INVESTIGATION OF CHLOROPHYLL AND STOMATAL CHLOROPLAST CONTENT IN DIPLOID AND TETRAPLOID BLACK WATTLE (*Acacia mearnsii* de Wild.)

By

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"*Es gibt zwei Arten Leute in der Welt;*
Forscher und Techniker"

ANON, 1979

PREFACE

The experimental work described in this thesis was carried out in the School of Molecular and Cellular Biosciences, University of Natal, Pietermaritzburg, under the supervision of Professor Annabel Fossey.

These studies represent original work by the author and have not otherwise been submitted in any other form to another University. Where use has been made of the work of others it is duly acknowledged in the text.

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Sadhna Mathura

I hereby certify that this statement is correct.

Professor Annabel Fossey

Supervisor

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

ABBREVIATIONS

$\bar{A}$	Mean absorbance
ANOVA	Analysis of variance
CCS	Chlorophyll-containing solution
C-mitosis	Colchicine-mitosis
df	Degrees of freedom
DNA	Deoxyribonucleic acid
F.pr	F-probability statistic
ICFR	Institute for Commercial Forestry Research
kg per m^3	Kilograms per cubic meter
LSD	Least significant difference
LT$\bar{A}$	Total mean absorbance for leaf age
ml	Millilitre
ms	Mean square
N	Sample size
nm	Nanometre
No.	Number
PEG	Polyethyleneglycol
PSO	Production seedling orchard
PT$\bar{A}$	Total mean absorbance within ploidy
p-value	p-probability statistic
Reps	Repeats
RuBPC	Ribulose-1,5-bisphosphate carboxylase
ss	Sum of squares
T$\bar{A}$	Total mean absorbance
TT$\bar{A}$	Total mean absorbance for tree age
Tδ	Total deviation from control
UV/vis	Ultraviolet light / visible light
vr	Variance ratio
WRI	Wattle Research Institute
2n	Diploid gametic number
2x	Diploid basic number
40 X	Forty times (magnification)

ABSTRACT

Black wattle (*Acacia mearnsii*) is one of South Africa's leading commercial exotic species comprising nearly seven percent of South African forestry plantations. The planting of black wattle has become increasingly popular, initially for its high-quality tannin content and in more recent times, for its wood and wood products. The industry also provides jobs for more than 36 000 people. Despite the commercial value of black wattle, if left unmanaged, it is one of South Africa's top invader species that aggressively colonise and rapidly out-competes indigenous vegetation. Thus, both plant breeders and environmentalists alike are faced with an interesting paradox of balancing the commercial significance of black wattle on the one hand with increasing environmental concern on the other.

At the Institute for Commercial Forestry Research (ICFR), black wattle breeding programmes are being designed and implemented in order to reduce invasiveness whilst still maintaining product quality. One way of minimising invasiveness is to decrease fertility through the introduction of semi-sterility; while at the same time leaving product yield and quality unaffected. A method of achieving semi-sterility is by the induction of autopolyploidy that results in unviable gametes. Autopolyploidy, tetraploidy, is induced chemically through doubling of the chromosomes of diploids. These induced tetraploids may then be crossed with diploids to produce triploids. Thus, an effective method to identify polyploids at the seedling stage would greatly facilitate the success of the abovementioned breeding programmes in the black wattle industry.

Polyploidy in plants is often associated with physiological and biochemical changes that become apparent as gigantism of organs which include fruits, flowers and leaves. Polyploidy is also associated with an increase in the number of organelles such as the number of stomatal chloroplasts and nucleoli, as well as an increased production of some proteins and pigments such as chlorophyll. These ploidy-related manifestations are often utilised in breeding programmes to increase the size and quality of plant products as well as a tool to discriminate between polyploids and diploids.

Two putative diagnostic procedures to differentiate between diploid and tetraploid black wattle were developed in this investigation. The study focused on the discriminating power of stomatal chloroplast numbers and arrangements as well as the chlorophyll content in the two different ploids. A number of associated experiments were initially conducted to establish the optimal conditions for chlorophyll content analyses such as the type of leaf material and storage conditions.

Stomatal chloroplast frequencies were determined in diploid and tetraploid black wattle and comprised three lines per ploidy level with five plants per line. A thin epidermal layer from the abaxial surface of a pinnule was stripped, stained, mounted and 15 stomatal guard cells per plant were viewed at 40X magnification. The mean number of chloroplasts per cell in diploids (9.89 ± 0.222) was found to be statistically different ($p < 0.001$) to that of tetraploids (22.43 ± 0.222) with no overlapping of the mean chloroplast values between the two ploidy levels. The ratio of diploid and tetraploid stomatal chloroplast numbers was roughly 1:2. An analysis of the least significant difference (LSD) was performed and indicated significant differences between plants within lines, between lines of different ploids (LSD = 0.6266), as well as between the different ploids (LSD = 0.2802).

Furthermore, stomatal chloroplasts spatial arrangements were distinctly different in diploids and tetraploids. In diploids, chloroplasts were clustered into two regions, each towards the extreme ends of the kidney shaped stomatal cells. In the tetraploids, no clustering of chloroplasts could be identified, with an even distribution around the convex curvature/perimeter of the cells.

There are a number of factors that influence chlorophyll content and degradation, which are either environmental or genetic in nature. Environmental factors that were considered are sample age and sample storage conditions. Genetic factors include genetic composition and, specifically, the number of sets of chromosomes, that is, the ploidy.

Chlorophyll content was investigated by chemically extracting chlorophyll from leaf material and obtaining absorbance spectra with a PerkinElmer UV/vis spectrometer for wavelengths from 400 nm to 700 nm. Chlorophyll absorbance spectra were generated in terms of leaves stored prior to chlorophyll extraction, leaves of different ages, trees of different ages and ploidy.

The effects of storage of leaves on chlorophyll content were determined in five non-identical two year-old nursery diploid black wattle genotypes. Fifteen leaf samples from each genotype were either oven dried and then stored for one week or one month at room temperature, or frozen for one week or one month at -4 ^{o}C, before chlorophyll was extracted and absorbance spectra determined. Chlorophyll absorbance values of chlorophyll extracted from leaf material on the day of collection (day-0) was used as the control. An analysis of variance (ANOVA) revealed that the chlorophyll absorbance values of the different storage treatments were all significantly lower than the chlorophyll absorbance values of the control ($p < 0.001$).

Assessment of the mean chlorophyll absorbance ($T\bar{A}$), sum of the three peak absorbance values at three wavelengths, namely, 433 nm, 456 nm and 663 nm, revealed significant differences ($p < 0.001$) from the control ($T\bar{A} = 1.275$) for all treatments. Dried leaves that were stored for seven days ($T\bar{A} = 1.132$) resulted in the least amount of chlorophyll degradation followed by 28 day ice storage ($T\bar{A} = 1.114$), seven day ice storage ($T\bar{A} = 1.103$) and lastly 28 day dried storage ($T\bar{A} = 1.093$). An analysis of least significant differences (LSD) revealed that chlorophyll absorbance values within lines and between wavelengths were significantly different (LSD = 0.005). Furthermore, LSD analysis revealed significant differences between all treatments (LSD = 0.003) which also supported the ANOVA findings.

Chlorophyll absorbance values within dried and frozen treatments were compared with respect to storage time periods of one week and one month. It was noted that whilst all treatments decreased from the control (day-0), dried samples responded differently to storage periods as compared to frozen samples. Chlorophyll absorbance values of dried material decreased steadily over time from control to

seven-day storage to one-month storage, whereas, in the case of frozen material, a similar trend could not be identified. A greater decrease from the control to seven day ice storage was recorded than for the decrease from the control to 28 day ice storage.

The effects of tree and leaf ages of diploid black wattle on chlorophyll content were determined. Two types of leaf flushes namely, old and new flush, were examined in relation to different tree ages; two, four, six, eight and nine year-old; in order to assess whether the choice of material impacts on chlorophyll absorbance values. Five leaf samples from each tree were collected, bagged and chlorophyll extracted within two hours of collection. These chlorophyll absorbance values were compared to young diploid seedling material as a base-value and as a control value.

An analysis of variance (ANOVA), revealed significant differences between tree ages and between leaf ages ($p < 0.001$). An analysis of least significant differences (LSD) revealed that new flush of all tree age groups were significantly different from the control (LSD = 0.006). This was mostly true for old flush, except that of six year-old old flush which was not significantly different from the control (LSD = 0.006).

The chlorophyll absorbance values of both old and new flush of different age groups produced spectral graphs for which no specific trends could be ascertained. Therefore, the data from the two flush types were pooled and revealed a marked increase in chlorophyll absorbance as trees became older. Moreover, this increase was more apparent in new flush than in old flush. Interestingly, juvenile characteristics were identified in two year-old black wattle trees, where a marked increase in chlorophyll content was noted.

The effects of the number of chromosome sets on chlorophyll content were assessed for diploid and tetraploid black wattle. Seedlings, bagged juveniles as well as two year-old field trees were analysed. Three genetic lines per ploidy level comprising of ten plants per line were used in the analysis. An analysis of variance (ANOVA) revealed significant increases of chlorophyll absorbance values

($p < 0.001$) for diploid seedlings ($T\bar{A} = 1.1086$) to bagged trees ($T\bar{A} = 1.149$) to field trees ($T\bar{A} = 1.224$). Similar significant increases were recorded for the tetraploid seedlings ($T\bar{A} = 1.886$) to bagged trees ($T\bar{A} = 1.931$) to field trees ($T\bar{A} = 2.059$). There were distinct differences in chlorophyll absorbance between the two levels of ploidy (LSD = 0.002). Furthermore, chlorophyll absorbance within lines, between wavelengths were found not to be significant ($p = 0.984$), which was supported by an analysis of least significant differences (LSD = 0.004). Moreover, the ratio of diploid to tetraploid chlorophyll absorbance was roughly 2:3. Additionally, the increase of chlorophyll content from seedlings to bagged juveniles to field material of both diploid and tetraploid black wattle further supported the findings in the previous age study that there was an increase in chlorophyll content as the tree matures.

Stomatal chloroplast frequencies and chlorophyll content have been identified as two methods that are able to effectively, and with ease, discern between diploid and tetraploid black wattle.

1 CHAPTER ONE INTRODUCTION

1.1 INTRODUCTION

Trees are of fundamental importance to sustaining life on earth and constitute a significant portion of global land cover. Figure 1.1 provides an indication of global tree coverage as reported by a recent satellite survey (DeFries, *et al.*, 2000). This tree population sustains both local communities and wildlife in providing food, shelter and fuel. In addition, forests help to maintain the delicate environmental balance by curbing soil erosion and enhancing soil properties. Trees also play a critical role in the reduction of atmospheric carbon dioxide thereby reducing damages caused by the Greenhouse Effect. Global forests consist of indigenous forests, which comprise naturally occurring tree species that exist through no human influence; and plantation forests, which have been developed for commercial purposes such as the production of timber and timber products.

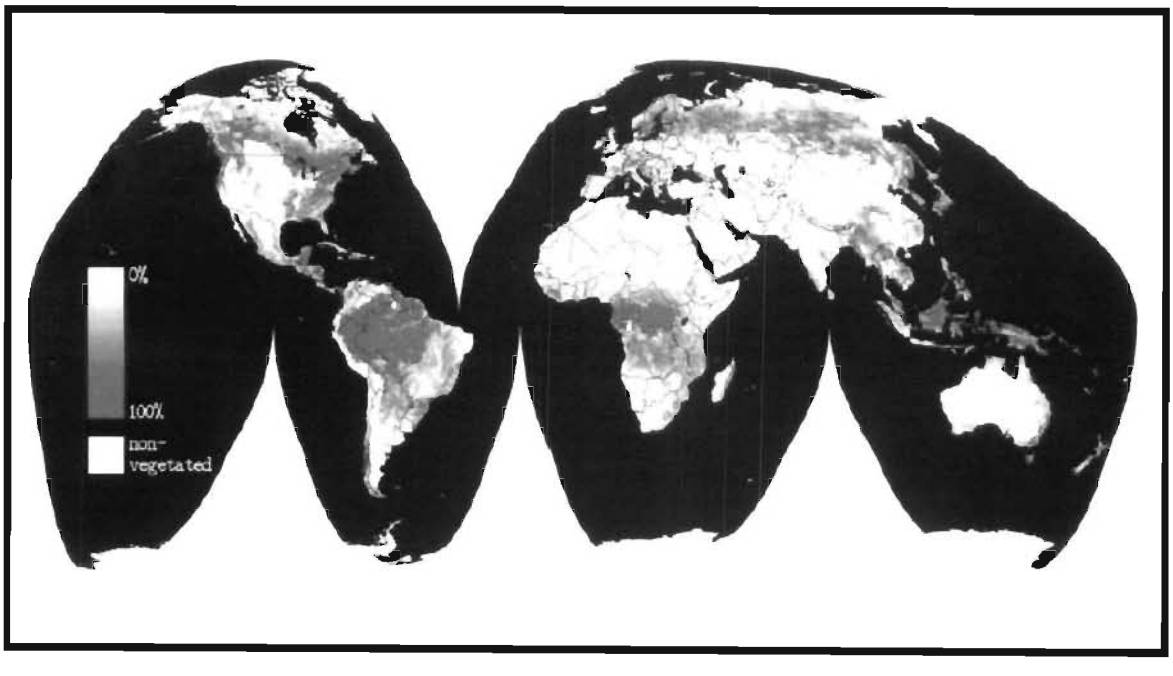

Figure 1.1 Global tree distribution (adapted from DeFries *et al.*, 2000)

Indigenous tree-coverage form a significant part of global forestry practices. Today indigenous forests consist of several habitat-specific species that have evolved over many centuries (FAO, 1997; CIDA, 1992). Many of these species are felled, to maintain the fuel, food and shelter needs of neighbouring populations. The world's indigenous forests are being depleted at an alarming rate with annual global tree-loss estimated at 17 million hectares (CIDA, 1992, FAO, 2000). It is therefore of great importance that countries manage their natural forests in a sustainable manner.

Commercial forestry is a significant global industry generating a considerable amount of employment and foreign exchange. The industry has grown considerably after World War II as the demands for wood and wood products increased (Tewari, 2000). Thirty percent of the world's land is under forest (FAO, 2000) with forestry land distribution percentages indicated in Table 1.1.

Table 1.1 Percentage of global land utilised for forestry practices (adapted from FAO, 2000).

Country	% Land utilisation
Japan	67.0
Russian Federation	46.0
Canada	39.0
U.S.A	30.0
European Union	29.0
R.S.A	1.1
Zimbabwe	0.3

South African natural forests cover a very small area of the total geographical region; about 0.1 %, predominantly concentrated towards the eastern coast (Tewari, 2000). Indigenous species include yellow- and stinkwood varieties. South African foresters recognize that the utilization of natural forests as a source of wood and wood products is limited mostly due to slower growth rates of the natural species (Tewari, 2000).

Species utilized for commercial purposes require attributes that make them suitable for forestry practices. Characteristics of importance include physiological fitness; disease and pest resistance; sustainability and adaptability to a variety of habitats; and most importantly, fast growth with high quality wood (Richardson, 1998). The demand for wood in South Africa is expected to double by 2005 (Tewari 2000), however, South African indigenous species generally do not display many of the abovementioned attributes, whereas exotic species are far more suitable for commercial exploitation. Since their introduction, exotic species have formed the basis of all commercial forests within South Africa (Richardson, 1998).

Exotic species planted in South Africa have mostly been obtained from Australia and California and consist of many species of hard- and softwood (Richardson, 1998; Tewari, 2000). These include species of *Pinus* (pines), *Eucalyptus* (eucalypts) and *Acacia* (wattles). Pines and other softwood species comprise 53 % of our country's plantation land, whilst hardwood species like eucalypts and wattle occupy 47 % (DWAF, 2000). In recent years the wattle species have attracted the interest of South African commercial foresters and the number of wattle plantations has since shown a steady increase (Dunlop and MacLennan, 2002).

1.2 SOUTH AFRICAN BLACK WATTLE INDUSTRY

1.2.1 Introduction

Acacia mearnsii (de Wild.), commonly called black wattle, is an exotic species that was introduced to South Africa from Australia (Sherry, 1971). Seeds from south eastern Australia were introduced to Camperdown in KwaZulu-Natal by John van der Plank in 1864 (Jarmain and Lloyd Jones, 1982). Black wattle was initially planted to provide shade for livestock, fuel and shelterbelts. It was soon discovered that these trees contain high quality tannins, a quality that initiated the wattle industry in South Africa (Figure 1.2) (Heiberg-Iurgensen, 1967; Sherry, 1971).

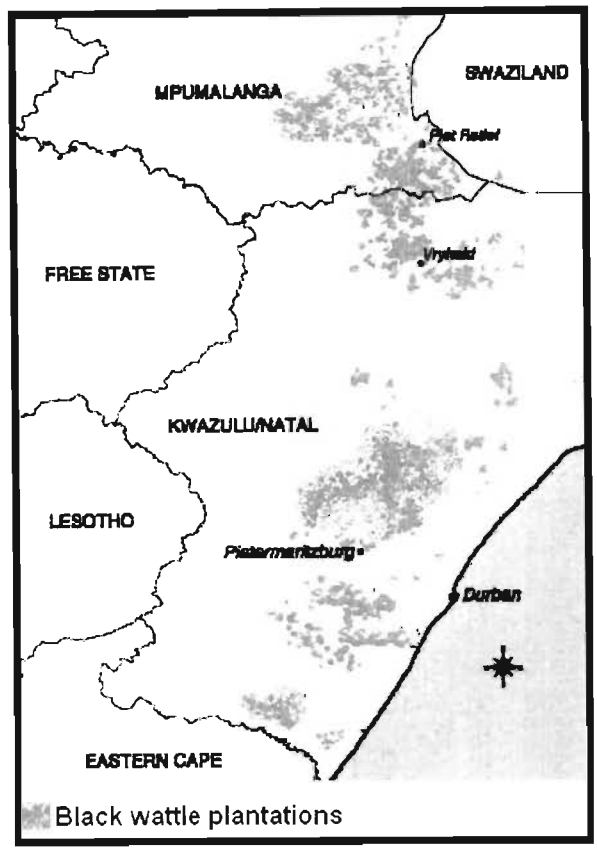

Figure 1.2 Distribution of black wattle plantations in South Africa. (adapted from Dunlop and MacLennan, 2002)

Today, the commercial significance of the black wattle forestry industry within South Africa is enormous. Black wattle comprises nearly seven percent of South African forestry plantations and provides jobs for more than 36 000 people (Dunlop and MacLennan, 2002). Black wattle was estimated to have fetched up to R 826 million per annum on the international market (Dobson, 1998). By the year 2000, black wattle was the most commonly planted species used for afforestation (46.9 %) (FSA, 2000). Black wattle's increasing value as a commercial tree is catapulting the South African black wattle industry to the forefront of a competitive global industry.

The significant commercial value of black wattle has led to extensive research aimed at promoting the use and profitability of black wattle in South Africa (Sherry, 1971). This research became the pinnacle task of the South African forestry industry in order to compete with international markets. Wattle research began in the early 1900s (Osborn, 1931) and continued at the Wattle Research Institute (WRI) upon its founding in 1947. The WRI was formed as a three-way partnership comprising

wattle growers, the South African government and the University of Natal; the purpose of the institute being to spearhead solely wattle research programmes[¥] (Dunlop and MacLennan, 2002).

In the 1940s the WRI initiated several wattle breeding programmes. The initial aims of these programmes were to increase the production of bark yields for the valuable tannin content as well as to increase resistance to gummosis (Dunlop and MacLennan, 2002). Many hybridisation experiments and progeny testing between black and green wattle were undertaken and by the 1960s South Africa was renowned for having the most advanced wattle breeding programme in the world (WRI, 1949; Nixon, 1992). Extensive research was underway in fields such as silviculture, genetics, chemistry and entomology (WRI, 1948; Dunlop and MacLennan, 2002).

In 1984 the WRI became the Institute for Commercial Forestry Research (ICFR), and expanded the scope of their research to include other commercial species such as eucalypts and pines, although wattle was retained as its primary focus. Furthermore, the change in market demands from tannin to wood caused a shift in the drive of black wattle breeding programmes (Dunlop and MacLennan, 2002). The aims have moved from the exclusive improvement of bark to the improvement of timber yield and quality whilst maintaining an acceptable bark quality. Improvements in yields are achieved through ongoing progeny tests and the successive selection of superior trees. The programme has lead to the establishment of Production Seedling Orchards (PSOs) that supply industries with improved seed. With the current increase in black wattle timber in the pulp industry, interests in black wattle timber production has grown extensively and so too has the research in that field (Dunlop and MacLennan, 2002).

The black wattle industry has faced many challenges over the years, the most pertinent of which, is the invasive nature of the species. The management of the *Acacia mearnsii* species is difficult due to its tendency to invade native woodlands and cultivated areas. The invasion of native woodlands by black wattle has various

[¥] Research programmes include many ways of improving tree physiology by breeding for certain traits. Hence, in this dissertation, the terms 'breeding programme' and 'improvement programme' are used interchangeably.

environmental consequences, which affect several parts of South Africa (Van Wilgen *et al.*, 1996). The consequences include clogged waterways and increased soil erosion as a result of its high water consumption (Versfeld *et al.*, 1998). Furthermore, some natural vegetation is being out-competed to the point of extinction thereby irreversibly altering the immediate environment (Cowling, 1992). The ICFR has taken a pro-active approach to addressing the invasiveness of black wattle by investigating various methods of control, such as felling, burning, chemical treatments, biological control and the introduction of genetic control mechanisms (Dunlop and MacLennan, 2002).

Black wattle research over the past 50 years has lead to important tree improvements. These improvements include an increase in the variety of black wattle and available provenances; an increase in the number of sites for establishment of plantations; a decrease in the incidence of gummosis and an increase in productivity due to tree improvement (Dunlop and MacLennan, 2002). These tree improvements coupled with the versatility of the species has enabled wattle growers to maintain their successful position in a thriving global industry.

1.2.2 Black wattle characteristics and growth habitat

Black wattle trees have a number of distinctive growth and physiological features. The tree may be described as a *legume* and is a member of the pea family (Fabaceae: Mimosoideae). It has seed-containing pods and nitrogen-fixing root nodules that enrich the soil. Furthermore, the thick roots also assist with the prevention of soil erosion. Trees grow to heights of between six and twenty meters with dense, deep green foliage that often serve as windbreaks and shelter. The inflorescence form fragrant pale yellow clusters that develop into grey dehiscing pods that contain smooth, black, elliptical seeds (Figure 1.3). Seeds can lie dormant for years and retain much of their moisture to grow when conditions turn favourable (Sherry. 1971; Jøker, 2000)

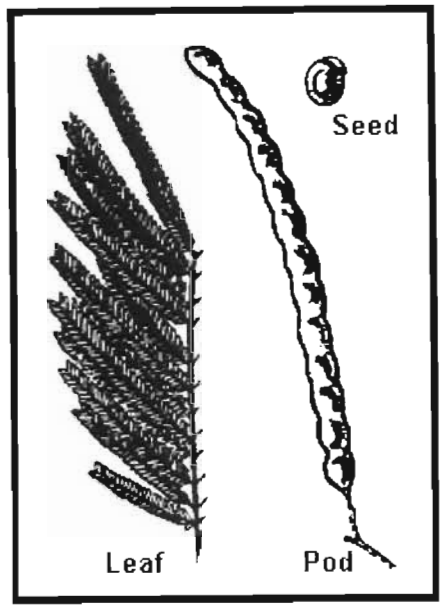

Figure 1.3 Illustration of a typical black wattle leaf, seed and pod composition

These trees are robust and grow in a variety of soil types, are frost resistant and can tolerate temperatures from 0 °C up to 28 °C, as well as a range of altitudes and rainfall. In tropical areas, black wattles favours highland areas with altitudes of 1500 m – 2500 m and with an average rainfall of about 900 mm – 1600 mm per year. These characteristics have promoted its extensive exploitation in the South African commercial forestry industry (Sherry, 1971; Jøker, 2000).

1.2.3 Uses of black wattle

Historically, black wattle's commercial purpose was its high grade export-quality tannin (Sherry, 1971). The highest concentration of tannin is localised in the bark, and when dried, 35 % of the bark's dry weight is tannin (Beard, 1957). Black wattle tannin is preferred for tanning leathers as it is fast absorbing and does not stain the leather, whereas other tannins often leave a red colouration and residue (Heiberg-Iurgensen, 1967). With the exception of mangrove species, black wattle produces more tannin per hectare than most other tanninferous plants. One ton of black wattle bark contains enough tannin to tan 2 530 hides (Sherry, 1971)

Currently black wattle trees are planted as much for their timber as for their bark (Stubbings and Schönau, 1983). Many new uses for its timber have gained prominence, shifting timber production to more than 75 % of total South African timber production, having at one stage only comprised about 50 %. The timber is of high quality and is utilised for mining, burnt for fuel, charcoal, and used in manufacturing paper (Table 1.2). It is also used in the making of parquet blocks and hardboard. The cellulose is used for the production of rayon (DWAF, 1998). It is also used for the production of adhesives and flocculants (Duke, 1981).

