South African Journal of Botany 2003, 69(4): 563–567
Printed in South Africa — All rights reserved

SOUTH AFRICAN JOURNAL OF BOTANY
EISSN 1727–9321

Evaluation of induced polyploidy in *Acacia mearnsii* through stomatal counts and guard cell measurements

SL Beck[1]*, RW Dunlop[1] and A Fossey[2]

[1] *Institute for Commercial Forestry Research, PO Box 100281, Scottsville, Pietermaritzburg 3209, South Africa*

[2] *School of Molecular and Cellular Biosciences, University of Natal Pietermaritzburg, Private Bag X01, Scottsville, Pietermaritzburg 3209, South Africa*

* *Corresponding author, e-mail: sascha@icfr.unp.ac.za*

Received 5 June 2003, accepted in revised form 13 August 2003

The black wattle, *Acacia mearnsii,* an important commercially grown tree in South Africa, is considered one of the top alien invader species within indigenous vegetation because of its abundant seed production and dispersal. One of the aims at the Institute for Commercial Forestry Research (ICFR) is to restrict the spread of wattle outside plantation boundaries. Current consideration has been given to polyploids, which could be semi-sterile or even sterile due to irregular meiosis that results in infertile gametes. Autotetraploids were produced by the scarification of seeds and treatment with different colchicine concentrations (0%, 0.01%, 0.03%, 0.05%, 0.07% and 0.1%) for various times (6h, 12h, 24h and 48h), rinsed and germinated in the dark at 25°C to induce chromosome doubling. Tetraploidy was successfully induced by applying 0.01% colchicine to chipped seeds for a period of 6h. This result was validated through percentage germination (90%), average seedling growth rate (16mm), seedling height (20mm) and percentage seedling survival (61%). Chromosome doubling was confirmed by stomatal guard cell length (39.9 ± 0.4 (SE) mm) and stomatal frequency measurements (12.0 ± 0.5 (SE)).

Introduction

Black wattle, *Acacia mearnsii* (de Wild) which is indigenous to South Australia, was introduced to South Africa in the early 19th century and has become one of the leading commercially grown forestry trees in South Africa. Black wattle timber has become extremely popular as a source of raw material for high quality pulp and its bark, which is rich in tannin, is used in the leather tanning industry, while other extracts are used in the manufacture of thermosetting adhesives. Wattle timber is also used as mine props, charcoal, fuelwood and building poles. Approximately 85% of the timber and 95% of bark products are exported, making wattle an important source of revenue for South Africa.

In South Africa, black wattle is classified as an alien invader of indigenous vegetation. For the wattle industry to remain competitive and accepted by the South African conservationists, it is in the industry's best interest to find a solution to contain the spreading of seeds beyond the boundaries of commercial plantations. One method of limiting the spread of wattle outside plantation boundaries might be to cultivate polyploids. Autopolyploids, induced or natural, usually display a variable meiosis with a range of pairing associations of chromosomes, that result in unbalanced, infertile gametes with a consequential reduction in seed set (Anon. 1974, Chaudhari and Barrow 1975, Ramsey and Schemske 1998). Often this is accompanied by an increased vegetative growth, which is of great use in plant breeding where vegetative parts are harvested. This has been successfully employed in tobacco (Borojevic 1990) and potato (Carputo *et al*. 1995). In the citrus industry, on the other hand, seed reduction in triploids has been exploited to produce seedless fruit (Geraci *et al*. 1982). Another primary advantage of polyploidy is that it often results in a greater tolerance of environmental stresses, enabling farmers to produce more robust crops with higher yields (Mohrdiek 1976, Dwivedi *et al*. 1989).

