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Comparison of Diploid *Swainsona formosa* and Their Tetraploid Relatives Obtained from Oryzalin Treatment

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Individu tetraploid berhasil diregenerasikan melalui penggandaan kromosom pada bibit *Swainsona formosa* menggunakan senyawa antimitosis, oryzalin (3,5-dinitro-N^4,N^4-dipropylsulfanilamide). Oryzalin dengan konsentrasi 0,0025 – 0,005% terbukti efektif menggandakan jumlah kromosom pada *S. formosa* yang ditumbuhkan dan dipelihara di rumah kaca. *S. formosa* tetraploid memiliki pertumbuhan yang lebih besar dan lebih kokoh dari pada kerabat diploidnya. Di samping itu, daun-daun tanaman tetraploid juga lebih lebar dibandingkan tanaman diploid. Diameter stomata tanaman tetraploid lebih besar namun kerapatan stomatanya per unit luas daun lebih rendah. Secara umum kualitas bunga mengalami peningkatan yang ditunjukkan oleh ukuran bunga yang lebih besar dengan tangkai yang lebih panjang pada individu tetraploid. *S. formosa* tetraploid juga memiliki serbuk sari yang lebih besar dari pada tanaman diploid, namun viabilitasnya lebih rendah sehingga menghasilkan lebih sedikit biji per polong.

INTRODUCTION

Swainsona formosa is one of Australia's native plants, and is one of the world's most spectacular flowering plants. The outstanding feature of *S. formosa* is its brilliant-coloured flowers, from white or pink through to dark red standard and keel with or without a distinctive boss. The great potential of *S. formosa* is as a container-grown ornamental, either in a flowering pot or hanging basket, or as a cut flower. However, the commercialisation of *S. formosa* as a cut flower is subject to a number of limitations such as colour variations, stem length, number of blooms per cluster and the production of large amounts of pollen in the flowers.

Research on the breeding of *S. formosa* has been conducted with the emphasis on the production of tetraploid plants for better flower quality. An antimitotic chemical, oryzalin, has been used to induce autotetraploid development.

Oryzalin or 3,5-dinitro-N^4,N^4-dipropylsulfanilamide, which was previously developed as a herbicide (Morejohn *et al.* 1987) has been reported to inhibit mitotic activity and proved to be useful to induce chromosome doubling in many plant species such as *Z. mays*, *Malus domestica*, *G. jamesonii*, *Actinidia deliciosa* and *Lilium longiflorum* (Morejohn *et al.* 1987; Wan *et al.* 1991; Bouvier *et al.* 1994; Tosca *et al.* 1995; Chalak & Legave 1996; Takamura *et al.* 2002). In *Nicotiana plumbaginifolia*, Verhoeven *et al.* (1990) reported that the inhibition of spindle formation was stronger with oryzalin than with colchicine, which resulted in a more efficient accumulation of metaphases with well-scattered chromosomes. Oryzalin proved to be a more efficient chromosome-doubling agent than colchicine in potato cell suspension culture (Ramulu *et al.* 1991). Following from this, Wan *et al.* (1991) apparently did not make a direct comparison between oryzalin and colchicine, but claimed that oryzalin very effectively induced chromosome doubling in anther-derived callus of maize but severely inhibited the growth of regenerable callus and plant regeneration.

This study attempted to induce chromosome doubling in *S. formosa* using oryzalin. The flowers of treated plants, upon pollination and fertilisation, produce seeds that are expected to grow into tetraploid plants. This study also compared the growth characteristics of diploid against their tetraploid relatives.

MATERIALS AND METHODS

Plant materials ($2n = 2x = 16$) were raised from seeds and routinely grown in a temperate glasshouse. Voucher specimen NE 79130 was lodged in the NCW Beadle Herbarium, University of New England, Australia. Oryzalin stock was prepared by dissolving 0.1 g oryzalin into 10 mL DMSO giving the concentration of 1%. The solution was then diluted in distilled water to final concentrations of 0, 0.001, 0.0025 and 0.005% plus 2% (v/v) of DMSO.