Black wattle timber has become a popular source of fibre for the pulp and paper industry. The density and pulp yield of black wattle make it an attractive alternative to other species such as *Eucalyptus globule* (Dunlop and MacLennan, 2002). It is believed to have bleaching and mechanical pulp properties that are almost on par with *E. grandis* (Nicholson, 1991). Furthermore, the mechanical pulp and bleach properties are thought to be equivalent to *Eucalyptus grandis* whereas pulp yield was found to be higher in black wattle (Muneri, 1997).

Table 1.2 Production of black wattle products (adapted from DWAF, 1998).

Product	Amount in Tonnes
Paper	416 098
Wood chips	242 561
Pulpwood	123 104
Firewood	81 692
Mining timber	42 856
Charcoal	27 778
Other by-products	21 926

Black wattle is a versatile species, which has been extensively utilized by the rural community in South Africa. Farmers use black wattle mostly for firewood and building material. Its ability to grow on poor soil also provides a crop for farmers living in such regions and thus an additional income to these farmers. Black wattle companion crop farming (agroforestry) is gaining popularity since it can be grown with other crops such as maize and beans due to its soil enriching abilities and coverage of foliage it provides (Dunlop and MacLennan, 2002).

1.2.4 Black wattle tree improvement programmes

Market demands and breeding problems have very often directed the focus of black wattle improvement programmes. Historically, black wattle improvement programmes were devised to improve bark yields for the tannin content and to breed for frost and disease resistance. This was due to the fact that black wattle bark was the major product of the industry during the initial years of its establishment in South Africa. Additionally, black wattle was found to be highly susceptible to frost and fungal diseases (Sherry, 1971; Jarmain and Lloyd Jones, 1982).

Today, the market demands of black wattle have shifted, and new breeding problems have arisen thus directing current improvement programmes towards addressing these issues. The black wattle market demands have diversified and now especially include new uses of timber and timber products (Nicholson, 1991; Muneri, 1997). Thus the focus of black wattle breeding programmes has expanded to include the improvement of timber quantity and quality whilst still maintaining an acceptable standard of bark yields (Dunlop and MacLennan, 2002). The improvement programmes also include the identification of other commercially useful *Acacia* species for progeny testing and hybridisation (Dunlop and MacLennan, 2002). Furthermore, black wattle has been flagged as one of South Africa's top invader species, the planting of which has raised many environmental concerns (Richardson, 1998). Currently, a major focus of black wattle breeding programmes is the introduction of an improved germplasm that would provide good quality black wattle products without exacerbating the invasive problem (Dunlop and MacLennan, 2002).

Previous research into improving germplasm started as an extensive study into the reproductive mechanisms of black wattle. Researchers investigated every facet of black wattle reproduction from pollination mechanisms and agents to seed properties and cytogenetics (WRI, 1950; Dunlop and MacLennan, 2002). However, perhaps the most noteworthy research at the time was in the area of seed improvement and cytogenetics. Moffett and Nixon (1960) explored polyploidy as a

method to improve seed and reported the first colchicine-induced tetraploids in black wattle. According to Blakesley *et al.*, (2002) it is the only report of chromosome doubling in the tribe Acacieae. Thus, cytology and induced tetraploids became key areas of research in black wattle improvement programmes (WRI, 1949; WRI, 1950; WRI, 1951; WRI, 1952).

Seed improvement is currently regarded as a critical aspect that requires ample research in black wattle tree improvement programmes. This is principally due to the prolific seeding ability which forms the crux of a serious black wattle invasion problem. This aggressive colonising ability is largely due to the high frequency and viability of black wattle seeds. Thus, various research projects are focussed in finding effective ways of reducing seed viability and seed production. The production of sterile or seedless black wattle varieties for distribution to the various farming sectors would greatly reduce the invasive problem. Two approaches to produce sterile seeds are being assessed at the ICFR; gamma irradiation of seeds and seedlings and the production of triploid trees. The production of triploid trees involves firstly, the production of artificial tetraploids which are then crossed with diploids to produce sterile triploids. If triploid production is successful, the distribution of sterile trees will rely upon the ability to clone individuals and to verify the level of ploidy before distribution (Dunlop and MacLennan, 2002).

1.3 POLYPLOIDY

1.3.1 Introduction

Polyploidy is a mutational phenomenon whereby the number of chromosomes in a cell increases (Stebbins, 1950). This increase is usually in multiples of the basic chromosome number, which is a characteristic of the genus to which that cell belongs and is typically because of some or other failure in the cell division cycle (Stebbins, 1950; Lewis, 1979). Whilst polyploidy is a relatively rare occurrence in animals (Vandel, 1938; Frankhauser, 1941), it is more common in the plant kingdom (Grant, 1981) and is often the mechanism whereby new plants evolve (Lewis, 1979).

Interest in polyploidy began in the early 1900s when some of the earliest natural polyploidy findings were observed in angiosperms. Inconsistencies in the basic chromosome number were first observed in various genera of *Liliaceae* (Jackson and Hauber, 1983) and thereafter in several species of *Chrysanthemum* (Tahara, 1915). DeVries' *Oenothera lamarickiana* is perhaps the most comprehensively documented example of a natural polyploid (Lutz, 1907; Gates, 1909). Winkler (1916) first employed the term *polyploidy* to describe the occurrence of duplicated chromosome sets that he found in *Solanum nigrum*. Subsequently, several examples of both natural and cultivated polyploids were reported in various species, thereby challenging what was known at that time about the constancy of chromosomes numbers and the evolution of higher plants (Winge, 1917; Stebbins, 1950; Jackson and Hauber, 1983; Soltis and Soltis, 1995).

Whilst polyploidy is widely distributed amongst some groups in the plant kingdom, it is often rare or absent in others (Federov, 1969; Lewis, 1979, Soltis *et al.*, 2003). Stebbins (1950) estimated that 30 - 35 % of angiosperm species were polyploid. Masterson (1994) estimated that 47 - 70 % of all angiosperm have experienced chromosome doubling at some stage of their evolutionary history, a figure that was later supported by Wendel (2000). A large proportion of polyploid angiosperms are found amongst the perennial herbs with the smallest concentration in the woody angiosperms (Stebbins, 1938; Leitch and Bennett, 1997). Some of the oldest known plants such pteridophytes and bryophytes as well as some common grass species have incredibly high levels of polyploidization (Manton, 1950; Burnham, 1962; Grant, 1981; Kellogg, 2001; Levy and Feldman, 2002). On the other hand, polyploidy is known to be rare or totally lacking in certain fungi and gymnosperm species, (Grant, 1981). Fewer than 5 % of gymnosperm species such as sequoia, juniper and conifers are polyploid whereas polyploidy is totally absent in cycads and ginkgo species (Stebbins, 1950; Lewis, 1979).

In general, polyploidy results as a failure in cell division which may occur in either somatic or gametic cells (White, 1942; Pierce, 2002). Generally the cell division failure occurs when the chromosomes divide (karyokinesis) without the subsequent cell division (cytokinesis). This is usually the case when the microtubules that

polarise the chromosomes are defective in some way having one of three outcomes: *C-mitosis*, *endomitosis* and *endoreduplication* (McDermott, 1975) (Figure 1.4).

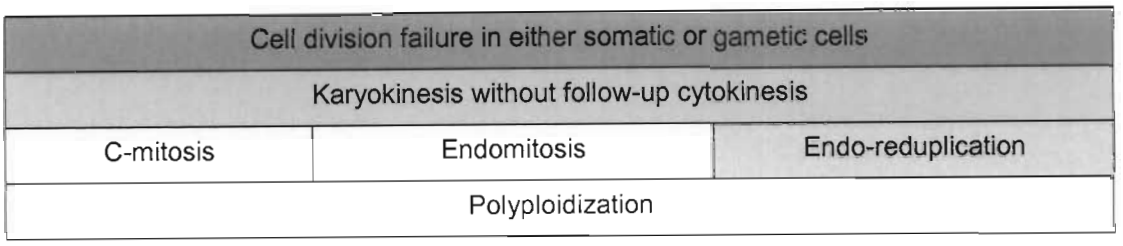

Figure 1.4 Occurrences of polyploidization.

Somatic and gametic cells that undergo polyploidization need to be differentiated from each other. Somatic cells usually have twice the basic chromosome number (2x) and are referred to as *diploid*. These somatic diploid cells may fail to divide after mitotic chromosome duplication thereby resulting in the cell having twice as many chromosomes as before (4x) which is referred to as *tetraploid* (White, 1942). This may also occur in gametic cells where a polyploid individual may result from the fusion of two unreduced gametes. Hence it is important to distinguish whether polyploidy arises from a somatic cell with duplicated chromosomes (2n) such as in tetraploid black wattle leaf tissue where 2n = 4x = 52 or whether it arises from unreduced gametic chromosomes (n) such as tetraploid black wattle pollen where n = 4x = 52 (Stebbins, 1950[f]; Beck *et al.*, 2003a).

The origin of natural polyploids is less well understood (Jones, 1970; Stebbins, 1971, Lewis, 1979). The two generally accepted modes of polyploid origin are that of somatic chromosome doubling and the fusion of unreduced (2n) gametes (Figure 1.5). (Harlan and de Wet, 1975; Vorsa and Bingham, 1979). Spontaneous chromosome doubling in natural polyploids is a rare event (Lewis, 1979; McCoy, 1982) which may occur either through meristematic chromosome doubling as reported in *Primula kewensis* (Digby, 1912; Newton and Pellow, 1929); or through zygotic chromosome doubling which was documented in several hybrids of *Nicotiana* (Clausen and Goodspeed, 1925; Clausen, 1941). The fusion of unreduced

[f] In several texts the terminology regarding x and n is reversed (Elliott, 1958). For the purposes of this dissertation, the format set out by Stebbins (1950) was followed.

gametes, on the other hand, is a more common mode of polyploid-formation in nature. Franke (1975) lists several examples of polyploidy originating by this mode. Unreduced gametes occur either as a failure in karyokinesis or more commonly as a failure in cytokinesis (Lewis, 1979).

Modes of formation of polyploidy			
In somatic cells		In gametic cells	
Through meristematic chromosome doubling	Through zygotic chromosome doubling	Unreduced gamete formation through cytokinesis	Unreduced gamete formation through karyokinesis

Figure 1.5 Origins of polyploidy

The agricultural significance of polyploidy has been of longstanding interest to plant breeders. This interest arises from the associated physiological and morphological changes exhibited by the polyploid plant such as enlarged fruits, leaves and flowers (Darrow, 1952; Kehr, 1996;) and increased variation due to the presence of multiple alleles, leading to hybrid vigour (Stebbins, 1950; Moore and Janick, 1983; Bonjean and Angus; 2001). Polyploidy is also used in the production of sterile cultivars (Darrow, 1952), and exploited in the fruit breeding industry to produce seedless fruits such as watermelon, citrus, kiwi, bananas and pawpaw (Stebbins, 1950; Pierce, 2002; McCuistion and Wehner, 2004).

1.3.2 Classification of polyploids

Several types of polyploidy exist. However, due to their cytogenetic complexity, the classification of these types has been long debated (Stebbins, 1947; 1950; Elliott, 1958). The system of classification proposed by Stebbins (1947; 1950) is the most widely accepted approach and was modified from Clausen *et al.* (1945). Figure 1.6 reveals that polyploidy is one facet of heteroploidy, where heteroploidy refers to any deviation from the normal chromosome number in a cell, tissue or organism (Jackson, 1971; 1976). Heteroploidy is subdivided into those cells that differ by individual chromosomes or part thereof (aneuploidy) or those cells that differ by

whole sets of chromosomes, which is referred to as euploidy and is of interest to this study (Burnham, 1962). Euploidy is segregated according to the number of chromosome sets that the cell contains (Stebbins, 1950). Haploidy, diploidy and polyploidy contain one, two and more than two chromosome sets, respectively.

Polyploidy can be further divided into allopolyploidy and autopolyploidy, depending on the source of their genomes. Allopolyploidy, occurs from the union of unreduced gametes of two distantly related species and have dissimilar or non-homologous chromosomes. Autopolyploidy occurs from the union of unreduced gametes of two closely related species and have similar or homologous chromosomes. Auto- and allopolyploidy represent two extremes of homology between which exist a series of intermediate individuals with varying degrees of homology (homoeologous) and are termed 'segmental allopolyploids' (Stebbins, 1947). Autopolyploidy, as classified by Stebbins (1950), is of significance to this work (Figure 1.6).

<table>
<tr><td colspan="8">Heteroploidy
(any change in normal chromosome number)</td></tr>
<tr><td colspan="4">Euploidy
(change in whole chromosome sets)</td><td colspan="4">Aneuploidy
(changes in single chromosomes or part thereof</td></tr>
<tr><td>Haploidy</td><td>Diploidy</td><td colspan="2">Polyploidy
(2n = 3x, 4x, 5x ...)</td><td colspan="3">Holaneuploidy
(changes in whole chromosomes)</td><td>Meroaneuploidy
(changes in parts of a single chromosome)</td></tr>
<tr><td>n = x</td><td>2n = 2x</td><td>Autopolyploidy</td><td>Allopolyploidy</td><td rowspan="2">Monosomy</td><td rowspan="2">Trisomy</td><td rowspan="2">Nullosomy</td><td rowspan="2">Centric fusion</td></tr>
<tr><td>Typical of gametic cells (n)</td><td>Typical of somatic cells (2n)</td><td colspan="2">Segmental allopolyploidy
(intermediate)</td></tr>
</table>

Figure 1.6 Classification of polyploidy

Autopolyploids

An autopolyploid essentially has three or more identical copies of the original parental genomes from the same species. The autopolyploid usually arises from spontaneous doubling of chromosomes in a shoot (somatically) or from the union of

unreduced gametes of closely related species (gametically). Autopolyploids are considerably less complex than allopolyploids and are designated the notation 'AAAA' to illustrate the identical genomes. In contrast to allopolyploids, autopolyploids have homologous or identical genomes that exhibit multivalent chromosomal pairing and polysomic inheritance patterns. These duplications of genomes may either occur spontaneously in nature or are induced artificially for agricultural exploitation.

Natural autopolyploids were thought to be less common in nature than allopolyploids. However, recent studies have shown that they are frequently misclassified and are perhaps more common than initially estimated (Muntzing, 1956; Soltis and Soltis, 2000). The most frequently occurring type of autopolyploid is the autotetraploids. Some of the earliest autopolyploid observations include *Liliceace* (Strasburger 1910), *Drosera* (Rosenberg, 1909), bananas (Tischler, 1910), *Chrysanthemums* (Tahara, 1915) and DeVries' much publicised tetraploid gigas mutant, *Oenothera lamarickiana* (Lutz, 1907; Gates, 1909). Since then, several natural autopolyploids were identified and interest in artificial autopolyploid induction has grown considerably.

Autopolyploids can also be artificially induced. One of the earliest reports of autopolyploidy induction was identified for mosses (Burnham, 1962). Perhaps the most well documented study on initial autopolyploid induction in higher plants was by Winkler (1916) when he produced tetraploid *Solanum nigrum* by the formation of calluses. The discovery of colchicine now provides a means to rapidly and effectively induce autopolyploidy. Today, several important crops are autopolyploid such as banana, coffee and alfalfa (Elliott, 1958).

Autoploidyploids have several conspicuous characteristics such as larger organs, increased production of certain enzymes; higher heterozygosity compared to diploids and most importantly, decreased fertility. The increased sterility is often exploited in the fruit breeding industries where the production of seedless fruits is economically desirable. It is important to note that these characteristics, particularly sterility, depend largely on the genotype of the diploid parents as well as the species of the plant.

Autopolyploids may or may not be fertile due to possible chromosome pairing difficulties during meiosis. Autopolyploids have multiple occurrences of a set of chromosomes; an autotetraploid (2n = 4x) has four copies of each homologous chromosome and an autotriploid (2n = 3x) has three copies. These multiple copies tend to form multivalents during meiosis, resulting in unequal segregation of chromosomes during anaphase 1 and the formation of unbalanced gametes. Unbalanced gametes are often not viable, thereby causing semi-sterility for the autopolyploid. Autotriploids may form trivalents, bivalents and monovalents during meiosis. Chromosome segregation in the following anaphase 1 will result uneven and incomplete chromosome distribution to the poles (aneuploidy) nearly always resulting in an imbalance of the chromosomes composition leading to lethality and thus extreme semi-sterility or even sterility. Autopolyploids having an odd number of chromosomes sets tend to be more sterile than those having an even number of chromosomes sets. The latter often form bivalents or tetravalents that display balanced segregation at anaphase 1. The poles thereby receive a diploid number of chromosomes (2x), resulting in high fertility (Stebbins, 1950).

Allopolyploids

Allopolyploidy refers to the hybridization of two or more genomes from different species and is designated 'AABB' to illustrate the different parental genomes. Stebbins (1950) defines allopolyploidy as 'the hybridization between two or more distantly related species, of which the chromosomes are so different that they are unable to pair in the diploid hybrid or form only a small number of loosely associated bivalents.' The allopolyploid usually arises from spontaneous doubling of chromosomes in a shoot (somatically) or from the union of unreduced gametes of different species (gametically). In practice, natural and cultivated allopolyploids are characterised by their non-homologous genomes, exhibition of bivalent pairing and disomic inheritance patterns, high fertility and hybrid vigour.

Naturally occurring allopolyploids are common and the most stable form being allotetraploid. Allopolyploidy may occur between two different species within the same genera for example, Digby's (1912) fertile *Primula kewensis* from *P.*

verticillata X *floribunda*; or between two different species between different genera the classic example being Karpechenko's (1927) sterile *Raphanobrassica* which was as a result of crossing a cabbage (*Brassica oleracea*) with a radish (*Raphanus sativus*). In nature, the production of allopolyploids is often the mechanism whereby new species evolve for example *Galeopsis tetrahit* (Müntzing,1930), *Rubus maximus* (Rozanova, 1934) and *Nicotiana rustica* (Goodspeed, 1934).

Scientists have sought to emulate natural speciation by creating new species under human observation. Clausen and Goodspeed (1925) were the first to synthesise a new partially fertile species by crossing *Nicotiana glutinosa* x *N. tabacum var purpurea* and called it *N. digluta.* It is interesting to note that whilst natural allopolyploids tend to be stable phenotypically and genotypically, synthetic allopolyploids are often unstable and tend to be sterile. This suggests that natural allopolyploids have evolved some mechanism whereby they are able to stabilize the effects of their multiple chromosomes sets (Henikoff and Comai, 1998; Comai, 2000; Comai *et al.,* 2000)

Allopolyploids have some striking characteristics that make them of prime interest to plant breeders. These include increased fertility and their ability to restore fertility to sterile diploid hybrids and increased heterozygosity. They are usually fertile because the parental genotypes arise from distinctly different genomes allowing for successful bivalent formations and pairing associations during meiosis and thus balanced, viable gametes (Stebbins, 1950). Furthermore, these different genomes usually introduce large degrees of heterozygosity, which is believed to be the crucial factor in growth, performance and adaptability, that is, in hybrid vigour (heterosis). True allopolyploids are isolated from and, as a rule, morphologically discontinuous from their close relatives. Agriculturally important allopolyploids include tobacco, *Nicotiana tabacum*, cotton, *Gossypium hirsutum* and strawberry (*Fragaria grandiflora*) (Stebbins, 1950; Elliot, 1958)

Segmental allopolyploids

Stebbins (1947) defines segmental allopolyploids as 'a polyploid containing two pairs of genomes which have a considerable number of homologous chromosomal

segments or even whole chromosomes in common, but differ from each other in respect to a sufficiently large number of genes or chromosome segments, so that their different genomes produce sterility when present together at the diploid level.'
Natural segmental allopolyploids are difficult to identify without appropriate hybridization experiments and many have been erroneously classified as autopolyploids for example *Galax aphylla* (Baldwin, 1941), *Sedum ternatum* (Baldwin, 1942) and *Fritillaria camschatceusis* (Matsuura, 1935). The fertility of segmental allopolyploids varies and the occurrence of this type of polyploidy is almost exclusively confined to segmental allotriploid or segmental allotetraploids. Stebbins (1950) has listed several artificially produced examples of these allopolyploids, many of which were so sterile that they would not have survived naturally, for example *Crepis foetida-rubra* and *Layia pentachaeta-platylossa*. On the other hand, *Tradescantia canaliculata-humilis* and *Nicotiana glauca-langsdorffii* have been regarded as highly fertile.

1.3.3 Effects of polyploidy

Increased gene dosage often has several dramatic consequences that affect the biological functioning of both natural and cultivated polyploids. These consequences include changes in heterozygosity, cell size and sterility (Stebbins, 1950) (Figure 1.7). Many morphological and physiological implications have arisen as a result of these consequences, several of which have been of longstanding interest to plant breeders (Lewis, 1979). The consequence and implications of polyploidy were thoroughly examined in artificially induced polyploid species (Blakeslee, 1939; Straub, 1940; Randolph, 1941; Larsen, 1943 and Noggle, 1946). However, the only biological generalisation that can be made about these consequences is that they are greatly dependant on the genotype of the original diploid parents as well as the species of the plant and type of polyploid (Stebbins, 1950).

Polyploids display a range of specific characteristics and are described as follows (Stebbins, 1950; Elliott, 1958; Thompson and Lumaret, 1992):

- Polyploids display a higher level of heterozygosity than diploids due to the presence of multiple alleles. This enhanced vigour and fertility is particularly true in the case of allopolyploids with their dissimilar genomes, whereas allopolyploid having similar genomes display a reduced vigour and a more inbred-type effect.
- Increased cell size to accommodate the extra chromosomes which eventually leads to enlargement of certain organs such as fruits and leaves. This increase in size is not proportional to ploidy level but rather passes through an optimum which is usually tetraploidy. Higher levels of ploidy tend to be marked with stunted growth and extensive chromosomal abnormalities.
- Increased water content of the cell due to the cell's increased size. This often leads to watery fruit and increased frost susceptibility.
- Increased frequency of certain organelles for maintaining efficiency of the enlarged cell. These organelles include stomatal chloroplasts and nucleoli.
- Slower growth rates are observed particularly in very high levels of ploidy since more energy is expended in the maintenance of larger fruits and flowers. This often leads to decreased metabolism and later flowering which can be to the detriment of the plant's reproduction if it depends on seasonal pollination agents such as insects and birds.
- Increased production of certain biochemicals as well as the production of new hybrid enzymes commonly observed in allopolyploids (enzyme multiplicity).
- Reduced fertility due to multivalent pairing associations and abnormal segregation at meiosis which leads to unbalanced gametes and ultimately less viable seeds. This is a characteristic of autopolyploids and commonly leads to a reduction in the number of viable seeds and pollen.
- Polyploids often have evolved alternative reproduction mechanisms such as the ability to undergo diploidization of the genome, to reproduce asexually as well as the ability to be more self-fertile than diploids. This is largely due to their reduced fertility and the fact that they are in constant competition over habitat with their diploid ancestors.
- Polyploids can hybridise with each other but usually not with their diploid parent-types which immediately sets up a 'reproductive barrier' between diploid parent-types, and polyploid progeny-types. This barrier creates a driving force towards speciation to ensure survival of the polyploid.

1.3.4 Agricultural significance of polyploidy

Plant breeders have shown considerable interest in the consequences and implications of polyploidy for crop improvement (Figure 1.7). Polyploidy has lead to the improvement of several agriculturally useful crops such as wheat, oats, potatoes and tomatoes (Pierce, 2002).

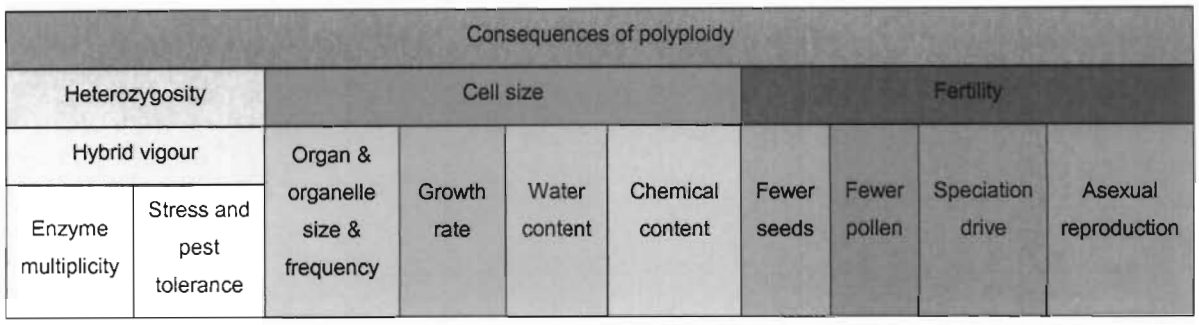

Figure 1.7 Consequences of polyploidy.

