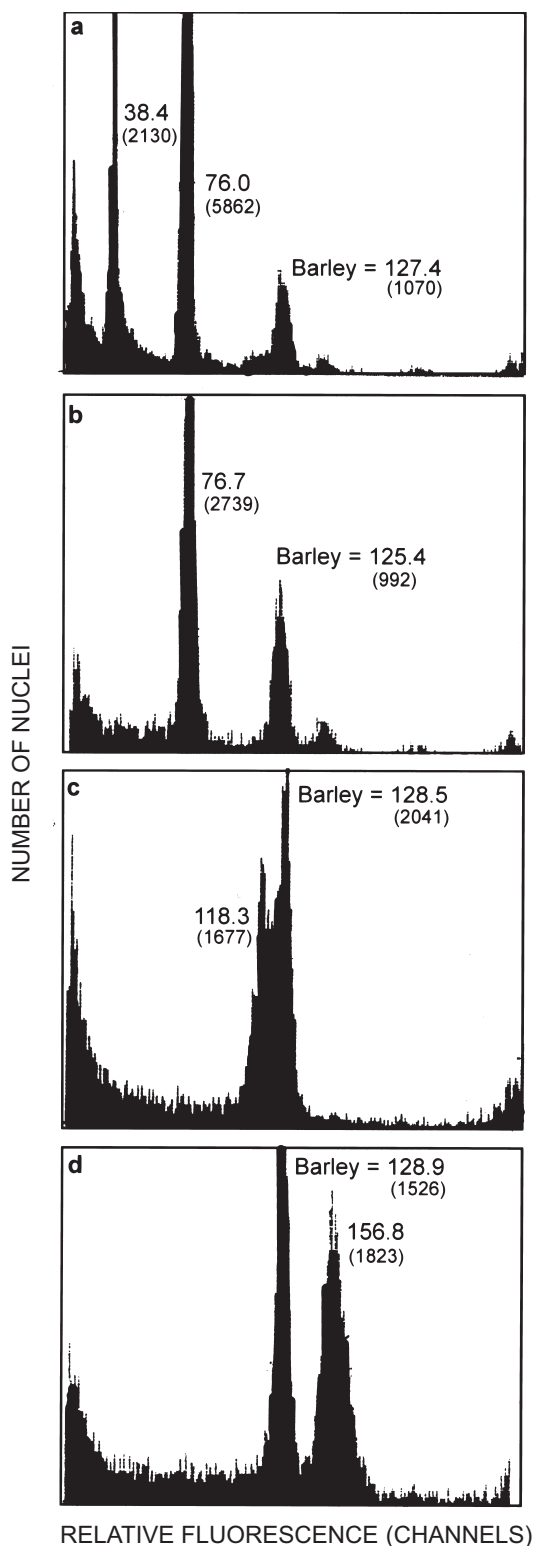In conclusion, triploid plants of *Sandersonia aurantiaca*

Figure 3: Histograms of linear amplified fluorescence (channel number) of nuclei from pollen (a), and diploid (b), triploid (c) and tetraploid (d) plants of *Sandersonia aurantiaca*. The mean 2C channel numbers of the internal standard (barley) are given on each histogram with the number of observations in the peak bracketed. The mean channel numbers for the various *Sandersonia* samples and the number of measurements comprising each peak are also provided

were produced by crossing diploid and tetraploid plants. Nuclear DNA contents, chromosome numbers and guard cell lengths of the triploid plants were all intermediate between those of the diploid and tetraploid plants. The short stature of the tetraploid plant may mean that it is of use as a potted plant and the strong stems of the triploid plants confer advantages for winter production in New Zealand greenhouses. Unfortunately, the very poor fertility observed in both triploid and tetraploid plants means that seed propagation is not possible and the costs of vegetative propagation would be prohibitive.

References

Alexander MP (1969) Differential staining of aborted and non-aborted pollen. Stain Technology **44**: 117–122

Bennett MD, Smith JB (1991) Nuclear DNA amounts in angiosperms. Philosophical Transactions of the Royal Society of London, Series B, Biological Sciences **334**: 309–345

Cohen D, Yao J-L (1996) *In vitro* chromosome doubling of nine *Zantedeschia* cultivars. Plant Cell, Tissue and Organ Culture **47**: 43–49

Conover WJ (1971) Practical non-parametric statistics. John Wiley and Sons, New York, pp 229–231

Galbraith DW, Harkins KR, Maddox JM, Ayres NM, Sharma DP, Firoozabady E (1983) Rapid flow cytometric analysis of the cell cycle in intact plant tissues. Science **220**: 1049–1051

Griesbach RJ, Bhat RN (1990) Colchicine-induced polyploidy in *Eustoma grandiflorum*. Hortscience **25**: 1284–1286

Kishi F, Kagami Y, Shinohara M, Hatano S, Tsurushima H (1994) Production of an interspecific hybrid in *Gypsophila* by ovule-embryo culture. Euphytica **74**: 85–90

Morgan ER, Burge GK, Seelye JF, Grant JE, Hopping ME (1995) Interspecific hybridisation between *Limonium perigrinum* Bergius and *Limonium purpuratum* L. Euphytica **83**: 215–224

Morgan ER, Burge GK, Seelye JF, Hopping ME, Grant JE (1998) Production of interspecific hybrids between *Limonium perezii* (Stapf) Hubb. and *Limonium sinuatum* (L.) Mill. Euphytica **102**: 109–115

Morgan ER, Burge GK, Seelye JF, Hopping ME, Grant JE (1999) Applying *in vitro* technologies to produce 'new' ornamental plants. In: Johnson KA, McFarlane IJ (eds) Proceedings of the Australian Branch of IAPTC&B Conference on Plant Tissue Culture at the Edge of the New Millennium

Morgan ER, Burge GK, Seelye JF (2002) *Sandersonia*: Towards the new generation. Acta Horticulturae **570**: 87–90

Morgan ER, Burge GK, Seelye JF, Hopping ME, Grant JE, Warren AGF, Brundell DJ (2001) Wide crosses in the Colchicaceae: *Sandersonia aurantiaca* (Hook.) x *Littonia modesta* (Hook.). Euphytica **121**: 343–348

Morgan ER, Hofmann BL, Grant JE (2003) Production of tetraploid *Gentiana triflora* var *japonica* 'Royal Blue' plants. New Zealand Journal of Crop and Horticultural Science (in press)

Nielsen KM, Lewis DH, Morgan ER (2003) Characterisation of carotenoid pigments and their biosynthesis in two yellow flowered lines of *Sandersonia aurantiaca* (Hook.). Euphytica **130**: 25–34

Van Tuyl JM, Van-Sande K, Van-Dien R, Straathof D (1990) Overcoming interspecific crossing barriers in *Lilium* by ovary and

embryo culture. Acta Horticulturae **266**: 317–322

Yao J-L, Cohen D (1996) Production of triploid *Zantedeschia* hybrids using embryo rescue. New Zealand Journal of Crop and Horticultural Science **24**: 297–301

Zou X, Fountain DW, Morgan ER (2000) Anatomical and morphological studies of seed development in *Sandersonia aurantiaca* (Hook.). South African Journal of Botany **67**: 183–192