Stomatal length and frequency as a measure of ploidy level in black wattle, *Acacia mearnsii* (de Wild)

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The length and frequency of stomata on leaf surfaces were examined as rapid techniques for future identification of ploidy level of *Acacia mearnsii* (de Wild). Diploid (*2n* = 26) and tetraploid (*2n* = 52) plants were germinated from chipped seed at 25°C and grown under nursery conditions. After one month, measurements showed that the mean stomatal length was 27.17 ± 0.474 µm for diploids and 40.24 ± 0.521 µm for tetraploids and these differed significantly from each other (*P* < 0.001). The frequency of stomata per leaf surface was shown to decrease significantly (*P* < 0.001) as the ploidy level increased, with a mean of 22.11 ± 0.495 for diploids and 10.26 ± 0.495 for tetraploids. It was concluded that stomatal length and stomatal frequency are rapid indirect methods to identify ploidy level in black wattle. © 2003 The Linnean Society of London, *Botanical Journal of the Linnean Society*, 2003, 141, 177–181.


INTRODUCTION

*Acacia mearnsii* (de Wild), commonly known as the black wattle, was introduced into South Africa in 1864 through seed collected in southern Australia. Despite the fact that only approximately 10% of the commercial plantation area in South Africa is planted with this species, it is one of the leading commercially grown forestry trees in this country. The black wattle is an extremely versatile and useful tree, not only to the forestry industry as a source of high quality raw material for pulp production and as a source of vegetable tannin, but also for firewood and building purposes by the rural communities and local farmers. The production of improved seed and the maintenance of a high level of productivity is therefore particularly important to the wattle industry and rural farmers in South Africa.

In order to meet the needs of the forestry industry, breeding and production programmes are constantly being adapted to provide suitable genotypes for particular purposes. Besides the more conventional breeding strategies, various biotechnological techniques are also employed (Durzan, 1988) and include mutation breeding, transformation (Hammat, 1992), tissue culture, hybridization, chromosome doubling (polyploidization) and clonal propagation.

Recently, polyploidization has been recognized as a valuable technique in breeding strategies and is being investigated as a procedure to increase bulk for the pulp industry and to introduce sterility in the black wattle to help restrict the spread of wattle outside plantation boundaries.

Polyploidization, through the use of colchicine, has produced promising results in various trees and other plant species (Blakeslee & Avery, 1937; WRI report, 1952; Johnsson, 1956; Pesina, 1963; Winton, 1968; Pundir, Rao & van der Maesen, 1983; Yang & Yang, 1989; Du Plooy *et al.*, 1992; Kunitake *et al.*, 1998; Sangduen & SinpatanAnon, 1998). For the successful utilization of synthetic polyploids, it is important to
confirm the status of the level of ploidy. This is most often done by counting the chromosomes in root tip squashes. In the case of black wattle, the chromosome numbers are for the diploids \(2n = 2x = 26\), and for the tetraploids \(2n = 4x = 52\) (WRI, 1951/1952).

In black wattle, especially the polyploids, the high number of chromosomes combined with their small size and the low number of dividing cells that can be obtained from root tip squashes, can lead to incorrect identification of polyploids. It is therefore necessary to find more accurate, rapid and reliable procedures to identify black wattle polyploids.

Pollen grains, stomata length and frequency and number of chloroplasts are a few of the most widely used indicators of polyploidy (Geok-Yong Tan & Dunn, 1973). Stomatal lengths have been successfully used as indicators of polyploidy (Geok-Yong Tan & Dunn, 1973). The pinnules of the first leaf were removed from one-month-old seedlings for the purposes of this investigation.

Material and Methods

Plant Material

Diploid and tetraploid Acacia mearnsii seedlings were grown from seed, under nursery conditions. The tetraploids used were obtained from experiments done in the 1950s at the Wattle Research Institute (WRI) where tetraploidy was induced using colchicine and confirmed through chromosome counts (WRI, 1951/1952). The pinnules of the first leaf were removed from one-month-old seedlings for the purposes of this investigation.