Polyploids maintain a higher level of heterozygosity than normal diploids. This increased heterozygosity allows polyploids to maintain a certain amount of adaptive and evolutionary advantage over their diploid progenitors such as enhanced vigour and adaptability, improved fertility, enzyme multiplicity as well as improved stress and pest resistance (Hagerup, 1932; Tischler, 1934; Manton, 1934; Roose and Gottlieb, 1976; Soltis and Soltis, 1993). It is for this increased heterozygosity and vigorous progeny that plant breeders often exploit polyploidization in plant improvement programmes.

Variation in cell size is another agriculturally exploitable consequence of polyploidization (Stebbins, 1950). Cellular enlargements often lead to eventual organ enlargements such as fruits, flowers and leaves, which is beneficial to certain plant breeding industries. For instance, larger cells often incorporate more water which tends to make fruits less intense in taste (Schlosser, 1944; Darrow, 1952) whereas this is of particular interest to the ornamental flower industry where the larger, fleshy petals have a longer shelf-life as reported in snapdragons, *Antirrhinum*

majus (Emsweller and Ruttle, 1941; Burnham, 1962; Levin, 1983; Kehr, 1996). Increase in cell size causes a species-specific increase in the frequency and dimensions of some organelles, namely stomatal chloroplasts which are often used as an indicator of ploidy level (Mochizuki and Sueoka, 1955). Certain polyploid crops produce higher concentrations of desirable chemicals such as alkaloids for drug development, valuable proteins, vitamins, pigments, cellulose, auxins and carbohydrates (Ruttle and Nebel, 1939; Burnham, 1962).

The increased sterility in polyploids is of major agricultural importance in the fruit and forestry industries. Fruit breeders utilise triploidy for the production of seedless fruits that are also larger than diploids such as bananas, watermelon, certain citruses and seedless grapes (see autopolyploidy; section 1.3.2). Sterile cultivars are also utilised in the forestry industry where the protection of indigenous species against invasive species is paramount. Development of sterile forms of important nursery crops is an ideal method of addressing this problem. In doing so, plants can be grown and used for landscaping while virtually eliminating any possibility that these plants could sexually reproduce and become invasive. The induction of polyploidy is perhaps one of the most rapid and cost effective approaches of inducing sterility. With the exception of reproductive biology, particularly meiosis, these plants function normally in most cases.

Polyploidy can also be used to enhance fertility in certain sterile diploid hybrids (Figure 1.8). For example in *Triticale* which results from the cross of wheat (*Triticum aestivum*) and rye (*Secale cereale*) (Müntzing, 1939). This type of crossing is ultimately dependant on the fertility of the diploid parents and may not always result in fertile offspring for example in millet (*Setaria*) hybrids (Li *et al.,* 1945).

AB	→	AABB
Sterile diploid hybrid genotype	Polyploidization	Fertile allotetraploid genotype

Figure 1.8 Schematic representation of fertility restoration through allopolyploidization.

Polyploids have evolved with outstanding colonising abilities as they are in constant competition over shared habitat with their diploid ancestors (Lewis, 1979). These abilities include high seed production and offspring adaptability; the ability to

undergo genome diploidization, asexual reproduction and they are more self-fertile than diploids (Gillie, 1912; Nygren, 1967; Dudley and Alexander, 1969; Pojar, 1973; deWet and Stalker, 1974; Dobzhansky *et al.*, 1977). These abilities cater for their minority disadvantages and polyploidy is often utilised in plant improvement programmes in areas that have stressful environments and with species that have low fertilization frequencies. (Briggs and Walters, 1997).

1.3.5 Induction of polyploidy

The doubling of chromosomes in cells is a common occurrence in both natural and cultivated species (Blakeslee and Avery, 1937). Polyploidy, generally occurs as a result of some failure in the cell division cycle. Several factors may induce cell division failure in natural plants (Figure 1.9). These factors are often studied and simulated under artificial conditions for the induction and implementation of polyploidy in plant improvement programmes (Figure 1.9). Factors that induce polyploidy may be classified as either environmental or genetic in nature. Environmental factors that induce polyploidy in nature include temperature shock, dehydration, UV radiation as well as certain mechanical injury caused by pests and bacteria. Genetic factors that induce natural polyploids may include the rare presence of a recessive gene that disrupts cell division or the even rarer presence of twinning.

Natural polyploids

Sudden changes in the environment have often been associated with the meiotic irregularities leading to the formation of natural polyploids. Changes in temperature, moisture and UV radiation due to ozone depletion have been correlated with diploid pollen formation, microtubule irregularities and chromosome segregation failures. Many natural polyploids are found in regions of extreme temperatures such as *Tradescantia* and *Solanum phureja* (Sax, 1937; McHale, 1983). Furthermore, polyploids tend to be more frequent in some seasons than in others (Blakeslee and Avery, 1937). Dehydration and an increase in environmental UV radiation have been associated with gross physiological disturbances in the cells resulting in

disruptions of normal cell division cycles (Giles, 1939; Darlington and La Cour, 1960).

Other environmental agents of natural polyploid induction such as mechanical injury due to pest infections have been observed (Sax, 1937). Many researchers have reported polyploid cells in galls that formed as a result of infections of bacteria, nematodes or chemically-induced tumours (Kostoff, 1930; Kostoff and Kendall, 1933; Burnham, 1962; Winge, 1927). Wipf and Cooper (1938) reported a common occurrence of tetraploid nodules in red clover as a result of bacterial infections. These pests disrupt the synchronization of normal nuclear and cellular division by inhibiting spindle formations or inhibiting normal segregation of chromosomes.

Genetic agents of polyploid induction are relatively rare and species-specific. Genetic agents that influence the formation of unreduced gametes such as genetic control by recessive genes, twinning and gene silencing (Pikaard, 2001) is a lesser understood topic and observed in only a few species (Mok and Peloquin, 1975 Veilleux *et al.*, 1982; McCoy, 1982; Qu and Vorsa, 1999). Some recessive genes exist that affect the harmonious functioning of cell division by causing asynapsis of chromosomes at meiosis resulting in the formation of polyploid gametes (Beadle, 1933). These have been observed in only a handful of species such as *Datura* cotton, wheat and corn (Burnham, 1962). The frequency and presence of twinning is another genetic agent of polyploidy induction and is often influenced by temperature and by the presence of certain genes (Burnham, 1962). Triploid and tetraploid twin seedlings are rare and usually only found in colder seasons (Burnham, 1962).

<table>
<tr><td colspan="12">Induction of polyploidy</td></tr>
<tr><td colspan="6">Natural</td><td colspan="6">Artificial</td></tr>
<tr><td colspan="4">Environmental</td><td colspan="2">Genetic</td><td colspan="4">Microtubule disruption</td><td colspan="4">Cellular manipulation</td></tr>
<tr><td rowspan="2">Temperature</td><td rowspan="2">UV radiation</td><td rowspan="2">Mechanical injury</td><td rowspan="2">Dehydration</td><td rowspan="2">Recessive genes</td><td rowspan="2">Twinning</td><td rowspan="2">Temperature</td><td rowspan="2">Mechanical</td><td rowspan="2">Radiation</td><td rowspan="2">Poisons</td><td colspan="3">Tissue culture</td><td rowspan="2">Protoplast fusion</td></tr>
<tr><td>Callus</td><td>Pollen</td><td>Endosperm</td></tr>
</table>

Figure 1.9 Methods of inducing polyploidy.

Artificial polyploids

It was often noticed that natural polyploid plants were larger and more vigorous than diploid plants of the same species. As a result, plant breeders became more interested in the induction of polyploidy for the utilisation in plant improvement programmes (Elliott, 1958). However, several natural methods of induction were explored with little success. The induction of polyploidy in cultivated crops using the methods known at the time proved to be unpredictable and unstable or produced polyploids in too low a frequency (Elliott, 1958; Burnham, 1962). Methods of artificial induction of polyploidy may be classified in two ways, those methods that cause disruption in cell division cycles and those that allow for actual manipulations of the cell and its contents. Methods of polyploid induction that occur due to a disruption in cell division include temperature shock, mechanical damage, irradiation and microtubule poisons, the latter being the most popular and effective method. Cellular manipulation methods include various tissue culture treatments and protoplast fusions. Often these methods are not used in isolation but rather in combination for optimal effect in a particular species.

Taking their lead from nature, Blakeslee and Belling (1924) explored temperature shock as a method of polyploid induction in plants. They found tetraploid branches in *Datura* as a result of cold treatments; however this treatment delivered inconsistent results. Randolph (1932) succeeded in obtaining polyploid cells by using electrical heat pads in early maize cells. High temperatures also produced polyploidy in barley, rye and rice (Burnham, 1962) and several others (Dorsey, 1936; Atwood, 1936; Peto, 1936; Sax, 1936; Lutkov, 1937). Specialized heating techniques were later described by Brink (1936) and Peto, (1936). However, temperature shock requires careful control of the experimental conditions and is not recommended as the primary method of polyploid induction (Blakeslee and Avery, 1937).

The induction of polyploidy by mechanical damage is perhaps a lesser-understood method. Kostoff (1935) attempted to disrupt cell division cycles in *Nicotiana* by centrifugation. He reported changes in chromosome numbers although they were

inconsistent in most cases. Attempts to apply the same technique in other species, such as barley and rye, were unsuccessful (Burnham, 1962).

Various types of radiation disrupt cell division cycles and may be utilised in the production of polyploid cells. These forms of radiation may be classified as ionising or non-ionising (Sharma and Sharma, 1965). Ionising radiation is commonly used in polyploidization and includes X-rays, gamma-rays, alpha-rays, beta-rays and fast neutrons whilst non-ionising radiation include UV-rays and IR-rays (Darlington and La Cour, 1960). Overall effects of radiation usage have been extensively reviewed by Gustafsson (1954). Ultimately, the type of radiation, treatment methods and dosage of radiation depend on the species and type of plant material used (Darlington and La Cour, 1960). This method often requires specialised equipment and radiation sources. Moreover, the extent of chromosomal damage cannot be controlled or predicted (Gustafsson, 1960). Radiation is attempted for induction of polyploidy and sterility in some species such as in *Datura* (Blakeslee and Avery, 1937) and in black wattle (Dunlop and McLennan, 2002).

Microtubule poisons have been perhaps the most significant finding in the field of artificial polyploidy induction. Nemec's classical polyploidization experiments in 1904 with chemicals such as chloral hydrate and other narcotics lead to the identification of several other microtubule-disrupting chemicals including oryzalin, amiprophos-methyl, N_2O gas and most significantly, colchicine (Blakeslee and Avery, 1937; Taylor *et al.*, 1976; van Tuyl *et al.*, 1992; Bouvier *et al.*, 1994) Induced polyploidy from colchicine treatments are very reproducible and occur in high frequencies (Blakeslee and Avery, 1937).

Colchicine is sourced from meadow saffron (*Colchicum autumnale*) and is a microtubule poison. The main biological action of colchicine is its ability to disrupt or inhibit[ϒ] microtubule or spindle fibre formations, which are the key structures responsible for the polarisation of doubled chromosomes in the dividing cell. Colchicine causes the doubled chromosomes to arrest at the metaphase plate instead of being moved towards the poles of the existing cell to form a new cell

[ϒ] The terms 'disruption' and 'inhibition' can be used interchangeably in this context.

(anaphase). Colchicine is remarkable in that it is strongly water-soluble and is very reactive even in small quantities (Sharma and Sharma, 1965). It is suggested that colchicine catalyses a reaction that brings about a change in the colloidal state of the cytoplasm. This altered cytoplasm is then so fluid that it disturbs the formation of spindle fibres (Sharma and Sharma, 1965). Another interesting property of colchicine is its ability to fluoresce, thereby allowing researchers to trace its movement through the plant (Burnham, 1962).

Several features have become evident since the use of colchicine was initiated. Colchicine appears to affect only rapidly dividing cells making its utilisation effective only in meristematic tissue such as root tips. The optimal dosage and length of treatment needs to be identified for a particular species since not all cells divide at the same time or at the same rate. The method of application also varies depending on the type of tissue available and is usually determined by trial and error. The chemical needs to be fully removed from the cells before fixing because it inhibits staining and thus visibility. Woody plants such as black wattle require stronger solutions over longer time periods for effective polyploidization. Successful polyploidy has been induced in plants such as *Carica papaya* (Hofmeyr and van Elden, 1942), *Sequoia gigantea* (Jensen and Levan, 1941) and apples (Dermen, 1952).

The production of polyploids via the tissue culture technique allows for the cultivation of polyploid cells *in vitro* in natural or as close to natural media as possible (Sharma and Sharma, 1965). Polyploid cells can be produced from various types of cell cultures such as callus-formations that undergo somaclonal variation (Winkler, 1916); pollen cultures (Chen, 1985); endosperm cultures which are already polyploid (Wang and Chang, 1978) as well as from protoplast cultures (Vardi, 1981; 1977). However, this procedure is technically difficult with varying levels of success, particularly since plants have cell walls and this cannot be exactly reproduced *in vitro* (Sharma and Sharma, 1965). Moreover, continued investigations have been conducted to refine culture media and to broaden the range of species and plant material that can be used to yield successful polyploids by this technique (Hidaka *et al.*, 1981)

Protoplast fusion is perhaps the more intriguing alternative to tissue culture. The technology involves the extraction of whole protoplasts and subsequent cellular fusion. This method has been used to produce several polyploid plants such as *Citrus* and related genera (Bravo and Evans, 1985; Grosser, 1986; Galun and Vardi, 1986). Typically, protoplasts are isolated from embryogenic tissue by the enzymatic breakdown of the cell wall. After protoplast isolation, cell fusion is induced by electric stimulation or by treating cells with polyethyleneglycol (PEG) and calcium (Grosser and Gmitter, 1990). Protoplast fusion has not gained much prominence because it is technologically demanding and requires skilled personnel. Furthermore, it is not guaranteed that the protoplasts of hybrid cells will combine once the cell has reformed, instead they could remain as two distinct protoplasts (Kao *et al.*, 1974). Several problems have arisen from the use of protoplast culture thereby yielding inconsistent results, many of which are not completely understood (Jones *et al.*, 1989; Peschke and Phillips, 1992; Burza and Malepszy, 1995)

1.3.6 Identification of polyploidy

The success of polyploidy in plant improvement programmes hinges on the fast and reliable techniques developed for the verification of ploidy levels. Historically, ploidy levels were identified by directly counting the number of chromosomes in the plant by slide preparations (Darlington and La Cour, 1960). Whilst this technique is very effective in some species, it is impractical in others. Several alternative techniques are being developed for the rapid discernment of ploidy levels in plant improvement programmes. Ultimately the breeder has to choose the appropriate ploidy detection technique that is best suited to the project concerned, based on the reason for ploidy quantification as well as on certain project limitations. These limitations include type and abundance of available material, the original basic number of the species used, the level of accuracy required, the amount of time available, cost factors, levels of expertise required and available equipment (Darlington and La Cour, 1960; Sharma and Sharma, 1965). The

technique of choice usually requires a degree of optimisation depending on the species and samples used in a particular project.

The techniques of quantification of ploidy level are numerous and are constantly evolving. For the purposes of this dissertation, detection techniques have been grouped into *direct-* , *intermediate* and *indirect* methods. For example, a direct method of ploidy quantification involves the counting of actual chromosomes using the metaphase smear technique whereas an indirect method infers the level of ploidy by counting some other visible ploidy marker such as the chloroplasts in the stomatal chloroplast technique. An intermediate method would imply the quantification of a more pronounced chromosome-related structure such as the nucleoli by the silver-staining technique (Table 1.3).

Table 1.3 Summary of ploidy identification methods

Method	Principle	Process		Technology	Advantages	Disadvantages
Direct	Actual counting of the chromosomes	• Obtain rapidly dividing cells • Fix cells in metaphase • Stain cells • View cells	Slide preparations	Metaphase smear preparation[1]	• 2 days preparation time • cheap • reliable • suitable for low levels of ploidy • directly assesses ploidy • can tell the type of ploidy	• use of hazardous chemicals eg colchicine • not suitable for high levels of ploidy typical of plants • cell walls digestion required • availability of rapidly dividing cells • not suitable for short, fat chromosomes eg in trees
			Block preparations	Paraffin preparation[1]	• Good for very small organs • Excellent visualisation • Sample does not have to be living	• time consuming • laborious • expensive equipment required • hazardous chemicals
Intermediate	Quantification of DNA content and some chromosome related structure	• Obtain living cells • Monodisperse cells • Stain • Pass through laser	Chromosome-related structure quantification	Nucleoli staining[1]	• Clearly visible structures • Relatively quick.	• the number of nucleoli is species-specific. • Specialised chemicals and techniques required.
			DNA quantification	Flow cytometry[2]	• Fast • Material does not have to be fixed • Reliable	• Requires expensive equipment • Requires technical expertise • Quantifies DNA. Increased DNA may not always be due to polyploidy
Indirect	Establishment of a relationship between some morphological characteristic and ploidy level	• Identify a stable characteristic • Quantify and compare with different ploidy levels of the same species	Macromorphological markers	Characterising various aspects of leaf, flower and fruit physiology[4] • size • colour • shape • trichome frequency	• Fast and reliable once tested for that species • Low technical expertise required • Low cost	• Technique needs to be tested for a particular species • Samples may not always be available • Not actually quantifying number of chromosomes • Cannot tell the type of ploidy
			Micromorphological markers	Measuring pollen size and germpore frequency[3] Measuring stomatal guard cell[5] • length • frequency Measuring guard cell chloroplasts[6] • frequency • size • arrangement Measuring chlorophyll content[4]	• Rapid • Reliable • Low cost • Low technical expertise required • Does not require expensive equipment • Useful for screening large populations	• Material may be seasonal such as pollen. • May be influenced by environmental factors such as light and moisture. • Is a species-specific tool therefore the tool needs to be tested for a particular species first.

[1]Darlington and La Cour, 1960; [2]Shapiro, 2003 [3]Evans, 1955; [4]Warner and Edwards, 1993; [5]Beck *et al.*,2000a; [6]Beck, Fossey and Mathura, 2003b.

Direct identification methods

Direct methods of ploidy identification involve the manual calculation of chromosomes in a particular sample and comparing this number to the basic number of chromosomes for that species. This is typically achieved by two distinct techniques; *metaphase smears* and *paraffin wax embedding* (Darlington and La Cour, 1960) (Table 1.3). Both these techniques share the same methodology but have distinctly different preparations. Several variations of these techniques exist depending on the type of material available (for example living or non-living material) and have been well-documented by Darlington and La Cour (1960).

Both metaphase smears and paraffin wax embedding techniques follow the same methodology (Darlington and La Cour, 1960). They both require samples to be fixed, stained and mounted before viewing and quantifying the chromosomes in these samples. Metaphase smears are relatively simple to perform with a reasonable level of accuracy. Rapidly dividing cells such as root tip cells are fixed with acetic acid and a microtubule poison such as colchicine (Beek, 1955). Cells are then stained with DNA-specific stains such as Feulgen stain or fluorescent stains such as Hoechst stain (Sharma and Sharma, 1965; Van't Hof, 1999). These cells are then mounted on a slide and viewed with a microscope (Darlington and La Cour, 1960). Paraffin wax embedding on the other hand requires the cells to undergo a series of preparatory steps such as stepwise dehydration, chemical fixing (osmium tetroxide) and subsequent embedding into wax before sectioning the sample using a microtome. These sections are then stained and mounted onto a grid, and finally viewed with an electron microscope (Rawlins and Takahashi, 1947; Foster and Gifford, 1947).

Metaphase smears and paraffin wax preparations have several advantages and disadvantages. Metaphase smears requires a relatively low level of skill as compared to paraffin wax embedding which are considerably more specialised particularly in terms of sectioning (Darlington and La Cour, 1960). The paraffin wax embedding technique requires expensive equipment such as electron microscopes and microtomes. Whilst metaphase smears require anything from a few hours to two days to prepare cells, paraffin preparation takes at least a week (Darlington and

La Cour, 1960). Both utilise hazardous chemicals such as colchicine and osmium tetroxide. However, once a sample has been fixed in the paraffin preparation, it has a longer shelf life than metaphase smears that deteriorate rapidly (Serra, 1947). Paraffin wax embedding is suitable for obtaining cells, either living or non-living, from small organs and for good quality visualisations with electron microscopy. Metaphase smears are suitable for rapid analysis of living cells where merely the level of ploidy is required (Sharma and Sharma, 1965). Visibility may easily be retarded in both techniques, depending on the staining technique that is employed.

Direct methods of quantifying ploidy level are very effective when utilised appropriately. The determination of actual chromosome numbers of a sample is more reliable and less time consuming than having to infer the level of ploidy by a phenotypic relationship. However, direct methods are not always practical and their utilisation requires some knowledge of the sample's cytogenetics. Forestry cytogenetics reveals that tree samples typically have high levels of ploidy with very small chromosomes, making good visibility and accurate chromosome quantification nearly impossible (Lesin, 1954). This difficulty was encountered in black wattle (WRI, 1950). Moreover, Li (1954) suggested that the high tannin content of trees such as black wattle may affect cytological handling and visibility. Furthermore, cell wall residues reduce visibility causing erroneous results (Lesins, 1954). The techniques mentioned here usually require actively dividing cells such as root tips, which are difficult to obtain from a fully grown tree. These cells need to be fixed at a certain phase in the cell cycle for maximum visibility and pinpointing the occurrence of this phase can depend on any number of factors (Sharma and Sharma, 1965). Where non-living cells are utilised, significant sample preparation time is required and screening a large number of progeny becomes tedious (Santen and Casler, 1986). Ample consideration should be given to each of these factors since they could make the utilisation of direct chromosome quantification techniques time-consuming and costly.

Intermediate identification methods

Intermediate methods of ploidy identification involve the quantification of some distinct chromosome-related structure as opposed to the actual chromosomes in

direct methods or a morphological marker not related to the chromosomes in indirect methods. In principle, there exists a correlation between the quantified chromosome-related structures including the amount of DNA, with the ploidy level for a particular species (Price, *et al.*, 1973; Vilhar *et al.*, 2002). The quantification of *nucleoli* by the silver-staining technique (Miller and Beatty, 1969) and the DNA quantification by the *flow cytometry* technique (Melamed *et al.*, 1990) are commonly used (Table 1.3). Both these techniques share the same methodology but are separate in terms of preparations. Several variations of these techniques exist, and have been extensively documented by Popescu *et al.*, (2000) and Shapiro (2003).

Both nucleoli silver-staining and flow cytometry share the same basic methodology. That is, if there are more chromosomes, as is the case with polyploidy, there should be more chromosome-related structures and DNA. A nucleolus (*pl.* nucleoli) is a ribosomal RNA-containing organelle found within the nucleus and produced by the nucleolar organiser region of the chromosomes (Darlington and La Cour, 1960; Vilhar *et al.*, 2002). These nucleoli may be quantified by the silver-staining method (Harrell and Heukelem, 1998). The amount of DNA may be quantified using flow cytometry which is swiftly becoming a popular ploidy diagnostic tool with several species (Carr and Shearer, 1998; Johnson *et al.*, 1998; Brummer *et al.*, 1999; Voglmayr, 2000; Collier, 2000; Bonos *et al.*, 2002; Wilhelm *et al.*, 2003). This technique is rapid and can quantify the amount of DNA in each stained cell of the sample within a few seconds by measuring the optical fluorescence intensities at several wavelengths (Shapiro, 2003). Perhaps the primary disadvantage of this technique is the expense and technical expertise associated with the sophisticated equipment, such as laser optics and electronic detectors. Moreover, cells must be mono-dispersed in suspension since individual cells are required to pass through the laser during a measurement.

The validity of intermediate methods depends on the existence of an accurate and reliable relationship between chromosome-related structures and level of ploidy. Whilst the ploidy level may be intuitively related to the amount of DNA, the reverse may not always be valid since an increase in DNA does not always imply polyploidy (McClintock, 1934; Poggio *et al.*, 1988; Doležel *et al.*, 1998). Furthermore, Whilst correlations exist for this relationship in some species, these techniques still require

testing for applicability in other species before being put into practise with confidence (Harrell and Heukelem, 1998).

Indirect identification methods

Polyploidy is often associated with various physiological and biochemical changes that manifest on the polyploid plant, many of which could be used as morphological markers for the indirect identification of ploidy level (Warner and Edwards, 1993). The underlying methodology is that the chosen marker is quantified by various preparations suitable to the sample and then correlated with the level of ploidy for that sample in order to ascertain any possible relationship. Morphological markers in polyploids are numerous, so for the purposes of this dissertation they may be classified into two distinct groups; *macromorphological markers* that are large enough to see with the naked eye such as fruits and leaves; and *micromorphological markers* that are less visible to the naked-eye such as stomatal guard cells (Vilhar *et al.*, 2002) (Table 1.3).

