Effects of leaf and tree age on chlorophyll absorbance in diploid black wattle (*Acacia mearnsii*)

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ABSTRACT

The invasiveness of black wattle (*Acacia mearnsii* de Wild) in South Africa has created a need to investigate ways to reduce seed production. The Institute for Commercial Forestry Research is investigating polyploidy production with the view to inducing reduced fertility. Accurate, reliable and affordable identification of ploidy level is necessary. An effective technique for ploidy identification quantifying total chlorophyll content using absorption spectra has been developed, however its accuracy could be compromised by a number of factors of which this investigation assesses leaf and tree age. Young leaf material (new flush) and older leaf material (old flush) were collected from 20 genetically unrelated plantation trees in five age classes (two, four, six, eight and nine years). Seedling reference leaf material comprising essentially new leaf flush, were collected from ten genetically unrelated eight-month old seedlings. Chlorophyll was extracted in 90% acetone from five leaf samples of each new and old leaf type and each tree age. Absorbance spectra were obtained by passing light of wavelength 400 to 700 nm through the solution, with absorbance resolution of 1 nm intervals. Absorbance spectra peaked at 433, 456 and 663 nm in each case. Significant differences in value of peak absorbance were recorded among leaf samples, according to leaf type and tree age respectively. Mean chlorophyll absorbance values of corresponding peak wavelengths for all leaf types and age groups were mostly significantly different from one another, and all values were significantly different from the mean values for seedling reference samples. All values of new versus old flush at a particular age also differed significantly ($p < 0.05$). These results demonstrate that tree age and leaf type affect chlorophyll content significantly ($p < 0.05$), and should be considered when chlorophyll absorbance is used to determine ploidy.

Keywords: black wattle; phase transition ; polyploidy ; total chlorophyll content

INTRODUCTION

Black wattle (*Acacia mearnsii*), was introduced into South Africa in 1864 (Beard, 1957). Since that time, this species has become one of the leading forestry species, constituting approximately 7% of South Africa's forestry plantations (Dunlop and MacLennan, 2002). Despite its significant commercial value, it is a prolific seed producer that tends to invade native woodlands and cultivated areas, posing a great threat to non-commercial forest environments (Blakesley *et al*., 2002). Therefore, the containment of this invader species has become the focus of research in the *Acacia* Tree Improvement Programme at the Institute for Commercial Forestry Research (ICFR) in South Africa. Currently, control of black wattle through polyploidization is being explored as a means of introducing seed sterility into the species in order to reduce the prolific seed production (Dunlop and MacLennan, 2002). It is well established that autotetraploids tend to have variable and abnormal meioses due to the presence of multiple genomes and variable chromosome synapsis. In the case of black wattle, induced autotetraploids are being crossed with diploids to produce triploids, which are expected to display a significant reduction in seed set (Blakesley *et al*., 2002).

Induced black wattle autotetraploids require accurate identification prior to their implementation in crosses. Chromosome counting has proven to be labour intensive and unreliable in black wattle due to poor chromosome spreading and the small size of the chromosomes (WRI, 1951; WRI, 1952; Beck *et al.,* 2003b). Alternate methods have been developed to identify polyploidy in a number of species. These include quantification of pollen grain dimensions (Evans, 1955; Najčevska and Speckmann, 1968), measurement of stomatal guard cell length (Speckmann *et al*., 1965; Tan and Dunn, 1975; Mishra, *et al*., 1991); and analysis of the numbers of chloroplasts present in stomatal guard cells (Hamada and Baba, 1930; Mochizuki and Sueoka, 1955; Bingham, 1968; Chaudhari and Barrow,

1975). The determination of stomatal guard cell length and frequency (Beck *et al*., 2003a), stomatal guard cell chloroplast frequency (Beck *et al*., 2003b) and the use of flow cytometry (Beck *et al*., 2005) have also been successfully implemented in the analysis and identification of polyploidy in black wattle.