The most commonly used chemical inducer of polyploidy is the alkaloid, colchicine (or colecemid, a synthetic equivalent), which prevents spindle formation during meiosis, preventing separation of homologous chromosomes. As it only affects actively dividing cells, it is generally applied to the actively growing meristematic regions of young plants or to germinating seedlings (Singh 1993). It has been used to successfully induce tetraploidy in a variety of plants and crops (Blakeslee and Avery 1937, WRI 1952, Johnsson 1956, Pesina 1963, Winton 1968, Pundir *et al.* 1983, Yang and Yang 1989, Du Plooy *et al.* 1992, Kunitake *et al.* 1998, Sangduen and SinpatanAnon 1998). Colchicine-induced tetraploidy in the *Acacia* species has only been reported for

A. mearnsii by Moffett and Nixon (1960). These studies were exploratory in nature, hence the need for further investigation.

Confirmation of polyploidy is most often done by counting the chromosomes in root tip squashes. In black wattle, especially the polyploids, the high number of chromosomes together with their small size and the low number of dividing cells that can be obtained from root tip squashes, often lead to incorrect identification of polyploids. However, alternative techniques using stomatal guard cell lengths and stomatal frequencies have recently been used to successfully distinguish between diploid and tetraploid black wattle (Beck *et al*. 2003). Beck *et al*. (2003) showed that the mean stomatal length was 27.2 ± 0.5µm for diploid and 40.2 ± 0.5µm for tetraploid *A. mearnsii*. The frequency of stomata per leaf surface was shown to decrease significantly ($P < 0.001$) as the ploidy level increased, with a mean of 22.1 ± 0.5 for diploid and 10.3 ± 0.5 for tetraploid *A. mearnsii*. These techniques were successfully applied to other plant species (Evans 1955, Moffett and Nixon 1960, Speckmann *et al.* 1965, Tan and Dunn 1973, Przywara *et al.* 1988) and have shown to produce accurate, rapid and reliable results for the confirmation of ploidy.

The purpose of this investigation was to double the number of chromosomes of diploid wattle with colchicine, to standardise the colchicine treatment conditions and treatment times and thereafter, determine the average number of stomata and stomatal guard cell lengths of colchicine treated diploids to confirm polyploidisation.

Materials and Methods

Induction of polyploidy

Unimproved commercially available diploid black wattle (*Acacia mearnsii*) seed collected in 1996 and stored at 18°C, was scarified by chipping and soaked in various concentrations of colchicine (0.01%; 0.03%; 0.05%; 0.07%; 0.1%) for 6h, 12h, 24h and 48h. Untreated diploid and tetraploid seeds soaked in water were used as the controls. Seeds were soaked in glass jars in the dark at room temperature. A total of 24 treatments were tested, with 50 seeds per treatment. After the colchicine treatment the seeds were rinsed in tap water, placed on moistened filter paper in petri dishes containing vermiculite and germinated in the dark at 25°C in an incubator. Based on the results of the initial experiment, the experiment was repeated using only 0%, 0.01%, 0.03% and 0.05% colchicine for 6h and 12h only.

Seed germination and seedling performance

Untreated diploid and tetraploid *A. mearnsii* seeds (25 diploid and 25 tetraploid), as well as colchicine treated diploid seeds were germinated and grown under nursery conditions. The tetraploid seed were obtained from earlier experiments done in the 1950s at the Wattle Research Institute (WRI) where tetraploidy was induced using colchicine and confirmed through chromosome counts (Moffett and Nixon 1960).

Germination of all the seed groups was monitored for three days. After this, half of the surviving seedlings of each colchicine treatment group and untreated diploid and tetraploid seedlings were planted out in the nursery, while the remainder were kept in the dark at 25°C in an incubator for viability studies. After seven days under incubator conditions, average growth rate, measured as average root length, was recorded. After 60 days under nursery conditions, the number of surviving colchicine treated seedlings and average seedling height of each treatment were recorded. The height of seedlings was determined by using a metre ruler and measuring from the soil surface to the apical bud.

Confirmation of chromosome doubling

After 60 days under nursery conditions five plants from each untreated and colchicine treated group were selected to determine stomatal length and frequency in order to confirm that chromosome doubling had occurred.