Macromorphological markers such as leaves, fruits and flowers are sometimes utilised in the identification of ploidy level (Hitchcock, 1971). However, in many instances, these traits are found to be variable in frequency and size and cannot be utilised to definitively discern ploidy levels (Bonos, *et al.*, 2002).

Micromorphological markers such as pollen, stomatal guard cells, stomatal chloroplasts as well as certain pigments such as chlorophyll are utilised in ploidy identification. The determination of pollen size and pollen germpore frequency is often correlated to ploidy level and involves the collection, staining and quantification of pollen size and germpores using a microscope (Evans, 1954; Vilhar *et al.*, 2002). Pollen size and germpores tend to increase with an increase in ploidy level although this relationship is species-specific (Blakeslee and Avery, 1937; Evans, 1954; Gould, 1957). Whilst this is a rapid technique, it depends upon the availability of pollen and can therefore only be utilised seasonally.

The frequency and dimensions of stomatal guard cells have often been used as morphological markers for identifying ploidy level in many plant species (Sax and

Sax, 1937; Mishkin and Rasmussen, 1970; Sapra *et al.*, 1975; Mishra, 1997). Stomatal guard cells are stained and viewed, similar to that of pollen quantification techniques (Evans, 1954). The length is measured using a micrometer eyepiece attached to the microscope (Evans, 1954). Typically stomatal guard cell dimensions increase with an increase in ploidy level whilst the frequency is found to decrease per unit leaf area (Mishra, 1997). This was found to be particularly true in the *Coffea* genus where different levels of ploidy were assessed and revealed the same trend (Franco, 1939; Mishra *et al.*, 1991). This trend is also present in black wattle where a positive correlation was discerned between stomatal guard cell length and ploidy level. At the same time an inverse relationship was found between stomatal guard cell frequency and ploidy level (Beck *et al.*, 2003a)

The quantification of the number and frequency of stomatal guard cells is a relatively rapid procedure and may be executed with ease. Certain lesser-understood hereditary and environmental factors may exist that could affect results such as the effect of neighbouring cells and growth conditions (Fernandez and Muzica, 1973). These factors need to be considered and compensated for when optimising this procedure for a particular species (Mishka, 1997). This technique has been successfully utilized in discerning ploidy levels in species such as alfalfa, *Medicago sativa* (Bingham, 1968), orchard grass, *Dactylis* (Santen and Casler, 1986), various species of rye grass (Speckman *et al.*, 1965), brome grass *Bromus inermis* (Tan and Dun, 1973) and most recently, and in this work, in black wattle, *Acacia mearnsii* (Beck *et al.*, 2003a)

Stomatal chloroplast frequency as a ploidy discerning tool has been of scientific interest since the beginning of the last century (Sakisaka, 1929; Mochizuki and Sueoka, 1955). Chloroplasts in stomatal guard cells are comparatively easy to study in epidermal tissue of plants. The technique involves stripping the lower epidermis of young leaves, staining the epidermis and then viewing the stained sample with an optical microscope (Chaudhari and Barrow, 1975). Young leaves are preferred since they are more supple and easier to handle.

The frequency of stomatal chloroplasts tends to change with a change in the number of chromosome sets present in a particular species. In 1930, Hamada and

Baba were the first to show that stomatal chloroplast number has a tendency of increasing with increasing levels of ploidy within the same species (Hamada and Baba, 1930). This relationship was thereafter observed in several species such as sugar beets (Mochizuki and Sueoka, 1955; Dudley, 1958) and in various clover species (Najcevska and Speckmann, 1968; Butterfass, 1973).

Attempts have often been made to fit a model to the distribution of stomatal chloroplasts in young leaves of different ploidy levels. Mochizuki and Sueoke (1955) identified a geometric relationship of stomatal chloroplasts and ploidy level in sugar beets. This relationship was disputed by Yudanova *et al.* (2002) who found a binomial relationship between stomatal chloroplasts and ploidy of the same species. Less complex relationships have been observed in other species. In black wattle, the frequency relationship between diploid stomatal chloroplast frequency and tetraploid stomatal chloroplast frequency was found to be 1:2 (Beck, Fossey and Mathura, 2003b). This type of relationship was also observed in watermelon cultivars (McCuiston and Wehner, 2004) and cotton plants (Chaudhari and Barrow, 1975). However, higher ratios were reported in species such as clover (Najcevka and Speckmann, 1968), potato (Hermsen and De Boer, 1971) and turnips (Speckmann *et al.*, 1967).

Whilst a relationship is often noted between stomatal chloroplast frequency and ploidy level, this is however, not a universal rule (Chaudhari and Barrow, 1975). Striking examples of plants that show no change in stomatal chloroplast frequency with an increase in ploidy level include *Bryum casepitium, Funaria hygrometrica, Physcomitrium piriforme* and *Dumortiera hirsute.* It is therefore strongly advised that the plant breeder tests the applicability of the stomatal chloroplast technique with the species in question.

The stomatal chloroplast frequency technique is rapidly becoming a popular means of discerning ploidy levels in various species. Plant breeders favour this technique because it is a rapid and reliable method by which to screen several samples efficiently. Minimal skill is required and the technique tends to be highly repeatable, displaying different stomatal chloroplast frequencies for each ploidy level. In

general, this technique is not confined to any particular type of leaf material although young leaf material is preferred.

Mochizuki and Sueoka (1955) have done extensive work on the various factors that may affect stomatal chloroplast frequencies in sugar beets. They discovered that the technique was not very effective in old or dead sugar beet leaf material. Moreover, this increase in plastids in relation to ploidy level was little affected by the type and position of leaf sample that was used (Mochizuki and Sueoka, 1955). Athough this technique is effective when dealing with autopolyploids, it may require some optimisation when dealing with chimeras or mixaploids (Chaudhari and Barrow, 1975). Moreover, this technique is only effective once the nature of the relationship between stomatal chloroplast frequency and ploidy level is established for that particular species. The success of this technique has already been established in species such as alfalfa, *Medicago sativa* (Bingham, 1968), mulberry, *Morus* spp. (Hamada and Baba, 1930), potato, *Solanum tuberisum* (Hermsen and De Boer, 1971), turnips, *Brassica rapa* (Speckmann, Dijkstra and Ten Kate, 1967) and in trees species such as black wattle (Beck, Fossey and Mathura, 2003b).

Polyploidy can also be associated with physiological and biochemical changes that affect the content and types of certain proteins in higher plants (Stebbins, 1950, Burnham, 1962). These changes tend to affect biochemical processes such as the rate of photosynthesis and the production of chlorophyll. The increased frequency of chloroplasts in polyploid cells may also lead to an associated increase in chlorophyll content (Joseph *et al.*, 1981; Meyers *et al.*, 1982; Warner and Edwards, 1993).

Research to establish relationships between chlorophyll content and ploidy level has been undertaken in a limited number of species. In *Medicago sativa* the activity and amount of proteins such chlorophyll and ribulose-1,5-bisphosphate carboxylase (RuBPC) were shown to almost double from diploid to tetraploid plants (Molin *et al.*, 1982). Warner *et al.*, (1987) reported a significant increase in chlorophyll content from tetraploid to octaploid. In tall fescue plants chlorophyll concentration increased significantly as a quadratic function from tetraploid to decaploid with the mean for octaploid genotypes representing the maximal chlorophyll concentration (Joseph

and Randall, 1981). A similar result was observed in C_4 grass *Panicum virgatum* where chlorophyll content and other soluble proteins increased from tetraploid to octaploid by 40 - 50 % (Warner *et al.*, 1987). Nitrogen and chlorophyll content was found to increase by 25 % per cell in diploid and tetraploid citrus, *Citrus sinensis* (Romero-Aranda *et al.*, 1997).

Interestingly, an increase in chlorophyll content is not always apparent. Warner and Edwards (1989) also showed that chlorophyll content remained constant in many levels of ploidy in *Atriplex confertifolia*, from diploid to decaploid, whereas other proteins were found to have increased. Similar evidence was found for the castor bean, *Ricinus communis,* where the relative distribution of chlorophyll content was found to be similar in haploid, diploid and tetraploid plants (Timko and Vasconcelos, 1981).

The utilisation of chlorophyll content as a ploidy indicator is a relatively new field of expertise thus requiring further research as to its usefulness as a diagnostic tool. It is important to predetermine the nature of the relationship between chlorophyll content and ploidy level, since not all species display a proportional increase in chlorophyll content (Molin *et al.,* 1982). Furthermore, environmental factors that affect chlorophyll degradation need to be assessed for a particular species before this technology can be utilised with confidence. Should chlorophyll content be correlated to ploidy level in a particular species, it would be of great value in the initial stages of plant improvement programmes since a large number of species of virtually any age could be assessed.

The crux of indirect methods of ploidy identification hinges on the establishment of a correlation between the quantification of some distinguishable morphological marker on the sample and the number of chromosome sets in that sample.

Ultimately, the selection of an appropriate ploidy discernment method, whether direct, indirect or intermediate, depends greatly on factors such as species, genotype, time, resources, accuracy, and type of cells available. Furthermore, the various techniques often need to be optimised for that particular species.

1.4 AIMS OF RESEARCH PROJECT

Black wattle is of prime commercial significance to the South African forestry industry. The exceptional utility of this exotic tree has created an overwhelming demand on both a local and global front thereby increasing revenue into the country as well as promoting job-creation initiatives. Thus, cutting-edge forestry research and tree improvement programmes are critical for the South African commercial forestry industry to remain competitive.

There are several facets to the South African black wattle tree improvement programme. The initiative that is relevant to this work is the reduction of viable seeds in order to decrease the colonizing ability of black wattle. Black wattle is one of South Africa's top invader species and wild black wattle is creating environmental difficulties that, in turn, impact negatively on the commercial industry. Thus, breeding for sterility has become an important focus in black wattle improvement programmes.

One of the simplest and most cost effective methods of producing sterile seeds is through the induction of autopolyploidy. The increased number of homologous chromosome sets cause multivalent associations during meiosis resulting in unbalanced chromosomal segregation during anaphase 1. Subsequently, the poles receive unbalanced chromosome sets, leading to lethality of the gametes thereby reducing the fertility of the seeds and minimising invasive potential of black wattle.

In black wattle, the intention is to induce autotetraploidy and backcross these tetraploids with diploids to produce sterile triploids. Breeders require an effective diagnostic tool to verify the level of ploidy before mass cultivations. Crossing two trees of incorrect ploidy level could be deleterious to the success of this programme. Hence, it is critical to have rapid and reliable ploidy diagnostic tools to discern between the different levels of ploidy in black wattle. In black wattle, however, the identification of an autotetraploid through chromosome counts is laborious and inaccurate, since the chromosomes are small and superimposed in

squash preparations. Furthermore, other methods of ploidy identification discussed previously require additional expertise and expensive apparatus. It has therefore become necessary to devise alternative methods to identify autotetraploids in black wattle so that they can be selected for subsequent crosses.

Many findings have suggested that the frequency of stomatal chloroplasts increase with an increase in ploidy. It is anticipated that this would be the case in black wattle as well. Also, it is hoped that the concentration of chlorophyll would increase with increasing numbers of chloroplasts.

To this end, two indirect methods of ploidy assessment were investigated, firstly, the quantification of stomatal chloroplasts and secondly the quantification of chlorophyll content, as follows:

- A comparative investigation of stomatal chloroplast frequency in diploid and tetraploid black wattle through light microscopy was undertaken, and

- A comparative analysis of the chlorophyll content in diploid and tetraploid black wattle through chlorophyll absorbance spectroscopy was undertaken.

A number of factors influence chlorophyll content and degradation. These factors are either genetic or environmental in nature. Environmental factors include sample age and sample storage conditions. It was therefore imperative to investigate how these factors affect the stability of the chlorophyll absorbance whilst sampling. Consequently, a supplementary study of chlorophyll content was undertaken in the form of two preliminary investigations:

- An investigation of the effect of storage conditions on chlorophyll content in diploid black wattle material that was either dried or frozen, and

- An investigation of the effect of tree and leaf age on chlorophyll content of two-year; four-year; six-year; eight-year and nine-year old trees as well as old and new leaf flushes in diploid black wattle.

2 CHAPTER 2 MATERIALS AND METHODS

2.1 INTRODUCTION

Polyploidization has been recognised as a valuable tool in plant breeding and is currently being investigated in black wattle breeding programmes to increase product yields and quality (Elliott, 1958). It can also be used to a large extent to contain the spread of black wattle trees outside plantation boundaries by limiting the number of viable seed. The utilisation of polyploidization in breeding is dependant on the accurate assessment of the level of ploidy as early as possible in the life of a growing tree in order to facilitate early selection of polyploids. In the black wattle industry autotetraploid production is the first of the polyploids that are produced. It has therefore become necessary to distinguish between diploid and tetraploid seedlings and trees.

The direct method to assess the ploidy of a plant is through chromosome quantification (Darlington and La Cour, 1960). This however, is not always accurate, as in the case of black wattle, where the chromosomes are small and tend to lie on top of one another in squash preparations. Therefore, two indirect methods were identified and explored in this project. Attempts were made to establish the relationship between stomatal chloroplast frequencies and chlorophyll content on the one hand, and the level of ploidy on the other hand.

There are however, a number of factors that influence chlorophyll content and degradation which are either genetic or environmental in nature. Genetic factors include genotype and number of chromosomes, whilst relevant environmental factors identified for this investigation are the age of leaves and trees as well as leaf storage conditions. It was therefore imperative to investigate how these factors affect stability of chlorophyll and hence the quantification of diploid and tetraploid black wattle. Chlorophyll absorbance was used as a measure for chlorophyll content on the basis of Beer's Law (Harwood and Moody, 1989). Consequently two additional investigations were undertaken.

This research project was undertaken in the following manner and illustrated accordingly (Figure 2.1):

Investigation 1

Quantitative assessment of stomatal chloroplast number in black wattle, which included a comparative analysis of the chloroplast number in stomatal guard cells of diploid (2n = 2x = 26) and tetraploid (2n = 4x = 52) black wattle, and

Investigation 2

Quantitative assessment of chlorophyll content in black wattle, which included a comparative analysis of the chlorophyll content in diploid (2n = 2x = 26) and tetraploid (2n = 4x = 52) black wattle.

This research was executed as three separate investigations:

Investigation 2.1
An analysis of the effect of storage conditions on chlorophyll content in diploid black wattle,

Investigation 2.2
An analysis of the effects of aging of leaves and trees on chlorophyll content in diploid black wattle, and

Investigation 2.3
An analysis of the effects of chromosome dosage on chlorophyll content in diploid (2n = 2x = 26) and tetraploid (2n = 4x = 52) black wattle.

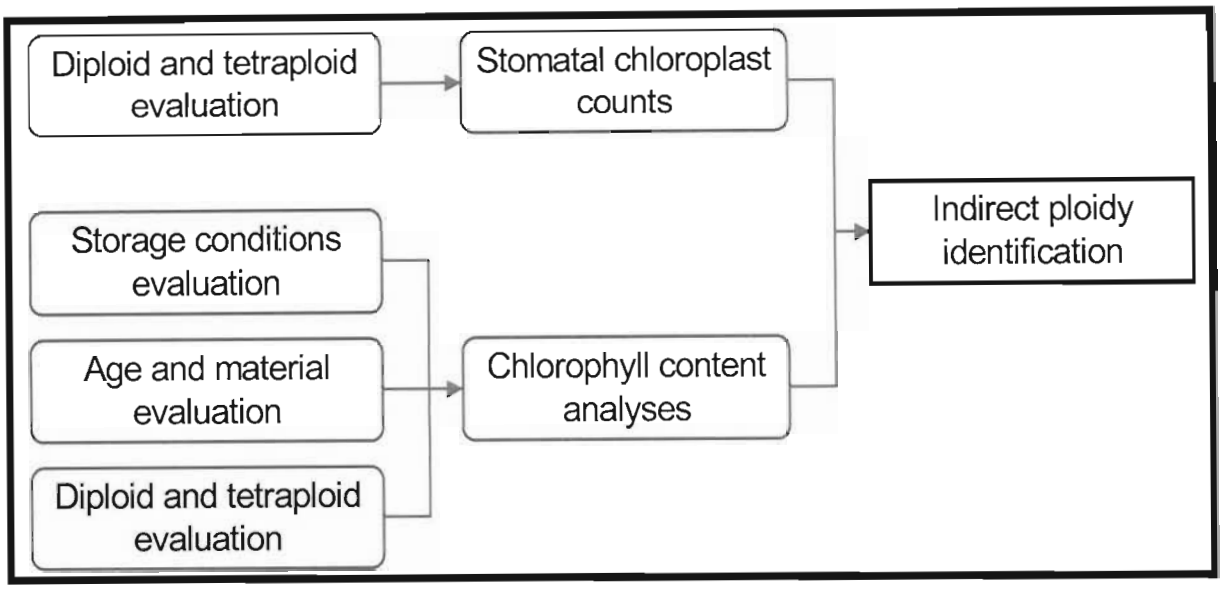

Figure 2.1 Flow diagram of the different analyses undertaken in this investigation.

2.2 QUANTIFICATION OF STOMATAL CHLOROPLASTS

2.2.1 Materials

The material required for the investigation of the relationship between stomatal chloroplast frequency and ploidy included diploid and tetraploid black wattle obtained from the Institute for Commercial Forestry Research (ICFR). Diploid and colchicine-induced tetraploid seeds from six different genetic black wattle lines, three lines per ploidy level and five trees per line were obtained and germinated under nursery conditions (Table 2.1). The tetraploids originated from colchicine-induced experiments conducted during the late 1940s by the Wattle Research Institute (WRI), and were confirmed at that time through root tip squashes (WRI, 1950).

Table 2.1 Diploid and tetraploid black wattle lines used to quantify stomatal chloroplasts.

Ploidy level	Genetic line	No. of trees per line	Origin
Diploid (2n = 26)	117	5	Bloemendal, KwaZulu-Natal
	272	5	Bloemendal, KwaZulu-Natal
	283	5	Bloemendal, KwaZulu-Natal
Tetraploid (2n = 52)	C 19/48/19	5	Colchicine-induced, WRI
	C 19/48/20	5	Colchicine-induced, WRI
	C 25/48/05	5	Colchicine-induced, WRI

2.2.2 Methods

Sample preparation

The diploid and tetraploid seeds obtained from the ICFR were germinated under nursery conditions. Fifty seeds per line were chipped using a sharp scalpel to promote germination of seeds. Thereafter, these chipped seeds were washed with a mild soap solution to reduce any fungal growth that may occur. Petrie dishes were prepared for the seeds by half-filling dishes with vermiculite (growth media) and covering with a single sheet of filter paper. No more than ten seeds were placed on the filter paper in each prepared petrie dish. Subsequently, petrie dishes containing seeds were moistened with distilled water, labelled with the date, genetic line and ploidy level. Thereafter, the petrie dishes were incubated for three to eight days, or until seeds were germinated in the dark at 25 °C. Once the seeds had germinated, the young seedlings were transferred into black plastic growth bags containing standard black wattle growing media and kept in the ICFR nursery for roughly eight months.

Large leaves from the healthy eight month-old seedlings were selected and temporarily stored in polytop vials that contained cooled distilled water. Slides were prepared within the hour of leaf collection by removing the thin epidermal layer of the abaxial surface of the pinnule. The epidermal layer was stripped gently with a very sharp razor blade, removed with a pair of fine tweezers or forceps and placed on top of a prepared slide with a drop of iodine-containing stain. The iodine stain was prepared by mixing 1 g of iodine and 3 g of potassium iodide in 100 ml of distilled water and with stirring until the contents had dissolved. The prepared stain was then poured into a dark bottle that was wrapped in tin foil, and stored in a dark cupboard to prevent oxidation. The material on the slide was covered with a clean cover slip, and viewed with an Olympus SZH Zoom microscope. The preparation was left for approximately five minutes to absorb stain and the slide viewed within the hour under 40 X magnification since stomatal guard cells shrank rapidly from the heat of the microscope lamp. The chloroplasts of 15 stomatal guard cells were counted and recorded on prepared datasheets and transferred to electronic spreadsheets using Microsoft® Excel 2002 spreadsheet software.

2.3 QUANTIFICATION OF CHLOROPHYLL CONTENT

2.3.1 Materials

Investigation 2.1: Storage conditions

Plant material is often transported from the field to the laboratory at which time chlorophyll degradation may occur. It was therefore necessary to investigate the most appropriate procedure of containment of the collected leaf material. Two classical preservation methods were selected namely drying and freezing. Chlorophyll content was investigated in leaf material that was dried overnight at 84 °C and stored at room temperature for either seven days or 28 days, or frozen for either seven days or 28 days. Chlorophyll of leaf material that was extracted on the day of collection was used as the control. Five non-identical two year-old

diploid trees from the ICFR nursery were selected with fifteen leaf samples per tree for each of the four storage treatments (Table 2.2).

Table 2.2 Materials used of the four different storage treatments.

Storage method	Storage time (days)	No. of trees	Samples per tree
Control	0	5	15
Dried	7	5	15
	28	5	15
Frozen	7	5	15
	28	5	15

Investigation 2.2: Leaf and tree age

The effect of age on chlorophyll content was investigated in terms of two different factors, namely tree age and leaf age. Firstly, trees of different ages were compared and secondly, leaf material of different ages on the same tree was compared. The material utilised in this investigation consisted of old and new flushes of leaf material of two, four, six, eight and nine year-old trees (Table 2.3). Initially, 20 trees per age group (that is, 100 trees in total) from the Bloemendal plantations in KwaZulu-Natal were selected and tagged. Old flush leaf samples were collected from all 100 trees. However, at the time of new flush sample collection it was found that some of the tags were lost, or some trees had been felled, and hence the number of new flush samples was only 90. Five leaf samples from old flush and subsequently from new flush were collected from each tree, placed in a black plastic bag and stored on ice whilst in transit from the collection site to the laboratory.

Table 2.3 Tree material used in chlorophyll quantification of diploid black wattle trees of different age groups.

Tree material	Age (years)	No. of trees per age
Old flush	2	20
	4	20
	6	20
	8	20
	9	20
New flush	2	20
	4	18
	6	16
	8	16
	9	20

Investigation 2.3: Ploidy level

The effects of an increase in the number of chromosome sets on chlorophyll content were determined in diploid and tetraploid black wattle. In this investigation the only previously identified tetraploid material that was available was also included (WRI, 1950). These were seedlings of approximately eight months of age, bagged juveniles of approximately one year of age and two year-old field material. Equivalent diploid material was also selected. Seedling and bag materials were kept under nursery conditions at the ICFR, whilst the field material was obtained from the Bloemendal black wattle plantation in KwaZulu-Natal.

Many seeds (~ 50) of three genetic lines per ploidy level were germinated to ensure that sufficient material was available for this experiment since tetraploid seeds germinated with difficulty. Leaf samples from ten germinated seedlings per line from each black wattle repeat were collected and stored temporarily in black plastic bags that were kept on ice whilst in transit from the nursery or the field to the laboratory (Table 2.4).

Table 2.4 Tree material used in chlorophyll analysis of diploid and tetraploid black wattle.

Plant material	Ploidy levels	No. of lines	No. of plants
Seedlings	2x	3	10
	4x	3	10
Bags	2x	3	10
	4x	3	10
Field	2x	3	10
	4x	3	10

2.3.2 Methods

Chlorophyll was extracted from all leaf samples collected for each of the different investigations involving the quantification of chlorophyll content and thereafter, the chlorophyll absorbance was determined.

Chlorophyll extraction

Chlorophyll was extracted from leaf samples obtained from the various leaf material as follows (method modified from Vernon and Seely, 1966)

1. Leaves were washed with distilled water to remove impurities.
2. Approximately 1 g of green leaf material was homogenized in liquid nitrogen to reduce degradation, using a pestle and mortar.
3. Subsequently, 1 g of this powdery homogenate was weighed out using a 4 decimal-place electronic mass balance.
4. Working in reduced light, this sample was re-homogenized in 5 ml of 90 % acetone.
5. The chlorophyll-containing solution (CCS) was then siphoned off using a Pasteur pipette and placed into a polytop vial covered with tin foil.
6. Steps 4 and 5 were repeated two more times.

7. At the end of the third extraction, the total of 15 ml of the CCS was used to make a standard solution and was poured into a 25 ml volumetric flask and made up to the 25 ml-mark with 90 % acetone.
8. The volumetric flask, now containing the standard solution, was then covered with tin foil, placed on ice and absorbance spectrum determined within 15 minutes by chlorophyll absorbance spectroscopy.*

Chlorophyll absorbance spectroscopy

Chlorophyll absorbance spectra were recorded by placing 1 ml of the standard 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 and was utilised to standardise chlorophyll absorbance measurements to compensate for any absorbance that the acetone may introduce. Both cuvettes were placed in a PerkinElmer Lambda 45 UV/vis spectrometer. Light ranging in wavelength from 400 nm to 700 nm (visible light) was passed through the sample in the cuvette. This light was absorbed to produce a chlorophyll absorbance spectrum for each sample. These spectra were recorded as ASCII files and analysed statistically.