The standard method for determining the amount of chlorophyll in a leaf sample is to distinguish between the different chlorophyll pigments, however, this method is time consuming, especially when there are numerous specimens to analyze. For this reason a fast and cost effective technique to accurately distinguish between diploid and tetraploid black wattle was developed by Mathura *et al*. (2006). This method involved the quantification of total chlorophyll content in leaf material, through chlorophyll absorbance at three prominent peaks (at wavelengths 433, 456 and 663 nm). For routine application of this technology, an understanding of factors that might compromise the accuracy and reliability of the technique is required.

Plant growth begins with vegetative development which has been associated with a juvenile phase, which progresses into the sexual reproductive adult phase (Huang *et al*., 2003). In woody species, especially trees this change in juvenility to maturation may take several years and is commonly associated with a range of anatomical and physiological changes (Huang *et al*., 2003). In *Eucalyptus globulus* ssp. *globulus* it has been reported that leaf anatomy and morphology undergo dramatic changes in development when changing from seedling to adult plants and was reported to take between one and three years (James *et al*., 1999). As black wattle trees also undergo a similar transition period from seedling to adult, it is suspected that comparable physiological changes such as changes in photosynthetic capacity, may occur.

This investigation was thus undertaken to establish the effects of leaf maturity and tree age on total chlorophyll absorbance spectra in diploid black wattle, as these are factors that could influence chlorophyll content.

MATERIALS AND METHODS

Plant material

Leaf material for chlorophyll extraction was collected from diploid *A. mearnsii* trees located at the ICFR's research farm Bloemendal, in KwaZulu-Natal, South Africa. Twenty unrelated trees each of age two, four, six, eight and nine years respectively were selected and tagged. On each tree, leaf samples of two contrasting maturity classes were further identified by visual examination, namely, young leaf material (referred to as "new flush") produced early in the growth season (September) and older leaf material (referred to as "old flush") produced in the previous seasons. Five leaf samples of each of these two maturity classes for each cohort of tree age, were collected in September and placed in sealed black plastic bags and stored on ice whilst in transit from the collection site to the laboratory. At the time of the collection of new flush samples, it was found that identification tags of some trees were lost and some trees had been felled, which reduced the number of trees available for sampling at the time. This resulted in 18 H 4 yr old trees, 16 H 6 and 16 H 8 yr old trees being sampled.

Young diploid seedlings, grown in a seedling nursery under 20 % shadecloth, were comprised of essentially the new flush and these were sampled in order to provide a reference value against which all other estimates were compared. Five foliar samples from each of ten seedlings aged eight months were collected from three unrelated families.

Chlorophyll extraction and spectroscopy

Chlorophyll was extracted from leaf material by employing the method of Vernon and Seely (1966) with a few modifications. Leaves were firstly washed with distilled water to remove water soluble impurities. Approximately 1 g leaf material was homogenized in liquid nitrogen to reduce degradation, using a pestle and mortar. One gram of the powdery homogenate was weighed (to 0.1 mg). Working in reduced light, this sample was re-homogenized in 5 ml of 90% acetone. The chlorophyll-containing solution (CCS) of volume 5 ml was then siphoned off using a Pasteur pipette and placed into a polytop vial covered with tin foil. A total sample volume of 15 ml of CCS was produced by combining CCS sourced from three respective 1 g leaf samples of common origin. This was poured into a 25 ml volumetric flask and made up to 25 ml-mark with 90% acetone to make up a sample solution. The sample solution was then covered with tin foil, placed on ice and the chlorophyll absorbance spectrum was determined within 15 min of sample preparation, using regular techniques of chlorophyll absorbance spectroscopy as described below. Although the extraction process may have allowed for the extraction of other pigments and chlorophyll degradation products, these were not identified in the samples.