For stomatal length measurements, one slide was made for each plant. A razor blade was used to strip a thin layer from the abaxial surface of the pinnule. Initially non-permanent mounts were prepared by floating the epidermal layers in a 1% aceto-carmine solution, covering with a cover slip. Examination with a light microscope followed, to check the quality of the epidermal sections. Permanent mounts were prepared by floating the epidermal layers in a 1% aceto-carmine solution and squashing with a cover slip. The cover slips were removed by soaking the slides in 45% acetic acid and depending on whether the epidermal layers adhered to the slide or cover slip, they were passed through a series of alcohol dehydration and mounted using Euparal essence and mountant (Beck *et al*. 2003). Stomata were measured under x40 magnification using a light microscope with an ocular scale bar where each ocular unit of the scale bar measured 2.5µm. Twenty stomatal guard cell lengths were measured per slide.

Stomatal frequency was determined for each plant by examining 15 microscopic fields (microscopic 'counts' of the stomatal complexes i.e. guard cells and pore, at a magnification of x500) using an Environment Scanning Electron Microscope (ESEM) to view the plant.

Statistical analysis

GENSTAT® Version 4.2 (Lane and Payne 1996) was used to analyse the data statistically. Differences in germination and seedling survival were analysed by ARCSIN transforming the data and conducting a general analysis of variance (ANOVA). Differences in average root length and seedling height were determined using residual maximum likelihood model (REML).

A general ANOVA was used to statistically analyse differences in stomatal guard cell length measurements. A three-way ANOVA was conducted, to compare concentration, time and plant replicates for stomatal frequency measurements.

Results

Seed germination and seedling performance

In all colchicine treatments, mortality increased with an increase in colchicine concentration as well with an

increase in exposure time. The greatest percentage germination of treated seeds was obtained from seeds treated for 6h (90%) and for 12h (86%). No significant differences ($P > 0.001$) between the concentrations tested, could be established for these time periods. Normal root development was noted for seeds treated for 6h and 12h with 0.01% (16mm and 14mm, respectively) and these did not differ significantly from the diploid control (15mm) but were significantly different from the tetraploid control (5mm). Normal root development was also noted for seeds treated for 6h and 12h with 0.03% (8mm and 7mm, respectively) colchicine. In general these did not differ significantly from the tetraploid and diploid controls, only seeds treated with 0.03% colchicine for 12h differed from the diploid control (Table 1).

After 60 days under nursery conditions, seedling survival was recorded. Second to the controls, the greatest survival was noted with the 0.01% and 0.03% colchicine concentrations at 6h and 12h (Table 1).

Seedling height was measured after 60 days and the seedlings treated with 0.01% and 0.03% colchicine for 6h showed normal growth development with heights of 29mm and 20mm, respectively. When compared to the controls, seedlings treated with 0.01% colchicine for 6h, showed comparable growth (31mm for diploid and 20mm for tetraploid control) (Table 1).

Confirmation of chromosome doubling

Stomatal guard cell length was used successfully as an indicator of tetraploid induction in *A. mearnsii* (Beck *et al*. 2003). Results from this study were compared with stomatal guard cell lengths of untreated diploid and tetraploid controls (Beck *et al*. 2003). A two-way ANOVA was conducted and all colchicine treatments had significantly larger stomatal guard cell lengths than the untreated diploid control ($P < 0.001$) (Table 2). When compared to the untreated tetraploid control, only the 0.01% colchicine treatment for 6h was not significantly different from the tetraploid control ($P > 0.001$), the other treatments all differed significantly ($P < 0.001$) (Table 2). From the results of this study, maximum average stomatal guard cell lengths of 39.9 ± 0.4µm were obtained from seeds treated for 6h and with 0.01% colchicine. The stomatal length of the pinnules from seeds treated with 0.01% colchicine for 6h, showed high variation, ranging from 36.4–45.0 ± 1.1µm.