Two-week old seedlings with fully developed cotyledons were treated with oryzalin. One drop of oryzalin was applied onto the apical meristem between the two cotyledons. Seedlings were kept in dark conditions for 24 hours to avoid the destructive effect of light on the oryzalin. The humidity was kept as high as possible as drying increases the concentration and treated plants may die. Seedlings were then transferred back into the shaded glasshouse. Intermittent mist spray in the glasshouse was used to wash off the excess of the chemicals. Two weeks later, the seedlings were transferred to larger pots in a temperate glasshouse for further growth and development.

Leaf Area and Stomatal Characteristics. Leaf area and stomatal characteristics (size and density) were used as initial parameters to assess the ploidy level of treated plants. Presumed tetraploids were examined for their chromosome numbers in the microspore mother cells.

Leaflet area was measured using a leaf area meter, model Delta-T. As this observation used a destructive sampling method, only ten mature leaflets were taken from each replicate resulting in a total of 100 leaflets for each treatment. For uniformity of the treatment, all plants had entered the reproductive period and the third flower was fully opened. At this stage the leaflets had already reached their maximum size. In addition, all leaflets were taken from the fourth or fifth node on the central stem and the axillary branches growing from the base.

Mature leaflets were used for stomatal evaluations. For the uniformity, only the terminal leaflet was used. As this was a destructive sampling method, three leaflets were sampled from each plant. All leaflets were taken from leaves arising at the fourth or fifth node on the central stem and axillary branches. Samples to be evaluated were taken from the vein-free epidermal layer in the middle section of the abaxial side (lower surface) of the leaflet. To avoid variation due to environmental changes and to ensure the stomata were open to their maximum aperture, stomatal sampling was undertaken between 9.00 and 10.00 a.m.

A thin layer of a clear nail polish was applied to the abaxial surface of the leaflets and left to dry. After several minutes, the leaflets were removed from the plant and placed between two moistened filter papers in a closed Petri dish. The samples were kept at 4^{o}C to keep them fresh before use on the same day.

By using forceps, the nail polish along with a layer of thin epidermis was peeled off and placed on a clean microscope slide. Observations were made with a light microscope at a magnification of 400x and the size and density of stomata were compared between non-treated and treated plants. Ten stomata from each leaflet were examined to obtain an average measurement.

For stomatal density, five places in the middle region of the abaxial surface of the leaflets were examined. The frequency of stomata was estimated under a magnification of 100x.

Other Characteristics. Having determined the tetraploid plants, the following parameters were examined: leaflet number per leaf, flower length, flag and keel widths, peduncle length, pollen diameter and viability, and number of seeds per pod. These characteristics were assessed on those tetraploids that resulted from the treatments and on the control plants.

Total leaflet number per leaf was calculated when the plants had reached flowering. At this stage the plants had developed the maximum number of leaflets on every leaf. For uniformity, only those leaves located under the first inflorescence were used as samples. Five leaves, starting below the inflorescence, were sampled down each of central stem and similarly down each axillary branches. The average number of leaflets from these 15 leaves (obtained from 3 stems) was then calculated.

For the assessment of floral characteristics, ten inflorescences from each plant were sampled by taking 4 umbels from the central stem and 3 umbels from each of the axillary branches. The umbels were sampled from the first inflorescence upwards until the required number of umbels was obtained. All umbels were isolated from the plants one day after anthesis to ensure that the flowers have reached their maximum size. The measurement of flower length was made from the tip of the flag petal through to the tip of the keel petal. The width of the flag petal was measured on the widest part from left to right. Similarly, the keel width of the same flower was also measured. Keel width was taken on the widest part of the keel, approximately 2/3 of the distance from the tip of the keel. At the same time as the floral features were measured, the length of peduncle was measured from the junction with the stem through to the junction with the pedicels.

Newly released pollen grains were collected from flowers at the time of anther dehiscence. Pollen grains were stained with one drop of 1% aceto-orcein on a microscope slide, covered with a cover glass (No. 1 thickness) and examined with a light microscope at a magnification of 400x. Pollen grains were sampled from five different inflorescences (5 – 7 flowers) of each plant. In order to obtain a reliable measurement, ten pollen grains were measured for each flower. A total of 250 - 350 pollen grains were examined from each plant, depending on the number of flowers. The viability of pollen was assessed using the same techniques as described by Prakash (Prakash 2000).