Total chlorophyll content was quantified in terms of absorbance spectra, by placing 1 ml of each sample solution into a 3 ml quartz cuvette, filled to the graduation mark on the cuvette with 90% acetone. A second quartz cuvette was filled with 90% acetone only and was utilised to standardise chlorophyll absorbance measurements in order to compensate for any absorbance that the acetone may introduce. Both cuvettes were placed in a PerkinElmer Lambda 45 UV/vis spectrometer. Visible light ranging in wavelength from 400 to 700 nm at 1 nm intervals was passed through the sample in the cuvette producing a chlorophyll absorbance spectrum for each sample. These spectra were recorded as ASCII files and

analysed statistically using the statistical package GenStat® 7.1 (Lane and Payne 2003). Standard means, ranges and deviations were calculated for wavelengths 433, 456 and 663 nm previously identified by Mathura *et al*. (2006). A general analysis of variance (ANOVA) was performed to assess the variation present among sample means and least significant differences were used to interpret the significance of such variation.

RESULTS

Chlorophyll absorbance values were determined for both new and old flush for 2-, 4-, 6-, 8- and 9-year old black wattle trees (Table 1). The chlorophyll absorbance spectra displayed the expected prominent peaks at 433, 456 and 663 nm where absorbance by the chlorophyll pigments was the greatest. The absorbance values at these peak wavelengths were used to determine the mean chlorophyll absorbance ($\bar{A}$) values as an indication of chlorophyll content. Among different trees and within a particular leaf type and age group, mean chlorophyll absorbance values at the respective three peak wavelengths differed significantly ($p < 0.05$). The grand mean of absorbance for the three peak wavelengths combined ($TT\bar{A}$) ranged from 0.378 to 0.474 for new flush leaves, and for old flush from 0.366 to 0.446 (Table 1). For each respective leaf type, mean absorbance values varied significantly with respect to age of trees, except for the new flush in 6-year and 8-year old trees and for the old flush in 8- and 9-year old trees ($p > 0.05$). For both new and old flush, mean chlorophyll absorbance of each age group differed significantly from the seedling reference group ($p < 0.05$) (Table 1). In most cases the mean absorbance of new flush differed significantly from old flush material of the same age group ($p < 0.05$), with the exception of the eight-year old group where there were no significant differences between new and old flush material ($p > 0.05$). At each age,

the old flush material displayed lower chlorophyll absorbance values than the new flush material, except for the two-year old group where the differences were reversed.

The effect of tree age was determined by comparing the grand mean absorbance (TTĀ) of the combined new and old flush absorbance values within each age group, across all tree ages (Figure 1). Chlorophyll absorbance data for all treatments, except for the two and four-year old leaf material, were all significantly greater ($p < 0.05$) than the seedling reference (Figure 1).

DISCUSSION AND CONCLUSION

Chlorophyll content in diploid black wattle displayed some general trends. Within each age group, the absorbance of the new flush leaves was higher than that of the old flush leaves, indicating diminishing chlorophyll content as leaves aged. Chlorophyll content in two year-old trees was, however, much greater in the old flush than in the new flush, a pattern that was not continued in older trees. This increased chlorophyll content in the older flush of the two-year old trees is probably indicative of the transitional process from juvenile to maturity, which agrees with James *et al*., (1999) in *E. globulus*.

The transition from juvenility to maturity is a lengthy process in trees (Huang *et al*., 2003) and in *E. globulus* it was found to take between one and three years (James *et al*., 1999). The results from this study in black wattle, showed that the grand mean absorbance (TTĀ) of the combined new and old flush absorbance values across the tree ages, remained essentially the same from seedling to four-years of age; indicating that the transition period in black wattle could be between four and six years.

This investigation revealed that leaf flush type and tree age should be considered when using chlorophyll absorbance spectra as a tool to distinguish ploidy levels in black

wattle, as both flush types and tree age have a significant effect on total chlorophyll content. For future application of this tool, sufficient plant material within different ploidy levels of black wattle are required for testing, and should be assessed for significant differences between ploidy levels, before this technology can be used as a standard technique.

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REFERENCES

Beard JS (1957) *A manual for growing wattle.* Natal Tanning Extract Company, Limited. Pietermaritzburg.

