

## Developing tetraploid perennial ryegrass (*Lolium perenne* L.) populations

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**Abstract** In a breeding programme aimed at developing tetraploids from Australian adapted germplasm, a root-tip squash technique was initially used for ploidy determination. The highest recovery of tetraploids (39.7%) was obtained by treatment of 1-week-old seedlings with 0.1% colchicine concentration. Significant cultivar  $\times$  colchicine concentration interaction was observed in seedling survival, highlighting the difference in response of the cultivars to colchicine treatment. Subsequently, flow cytometric estimation of the nuclear DNA content in young leaf tissues was used to screen the  $C_1$  and  $C_2$  generations. The speed and the efficiency of this method make it possible to examine a large number of plants. In the  $C_1$  population the percentage of tetraploids, diploids, and aneuploids was 25, 72.7, and 2.3% respectively, while in the  $C_2$  generation the percentage of tetraploids, diploids, and aneuploids was 43, 2, and 55% respectively.

**Keywords** *Lolium perenne*; colchicine; tetraploid; root-tip squash technique; flow cytometric technique

## INTRODUCTION

Perennial ryegrass (*Lolium perenne* L.) occurs in nature as a diploid ( $2n = 2x = 14$ ). Induced polyploidy by colchicine treatment was reported by Myers (1939). In Europe, tetraploid ryegrass cultivars ( $2n = 4x = 28$ ) developed have been widely sown, mainly due to their beneficial effects on animal intake and performance (Castle & Watson 1971; Hageman et al. 1993).

One of the key changes induced by polyploidy is the change in the DNA content (c-value), which has been associated with an adaptive advantage (Bennett 1987). The DNA c-value is positively correlated with cell size and minimum cell doubling time, which interact to determine growth rate and generation time (Humphreys 1991a). This DNA c-value may be strongly associated with “fitness” (survival and reproductive success). Superior spring growth at low temperatures has been found in species with higher DNA content, and a positive correlation between DNA content and altitude has been found in a number of plant species (Humphreys 1991b).

Chromosome doubling directly affects plant performance through increases in cell and vacuole size. This increases water and soluble carbohydrate content and enhances palatability and feeding values compared with the diploids. Tetraploids often have better winterhardiness, tolerance to drought and disease resistance than diploids. Seed and leaf size are also increased, thus improving seedling vigour and establishment potential, but tiller production is often reduced (Humphreys 1991a).

As European perennial ryegrass cultivars (both diploid and tetraploid) have displayed poor persistence and winter growth in Australia, having been bred for winter dormancy (winter hardiness), Cunningham et al. (1994) and Reed (1994) emphasised the need to develop tetraploid cultivars from adapted Australian material in order to improve herbage quality.

The standard method of colchicine treatment involves treatment of germinating seedlings with 0.2% aqueous solution of colchicine (Alhoowalia

1967; Simonsen 1973; Hague & Jones 1987; Hassan et al. 1989). However, some studies have clearly indicated differences in cultivar response to colchicine treatment (Ahloowalia 1967). Little is known about the response of genotypes adapted to Australia to colchicine treatment. In this study, colchicine has been used at different concentrations, with the objective of optimising a protocol for treatment of Australian and New Zealand perennial ryegrass cultivars to generate tetraploids to initiate a tetraploid breeding programme.

Ploidy determination in *Lolium* has been conventionally done by the root-tip squash technique (Myers 1939; Ahloowalia 1965). Easton (1973) used the modified leaf squash technique for chromosome counting. Morgan & Meredith (1983) developed a technique for identification of polyploid sectors in grass inflorescences. Microscopic chromosome counting is a very reliable method for ploidy determination but it is very time consuming. Flow cytometry has emerged as a new technique for ploidy determination, especially for screening large populations (Dolezel 1997). It offers an accurate and rapid method of ploidy determination of either single plants or plant populations (Galbraith et al. 1983; De Laat et al. 1987). Flow cytometry is now well accepted by turf and forage grass researchers (Barker et al. 1998). This paper describes the use of root-tip squash technique in the ploidy determination of tetraploid populations. Subsequently, flow cytometric estimation of the DNA content has been used, after the two methods were shown to give similar results.