2.4 STATISTICAL ANALYSES

All the data in this research project was recorded in Microsoft® Excel 2002 spreadsheets. Statistical analyses were undertaken with the statistical package GenStat® 7.1 (Lane and Payne, 2003).

The following statistical analyses were conducted:

1. The means, ranges and standard deviations were calculated.
2. Analysis of variance (ANOVA) was performed to assess the variation present in the different investigations.
3. Least significant differences (LSD) was performed to interpret the variation.

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* Although the extraction process may have allowed for the extraction of other pigments and chlorophyll degradation products, these were difficult to separate out and were identified as consistent standard additions throughout the several chlorophyll experiments and did not affect the overall trends established.

2.5 SUMMARY OF MATERIALS UTILISED IN THIS RESEARCH PROJECT

Table 2.5 provides a summary of all the materials, their sample sizes and repeats utilised in the investigation of stomatal chloroplast frequency and chlorophyll content in relation to the discernment of diploid and tetraploid black wattle.

Table 2.5 Materials, sample sizes and number of repeats utilised in this research project.

Experiment	Investigation	Parameters	Materials	No. of lines	No. of trees per line
Stomatal chloroplast analyses	Stomatal frequency	Diploid	2x	3	5
		Tetraploid	4x	3	5
	Stomatal arrangement	Diploid	2x	3	5
		Tetraploid	4x	3	5
Chlorophyll content analyses	Storage pre-assessment	Control	0 days	5	15
		Ice treatment	7 days	5	15
			28 days	5	15
		Dry treatment	7 days	5	15
			28 days	5	15
	Age pre-assessment	Leaf Age	Old flush	100	1
			New flush	90	1
		Tree Age	2 years	20	1
			4 years	20	1
			6 years	20	1
			8 years	20	1
			9 years	20	1
	Ploidy comparative analysis	Diploid 2x	Seeds	3	10
			Bags	3	10
			Field	3	10
		Tetraploid 4x	Seeds	3	10
			Bags	3	10
			Field	3	10

3 CHAPTER 3 RESULTS

3.1 INTRODUCTION

This investigation focused on the discriminating power of two putative diagnostic procedures to differentiate between diploid and tetraploid black wattle. The stomatal chloroplast arrangements and number were compared as well as chlorophyll content in the different ploids. A number of associated experiments were undertaken to establish the best materials and storage conditions for the chlorophyll content analyses.

3.2 STOMATAL CHLOROPLAST ANALYSES

Stomatal chloroplasts were investigated in terms of the following:

- A comparative investigation of stomatal chloroplast frequency in diploid and tetraploid black wattle. This included a comparison of variation between plants within the same line, between lines within the same ploidy level as well as between ploids (Figure 3.1).

- A comparative investigation of chloroplast arrangement in stomatal cells in diploid and tetraploid black wattle.

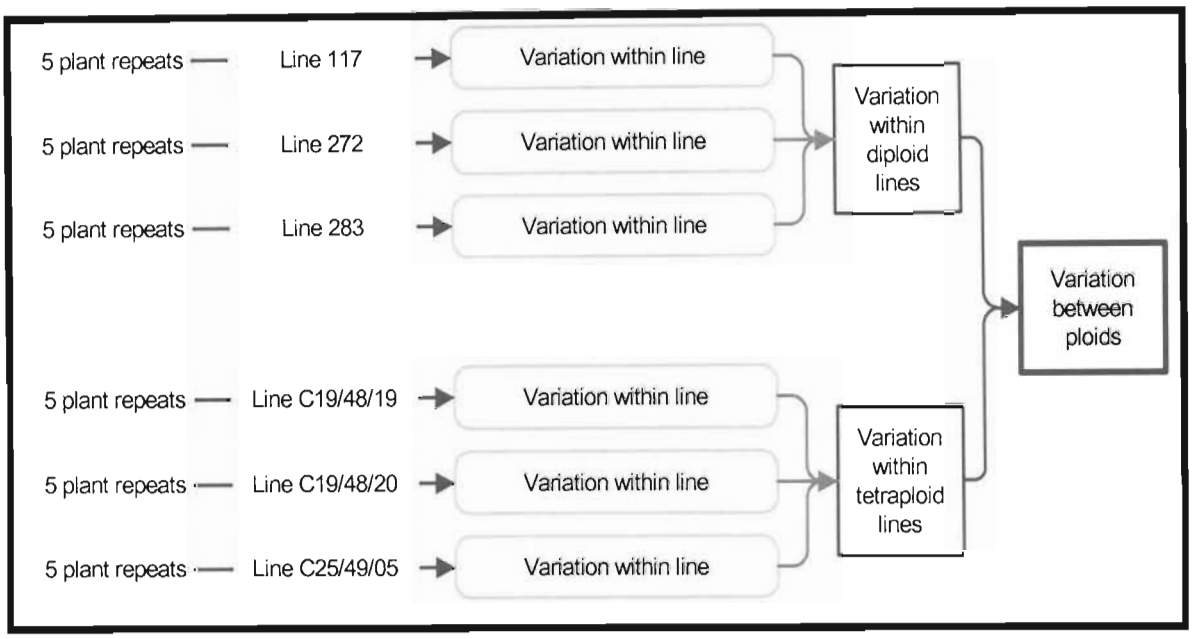


Figure 3.1 Flow diagram exhibiting expected levels of variation in stomatal chloroplast frequency experiments.

3.2.1 Stomatal chloroplast frequency

Stomatal chloroplast frequencies were determined in diploid and tetraploid black wattle. Three genetic lines per ploidy level with five plants per line were used for this experiment. Stomatal chloroplasts of 15 cells per plant were counted using an optical microscope at 40 X magnification. Chloroplast frequency data were captured in Microsoft Excel® 2002 and analyzed using GenStat® 7.1 (Lane and Payne, 2003).

The number of chloroplasts per cell was determined. These data indicated that the mean number of chloroplasts in diploids was distinctly different to that of the tetraploids, with no overlap of numbers between the different ploids (Table 3.1). The mean number of chloroplasts in the diploid lines was in the order of 10 per cell (9.89 ± 0.222), ranging, on average, from 7.5 to 11.7 per cell. In the tetraploids, on the other hand, the mean number of chloroplasts per cell was in the order of 22 (22.43 ± 0.222), ranging, on average, from 16.7 to 26.1.

Table 3.1 Mean stomatal chloroplast numbers of diploid and tetraploid black wattle seedlings.

Ploidy level	Genetic line	Mean number of chloroplasts within line	Range of mean number of chloroplasts within line	Mean number of chloroplasts within ploidy level	Range of mean number of chloroplasts within ploidy level
Diploid	117	09.093	07.533 – 10.600	09.989	07.533 – 12.067
	272	10.507	08.867 – 12.067		
	283	10.080	08.667 – 11.733		
Tetraploid	C19/48/19	23.307	16.867 – 26.067	22.427	16.867 – 26.067
	C19/48/20	22.347	18.267 – 25.733		
	C25/48/05	21.627	18.867 – 25.000		

Figure 3.2 provides a graphical representation showing the differences between mean number of stomatal chloroplasts within lines and between lines of diploid and tetraploid black wattle. The graphical representation clearly shows that the number of chloroplasts in the diploids do not overlap with that of the tetraploids. The ratio (of ~1:2) between the number of chloroplasts in the diploids and tetraploids is also clear.

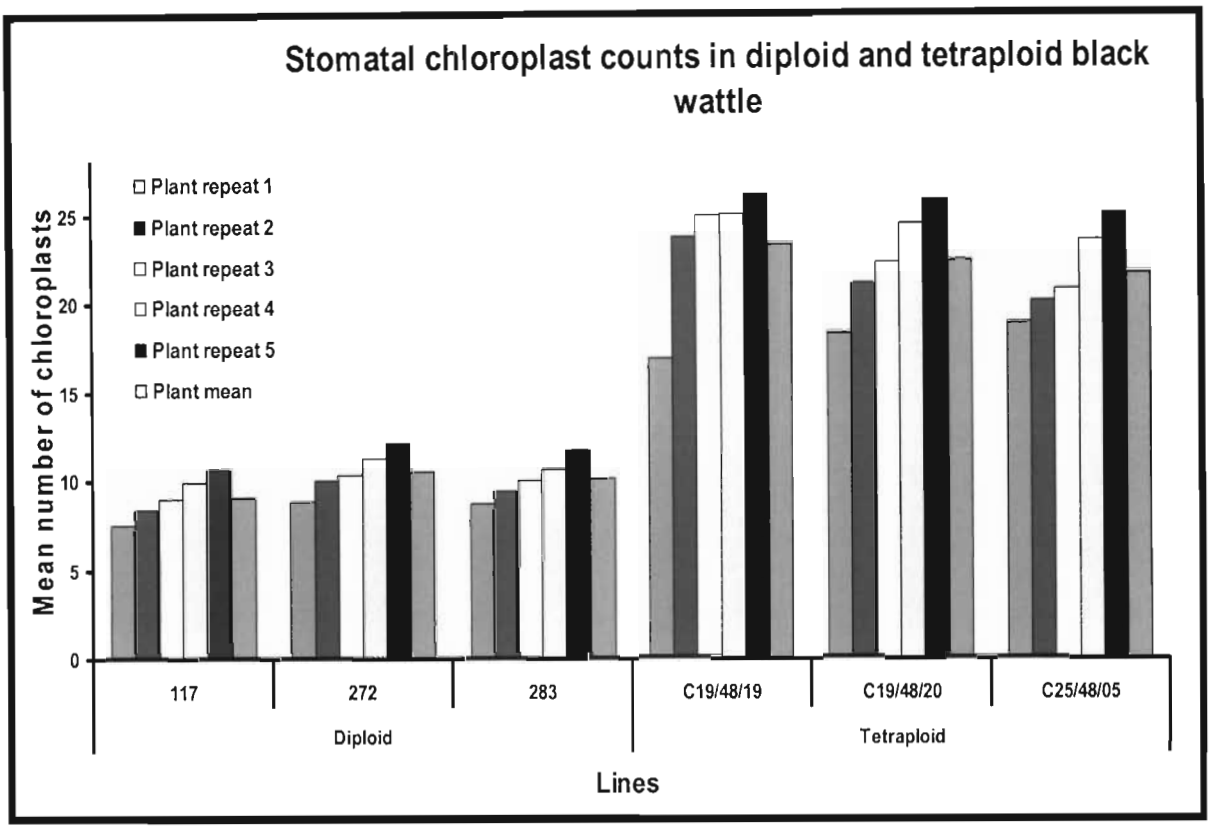


Figure 3.2 Mean number of stomatal chloroplasts in the different diploid (117, 272, 283) and tetraploids (C19/48/19, C19/48/20, C25/48/05) lines.

3.2.2 Stomatal chloroplast arrangement

An interesting finding was that stomatal chloroplasts in diploid cells displayed a distinctly different spatial arrangement when compared to the arrangement in tetraploids cells (Figure 3.3). In diploids, the chloroplasts were clustered into two regions, each towards the extreme ends of the kidney shaped stomatal cells. In the case of the tetraploids, no clustering of chloroplasts could be identified, however, the chloroplasts showed a more ordered, even distribution around the convex curvature/perimeter of the cells. These distinct chloroplast arrangements could be used to distinguish between diploid and tetraploid black wattle without quantifying chromosomal or stomatal chloroplast numbers.

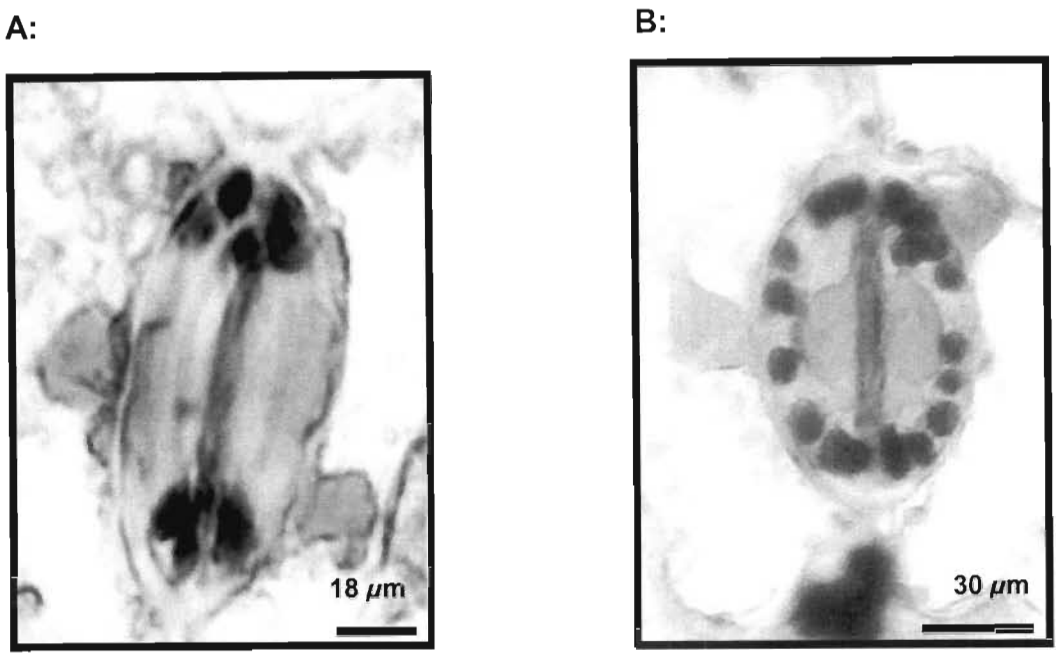


Figure 3.3 Chloroplast arrangement in A: diploid and B: tetraploid black wattle.

Statistical analysis of stomatal chloroplast frequencies

Stomatal chloroplast frequencies were compared between diploid and tetraploid black wattle. Six genetic lines; three per ploidy level, with five plant repeats per genetic line were included in this comparison. An analysis of variance (ANOVA) was conducted in order to investigate the sources of variation in stomatal chloroplast numbers. Table 3.2 presents the sources of variation within both diploids and tetraploids collectively. Significant differences in stomatal chloroplast numbers were identified between the different plants within each genetic line, between each genetic line within diploids, as well as within tetraploids ($p < 0.001$).

Table 3.2 Summary of analysis of variance of stomatal chloroplast numbers between genetic lines, within diploid and tetraploid black wattle.

Source of variation	df	ss	ms	vr	F.pr
Line	5	17857.3867	3571.4773	4685.61	< 0.001
Plant	4	1511.7467	377.9367	495.84	< 0.001
Line.Plant	20	477.2133	23.8607	31.30	< 0.001
Residual	420	320.1333	0.7622		
Total	449	20166.4822			

df = degrees of freedom; ss = sum of squares; ms = mean square; vr = variance ratio; F.pr = F-statistic.

An ANOVA was also conducted to compare the variation in stomatal chloroplast numbers between all diploid and tetraploid measurements. Table 3.3 illustrates that the number of stomatal chloroplasts in diploid black wattle was significantly different from the number in tetraploids ($p < 0.001$).

Table 3.3 Summary of analysis of variance of stomatal chloroplast numbers between diploid and tetraploid black wattle.

Source of variation	df	ss	ms	vr	F.pr
Ploidy	1	17672.000	17672.000	3173.83	< 0.001
Residual	448	2494.480	5.568		
Total	449	20166.480			

df = degrees of freedom; ss = sum of squares; ms = mean square; vr = variance ratio; F.pr = F-statistic.

An analysis of the least significant differences (LSD) was conducted to interpret the variation of stomatal chloroplast numbers at the level of ploidy, genetic line and plant repeat (Table 3.4). The LSD revealed significant differences between the

different plants within each genetic line; between each genetic line within diploids, between each genetic line within tetraploids, and lastly, between diploids and tetraploids. These differences suggest that trees of different genotypes should be sampled to establish whether the range of chloroplasts numbers lie within the currently known range.

Table 3.4 LSD analysis of the mean stomatal chloroplast numbers in diploid and tetraploid in black wattle.

Ploidy level	Genetic line	Plants within lines					Mean number of chloroplasts within line	Mean number of chloroplasts within ploidy
		1	2	3	4	5		
Diploid	117	07.533 **a**	08.400 **b**	09.000 **b**	09.933 **c**	10.600 **d**	09.093 **a**	
	272	08.867 **a**	10.000 **b**	10.333 **b**	11.267 **c**	12.067 **d**	10.507 **c**	09.989 **A**
	283	08.667 **a**	09.400 **b**	10.000 **bc**	10.600 **c**	11.733 **d**	10.080 **b**	
Tetraploid	C19/48/19	16.867 **a**	23.667 **b**	24.933 **c**	25.000 **c**	26.067 **d**	23.307 **f**	
	C19/48/20	18.267 **a**	21.000 **b**	22.267 **c**	24.467 **d**	25.733 **e**	22.347 **e**	22.427 **B**
	C25/48/05	18.867 **a**	20.000 **b**	20.733 **c**	23.533 **d**	25.000 **e**	21.627 **d**	

Treatments denoted by different letters (within each line) are significantly different ($P < 0.001$); (LSD = 0.6266). Treatments denoted by different capital letters (between grand means for each line) are significantly different ($P<0.001$); (LSD = 0.2802).

This investigation demonstrated that the assessment of chloroplast numbers has the potential to be utilized as a rapid and reliable means to distinguish between diploid and tetraploid black wattle.

3.3 CHLOROPHYLL CONTENT ANALYSES

There are a number of factors that influence chlorophyll content and degradation. These factors are either genetic or environmental in nature. Genetic factors include the variation in genetic composition, the genotype, and the level of ploidy. Environmental factors on the other hand, include sample age and sample storage conditions. It was therefore imperative to investigate how these factors affect the stability of the chlorophyll absorbance whilst sampling. Consequently, chlorophyll content was investigated in terms of the following:

- An investigation of the effect of storage conditions on chlorophyll content in diploid black wattle was conducted. Storage conditions included the storage of dried leaf material kept at room temperature as well as frozen leaf material; both stored for either a period of seven days or a period of 28 days, before chlorophyll was extracted.

- An investigation of the effect of tree age on chlorophyll content in diploid black wattle. Tree material from two-year; four-year; six-year; eight-year and nine-year old trees was used.

- An investigation of the effect of leaf age of the same tree on chlorophyll content was conducted. Leaf material included new flush and old flush.

- A comparative analysis of the chlorophyll content in diploid and tetraploid black wattle was undertaken.

A summary of the different factors that may affect chlorophyll content in black wattle as well as the methods of investigation is presented in Figure 3.4.

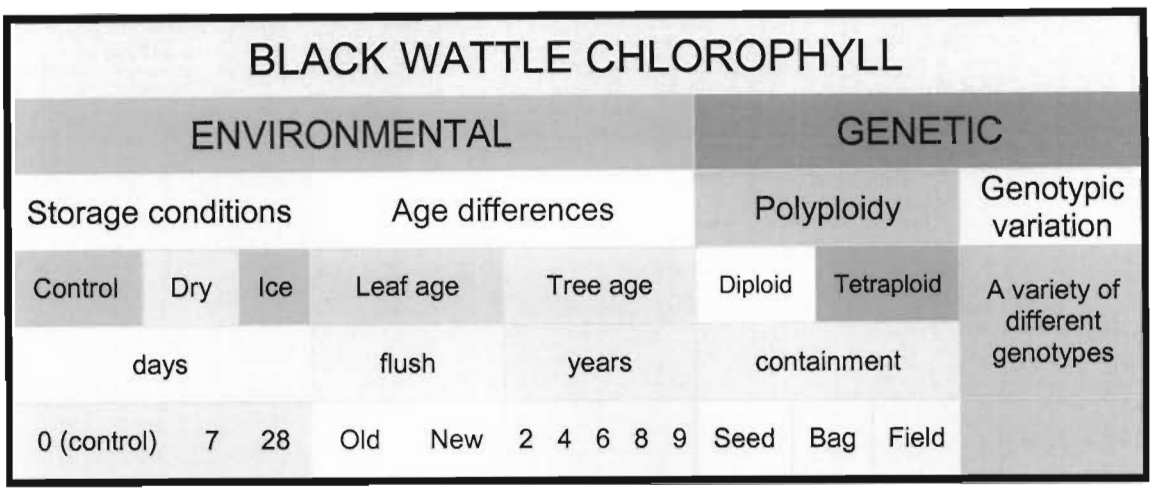


Figure 3.4 Factors that may affect chlorophyll content as well as methods of investigation.

3.3.1 Effects of storage on chlorophyll content

Storage effects on chlorophyll content as an indicator of possible degradation were determined for five non-identical two-year-old nursery diploid black wattle genotypes. Fifteen leaf samples from each genotype were exposed to a number of storage treatments before the chlorophyll was chemically extracted. Two classical methods of sample-preservation were selected, namely drying and freezing. Samples were either oven dried overnight at 84 °C and kept at room temperature, or frozen at - 4 °C. Two storage periods were applied; a seven-day period or a 28-day period. Therefore, four different storage treatments were tested in this investigation; dried for seven days, dried for 28 days, frozen for seven days and frozen for 28 days. Thereafter, chlorophyll was extracted from the various leaf samples and absorbance measured between 400 nm to 700 nm (visible light) in a standard UV/vis absorbance spectrometer. The absorbance of light by the chlorophyll over this wavelength range resulted in characteristic peaks at wavelengths of 433 nm, 456 nm and 663 nm. The amplitude of these peaks was used as an indicator of chlorophyll content in each sample (Beer's Law, See Harwood and Moody, 1989). Subsequently, chlorophyll absorbance values and absorbance spectra were recorded for each stored sample using Microsoft® Excel

2002 and statistically analysed with the software package GenStat® 7.1 (Lane and Payne, 2003).

The effects of storage treatments on chlorophyll absorbance were determined in terms of the following (see figure 3.5 for schematic representation):

1. Chlorophyll absorbance values were holistically compared in order to identify any trends,

2. Comparative analyses between chlorophyll absorbance values within each of the four storage treatment was undertaken, and

3. Comparative analysis of chlorophyll absorbance values between each of the four storage treatments and the control was undertaken.

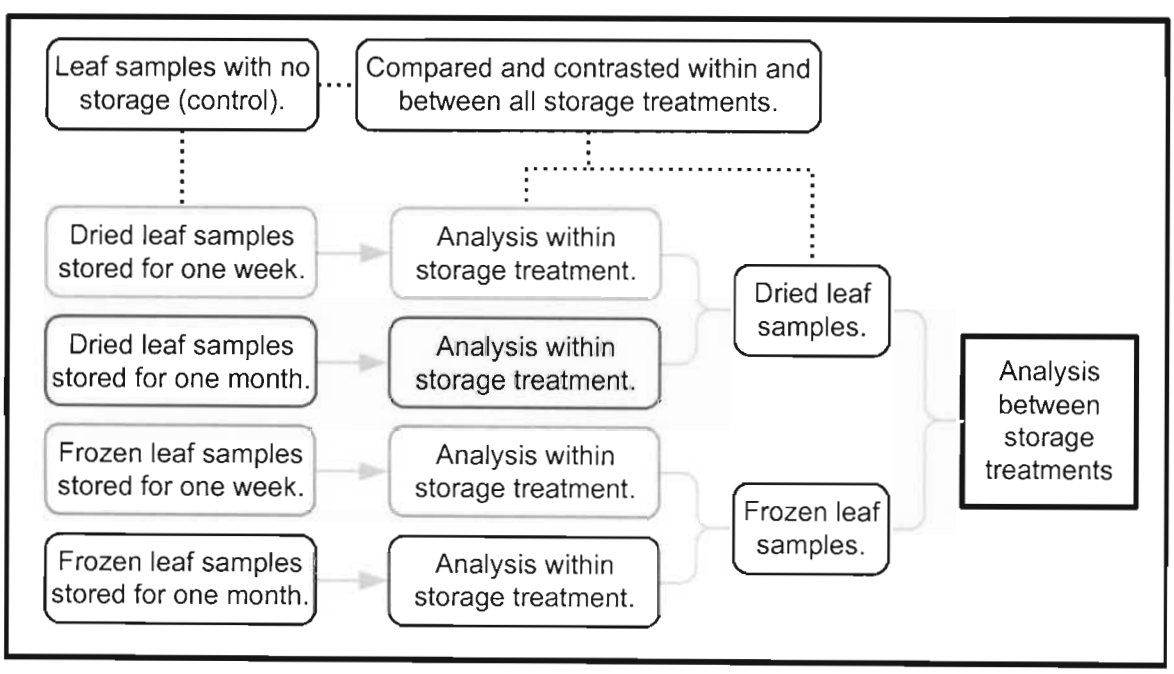


Figure 3.5 Flow diagram exhibiting the comparative analyses of the effects of storage on chlorophyll content undertaken in this investigation.