Beck SL, Dunlop RW and Fossey A (2003a) 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

Beck SL, Fossey A and Mathura S (2003b) Ploidy determination of black wattle (*Acacia mearnsii*) using stomatal chloroplast counts. *South African Forestry Journal* 192: 79-82

Beck SL, Visser G and Dunlop RW (2005) A comparison of direct (flow cytometry) and indirect (stomatal guard cell lengths and chloroplast numbers) techniques as a

measure of ploidy in black wattle, *Acacia mearnsii* (de Wild). *South African Journal of Botany* 71 (3&4): 359-363

Bingham ET (1968) Stomatal chloroplasts in alfalfa at four ploidy levels. *Crop Science* 8: 509–510

Blakesley D, Allen A, Pellny TK and Roberts AV (2002) Natural and induced polyploidy in *Acacia dealbata* Link. and *Acacia mangium Willd. Annals of Botany* 90: 391–398

Chaudhari HK and Barrow JR (1975) Identification of cotton haploids by stomatal chloroplast-count technique. *Crop Science* 15: 760–763

Dunlop RW and Maclennan LA (2002) *Black wattle: the South African research experience.* Institute for Commercial Forestry Research. Pietermaritzburg, South Africa

Evans AM (1955) The production and identification of polyploids in red clover, white clover and lucerne. *New Phytologist* 514: 149–162

Hamada N and Baba H (1930) On the number of chloroplasts in the guard cells in mulberry. *Journal of Sericultural Science* 1: 305–309

Huang LC, Weng JH, Wang CH, Kuo CI and Shieh YJ (2003) Photosynthetic potentials of *in vitro* grown juvenile, adults and rejuvenated *Sequoia sempervirens* (D.Don) Endl. Shoots. *Botanical Bulletin of Academia Sinica* 44: 31–35

James SA, Smith WK and Vogelmann TC (1999) Ontogenetic differences in mesophyll structure and chlorophyll distribution in *Eucalyptus globulus* ssp. *globulus* (Myrtaceae). *American Journal of Botany* 86: 198-215

Lane PW and Payne RW (2003) GenStat® 7.1: Genstat for Windows. The Numerical Algorithms Group. London.

Mathura S, Fossey A and Beck SL (2006) Comparative study of chlorophyll content in diploid and tetraploid black wattle (*Acacia mearnsii*). *Forestry* 79: 381-388

Mishra MK, Prakash NS and Sreenivasan MS (1991) Relationship of stomatal length and frequency to ploidy level in *Coffea* L. *Journal of Coffee Research* 21: 32–41

Mochizuki A and Sueoka N (1955) Genetic studies on the number of plastids in stomata. I. Effects of autopolyploidy in sugar beets. *Cytologia* 50: 358–366

Najcevska CN and Speckmann GJ (1968) Number of chloroplasts and pollen grain pores in diploid and tetraploid varieties of some *Trifolium* species. *Euphytica* 17: 357–362

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

Tan G-Y and Dunn GM (1975) Stomatal length, frequency and distribution in *Bromus inermis* Leyss. *Crop Science* 15: 283–286

Vernon LP and Seely GR (1966) *The chlorophylls.* Academic Press Inc. New York.

WRI (1951) Wattle Research Institute Report. Third year. Pietermaritzburg.