## MATERIALS AND METHODS

### Colchicine treatment

Twelve Australian and New Zealand cultivars of perennial ryegrass were used in this experiment, which commenced in 1997. These cultivars—'Banks', 'Boomer', 'Brumby', 'Camel', 'Ellett', 'Embassy', 'Grasslands Nui', 'Jackaroo', 'Kangaroo Valley', 'Vedette', 'Victorian' and 'Yatsyn 1'—represent a broad range of perennial ryegrass cultivars sown in contrasting Australian environments.

Three replicates of 20 seeds of each cultivar were germinated on moist filter paper in Petri dishes. One-week-old seedlings were treated at room temperature ( $20^{\circ}\text{C} \pm 2^{\circ}\text{C}$ ) with 0.1, 0.2, 0.3 or 0.4% aqueous concentrations of colchicine. The period of treatment was for 3 h (Wit & Speckmann 1955). A control

treatment was also included in which the seeds were kept moist with distilled water.

After treatment the seedlings were washed five times in distilled water and were left in a thin layer of water overnight. The seedlings were then placed on fresh filter papers and kept in the Petri dishes. After a week the seedlings were transplanted into seedling trays and maintained in a glasshouse at  $20\text{--}25^{\circ}\text{C}$ . The number of seedlings that survived colchicine treatment was expressed as a proportion of the initial 20 seeds germinated.

### Root-tip squash technique

When the surviving plants had at least 3–5 tillers, they were separated into single tillers and their root tips were examined for the ploidy level. The root-tip squash method reported by Ahloowalia (1965) in *Lolium* was used to determine the chromosome number. The tetraploids recovered were expressed as a proportion of the surviving seedlings.

Seventy-five  $C_0$  tetraploids selected were vernalised at  $5\text{--}6^{\circ}\text{C}$  in a growth room with 8 h photoperiod for 4 weeks to induce flowering, and were allowed to interpollinate in a crossitron so as to build a base population.

### Flow cytometric technique

The  $C_1$  tetraploids were confirmed by using a Partec Ploidy Analyser which works on the principle of flow cytometry. Fresh, young leaf samples were collected from the plant to be examined and finely chopped with a sharp razor blade (Galbraith et al. 1983) in 200  $\mu\text{l}$  of ice-cold commercial Partec buffer solution for 1.5 min. The suspension was poured through a 30  $\mu\text{m}$  filter to remove debris. To the filtrate was added 800  $\mu\text{l}$  of ice-cold commercial Partec DAPI solution. The suspension was analysed on a Partec PA flow cytometer. Ploidy level was determined based on position of the G1 peaks of unknowns in relation to the internal standard G1 peak (De Laat et al. 1987). The G1 peak of nuclei isolated from a diploid *L. perenne* genotype (used as the standard) was set at channel (relative fluorescence intensity) 100.

A total of 216 plants were examined for their ploidy. To validate the results obtained from flow cytometry, the ploidy level of  $C_1$  plants randomly selected from each class namely tetraploids (20 plants), diploids (20 plants) and aneuploids (4 plants) were examined. The stability of ploidy readings of excised leaves stored at  $4^{\circ}\text{C}$  for up to 2 days was also evaluated. The  $C_1$  tetraploids identified were grown in a glasshouse at  $20\text{--}25^{\circ}\text{C}$  and, following

vernalisation, polycrossed in a crossitron unit; the seed from individual parent plants in the polycrosses were kept as separate half-sib families.

Forty-three half-sib families were grown in a glasshouse and 51 plants were randomly selected from the C<sub>2</sub> population and their ploidy levels determined by the flow cytometric method.

### Statistical analysis

The comparison of the proportions of surviving seedlings and tetraploids recovered was carried out by fitting a generalised linear model (binomial distribution) with a logit link (Collet 1991). Where comparison of treatment proportions was not possible, due to low or zero proportions, Fisher's exact test was used to provide an estimate of the probabilities. All statistical analyses were performed using Genstat 5.32 software (Genstat 5 Committee 1994).