The frequency of stomata on the leaf surface has also been used as an indicator of a change in ploidy level for black wattle (Beck *et al*. 2003). Stomatal frequencies from known untreated tetraploid and diploid plants showed significant differences. Untreated diploid seedlings displayed a frequency of 22.1 ± 0.5, whereas untreated tetraploids displayed a frequency of 10.3 ± 0.5 (Table 3) (Beck *et al*. 2003). For all treatment combinations tested in this study, there was some variation between the plants. When compared with the untreated tetraploid and diploid controls (Table 3), all the treatments had significantly smaller frequencies than the known diploid standard, however, they were not as small as the tetraploid control. The smallest average frequency of stomata was obtained from seeds treated with 0.01% colchicine for 6h (12.0 ± 0.4) and from seeds treated with 0.05% colchicine for 12h (12.8 ± 0.4) (Table 3).

Discussion

Tetraploidy was successfully induced through the use of colchicine for all concentrations (0.01%, 0.03%, 0.05%, 0.07% and 0.10%) and with all time periods (6h, 12h, 24h and 48h) tested. This was confirmed using stomatal length measurements and stomatal frequency counts. Stomatal lengths of seedlings originating from colchicine treated seed were all significantly longer than the untreated diploid con-

Table 1: Table of means showing differences in average root length (mm) (after seven days incubation at 25°C), survival (%) and seedling height (mm) (after 60 days in the nursery) of seeds treated with concentrations of colchicine for various treatment times. Treatments denoted by the same letters are not significantly different ($P > 0.01$)

Seedling performance	Colchicine conc. (%)	Treatment time (h)				Untreated controls	
		6	12	24	48	Diploid	Tetraploid
Average root length (mm)	0.01	16 ^{e}	14 ^{cde}	5 ^{ab}	6 ^{ab}	15 ^{de}	5 ^{ab}
(LSD = 7)	0.03	8 ^{bcd}	7 ^{abc}	4 ^{ab}	2 ^{ab}		
	0.05	6 ^{ab}	6 ^{ab}	3 ^{ab}	3 ^{ab}		
	0.07	5 ^{ab}	5 ^{ab}	3 ^{ab}	3 ^{ab}		
	0.10	5 ^{ab}	5 ^{ab}	1 ^{a}	2 ^{ab}		
Seedling height (mm)	0.01	29 ^{bc}	20 ^{abc}	20 ^{abc}	–	31 ^{c}	20 ^{abc}
after 60 days	0.03	20 ^{abc}	12 ^{ab}	15 ^{abc}	–		
(LSD = 17)	0.05	13 ^{ab}	12 ^{ab}	–	–		
	0.10	10 ^{a}	7 ^{a}	–	–		

Seedling performance	Colchicine conc. (%)	Treatment time (h)				Untreated controls			
		6		12		Diploid		Tetraploid	
		Raw data	Transformed (ARCSIN)	Raw data	Transformed (ARCSIN)	Raw data	Transformed (ARCSIN)	Raw data	Transformed (ARCSIN)
Seedling survival (%)	0.01	61	0.9 ^{bcd}	750	0.9 ^{cd}	89	1.2 ^{a}	90	1.2 ^{a}
(LSD = 0.4)	0.03	57	0.7 ^{abc}	45	0.5 ^{a}				
	0.05	8	0.3 ^{a}	5	0.3 ^{a}				

Table 2: Two-way table of treatment means of stomatal lengths (µm) of pinnules from untreated tetraploid and untreated diploid seedlings and seedlings grown from seed, treated with various concentrations of colchicine (%) for 6h, 12h and 24h, after 60 days under nursery conditions (LSD = 1.03). Treatments denoted by the same letters are not significantly different (P > 0.01)

Time (h)	Colchicine Concentration (%)			Untreated Controls	
	0.01	0.03	0.05	Tetraploid	Diploid
6	39.9^{f}	36.9^{e}	36.4^{e}	40.2^{f}	27.2^{b}
12	30.7^{a}	34.3^{d}	35.0^{d}		
24	32.7^{c}	34.0^{d}	34.6^{d}		

Table 3: Two-way table of treatment means of stomatal frequencies from pinnules of untreated diploid and untreated tetraploid-seedlings and seedlings grown from seed treated with various concentrations of colchicine (%) for 6h, 12h and 24h, after 60 days under nursery conditions (LSD = 0.8). Treatments denoted by the same letters are not significantly different (P > 0.01)