Both tetraploids and diploids were self-pollinated. All pods formed on each plant of each treatment were harvested and the number of seeds within each pod was counted. Only normal seeds were counted with abnormal seeds, which are smaller, thinner and whitish in colour, not included.

RESULTS

Rate of Conversion. The results showed that oryzalin, when applied to seedlings, effectively induced tetraploidysation in S. formosa. The response, however, was selectively dependent upon the concentration of the treatment (Table 1). Higher frequencies of tetraploid induction were seen with 0.0025 – 0.005% oryzalin. Oryzalin concentration lower than 0.0025% was not effective.

Table 1. Effects of oryzalin on the induction of tetraploids from seedling of *S. formosa.*

Oryzalin (%)	No. of seedlings	No. of plants	% of plants with ploidy	
	treated	examined	Diploid	Tetraploid
0	10	10	100	0
0.001	10	10	70	30
0.0025	10	10	60	40
0.005	10	10	60	40

Chromosome counts in the microspore mother cells, which showed $2n = 4x = 32$, confirmed the determination of tetraploids using morphological indicators.

Leaflet Area and Leaflet Number Per Leaf. The area of tetraploid leaflets was significantly larger than the leaflet of diploid leaves. However, the number of leaflets per leaf was not significantly different between tetraploids

and diploids. Table 2 shows the comparison of leaflet area and leaflet number per leaf between diploids and tetraploids resulted from oryzalin treatment.

This study showed that in addition to a significantly larger area, leaflets of tetraploids were also thicker than leaflets in diploids. Histological examination showed less spongy parenchyma in diploid leaflets (Figure 2A) than in the tetraploid leaflets (Figure 2B), resulting in thinner leaflets.

Table 2. Leaflet area and leaflet number per leaf between diploid and tetraploid *S. formosa.*

Ploidy level	Leaflet area	Leaflet number per leaf
Diploid	673.99 ± 35.41	12.13 ± 0.31
Tetraploid	1443.38 ± 33.76	12.41 ± 0.29

Comparing the tetraploid and diploid leaf, both had the rachis much the same length, but because the tetraploid leaflets were larger in area, they overlapped on the leaf (Figure 3A) compared to diploid leaflets (Figure 3B).

Stomatal characteristics. Subsequent analysis revealed that the induced tetraploids had a significantly lower stomatal density per unit area than diploids. In addition, stomatal size in the tetraploids was significantly larger than in the diploids (Table 3 and Figure 5).

Table 3. Aperture length and stomatal density between leaflets of diploid and tetraploid *S. formosa*.

Ploidy level	Aperture length (μm)	Stomatal density per mm^2
Diploid	23.45 ± 0.45	116.66 ± 2.10
Tetraploid	36.39 ± 0.43	38.49 ± 2.00

Flower characteristics. The difference in flower length, keel width, flag width and peduncle length are shown in Table 4. The keel and flag width, as well as the peduncle length were larger in tetraploids than in diploids. The flower length, however, did not show a significant difference between tetraploids and diploids. In general it can be said that tetraploydisation effectively improved the flower quality of *S. formosa* as indicated by the wider flag and keel as well as the longer peduncle, compared to the diploid flowers (Figure 4).

Table 4. Flower length, keel width, flag width and peduncle length between diploid and tetraploid *S. formosa*.

Ploidy level	Flower length (mm)	Keel width (mm)	Flag width (mm)	Peduncle length (mm)
Diploid	85.98±0.24	32.61±0.13	32.78±0.20	125.75±1.24
Tetraploid	86.11±0.22	37.95±0.13	42.68±0.19	150.14±1.19

Pollen size and viability and number of seed per pod. Pollen diameter in tetraploids was larger than in diploids. However, the induced tetraploids had lower pollen viability. As a consequence, self-pollinated tetraploids produced fewer normal seeds per pod compared to diploids (Table 5).