## RESULTS

### Effect of colchicine

The effects of cultivar, concentration of colchicine and cultivar  $\times$  concentration interaction were significant ( $P < 0.001$ ). The use of colchicine resulted in a significant reduction in the survival of seedlings. Increasing the concentration of colchicine caused a highly significant ( $P < 0.001$ ) decline in seedling survival (0.1:41.8%–0.4:3.2%).

The response of cultivars to changes in colchicine treatment varied, particularly between 0.1 and 0.2%

concentrations (Table 1). Some cultivars, namely 'Brumby' and 'Yatsyn 1', showed no reduction in survival when the concentration of colchicine was increased from 0.1 to 0.2%.

### Recovery of tetraploids

#### Root-tip squash technique

The effects of both cultivar and concentration of colchicine on the recovery of tetraploids were significant ( $P < 0.05$ ), whereas the cultivar  $\times$  concentration interaction was not (data not presented). The surviving plants were found to have a mixture of diploid and tetraploid tillers. In general, the tetraploids isolated showed the gigas response being larger than the diploids (Hague & Jones 1987). Plants treated with 0.1% concentration of colchicine yielded 39.7% tetraploids. This concentration resulted in a significantly greater number of tetraploid plants ( $P < 0.001$ ) than other concentrations (Table 2). Overall, in both recovery of tetraploids and the mean survival of seedlings, 0.1% was considered the optimal concentration of colchicine for the treatment of these Australian and New Zealand cultivars.

#### Flow cytometric technique

DNA-histograms from nuclear preparations of leaf tissues of tetraploid plants showed a peak representing G1 nuclei at channel 200 compared to channel 100 of the diploid plants. Plants which recorded peaks in between channel 100 and 200 were classified as aneuploids. In the C<sub>1</sub> population the percentage of tetraploids, diploids, and aneuploids

**Table 1** The survival proportions of ryegrass cultivars at each concentration level (%) and the control after application of colchicine.

Cultivar	Colchicine concentration				
	0	0.1	0.2	0.3	0.4
'Banks'	0.733	0.400	0.100	0.133	0.167
'Boomer'	0.700	0.433	0.300	0.117	0.000
'Brumby'	0.800	0.550	0.583	0.333	0.167
'Camel'	0.767	0.400	0.233	0.117	0.000
'Ellett'	0.767	0.433	0.300	0.000	0.000
'Embassy'	0.633	0.500	0.267	0.000	0.050
'Grasslands Nui'	0.800	0.283	0.083	0.133	0.000
'Jackaroo'	0.900	0.583	0.283	0.333	0.000
'Kangaroo Valley'	0.700	0.467	0.233	0.000	0.000
'Vedette'	0.717	0.433	0.317	0.033	0.000
'Victorian'	0.700	0.333	0.150	0.083	0.000
'Yatsyn 1'	0.683	0.200	0.283	0.000	0.000
Mean	0.741	0.418	0.261	0.107	0.032

was 25, 72.7, and 2.3% respectively. In the following  $C_2$  generation the percentage of tetraploids, diploids, and aneuploids was 43, 2, and 55% respectively.

The flow cytometric estimation was validated by root-tip analysis, and the two methods gave identical results. On an average, 20 samples were examined per day by root-tip analysis compared with more than 200 samples by flow cytometry. Leaf preparations stored at 4°C for up to 2 days showed consistent results compared with fresh samples.