Time (h)	Colchicine Concentration (%)			Untreated Controls	
	0.01	0.03	0.05	Tetraploid	Diploid
6	12.0^{b}	14.8^{f}	17.8^{g}	22.1^{h}	10.3^{a}
12	13.2^{cd}	18.5^{g}	12.8^{bc}		
24	13.8^{de}	14.6^{ef}	15.1^{f}		

trol. These results agreed with those obtained by Beck *et al.* (2003) who showed that stomatal lengths could be used to distinguish between diploid and tetraploid *A. mearnsii*. Przywara *et al*. (1988) showed that stomatal lengths of kiwifruit increased significantly as ploidy increased and Tan and Dunn (1973) used stomatal length to distinguish between ploidy levels in *Bromis inermis*. However, from the results only the stomatal lengths from the 0.01% colchicine for 6h treatment were not significantly different to the untreated tetraploid control's stomatal length. From this one can predict that tetraploid induction is most likely to occur with a 6h treatment with 0.01% colchicine.

Stomatal frequency results indicated that tetraploid induction was occurring at all concentrations tested and at all times. When compared with untreated tetraploid and diploid controls, all the treatments had a significantly lower frequency than the untreated diploid control. This agrees with results obtained by Beck *et al*. (2003) with *A. mearnsii* and by Tan and Dunn (1973) in *Bromis inermis*, who showed that that stomata decreased per leaf surface with increasing polyploidy. However, the frequencies were still greater than untreated tetraploid control. Overall it is evident that as the colchicine concentration and treatment time increase, so the stomatal frequency increases and thus the chances of tetraploid induction are reduced.

The results obtained from this investigation are in contrast to preliminary studies by Moffett and Nixon (1960) where *Acacia mearnsii* tetraploids were induced by soaking seeds in 0.02–0.03% colchicine for 24h or 0.01–0.02% colchicine for 48h. In comparison, Fraser *et al*. (1991) successfully induced tetraploidy in *Actinidia chinensis* by soaking stratified seeds for 6h in 0.1% colchicine. Brewbaker (1952) produced polyploids in red clover by treating root tips with 0.15% colchicine for 3h. Various investigations indicate that the most commonly used range of colchicine concentrations for tetraploid induction varies from 0.006–3% colchicine, where the optimal choice of treatment time and concentration varies from species to species and depends on what meristematic explant is used.

This investigation has shown that chromosome doubling through colchicine treatment of diploids can be achieved with a high rate of success. However, in order induce tetraploidy in *Acacia mearnsii* through colchicine treatment and have a high rate of germination and nursery survival, it is recommended that chipped seed be treated with 0.01% colchicine for 6h. The tetraploids produced in this study will now be grown until flowering in order to assess seed production and usefulness as a commercial forestry tree.

Acknowledgements — We thank the South African Wattle Growers Union for their financial support. We also would like to thank the University of Natal, Pietermaritzburg Research Centre for Plant Growth and Development, School of Botany and Zoology and the Electron Microscopy Unit for the use of their microscopes.

References

Anonymous (1974) Abstracts of International Symposium. Haploids in higher plants — advances and potentials. 10–14 June 1974, Department of Crop Science, University of Guelph, Guelph, Ontario, Canada

Beck SL, Dunlop RW, Fossey A (2003) Stomatal length and frequency as a measure of ploidy level in black wattle, *Acacia mearnsii* (de Wild). *Botanical Journal of the Linnean Society* **141**: 177–181

Blakeslee AF, Avery AG (1937) Methods of inducing doubling of chromosomes in plants by treatment with colchicine. *Journal of Hereditary* **28**: 393–411

Borojevic S (1990) Principles and Methods of Plant Breeding. Developments of Crop Science 17. Elsevier, Amsterdam, The Netherlands, pp 13–17 and 267–290

Brewbaker JL (1952) Colchicine induction of tetraploids in *Trifolium* species. *Journal of American Society of Agronomy* **44**: 592