Table 5. Diameter and viability of pollen and seed number per pod of diploid and tetraploid *S. formosa.*

Ploidy level	Pollen diameter (μm)	Pollen viability (%)	Normal seeds per pod (%)
Diploid	21.48 ± 0.24	65.16 ± 0.67	98.95 ± 0.52
Tetraploid	28.45 ± 0.23	47.51 ± 0.64	53.91 ± 0.50

DISCUSSION

Significant morphological differences exist between diploid and tetraploid *S. formosa.* In general, tetraploid plants are bigger and stouter than diploid plants (Figure 6). This is similar to the observation of Griesbach (1990) on tetraploid *Anigozanthos* hybrid produced by colchicine treatment. Furthermore, the status of ploidy level of *S. formosa* correlates positively with stomatal size; the higher the chromosome numbers the larger the stomatal size. Meanwhile, a negative correlation is found with stomata density; the higher the chromosome numbers the fewer stomata per unit area. Therefore, stomatal size and density are useful indicators to distinguish diploids from tetraploids resulted from colchicine and oryzalin treatments. Proportional relationships between ploidy level and stomatal size and density are also found in *Bromus inermis* (Tan & Dunn 1973), *Hordeum vulgare* (Borrino & Powell 1988), *C. persicum* (Takamura & Miyajima 1996) and *Musa* spp. (Hamill *et al.* 1992; Azhar *et al.* 2002). Stomatal characteristics have been used effectively to distinguish tetraploids from diploids in these species.

The differences between tetraploids and diploid in flower size shown in Table 4 indicate that the flower lengths of the tetraploids are not significantly different from those of the diploids. However, the widths of the keel and flag petals in tetraploids are larger than in diploids. Similarly, Takamura and Miyajima (1996) reported that the petal size in tetraploid *C. persicum* was larger than in diploid plants. Larger flower size was also reported by Griesbach (1990) on tetraploid *Anigozanthos* hybrid produced by colchicine treatment.

A positive relationship exists between pollen diameter and ploidy level in *S. formosa.* As with *B. inermis* (Tan & Dunn 1973), *Musa* spp. (Tenkouano *et al.* 1998) and *C. persicum* (Takamura & Miyajima 1996) tetraploidysation of *S. formosa* results in larger pollen size than in diploid plants. However, the viability of tetraploid pollen is significantly lower than that of diploid pollen. The results of this research confirmed the negative relationship between ploidy level and pollen viability as reported by Takamura and Miyajima (1996) in *C. persicum*.

The techniques reported here produced tetraploids quickly and simply, and should find applications in the breeding programmes of *S. formosa*. It is clear that oryzalin may be used to induce polyploidysation in *S. formosa*. The variables such as leaflet number and area, stomata size and density, flower length, keel width, flag width and peduncle length, and pollen diameter and viability as well as seed number per pod can be used effectively to distinguish diploid and tetraploid plants obtained from oryzalin application.

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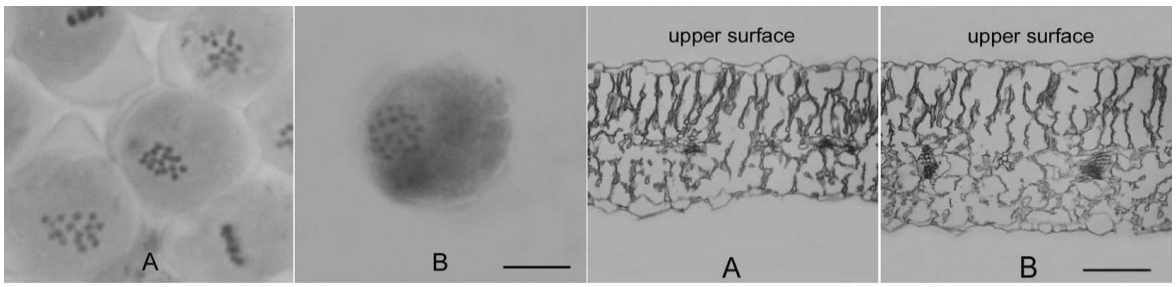
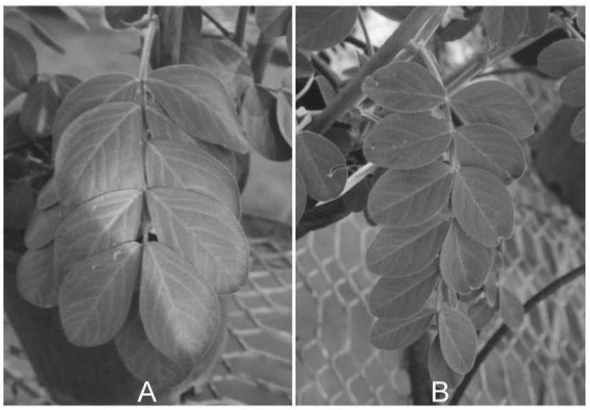