Trends in chlorophyll absorbance spectra

Chlorophyll absorbance spectral charts for the four different storage treatments were compared to identify possible trends (Figure 3.6). In general, chlorophyll absorbance profiles for all four storage treatments were distinctly lower than that of the control (0 days of storage), whilst still maintaining a similar profile with peaks at wavelengths of 433 nm, 456 nm (shoulder) and 663 nm.

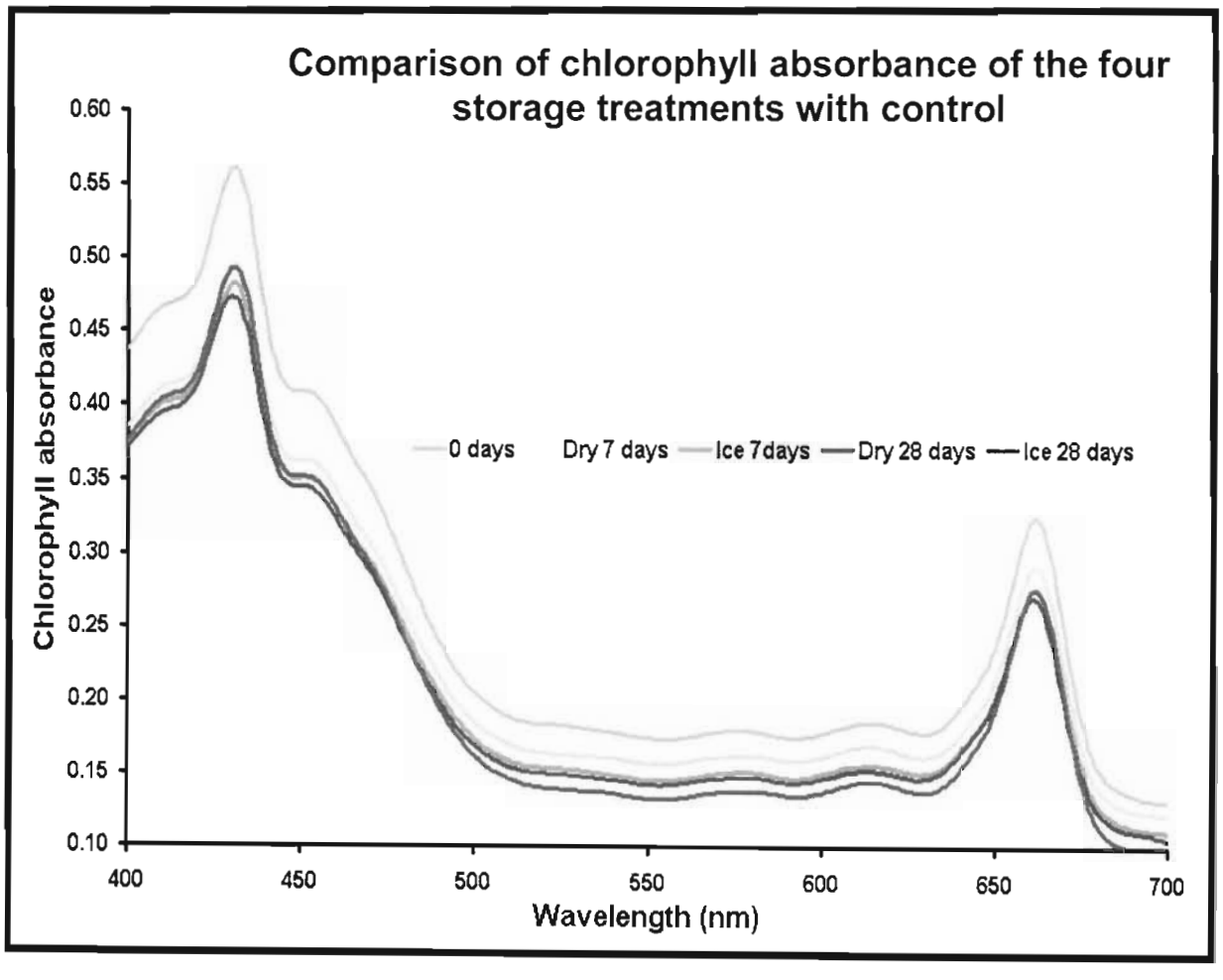


Figure 3.6 Chlorophyll absorbance of dried and frozen leaf material for 0 days, 7 days and 28 days.

Comparison of chlorophyll absorbance values

A detailed analysis was undertaken of the chlorophyll absorbance values for all four storage treatments (Table 3.5). The mean chlorophyll absorbance values ($\bar{A}$) for the different storage treatments at each of the three wavelengths 433 nm, 456 nm and 663 nm were compared. The $\bar{A}$ values of all the treatments at each wavelength were lower than that of the control. The order of the decrease in $\bar{A}$ of the four storage treatments differed for each of the wavelengths; therefore the total mean absorbance ($T\bar{A}$) value was determined as the sum of the $\bar{A}$ at 433 nm, 456 nm and 663 nm. The assessment of $T\bar{A}$ for the four different storage treatments revealed that dried leaves for seven days ($T\bar{A}$ = 1.132) resulted in the least amount of chlorophyll degradation when compared to the control ($T\bar{A}$ = 1.275), followed by 28 day ice storage ($T\bar{A}$ = 1.114), seven day ice storage ($T\bar{A}$ = 1.103) and lastly 28 day dried storage ($T\bar{A}$ = 1.093).

The magnitude of the decrease of the $\bar{A}$ values of each of the four storage treatments from the control was determined; the total deviation from the control ($T\delta$). The $T\delta$ for dried material was 11.2 % for material dried for seven days while for material dried for 28 days it was 14.3 %. On the other hand, in the case of frozen material, the $T\delta$ was 13.5 % for material frozen for seven days but was only 12.6 % for material frozen for 28 days. These data indicated that material dried and stored for seven days displayed the least amount of deviation from the control. On the other hand, material dried for a period of 28 days displayed the greatest deviation from the control.

Table 3.5 Mean chlorophyll absorbance for the four different storage treatments.

Treatment Type	Storage (days)	N [1]	Reps [2]	Wavelength (nm)	Absorbance range	Mean absorbance ($\bar{A}$) (%) [3]	Total mean absorbance [4] ($T\bar{A}$) (%) [3]	Deviation from control (δ) [5] (%) [3]	Total deviation from control ($T\delta$) (%) [3]
Control	0	5	15	433	0.5614 – 0.5421	0.5523			
		5	15	456	0.4107 – 0.3914	0.4016			
		5	15	663	0.3310 – 0.3117	0.3219	*1.2758 (100.0)*		
Dry	7	5	15	433	0.4978 – 0.4722	0.4874		0.0649 (11.8)	
		5	15	456	0.3662 – 0.3414	0.3563		0.0453 (11.3)	
		5	15	663	0.2986 – 0.2742	0.2890	*1.1327 (88.7)*	0.0329 (10.2)	*0.1431 (11.2)*
	28	5	15	433	0.5100 – 0.4430	0.4746		0.0777 (14.0)	
		5	15	456	0.3778 – 0.3208	0.3443		0.0573 (14.3)	
		5	15	663	0.3070 – 0.2563	0.2741	*1.0930 (85.6)*	0.0478 (14.8)	*0.1828 (14.3)*
Ice	7	5	15	433	0.5778 – 0.4388	0.4849		0.0674 (12.2)	
		5	15	456	0.4125 – 0.3080	0.3451		0.0565 (14.0)	
		5	15	663	0.3276 – 0.2408	0.2733	*1.1033 (86.4)*	0.0486 (15.1)	*0.1725 (13.5)*
	28	5	15	433	0.5821 – 0.4422	0.4885		0.0639 (11.5)	
		5	15	456	0.4168 – 0.3114	0.3487		0.0529 (13.1)	
		5	15	663	0.3319 – 0.2442	0.2769	*1.1141 (87.3)*	0.0450 (13.9)	*0.1617 (12.6)*

[1] N = number of samples.

[2] Reps = number of repeats or replicates per sample.

[3] Percentage of the control where the control is 100 %.

[4] Total mean absorbance = mean absorbance$_{433nm}$ + mean absorbance$_{456nm}$ + mean absorbance $_{663nm}$ = $T\bar{A} = \bar{A}_{433\ nm} + \bar{A}_{456\ nm} + \bar{A}_{663\ nm}$.

[5] Percentage difference from control that is, how different that value is from the control (0%) expressed as a percentage.

A graphical representation of the data showed the general trends displayed by the four storage treatments with greater clarity (Figure 3.7). Chlorophyll absorbance values within dried and frozen treatments were compared with respect to storage time periods of one week and one month. It was noted that whilst all treatments decreased from the control (day-0), dried samples responded differently to storage periods compared to frozen samples. Chlorophyll absorbance values of dried material decreased steadily over time from the control to seven-day storage to one-month storage, whereas, in the case of frozen material, a similar trend could not be identified. A greater decrease from the control to seven-day ice storage was recorded than for the decrease from the control to 28 day ice storage. This could be indicative of a type of 'chlorophyll recovery system' in response to cold temperatures over long periods over time (see for example Strand and Lundmark, 1987).

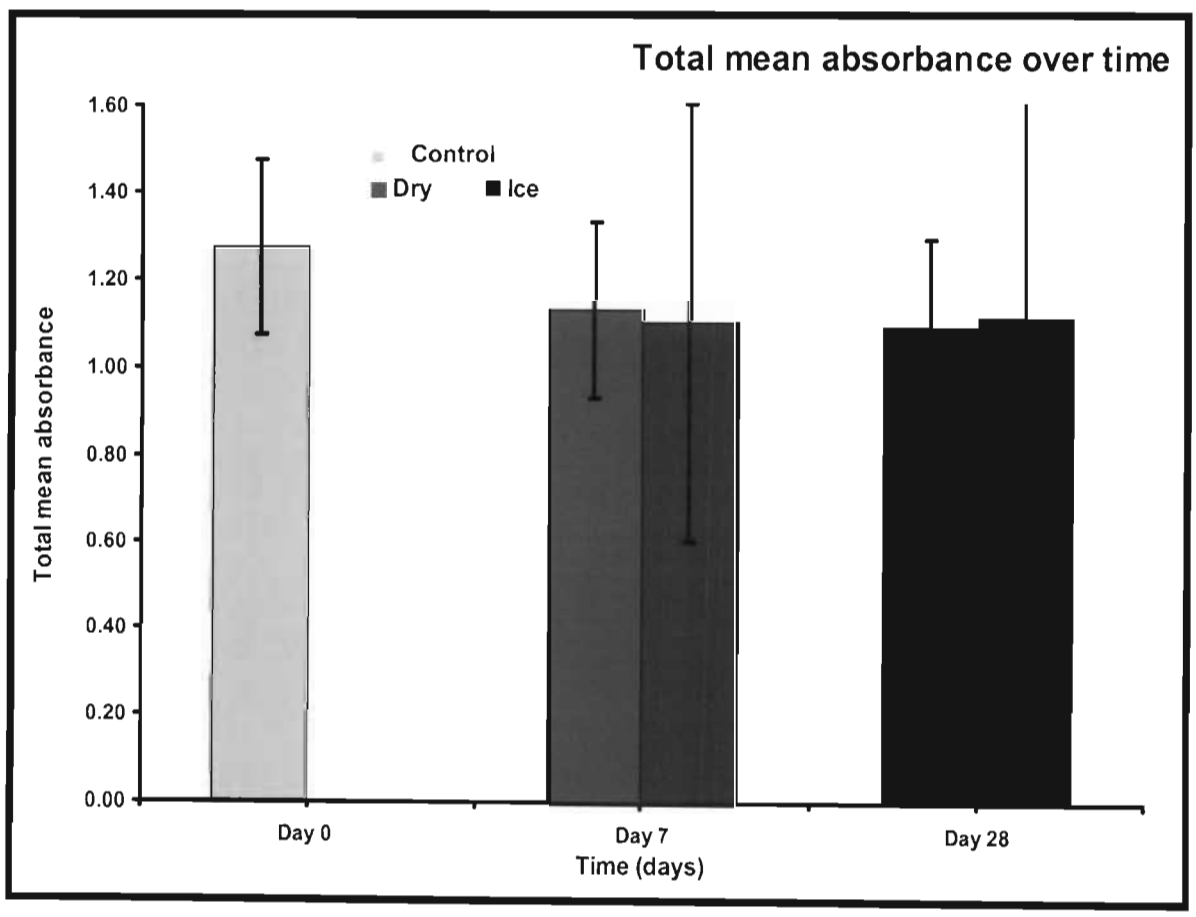


Figure 3.7 Comparison of total mean chlorophyll absorbance trends of the different storage treatments against the control.

Statistical analysis of chlorophyll absorbance

Chlorophyll absorbance for all four storage treatments were compared in five non-identical two year-old diploid black wattle genotypes (trees) in the nursery. Fifteen leaf samples (repeats) per storage treatment, per tree, were included in this comparison. An analysis of variance (ANOVA) was conducted in order to investigate the sources of variation in the different storage treatments (Table 3.5). Significant differences in chlorophyll absorbance values were identified between the different trees utilized, between each storage treatment within a tree as well as between the different wavelengths within a treatment ($p < 0.001$).

Table 3.6 Summary of analysis of variance for chlorophyll absorbance and storage treatments.

Source of variation	df	ss	ms	vr	F.pr
Tree	4	0.0870	0.0217	71.76	< 0.001
Tree. storage treatment	4	0.5655	0.1413	466.21	< 0.001
Wavelength	2	8.5814	4.2907	14150.00	< 0.001
Storage treatment. wavelength	8	0.0251	0.0031	10.35	
Residual	1106	0.3354	0.0003		
Total	1124	9.5946			

df = degrees of freedom; ss = sum of squares; ms = mean square; vr = variance ratio; F.pr = F-statistic.

An analysis of least significant differences (LSD) was calculated to interpret the variation in chlorophyll absorbance values for each of the four storage treatments at the three wavelengths that were examined (Table 3.7). The LSD revealed that chlorophyll absorbance for all treatments were significantly different from the control within each wavelength. However, it was interesting to note that within the

different storage treatments at a particular wavelength, not all treatments differed significantly from one another (as denoted by the same letter in table 3.7). The mean chlorophyll absorbance between the different storage treatments and the control was also significantly different ($p < 0.001$).

Table 3.7 LSD analysis of the mean chlorophyll content in diploid black wattle with various storage treatments.

Treatments	Wavelengths (nm)			
	433	456	663	Mean
Control 0 days	0.55226 **c**	0.40155 **d**	0.32185 **g**	0.42522 **E**
Dry one week	0.48740 **b**	0.35633 **e**	0.28900 **h**	0.37757 **D**
Dry one month	0.47459 **a**	0.34429 **f**	0.27411 **i**	0.36433 **A**
Ice one week	0.48486 **b**	0.34507 **f**	0.27326 **i**	0.36773 **B**
Ice one month	0.48849 **b**	0.34871 **f**	0.27690 **i**	0.37137 **C**

Treatments denoted by different lower case letters are significantly different from each other ($p < 0.005$); (LSD = 0.00558). Treatments denoted by different capital letters are significantly different from each other ($p < 0.005$); (LSD = 0.00322).

This investigation into the effects of storage of leaf material on the chlorophyll content indicated distinct degradation of chlorophyll, thereby reducing the amount of chlorophyll absorbance. Therefore, chlorophyll extractions should ideally be executed on the day of collection. However, if circumstances do not permit immediate extraction and spectral analysis, then storage of material is suggested. The application of the type of storage depends on the length of time required to store the material. Drying of the material is advised should storage time be for a week or less. However, it is advisable that if material is frozen, if a longer period of storage is required.

3.3.2 Effects of age on chlorophyll content

The effect of age on chlorophyll content was investigated in terms of two different features, namely tree age and leaf age. Firstly, trees of different ages were compared and secondly, leaf material of different ages on the same tree was compared. The material utilized in this investigation consisted of old and new flushes of leaf material in two, four, six, eight and nine year-old trees. Initially, a total of 100 trees of different ages with 20 trees per age group were selected and tagged. However, at the time of sample collection, it was found that some of the tags were lost or some trees had been felled, thereby decreasing the effective sample number to 90 in the new flush study. Five leaf samples from each tree were collected, placed in a black plastic bag and stored whilst in transit from the collection site to the laboratory. Chlorophyll was subsequently extracted from the various leaf samples and exposed to a wavelength range of 400 nm to 700 nm (visible light) in a standard UV/vis absorbance spectrometer. Thereafter, the chlorophyll absorbance data were recorded and analyzed in the same manner as that of the storage investigation (see section 3.3.1).

The effects of tree and leaf age on chlorophyll absorbance were determined in terms of the following (see figure 3.8 for schematic representation):

1. Chlorophyll absorbance values were holistically compared in order to identify any obvious trends,

2. Comparative analyses of chlorophyll absorbance values of trees of different ages were undertaken.

3. Comparative analyses of chlorophyll absorbance values of leaf material from the same tree of different ages were undertaken, and

4. Comparative analyses of all tree ages, as well as leaf ages were undertaken.

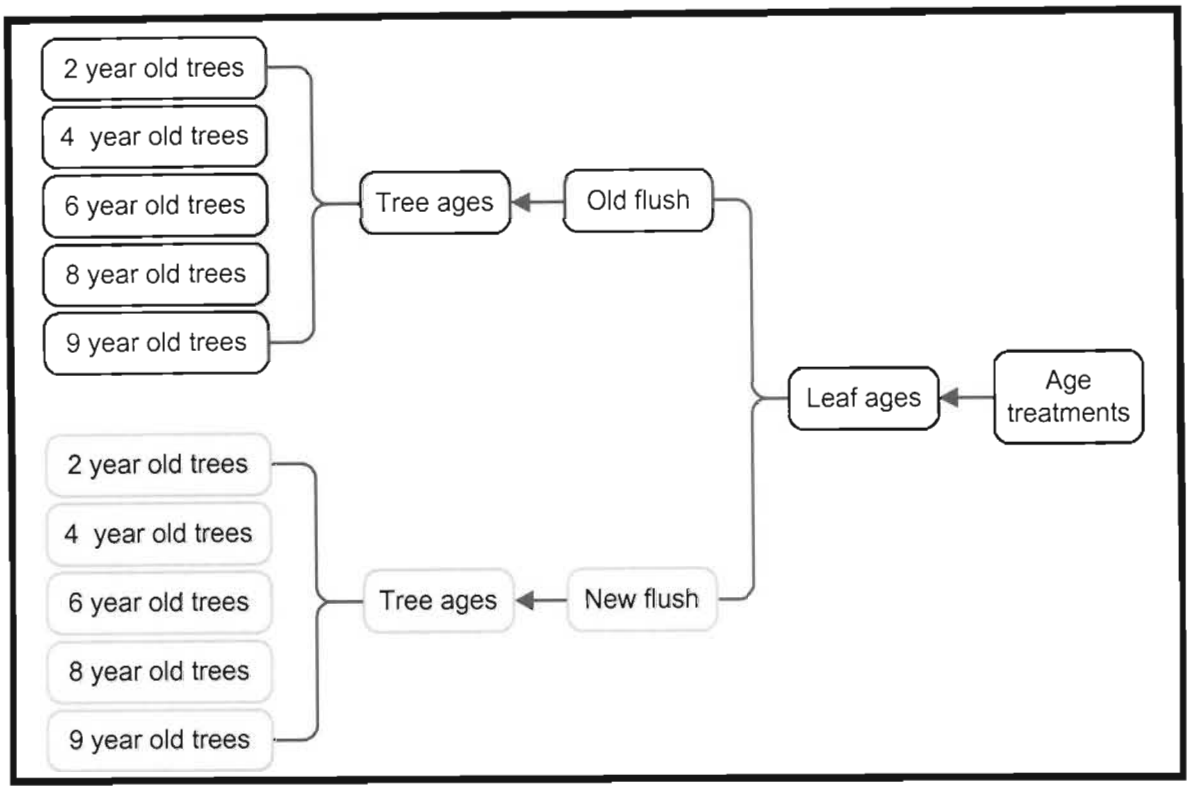
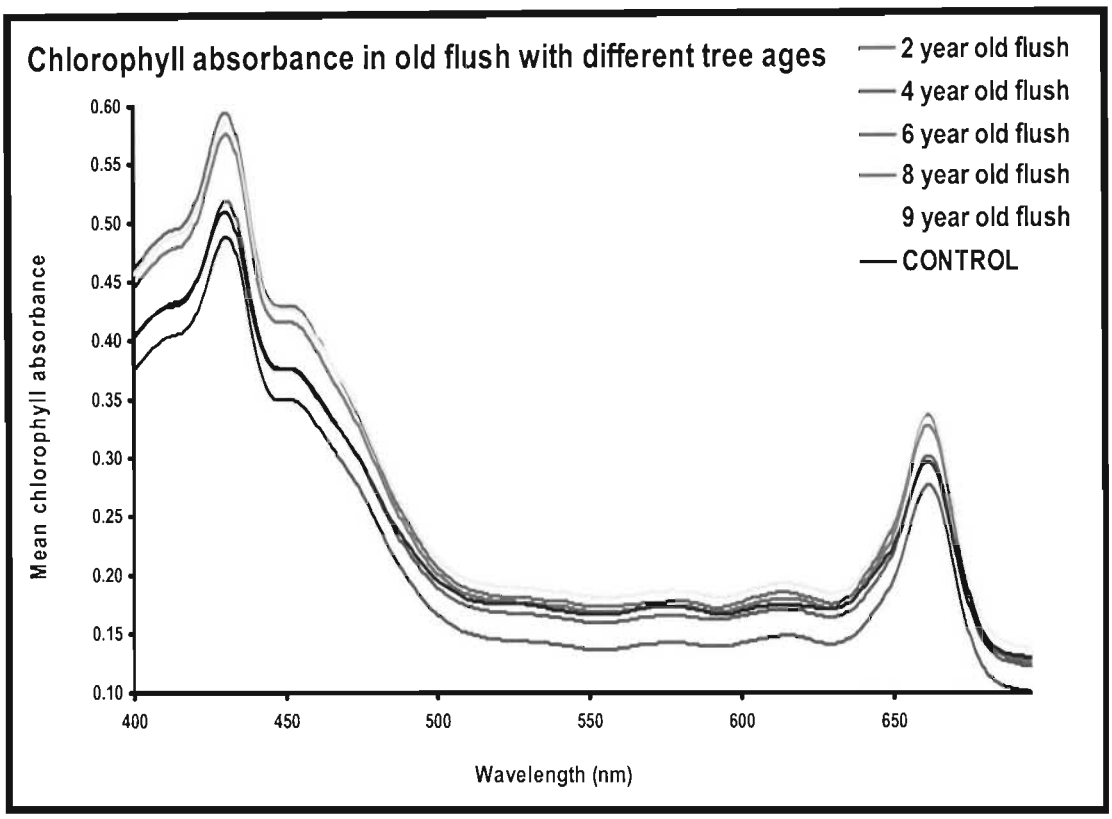


Figure 3.8 Flow diagram of the comparative analyses undertaken to study the effects of age on chlorophyll content.

Trends in chlorophyll absorbance spectra

Chlorophyll absorbance spectral charts for the different tree and leaf age groups were examined to identify typical trends. These spectral charts were compared to a control value, which was the chlorophyll absorbance of young diploid seedlings, that was chosen as an initial estimate of chlorophyll content since seedlings have, in essence only one type of flush (Figure 3.9). In general, chlorophyll absorbance profiles for all age groups and flush treatments maintained the expected graphical profile with peaks at wavelengths of 433 nm, 456 nm (shoulder) and 663 nm. The chlorophyll absorbance values of both old and new flush of different age groups produced spectral graphs for which no specific trends could be ascertained.