## DISCUSSION

Even though a 0.2% concentration of colchicine has been used as the standard method for the production of tetraploids in *Lolium perenne*, the reports of cultivar × colchicine concentration interaction suggested the need to develop a protocol for treatment of Australian and New Zealand cultivars. The 0.1% concentration of colchicine was found to be optimal for the recovery of tetraploids from the Australian and New Zealand perennial ryegrass cultivars used. The significance of using 0.1% concentration of colchicine is that, in addition to the greater recovery of tetraploids, it had the highest seedling survival. The variability in survival of perennial ryegrass cultivars after colchicine treatment observed in this experiment is similar to that found by Ahloowalia (1967). Ahloowalia (1967) observed that cultivars originating from colder regions showed a lower percentage of seedling mortality following colchicine treatment than those from relatively warmer climates, and concluded that a large number of seeds should be treated with colchicine to circumvent the differences in response to ploidy manipulation, particularly when the diploid material is from diverse sources of origin. The different result obtained in this study may be attributed to the Australian and New Zealand

cultivars being more sensitive to colchicine than cultivars used in other studies. The colchicine-treated plants produced a mixture of  $2n$  and  $4n$  tillers as reported by various authors, including Hague & Jones (1987) and Hassan et al. (1989).

The root-tip squash technique was initially adopted for ploidy determination. Subsequently, flow cytometric estimation of the DNA content was employed. Flow cytometry was found to be not only quick but also reliable in our studies, as also reported by Barker et al. (1998) in *Lolium perenne*. Barker et al. (1998) in their study had used tall fescue (*Festuca arundinacea* Schreb.) as the internal standard, whereas in this study a diploid genotype of *Lolium perenne* was used. Collection of samples for flow cytometric estimation was done from young, fully extended leaves (Barker et al. 1998). The fact that analysis by flow cytometric estimation was not affected by storage of fresh samples up to 2 days would allow researchers to make preparations on site and send them offsite for analysis (Farnham et al. 1998).

The use of a rapid technique such as flow cytometric determination of the ploidy level is very useful in the detection of aneuploids, especially when screening large numbers of plants. Simonsen (1973) confirmed the results of Ahloowalia (1967) that univalents, trivalents, and quadrivalents with linear and indifferent orientation at first metaphase were the major cytological causes of aneuploid gametes in autotetraploid ryegrass. Fertility expressed by seed set has been frequently used as a selection criterion to improve meiotic regularity. However, Simonsen (1973) found that seed set was not the most reliable character as some aneuploid individuals were fertile, at least of those with 27 and 29 chromosomes, and aneuploid gametes with one or more missing chromosomes, were nearly fully viable. The aneuploids studied by Simonsen (1973) were low yielding, and hence selection of the highest herbage yielding individuals was suggested as an effective method to eliminate the majority of aneuploid individuals, followed by cytological examination. In this study, aneuploid frequency was 2.3% in the  $C_1$  generation while it was 55% in the  $C_2$ . Ahloowalia (1967) reported an aneuploid frequency of 7% in the  $C_1$  generation. Easton (1973) and Simonsen (1973) found the frequencies to be 55% and 48.2% respectively in the  $C_2$  generation. Aneuploids identified in each generation were removed from the tetraploid populations before crossing, resulting in the improvement in the recovery of tetraploids from 25% in the  $C_1$

**Table 2** Mean proportion of tetraploids recovered after colchicine treatment determined by root-tip squash technique. Concentrations that are followed by different superscript letters differ significantly ( $P < 0.05$ ).

Colchicine concentration (%)	Proportion
0.1	0.397 <sup>a</sup>
0.2	0.185 <sup>b</sup>
0.3	0.157 <sup>bc</sup>
0.4	0.000 <sup>c</sup>

generation to 43% in the C<sub>2</sub> generation. The detection and further elimination of the aneuploids, especially in the early stages of the breeding programme, helps in maintaining stability in the populations developed, as aneuploids lead to decreased seed yields, but more importantly, to genetic shift as pointed out by Easton (1973) and Elgersma (1991). Flow cytometry offers not only a rapid but also an accurate method of ploidy estimation, which is vital for developing stable tetraploid perennial ryegrass populations.