Figure 1

Figure 2



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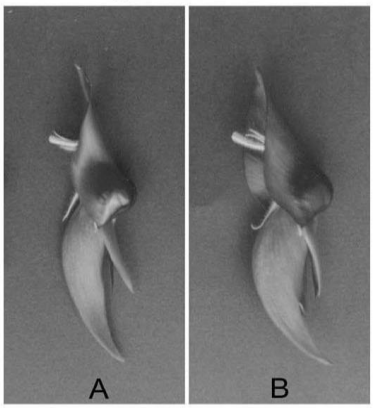
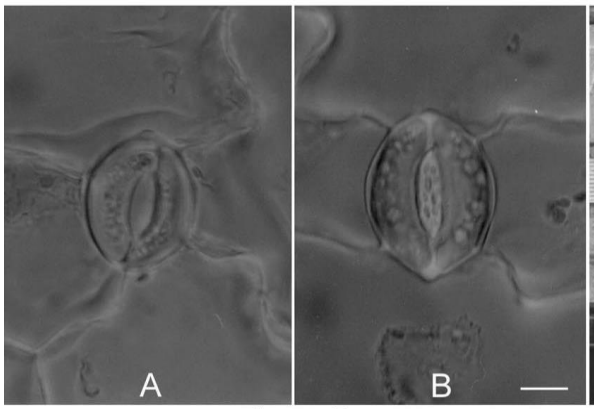


Figure 4



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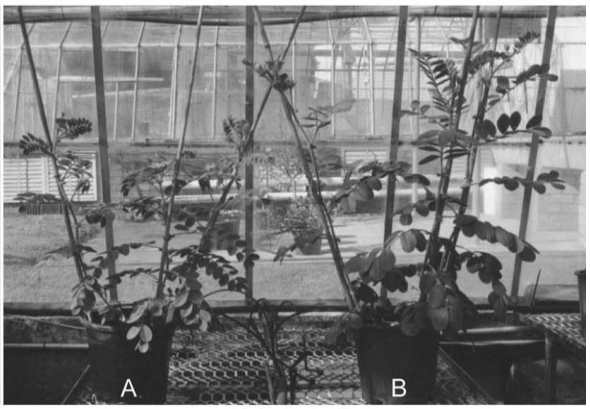

Figure 6

Figure 1. Number of chromosomes in the microspore mother cells of *S. formosa*. A, diploid $2n = 2x = 16$; B, tetraploid $2n = 4x = 32$. Bar = 10 µm.

Figure 2. Transverse sections of leaflets of diploid and tetraploid plants of *S. formosa* (arrows indicate stomata). A, diploid leaflet; B, tetraploid leaflet. Bar = 25 µm.

Figure 3. Comparison of leaflets in tetraploid and diploid plants of *S. formosa*. A, tetraploid; B, diploid.

Figure 4. Comparison of florets from diploid and tetraploid plants of *S. formosa*. A, floret from diploid plant; B, floret from tetraploid plant.

Figure 5. Comparisons of stomata from diploid and tetraploid leaflets of *S. formosa*. Bar = 10 µm.

Figure 6. Growth habits of diploid and tetraploid plants of *S. formosa*. A, diploid; B, tetraploid.