A



B

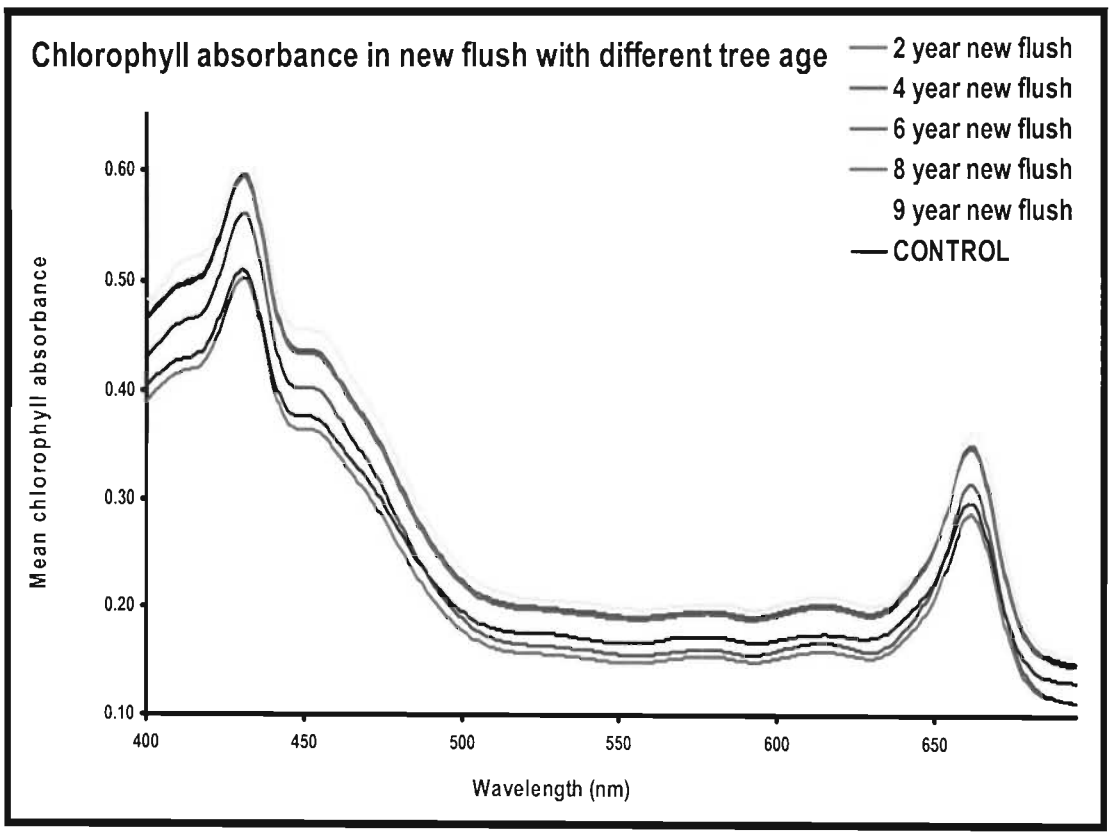


Figure 3.9 Chlorophyll absorbance of different leaf treatments at two, four, six, eight and nine year-old tree age: A: old flush and B: new flush.

The identification of old and new flush was based solely upon a visual selection process that may not have accurately distinguished between the two flush types. Therefore, the data of old flush and new flush were pooled.

Chlorophyll absorbance spectral charts for the combined flush over all ages were compared to identify possible trends (see figure 3.10). In general, chlorophyll absorbance profiles of all tree ages were distinctly greater than that of the control, whilst still maintaining the expected spectral profile, peaking at the wavelengths of 433 nm, 456 nm (shoulder) and 663 nm.

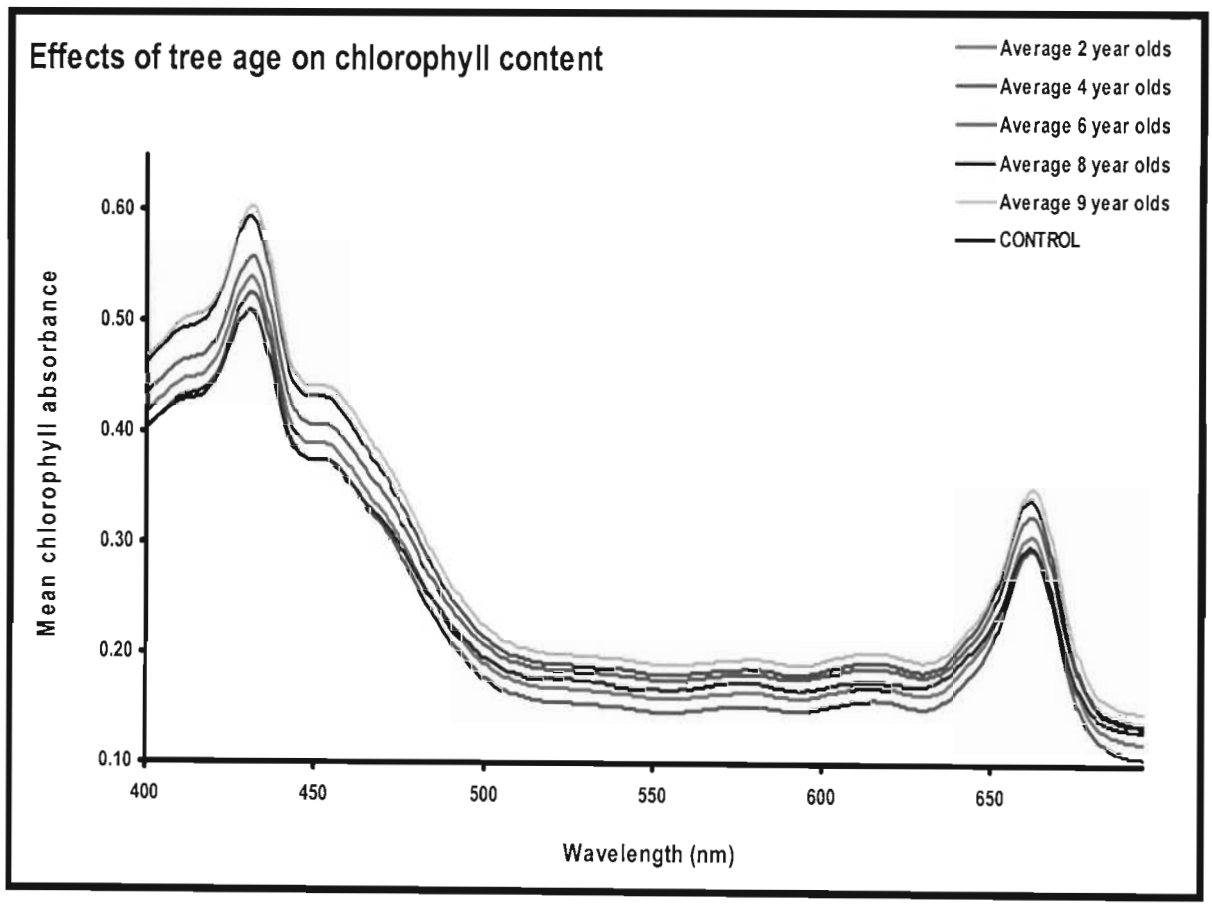


Figure 3.10 Chlorophyll absorbance of combined flush treatments over all tree ages.

Comparison of chlorophyll absorbance values

A detailed analysis was undertaken of the chlorophyll absorbance values for all age treatments (Table 3.8). The mean chlorophyll absorbance values ($\bar{A}$) for each of the different age treatments at the three wavelengths 433 nm, 456 nm and 663 nm were compared. The total mean absorbance values for tree age within flush type ($TT\bar{A}$), sum of the $\bar{A}$ values at 433 nm, 456 nm and 663 nm, were determined. The $TT\bar{A}$ values of the different age groups within the old flush as well as within new flush did not reveal any distinct patterns. However, it was noted that the $TT\bar{A}$ values for the older trees in old flush (eight year-old $TT\bar{A}$ = 1.34, nine year-old
$TT\bar{A}$ = 1.33) as well as in new flush (six year-old $TT\bar{A}$ = 1.36, eight year-old $TT\bar{A}$ = 1.35, nine year-old $TT\bar{A}$ = 1.42) were distinctly higher than that of the control and young trees.

A total mean absorbance ($LT\bar{A}$) of all ages within old flush as well as within new flush was determined. The $LT\bar{A}$ of old flush ($LT\bar{A}$ = 1.22) was found to be marginally less than that of new flush ($LT\bar{A}$ = 1.24).

Table 3.8 Mean chlorophyll absorbance for different age treatments.

Treatment type	Age (years)	Wavelength (nm)	Absorbance range	Mean absorbance (Ā)	Total mean absorbance for tree age (TTĀ)	Total mean absorbance for leaf age (LTĀ)
Control	2/3	433.0	0.5182 – 0.4799	0.4994		
		456.0	0.3849 – 0.3492	0.3683		
		663.0	0.3098 – 0.2756	0.2931	*1.1608*	
Old flush	2	433.0	0.7131 - 0.4415	0.5664		
		456.0	0.5624 - 0.2845	0.4086		
		663.0	0.4827 - 0.1998	0.3233	*1.2983*	
	4	433.0	0.5082 - 0.4423	0.4812		
		456.0	0.3677 - 0.3115	0.3438		
		663.0	0.2954 - 0.2443	0.2732	*1.0982*	
	6	433.0	0.5163 - 0.4982	0.5108		
		456.0	0.3758 - 0.3555	0.3703		
		663.0	0.3035 - 0.2564	0.2980	*1.1792*	
	8	433.0	0.6038 - 0.5279	0.5836		
		456.0	0.4405 - 0.3908	0.4222		
		663.0	0.3508 - 0.2925	0.3315	*1.3372*	
	9	433.0	0.5931 - 0.5423	0.5767		
		456.0	0.4361 - 0.3658	0.4197		
		663.0	0.3514 - 0.3098	0.3350	*1.3314*	*1.22136*
New flush	2	433.0	0.5815 - 0.4422	0.4935		
		456.0	0.4182 - 0.3114	0.3589		
		663.0	0.3285 - 0.2442	0.2830	*1.1354*	
	4	433.0	0.5751 - 0.5453	0.5514		
		456.0	0.4181 - 0.3327	0.3944		
		663.0	0.3334 - 0.2922	0.3097	*1.2555*	
	6	433.0	0.5984 - 0.5799	0.5868		
		456.0	0.4414 - 0.4277	0.4298		
		663.0	0.3567 - 0.3388	0.3451	*1.3616*	
	8	433.0	0.5974 - 0.5614	0.5838		
		456.0	0.4404 - 0.4055	0.4268		
		663.0	0.3557 - 0.3525	0.3421	*1.3527*	
	9	433.0	0.6294 - 0.5926	0.6116		
		456.0	0.4661 - 0.4235	0.4502		
		663.0	0.3764 - 0.3365	0.3606	*1.4224*	*1.2493*

A graphical representation of the data illustrates with greater clarity the general trends displayed by old flush and new flush in relationship to the control (seedling flush). The seedling flush was used as an initial estimate of chlorophyll content in young material (Figure 3.11). Chlorophyll absorbance in four year-old and older trees of both flushes displayed a tendency to increase with tree age, indicating an increase in chlorophyll content as the trees became more established. In these, trees the absorbance of the new flush was higher than that of the old flush indicating diminishing chlorophyll content as flush aged. It was interesting to note that the chlorophyll content in two year-old trees was 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 an indication of the growth stage of the tree. Black wattle trees undergo a transition period from seedling to adult known as the juvenile phase, which is associated with physiological and anatomical changes. Anatomical changes include various changes such as variation in leaf structure (James *et al.*, 1999), whilst physiological changes include increases in rates of photosynthesis and respiration (Huang *et al.*, 2003). These changes therefore increase the demand for many housekeeping functions; one of which was identified in this investigation as a marked increase in the chlorophyll content of old flush that is often associated with an increase in the photosynthetic rate.

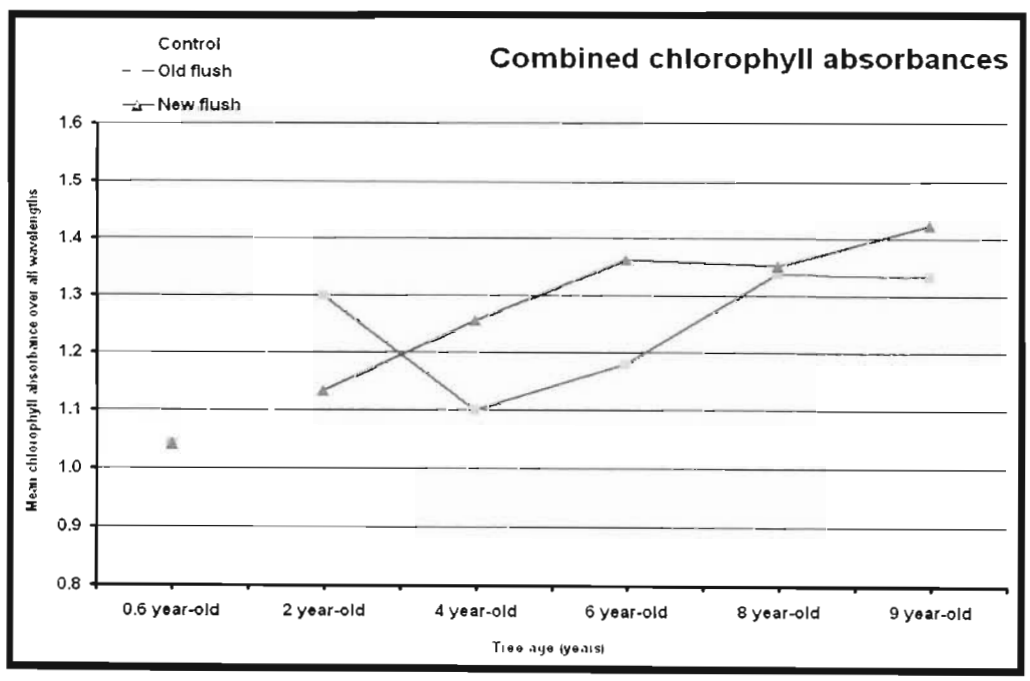


Figure 3.11 Mean chlorophyll absorbance of old and new flush in all tree ages.

Statistical analysis of chlorophyll absorbance

Chlorophyll absorbance for all leaf and tree age treatments was compared in non-identical diploid black wattle genotypes (that is, different trees) from the field. Five age groups with 20 trees per age group were included in this comparison. An analysis of variance (ANOVA) was conducted in order to investigate the sources of variation in the different leaf and tree age treatments (Table 3.9). Significant differences in chlorophyll absorbance values were identified between the different trees utilized, between the different age groups and leaf types, as well as between the different wavelengths within leaf and tree age treatments ($p < 0.001$).

Table 3.9 Summary of analysis of variance of chlorophyll absorbance and age treatments.

Source of variation	df	ss	ms	vr	F.pr
Tree	19	0.03807	0.00200	5.44	
Tree. age	12	1.02435	0.08536	231.63	< 0.001
Wavelength	2	7.59635	3.79817	10310.00	< 0.001
Age. wavelength	24	0.35446	0.01476	40.08	< 0.001
Residual	602 (120)	0.22185	0.00036		
Total	659 (120)	8.05542			

df = degrees of freedom; ss = sum of squares; ms = mean square; vr = variance ratio; F.pr = F-statistic.

An analysis of the least significant differences (LSD) was calculated to interpret the variation in chlorophyll absorbance values for all leaf and tree age treatments at the three wavelengths examined (Table 3.10). The LSD revealed that the chlorophyll absorbance data for all treatments were significantly different from the control (seedling data) within each wavelength, with the exception of six year-old old flush. Interestingly, not all age treatments were statistically different from one another (Table 3.10). Letters have been used to distinguish statistical differentiation with the chlorophyll absorbance ranked from lowest (a) to highest (f).

The LSD indicated in most cases that chlorophyll absorbance values of old flush and new flush at a particular age did not differ significantly. However, those of two year-olds were significantly different as indicated by the distance in LSD ranking between two year-old new flush and old flush.

Table 3.10 LSD analysis of the mean chlorophyll content in diploid black wattle with various age treatments.

Treatments	Mean chlorophyll absorbance
Control	0.38674 **c**
New flush 2 years	0.37847 **b**
New flush 4 years	0.36929 **a**
New flush 6 years	0.45189 **e**
New flush 8 years	0.45046 **e**
New flush 9 years	0.47412 **f**
Old flush 2 years	0.43683 **d**
Old flush 4 years	0.36606 **a**
Old flush 6 years	0.39310 **c**
Old flush 8 years	0.44575 **e**
Old flush 9 years	0.44383 **e**

Treatments denoted by different letters are significantly different from each other ($p < 0.005$); (LSD = 0.006883).

This investigation into the effects of leaf and tree age on the chlorophyll content indicated a marked increase in chlorophyll absorbance as trees became older. Moreover, this increase in chlorophyll content was more apparent in new flush than in old flush. However, juvenile characteristics have been identified in two year-old black wattle trees, one of which was a marked increase in chlorophyll content. This distinctly higher level of chlorophyll in two year-olds was supported by statistical findings and was likely due to a biological accommodation for the increased physiological activities associated with this growth stage.

3.3.3 Comparative analysis of ploidy on chlorophyll content

The effects of increased number of chromosome sets on chlorophyll content were determined in diploid and tetraploid black wattle. For this investigation the only previously identified tetraploid material that was available included seedlings, bagged juveniles and two year-old trees, which was compared to equivalent diploid material.

Three genetic lines per ploidy level with ten plants per line (repeats) were used for this experiment. The repeats were cloned material and regarded as genetically identical. Leaf samples from each plant were collected and stored temporarily. Chlorophyll was subsequently extracted from the various leaf samples and absorbance measured from 400 nm and 700 nm (visible light) in a standard UV/vis absorbance spectrometer. Thereafter, the chlorophyll absorbance data were recorded and analyzed in the same manner as that of the storage investigation (see section 3.3.1).

The effects of ploidy on chlorophyll absorbance were determined in terms of the following (see figure 3.12 for a schematic representation):

1. Chlorophyll absorbance values were holistically compared in order to identify any obvious trends between ploidy levels,

2. Comparative analyses of chlorophyll absorbance values within diploid and tetraploid black wattle were undertaken, and

3. Comparative analyses of chlorophyll absorbance values between diploids and tetraploid black wattle were undertaken.

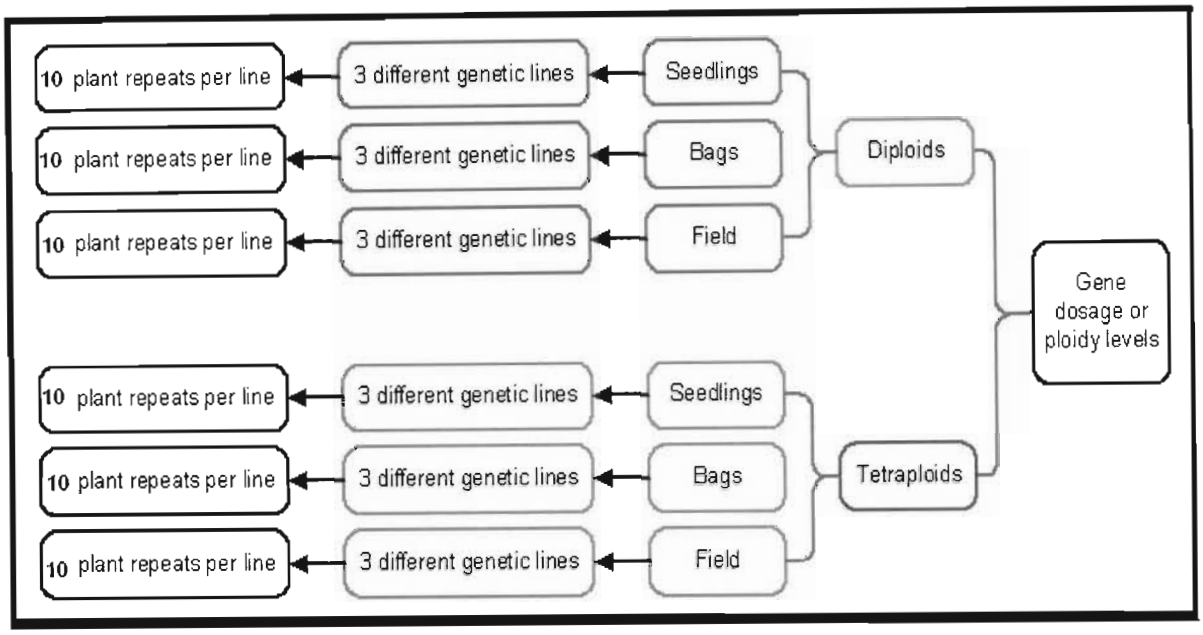


Figure 3.12 Flow diagram of the comparative analyses undertaken to study the effects of chromosome dosage on chlorophyll content.

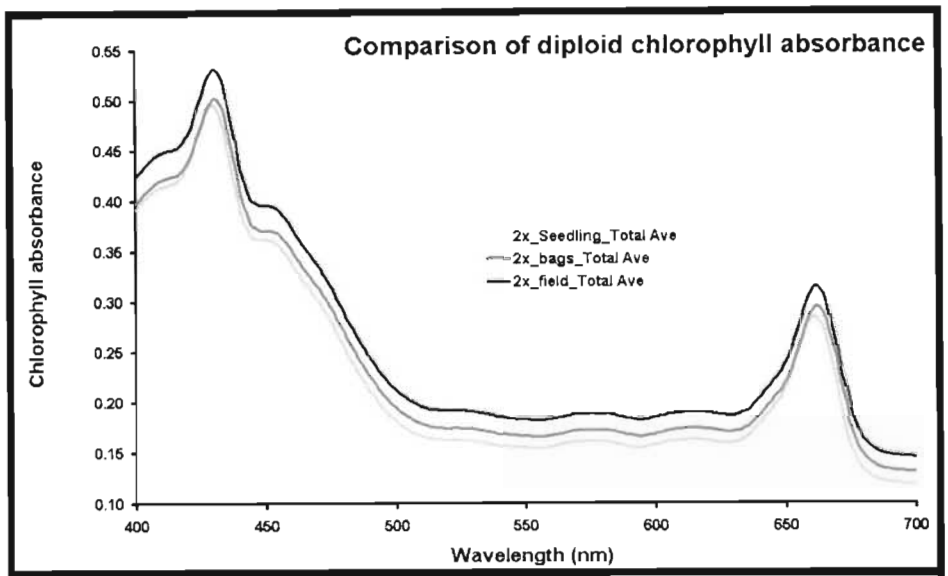
Trends in chlorophyll absorbance spectra

Chlorophyll absorbance spectra of diploid and tetraploid black wattle were compared to identify possible trends. In general, chlorophyll absorbance profiles for both levels of ploidy maintained the expected profile, with peaks at 433 nm, 456 nm (shoulder) and 663 nm (Figure 3.13).

In diploids, chlorophyll absorbance values of seedling material was distinctly lower than that of bagged and field material, with field material displaying the highest chlorophyll absorbance values at all wavelengths (Figure 3.13A). Similar trends were identified in tetraploid black wattle (Figure 3.13B).

Different chlorophyll absorbance values of the three different sample types for both diploids and tetraploids were pooled in order to obtain an improved understanding of the chlorophyll absorbance trends between the two ploidy levels (Figure 3.13C). The pooled chlorophyll absorbance values of diploids were distinctly lower than those of the tetraploids.

A



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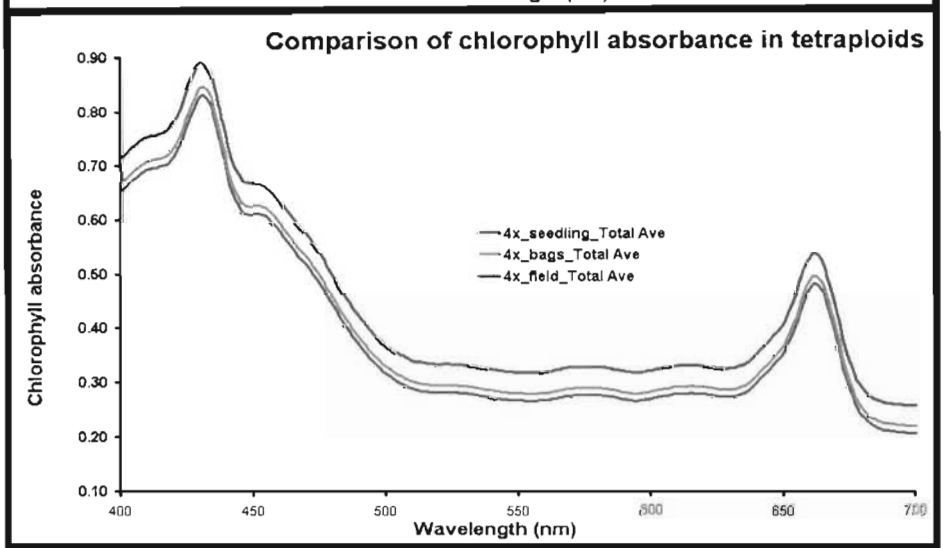

C

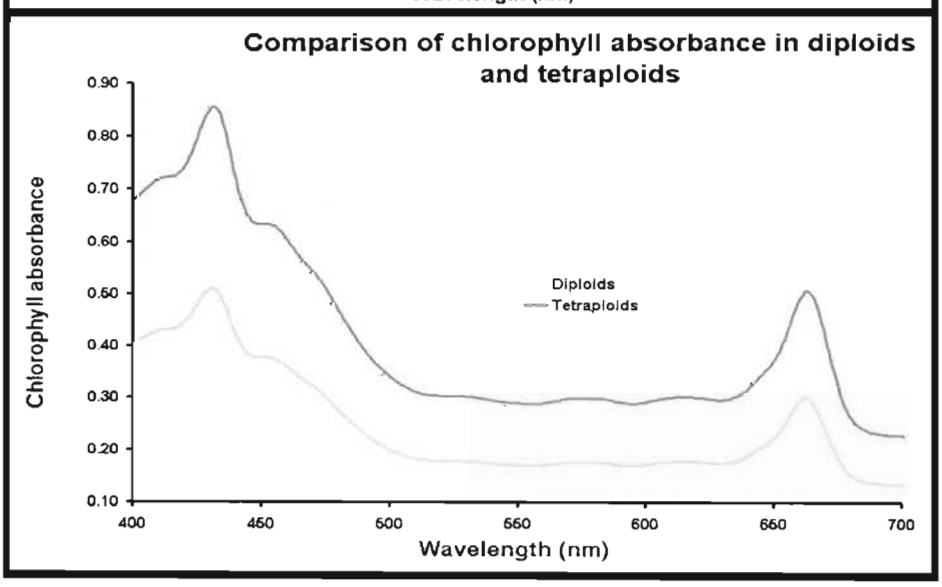

Figure 3.13 Chlorophyll absorbance of seedling, bagged and field material of dfferent ploids: A: diploids and B: tetraploids and, C: overall comparison of diploids vs tetraploids.