Carputo D, Cardi T, Frusciante L, Peloquin SJ (1995) Male fertility and cytology of triploid hybrids between tetraploid *Solanum commersonii* (2n=4x=48, 2EBN) and *Phureja-Tubersum* haploid hybrids (2n=2x=24, 2EBN). *Euphytica* **83**: 123–129

Chaudhari HK, Barrow JR (1975) Identification of cotton haploids by stomatal chloroplast-counts technique. *Crop Science* **15**: 760–762

Du Plooy CP, Breedt HJ, Human CF, Koekemoer PJJ, Coetzer E (1992) *Inligtingsbulletin Instituut vir Tropiese and Subtropiese Gewasse* **243**: 16–20

Dwivedi NK, Suryanarayana N, Sikdar AK, Susheelamma BN, Jolly MS (1989) Cytomorphological studies in triploid mulberry evolved by diploidization of female gamete cells. *Cytologia* **54**: 13–19

Evans A (1955) The production and identification of polyploids in red clover, white clover and lucerne. *New Phytologist* **54**: 149–162

Fraser LG, Harvey CF, Kent J (1991) Ploidy manipulations of kiwifruit in tissue culture. *Acta Horticulturae* **297**: 109–114

Geraci G, Starrantino A, Recupero GR, Russo F (1982) Spontaneous triploidy in progenies of monoembryonic hybrids of clemetine comune x avan mandarin and clementine comune x king of Siam. *Genetica Agraria* **36**: 113–118

Johnsson H (1956) Auto and allotriploid Betula families, derived from colchicine treatment. Zeitschrift Forestgenetics **5**: 65–70

Kunitake H, Nakashima T, Mori K, Tanaka M (1998) Somaclonal and chromosomal effects of genotype, ploidy and culture duration in *Asparagus officinalis* L. Euphytica **102**: 309–316

Lane PW, Payne RW (1996) Genstat for Windows. The Numerical Algoriths Group, London

Moffett AA, Nixon KM (1960) Induced tetraploidy in black wattle *(Acacia mearnsii* De Willd). Wattle Research Institute Report 1959–60. Wattle Research Institute, South Africa

Mohrdiek O (1976) Progeny Studies in Poplars of the Sections Aigeiros, Tacamahaca and Leuce, With Recommendations for Further Breeding Work. Georg-August-Universitat Göttingen, German Federal Republic

Pesina K (1963) Experimental induction of polyploidy in poplars. Presila **35**: 101–109

Przywara L, Pandey KK, Sanders PM (1988) Length of stomata as an indicator of ploidy level in *Actinidia deliciosa*. *New Zealand Journal of Botany* **26**: 179–182

Pundir RPS, Rao NK, Van der Maesen LJG (1983) Induced autotetraploidy in chickpea (*Cicer arietinum* L.). *Theoretical and Applied Genetics* **65**: 199–122

Ramsey J, Schemske DW (1998) Pathways, mechanisms and rates of ploidy formation in flowering plants. *Annual Reviews of Ecology and Systematics* **29**: 467–501

Sangduen N, SinpatanAnon A (1998) Tetraploid mulberry induction by colchicine treatment through tissue culture. Kasetsart *Journal Natural Sciences* **32**: 424–430

Singh RJ (1993) Plant Cytogenetics. CRC Press, Boca Raton, Ann Arbor, California, pp 122–123

Speckmann GJ, Post J Jr, Dijkstra H (1965) The length of stomata as an indicator for polyploidy in rye-grasses. *Euphytica* **14**: 225–230

Tan GY, Dunn GM (1973) Relationship of stomatal length and frequency and pollen-grain diameter to ploidy level in *Bromus inermis* Leyss. *Crop Science* **13**: 332–334

Winton LL (1968) Fertilization in forced quaking aspen and cottonwood. *Silvae Genetica* **17**: 20–21

WRI (1952) Genetics. Report from the Wattle Research Institute, South Africa

Yang JH, Yang XH (1989) Breeding of artificial triploid in mulberry. *Acta Sericologia Sinica* **15**: 65–70

Edited by CEJ Botha