Stomatal Length

Diploid and tetraploid seeds were germinated and grown in the nursery. From these seedlings, five diploid and five tetraploid seedlings (the same plants used for stomatal length determination) were selected from a tray to determine the average number of stomata per unit area of abaxial leaf surface, for each ploidy level. An Environment Scanning Electron Microscope (ESEM) was used to view the stomata. Stomata were counted and recorded in 20 microscopic fields (microscopic ‘counts’, at a magnification of \(\times400\)).

Stomatal Frequency

Diploid and tetraploid seedlings were germinated and grown in the nursery. From these seedlings five diploid and five tetraploid seedlings (the same plants used for stomatal length determination) were selected from a tray to determine the average number of stomata per unit area of abaxial leaf surface, for each ploidy level. An Environment Scanning Electron Microscope (ESEM) was used to view the stomata. Stomata were counted and recorded in 20 microscopic fields (microscopic ‘counts’, at a magnification of \(\times500\)).

Chromosome Analysis

The ploidy levels were confirmed by chromosome counts. Roots tips were taken from seeds germinated in an incubator at 25 °C. Young growing roots were harvested from three-day-old seedlings at approximately 10:00 h to ensure root cells were actively dividing. A root tip of approximately 10 mm was excised and placed in a fixative comprising a freshly prepared mixture of methanol : chloroform : acetic acid (6 : 3 : 1). Root tips were stained in Feulgen for 4 hours in the dark, prior to being squashed and mounted onto slides (one root tip per slide) for examination of chromosome number. Five slides were prepared for each ploidy level and five cells of each slide were counted, thus the chromosomes of 25 cells were counted for each ploidy level.

Statistical Analysis

GENSTAT Version 4.2 (Lane & Payne, 1996) was used to analyse the data statistically. Differences in stomatal length among plants of the same ploidy level and among slides within plants were analysed using a general nested ANOVA. A general nested ANOVA was then used to determine stomatal length differences between ploidy levels.
The statistical analysis of stomatal frequency was conducted to determine within and among plant variation and between ploidy variation. A one-way ANOVA was carried out to determine between ploidy variation. A general nested ANOVA was used to determine differences between plants within each ploidy, and a general one-way ANOVA was conducted for each ploidy separately to confirm these results. Differences between counts within plants within each ploidy level, i.e. within-plant variation, was analysed by accounting for the percentage variation each factor contributed, using values obtained from a general nested ANOVA.

RESULTS AND DISCUSSION

STOMATAL LENGTH

A positive relationship was found between ploidy level and stomatal length. On average, the tetraploids had a mean length of 40.24 ± 0.521 μm which was found to be significantly longer (P < 0.001) than their diploid counterparts (Table 1) that had a mean length of 27.17 ± 0.474 μm. It was noted that on examination of both the diploid and tetraploid plants separately, there were significant differences (P < 0.001 and P = 0.002, for tetraploid and diploid plants, respectively, Table 1) among the five plants tested. However, there were no significant differences (P = 0.479 and P = 0.836, for tetraploid and diploid plants, respectively, Table 1) among the slides within each plant. Thus there was only between-plant and no within-plant variation for both ploidy levels.

STOMATAL FREQUENCY

It was found that an inverse relationship existed between the frequency of stomata and ploidy level. It was noted that the frequency in the diploid leaves was significantly greater than in the tetraploid leaves (Table 2, Figs 1,2). Significant differences were noted between plants within each ploidy level (P = 0.008). A general one-way ANOVA was conducted for each ploidy separately to confirm these results (Table 2). Differences within each plant (i.e. microscopic ‘counts’) were analysed by accounting for the percentage variation each factor contributed to the total variation. The majority of the variation was associated with ploidy level (94%) and a small percentage of the total variation was associated with the interaction between counts within plants within ploidy level (Table 3).