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#### REFERENCES

- Ahloowalia, B. S. 1965: A root tip squash technique for screening chromosome number in *Lolium*. *Euphytica* 14: 170–172.
- Ahloowalia, B. S. 1967: Colchicine induced polyploids in ryegrass. *Euphytica* 16: 49–60.
- Barker, R. E.; Kilgore, J. A.; Cook, R. L.; Garay, A. E. 1998: Validity of flow cytometry for testing ploidy in ryegrasses. In: Young, W. C. III ed. Seed production research at Oregon State University, USDA-ARS Cooperating, Department of Crop Science and Soil Science Ext/CrS 112, 4/99. Pp. 40–44.
- Bennett, M. D. 1987: Variation in genomic form in plants and its ecological implication. *New Phytologist* 106: 93–111.
- Castle, M. E.; Watson, J. N. 1971: A comparison between a diploid and a tetraploid perennial ryegrass for milk production. *Journal of Agricultural Science, Cambridge* 77: 69–76.
- Collet, D. 1991: Modelling binary data. London, Chapman & Hall.
- Cunningham, P. J.; Blumenthal, M. J.; Anderson, M. W.; Prakash, K. S.; Leonforte, A. 1994: Perennial ryegrass improvement in Australia. *New Zealand Journal of Agricultural Research* 37: 295–310.
- De Laat, A. M. M.; Gohde, W.; Vogelzang, M. J. D. C. 1987: Determination of ploidy of single plants and plant populations by flow cytometry. *Plant Breeding* 99: 303–307.
- Dolezel, J. 1997: Application of flow cytometry for the study of plant genomes. *Journal of Applied Genetics* 38(3): 285–302.
- Easton, H. S. 1973: Performance of aneuploids in an autotetraploid ryegrass population. *New Zealand Journal of Agricultural Research* 16: 35–37.
- Elgersma, A. 1991: Seed yield and seed yield selection in polyploid forage crops. Proceedings of the XVII meeting of the fodder crops section of Eucarpia, Alghero (Italy) 14–18 October. Pp. 124–129.
- Farnham, M. W.; Caniglia, E. J.; Thomas, C. E. 1998: Efficient ploidy determination of anther-derived broccoli. *HortScience* 33(2): 323–327.
- Galbraith, D. W.; Harkins, K. R.; Maddox, J. M.; Ayres, N. A.; Sharma, D. P.; Firoozabady, E. 1983: A rapid flow cytometric analysis of the cell cycle in intact plant tissues. *Science* 220: 1049–1051.
- Genstat 5 Committee 1994: Genstat 5 Release 3.2 reference manual. Oxford, Clarendon Press.
- Hageman, I. W.; Lantinga, E. A.; Schlepers, H.; Neuteboom, J. A. 1993: Herbage intake, digestibility characteristics of milk production of a diploid and two tetraploid cultivars of perennial ryegrass. Proceedings of XVII International Grassland Congress, Palmerston North, New Zealand. Pp. 459–460.
- Hague, L. M.; Jones, R. N. 1987: Cytogenetics of *Lolium perenne* 4. Colchicine induced variation in diploids. *Theoretical and Applied Genetics* 74: 233–241.
- Hassan, L.; Jones, R. N.; Posselt, U. K. 1989: A novel source of genetic variation in ryegrass (*Lolium multiflorum* and *L. perenne*). *Heredity* 63: 339–342.
- Humphreys, M. O. 1991a: The value of polyploidy in breeding hybrid grasses. Proceedings of the XVII meeting of the fodder crops section of Eucarpia, Alghero (Italy) 14–18 October. Pp. 37–44.
- Humphreys, M. O. 1991b: Genetic control of physiological response – a necessary relationship. *Functional Ecology* 5: 213–221.
- Morgan, W. G.; Meredith, M. R. 1983: A technique for identifying polyploid sectors in grass inflorescences. *Euphytica* 32: 125–127.
- Myers, W. M. 1939: Colchicine induced tetraploidy in perennial ryegrass. *Journal of Heredity* 30: 499–504.
- Reed, K. F. M. 1994: Improved grass cultivars increase milk and meat production – a review. *New Zealand Journal of Agricultural Research* 37: 277–286.
- Simonsen, O. 1973: Cyto-genetic investigations in diploid and autotetraploid populations of *Lolium perenne* L. *Hereditas* 75: 157–188.
- Wit, F.; Speckmann, G. J. 1955: Tetraploid westerwolthys ryegrass. *Euphytica* 4: 245–253.