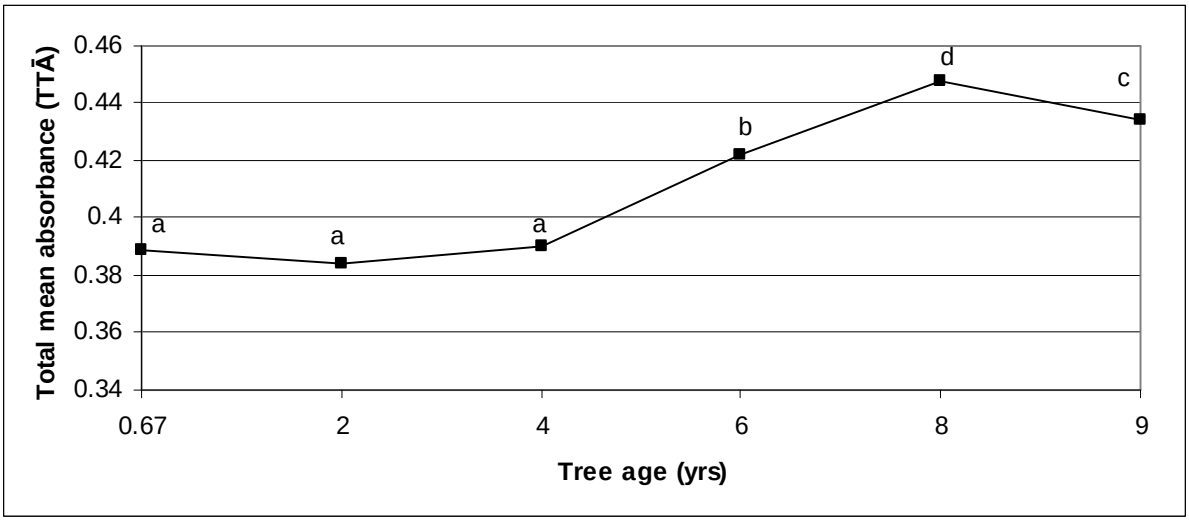
WRI (1952) Wattle Research Institute Report for 1951 – 1952. Pietermaritzburg.

Legend to figure

Figure 1. Comparison of diploid black grand mean absorbance ($TT\bar{A}$) for the seedling reference and new and old flush foliage, over different age groups. ($TT\bar{A}$ for pooled flush data, LSD = 0.005). Treatments denoted by the same letter are not significantly different from each other ($p > 0.05$).

1 Figure 1

2


Total mean absorbance (TTĀ)
0.46
0.44
0.42
0.4
0.38
0.36
0.34
a
a
a
b
d
c
0.67
2
4
6
8
9
Tree age (yrs)

3

Table 1. Mean chlorophyll absorbance of diploid black wattle at the three major peaks for the seedling reference and new and old leaf flush material, across the different tree ages.

Treatment	Age (yr)	Wavelength (nm)	Absorbance range	Mean absorbance ($\bar{A}_\lambda$) (LSD = 0.012)	Grand mean absorbance per tree age (TT$\bar{A}$) (LSD = 0.008)
Seedling	0.67	433	0.518 – 0.480	0.503	0.389 [c]
		456	0.385 – 0.349	0.370	
		663	0.310 – 0.276	0.295	
New flush	2.00	433	0.582 - 0.442	0.494	0.378 [b]
		456	0.418 - 0.311	0.359	
		663	0.323 - 0.244	0.283	
	4.00	433	0.575 – 0.545	0.551	0.418 [d]
		456	0.418 – 0.333	0.394	
		663	0.333 – 0.292	0.310	
	6.00	433	0.598 – 0.578	0.587	0.452 [g]
		456	0.441 – 0.428	0.430	
		663	0.357 – 0.339	0.345	
	8.00	433	0.597 – 0.561	0.584	0.451 [fg]
		456	0.440 – 0.406	0.427	
		663	0.356 – 0.353	0.342	
	9.00	433	0.629 – 0.593	0.612	0.474 [h]
		456	0.466 – 0.424	0.450	
		663	0.376 – 0.337	0.361	
Old flush	2.00	433	0.713 - 0.442	0.566	0.433 [e]
		456	0.562 - 0.285	0.409	
		663	0.483 - 0.120	0.323	
	4.00	433	0.508 - 0.442	0.481	0.366 [a]
		456	0.368 - 0.312	0.344	
		663	0.295 - 0.244	0.273	
	6.00	433	0.516 - 0.498	0.511	0.391 [c]
		456	0.376 - 0.356	0.370	
		663	0.304 - 0.256	0.298	
	8.00	433	0.604 – 0.528	0.584	0.446 [fg]
		456	0.441 – 0.391	0.422	
		663	0.351 – 0.293	0.332	
	9.00	433	0.593 – 0.542	0.577	0.444 [f]
		456	0.436 – 0.366	0.420	
		663	0.351 – 0.310	0.335	

Treatments denoted by the same letter are not significantly different from one another ($p > 0.05$).