CHROMOSOME ANALYSIS

The small size of the chromosomes made it difficult to obtain accurate chromosome counts, due to not all the chromosomes being equally visible. A mean of 16.08 ± 0.918 chromosomes was recorded for the diploid cells and a mean of 30.24 ± 1.748 chromosomes was recorded for the tetraploid cells. In comparison with what was expected (2n = 2x = 26 chromosomes; 2n = 4x = 56 chromosomes), these results are rather inaccurate. However, statistical analysis of the results did show significant differences between the diploids and tetraploids (P < 0.001), which concurred with results obtained from previous studies (WRI, 1950).

Table 1. Differences in stomatal length measurements among plants within each ploidy level and between diploids and tetraploids in general. Least significant difference (LSD) between means was obtained from the ANOVA

<table>
<thead>
<tr>
<th>Ploidy</th>
<th>Source of variation</th>
<th>df</th>
<th>F-prob</th>
<th>LSD</th>
<th>Ploidy mean length (μm; among plants)</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diploid (2x)</td>
<td>Plant</td>
<td>4</td>
<td>0.002</td>
<td>0.760</td>
<td>27.17</td>
<td>0.474</td>
</tr>
<tr>
<td></td>
<td>Slide</td>
<td>2</td>
<td>0.836</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tetraploid (4x)</td>
<td>Plant</td>
<td>4</td>
<td>&lt;0.001</td>
<td>1.073</td>
<td>40.24</td>
<td>0.521</td>
</tr>
<tr>
<td></td>
<td>Slide</td>
<td>2</td>
<td>0.479</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Differences in stomatal frequency per leaf surface of diploid and tetraploid plants (per field of view). Least significant difference (LSD) between means was obtained from the ANOVA

<table>
<thead>
<tr>
<th>Ploidy</th>
<th>Source of variation</th>
<th>df</th>
<th>F-prob</th>
<th>LSD</th>
<th>Ploidy mean stomatal frequency</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diploid (2x)</td>
<td>Plant</td>
<td>4</td>
<td>&lt;0.001</td>
<td>1.565</td>
<td>22.11</td>
<td>0.495</td>
</tr>
<tr>
<td>Tetraploid (4x)</td>
<td>Plant</td>
<td>4</td>
<td>0.008</td>
<td>1.311</td>
<td>10.26</td>
<td>0.495</td>
</tr>
</tbody>
</table>
CONCLUSION

In the case of black wattle, it is recommended that stomatal length and frequency be used as the preferred technique to determine the level of ploidy, rather than the tedious and rather inaccurate procedure of root tip chromosome counts.

ACKNOWLEDGEMENTS

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REFERENCES


Geok-Yong Tan, Dunn GM. 1973. Relationship of stomatal

Table 3. Determination of percentage variation attributed by ploidy levels, plants and counts, for differences noted in stomatal frequency between ploidy levels in A. mearnsii, as determined by a general nested ANOVA

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>df</th>
<th>SS</th>
<th>MS</th>
<th>Estimated (MS)</th>
<th>% contribution to variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>ploidy</td>
<td>1</td>
<td>7021.125</td>
<td>7021.125</td>
<td>684.409</td>
<td>94.53</td>
</tr>
<tr>
<td>ploidy:plant</td>
<td>8</td>
<td>1416.280</td>
<td>177.035</td>
<td>34.340</td>
<td>4.74</td>
</tr>
<tr>
<td>ploidy:plant:count</td>
<td>190</td>
<td>1004.750</td>
<td>5.288</td>
<td>5.288</td>
<td>0.73</td>
</tr>
<tr>
<td>Total</td>
<td>199</td>
<td>9442.155</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 1. Stomatal frequency in diploid A. mearnsii leaves. Scale bar = 50 μm.

Figure 2. Stomatal frequency in tetraploid A. mearnsii leaves. Scale bar = 50 μm.


**WRI report. 1950.** *Genetics*. Report from Wattle Research Institute, South Africa.

**WRI report. 1951/1952.** *Genetics*. Report from Wattle Research Institute, South Africa.

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