Comparison of chlorophyll absorbance values

A detailed analysis was undertaken of the chlorophyll absorbance values obtained for both diploid and tetraploid black wattle (Table 3.11). When the mean chlorophyll absorbance ($\bar{A}$) for each of the different ploidy levels at the three wavelengths 433 nm, 456 nm and 663 nm were compared, it was found that the $\bar{A}$ values of the tetraploids were roughly 40 % greater than that of the diploids. Not surprisingly, this trend was also reflected by the total mean chlorophyll absorbance ($T\bar{A}$), calculated as the sum of the absorbance values at 433 nm, 456 nm and 663 nm.

The total mean absorbance within a ploidy level ($PT\bar{A}$), calculated as the mean of $T\bar{A}$ of all the genetic lines within the level, showed a distinct difference of 40 % between the diploid and tetraploid groups.

Table 3.11 Mean chlorophyll absorbance of diploids and tetraploids from different sources.

Ploidy level	Source of materials [1]	Wavelength (nm)	Range of mean absorbance	Mean absorbance ($\bar{A}$)	Total mean absorbance ($T\bar{A}$)	Mean total mean absorbance within ploidy ($PT\bar{A}$)
Diploid	seedling	433	0.4778 – 0.4810	0.4797		
		456	0.3504 – 0.3535	0.3523		
		663	0.2748 – 0.2777	0.2766	*1.1086*	
	bags	433	0.4871 – 0.5089	0.4948		
		456	0.3564 – 0.3782	0.3641		
		663	0.2828 – 0.3046	0.2905	*1.1494*	
	field	433	0.5184 – 0.5268	0.5235		
		456	0.3841 – 0.3910	0.3885		
		663	0.3084 – 0.3145	0.3123	*1.2243*	*1.1607*
Tetraploid	seedling	433	0.8095 – 0.8184	0.8151		
		456	0.5921 – 0.5996	0.5969		
		663	0.4697 – 0.4746	0.4740	*1.8860*	
	bag	433	0.8185 – 0.8400	0.8314		
		456	0.6005 – 0.6163	0.6119		
		663	0.4778 – 0.4954	0.4883	*1.9316*	
	field	433	0.8696 – 0.8800	0.8762		
		456	0.6514 – 0.6572	0.6548		
		663	0.5239 – 0.5317	0.5287	*2.0597*	*1.9591*

[1] Materials sourced consisted of three genotypes, each containing ten clones (repeats).

A graphical representation of the data illustrates the general trends displayed by diploids and tetraploids with respect to chlorophyll absorbance, (Figure 3.14). Chlorophyll absorbance values clearly displayed the difference in chlorophyll absorbance between the different genetic lines of the diploids and tetraploids at each of the three wavelengths.

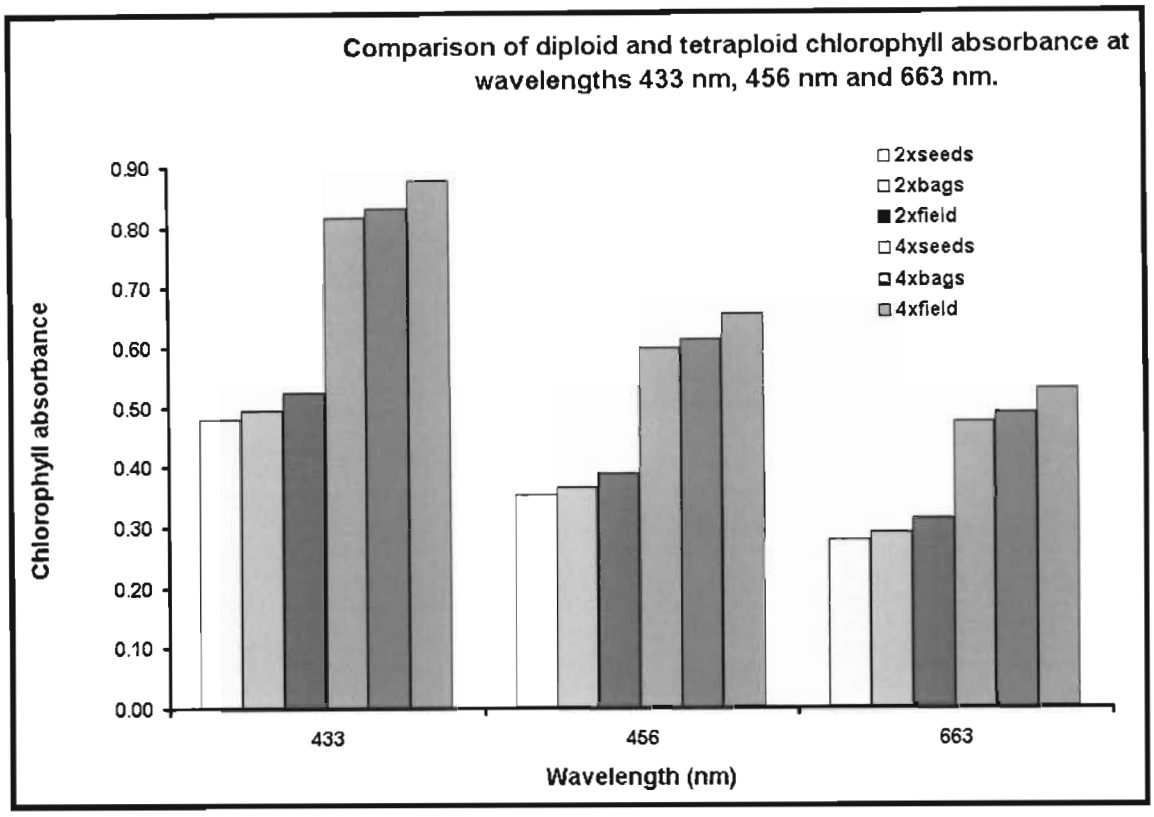

Figure 3.14 Comparison of mean chlorophyll absorbance trends at wavelengths 433 nm, 456 nm and 663 nm of diploids and tetraploids.

Statistical analysis of chlorophyll absorbance

Chlorophyll absorbance values were compared in diploid and tetraploid black wattle. Three genetic lines per ploidy level with ten plants per line (repeats) were included in this comparison. An analysis of variance (ANOVA) was conducted in order to investigate the sources of variation in the different ploidy levels (Table 3.12). Significant differences in chlorophyll absorbance values were identified between all sources of variation ($p < 0.001$), except for chlorophyll absorbance between lines at a particular wavelength within the same ploidy level ($p = 0.984$), and for chlorophyll absorbance between sample types within lines at a particular wavelength within the same ploidy level ($p = 1.000$).

Table 3.12 Summary of analysis of variance for chlorophyll absorbance at different ploidy levels.

Source of variation	df	ss	ms	Vr	F.pr
Repeats	9	0.785 E-02	0.873E-03	9.94	
Repeats. sample type	5	1.077E+01	2.154E+00	2.454E+04	< 0.001
Wavelength	2	7.965E+00	3.982E+00	4.536E+04	< 0.001
Line	2	0.722E-02	0.361E-02	41.12	< 0.001
Sample type. wavelength	10	0.436E+00	0.436E-01	496.24	< 0.001
Sample type. line	10	0.118E-01	0.118E-02	13.45	< 0.001
Wavelength. line	4	0.333E-04	0.833E-05	0.09	0.984
Sample type. wavelength. line	20	0.114E-03	0.571E-05	0.06	1.000
Residual	447	0.419E-01	0.878E-04		
Total	539	1.924E+01			

df = degrees of freedom; ss = sum of squares; ms = mean square; vr = variance ratio; F.pr = F-statistic.

An analysis of the least significant differences (LSD) was calculated to interpret the variation in chlorophyll absorbance values for each of the two ploidy levels at the three wavelengths examined (Table 3.13). The LSD revealed that chlorophyll absorbance values for seedlings, bagged material and field material (sample types) were all significantly different within the diploids as well as within the tetraploids.

Table 3.13 LSD analysis of the mean chlorophyll content in diploid and tetraploid black wattle.

Sample types	Wavelengths (nm)				Ploidy Mean
	433	456	663	Mean	
2x seed	0.48776 **b**	0.35535 **g**	0.28079 **m**	0.37463 **s**	3.4821 **A**
2x bag	0.49462 **a**	0.36390 **h**	0.17047 **n**	0.34300 **t**	
2x field	0.52360 **c**	0.38857 **i**	0.31230 **o**	0.40816 **u**	
4x seed	0.81523 **e**	0.59700 **j**	0.47411 **p**	0.62878 **v**	5.8773 **B**
4x bag	0.83162 **f**	0.61213 **k**	0.48854 **q**	0.64410 **w**	
4x field	0.87597 **d**	0.65469 **l**	0.52855 **r**	0.68640 **x**	

Treatments denoted by different lower case letters are significantly different from each other ($p < 0.005$); (LSD = 0.004754). Treatments denoted by different capital letters are significantly different from each other ($p < 0.005$); (LSD =0.002745).

This investigation into the effects of chromosome dosage on chlorophyll content identified a distinct difference between the two levels of ploidy, thereby indicating the potential to employ chlorophyll absorbance assessments as a means to identify and differentiate between diploid and tetraploid black wattle trees.

Additionally, the increase in chlorophyll content from seedlings to bagged juveniles to field material of both diploid and tetraploid black wattle lends further to the findings of the previous age studies (Section 3.3.2) where chlorophyll content was shown to increase as the tree matured.

3.4 SYNOPSIS OF COMPARATIVE CHLOROPHYLL CONTENT ANALYSES

The aim of this research project was to find rapid and reliable technologies to discern between diploid and tetraploid black wattle. The two technologies that were investigated in this research project were the quantification of stomatal chloroplasts and chlorophyll content in relation to ploidy level. Whilst chloroplast quantification could be undertaken without preliminary experimentation, the chlorophyll content quantification, required initial assessments regarding chlorophyll degradation (Sartory and Grobbelaar, 1984). The tendency of chlorophyll to degrade within black wattle needed to be assessed before the ploidy discriminating power of the technology could be investigated.

There are several factors that affect chlorophyll degradation. The two factors that were identified and assessed in this research project were that of storage conditions and the ages of leaves and trees. These factors were only assessed in diploid material due to a lack of equivalent tetraploid material. However, to facilitate comparative assessments, tetraploid data generated by the comparative ploidy assessment studies (Section 3.3.3) were included. Chlorophyll absorbance values for these tetraploid samples were introduced into comparative analyses with the diploid storage data, as well as with the diploid age data. These comparisons could provide valuable insights about how tetraploid chlorophyll absorbance would react under different storage treatments and different age groups.

3.4.1 Comparative overview between diploid and tetraploid chlorophyll absorbance

Diploid chlorophyll absorbance values of stored material were compared to mean tetraploid chlorophyll absorbance values of fresh material. It was found that all absorbance values of the three different methods of storage did not overlap with that of the tetraploid spectral graph (Figure 3.15). When the differences in

chlorophyll absorbance values of the different storage treatments of the diploids were superimposed on the tetraploid spectral graph, there was no overlap. This implies that if tetraploid material requires storage, chlorophyll absorbance values will still be distinctly different to any diploid absorbance values.

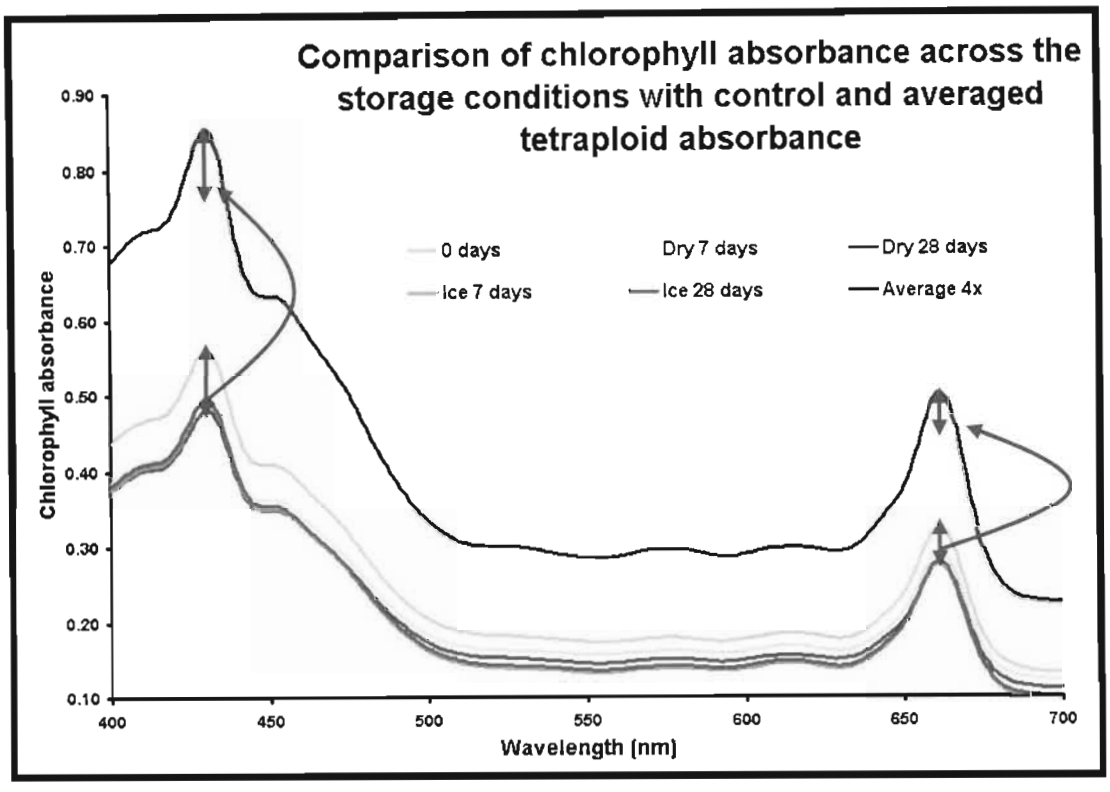

Figure 3.15 Chlorophyll absorbance of the various diploid storage treatments as compared to mean tetraploid chlorophyll absorbance.

Diploid chlorophyll absorbance values of material of different leaf and tree ages were compared to mean tetraploid chlorophyll absorbance values of fresh material. The chlorophyll absorbance spectra obtained from combined flush trees of different ages were clearly distinct from those of the tetraploid absorbance spectra (Figure 3.16). When the chlorophyll absorbance of the differently aged material of the diploids was superimposed on the tetraploid spectral graph, there was no overlap. Thus, chlorophyll absorbance values of tetraploid material of any tree age or leaf type would still be distinctly different to any diploid absorbance values.

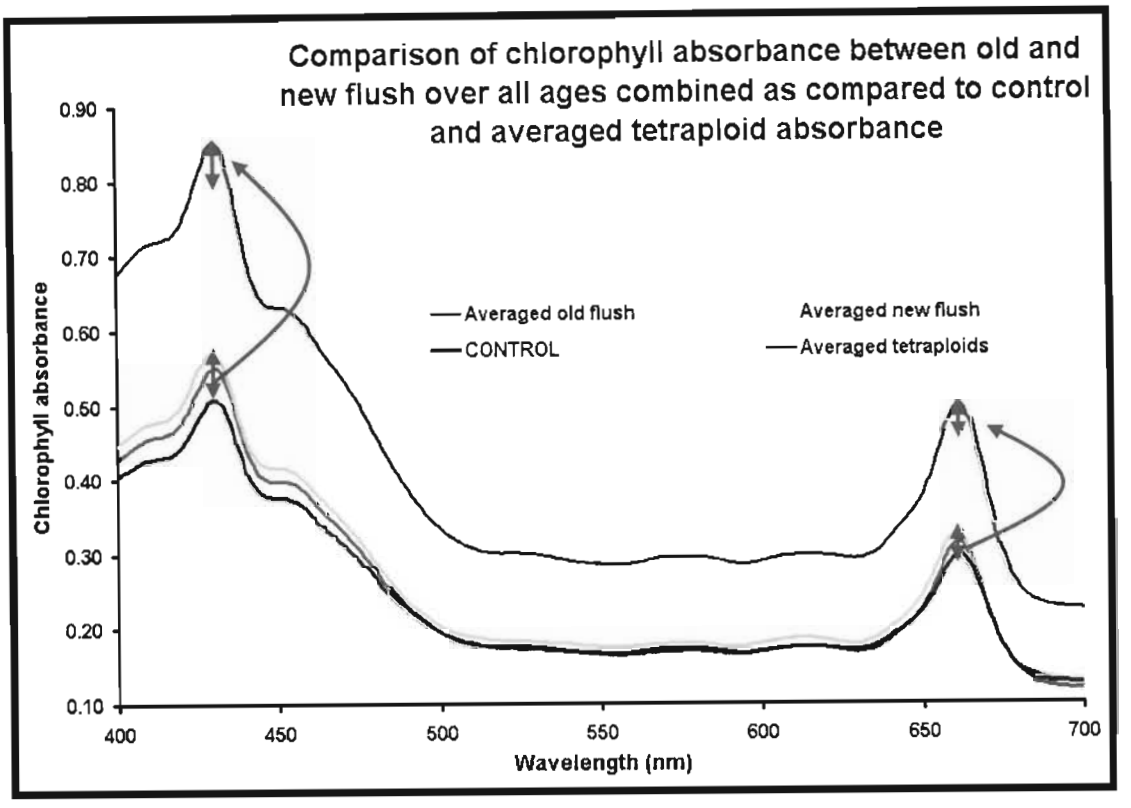

Figure 3.16 Chlorophyll absorbance of the various types of diploid material as compared to mean tetraploid absorbance.

3.4.2 Comparative summary between diploid and tetraploid diagnostic tools

Three different diagnostic tools namely, quantification of chloroplasts, chloroplast arrangements and quantification of chlorophyll content were investigated. All these procedures displayed high discriminatory power between diploid and tetraploid black wattle, were non-overlapping and easy to perform at low cost (Table 3.14). A possible limitation was that of material type, where the quantification of stomatal chloroplasts and stomatal chloroplast arrangements required the use of only young, fresh leaves. The quantification of chlorophyll content, on the other hand, could utilize fresh leaf material of any age as well as leaf material preserved for a limited period of time.

Table 3.14 Comparative assessment of the various diagnostic methods in black wattle.

Diagnostic tool	Discriminating ability	Non overlap	Difficulty rating	Preparation Time	Cost factor	Age of material required
Stomatal chloroplast arrangements	Yes	Yes	Very low	15 minutes	Very inexpensive	Young, fresh leaves
Stomatal chloroplast frequency	Yes	Yes	Low	30 minutes	Very inexpensive	Young, fresh leaves
Chlorophyll absorbance	Yes	Yes	Intermediate	1 hour	Intermediate	Any tree and leaf age *

* fresh preferred

4 CHAPTER 4
DISCUSSION AND CONCLUDING REMARKS

4.1 INTRODUCTION

The effects of polyploidy on plant physiology have been of longstanding interest to plant-breeders worldwide. This interest in polyploidy arises from the associated physiological and biochemical changes that the polyploid plant exhibits. Physiological changes often manifest on the plant as gigantism of organs which include fruits, flowers and leaves. It is also associated with an increase in the number of organelles such as the number of stomatal chloroplasts and nucleoli. Also, the number of structures such as stomata is often increased (Warner and Edwards, 1993). Furthermore, these physiological changes typically have biochemical implications such as an increased production of some proteins and pigments as well as increased rates of enzyme-related activities, for instance respiration and photosynthesis (Warner and Edwards, 1993). These ploidy-related manifestations are often utilised in breeding programmes to increase the size and quality of plant products as well as a tool to discriminate between polyploids and diploids.

4.2 POLYPLOIDY AND STOMATAL CHLOROPLASTS

Cellular volumes are correlated to the amount of nuclear DNA in both diploid and polyploid plants (Price *et al.*, 1973). Typically, cell size increases with an increase in DNA material since more space is usually required to house the additional chromosomes. The optimization of physiological efficiency in larger cells is required to accommodate the increased cell size. This is usually accomplished by increasing the number of organelles, such as nucleoli and chloroplasts within the cell for more efficient cellular management (Butterfass, 1973; Warner and Edwards, 1993).

An important anatomical consideration of polyploidy is the effect of genome size on the size and number of stomatal chloroplasts (Butterfass, 1973). There is a strong correlation between the amount of nuclear DNA and the number of chloroplasts in stomatal guard cells in many polyploids; however, the actual nature of this relationship may vary (Butterfass, 1973). Ellis and Leech (1985) suggested that the increase in nuclear material results in an accompanying increase in cell size. In the same way the number of chloroplasts also increases thereby compensating for the increased cell size. The increase in the number of stomatal chloroplasts is however, not universal; stomatal chloroplasts may increase in proportion to ploidy level, or, the increase may be random depending on the species (Pyke and Leech, 1987). These relationships are useful in discerning levels of polyploidy in various species. It is however a requirement that the nature of the relationship is established prior to its utilization.

Stomatal chloroplast number, an indirect method of discerning polyploidy, has been of scientific interest since the beginning of the previous century (Mochizuki and Sueoka, 1955). Chloroplasts in stomatal guard cells are comparatively easy to study in epidermal tissue of plants. In 1930, Hamada and Baba were the first to show that stomatal chloroplast number has a tendency to increase with an increase in ploidy. Furthermore, in 1955, Mochizuki and Sueoka showed that the tendency of chloroplast number to increase with an increase in ploidy was little affected by the type and position of leaf sample used. Since these early experiments, the quantification of stomatal chloroplast numbers has become a popular technique in discerning ploidy levels with success in several species such as alfalfa, *Medicago sativa* (Bingham, 1968), mulberry, *Morus* spp. (Hamada and Baba, 1930), potato, *Solanum tuberosum* (Hermsen and De Boer, 1971), red clover, *Trifolium pratense* (Nuesch, 1966), white clover, *T. repens* (Najcevska and Speckmann, 1968), sugar beet, *Beta vulgaris* (Mochizuki and Sueoka, 1955), and turnips, *Brassica rapa* (Speckmann, *et al.*, 1967).

In black wattle, the number of stomatal chloroplasts was found to be highly correlated to the level of ploidy. In diploid and tetraploid guard cells the mean number of stomatal chloroplasts was significantly different ($p < 0.01$);

approximately 9.89 ± 0.170 in diploids and 22.75 ± 0.170 in tetraploids (Beck, Fossey and Mathura, 2003b). It was interesting to note that the mean number of chloroplasts in tetraploids was more or less twice that of the diploids, displaying little variation between the different guard cells of a black wattle plant. However, between plants of the same line, significant differences in the number of chloroplasts were noted ($p < 0.01$). This suggests that trees of different genotypes should be sampled to establish whether the range of chloroplast numbers lies within the currently known range. However, whilst the range of measurements of the different plants within a ploidy level showed significant differences ($p < 0.01$), the range of chloroplast numbers of the diploids did not overlap with the range of chloroplast numbers of the tetraploids, and were thus found to be significantly different from each other. An analysis of least significant differences (LSD) was performed and supported the findings of significant differences between plants within lines (LSD = 0.6266), between lines of different ploids (LSD = 0.6266), as well as between different ploids (LSD = 0.2802).

In general, attempts have been made to fit a model to the distribution of stomatal chloroplasts with varying levels of complexity. Mochizuki and Sueoke (1955) suggested that in sugar beets the relationship between the number of stomatal chloroplasts and the level of ploidy was more geometric in nature than arithmetic whilst on the other hand, Yudanova *et al.* (2002) suggested that stomatal chloroplasts in sugar beets has a binomial relationship.

In black wattle the nature of the relationship between the number of chloroplast in the stomatal guard cells and ploidy level was found to be less complex. This investigation revealed that the ratio of the mean number of chloroplasts in diploids to that of tetraploids was approximately 1:2, which is supported by findings in other species such as alfalfa, *Medicago sativa* (Skinner, 1994), watermelon, *Citrullus lanatus* (McCuiston and Wehner, 1994) and cotton, *Gossypium* spp. (Chaudhari and Barrow, 1975), where the ratio of the number of chloroplasts in diploids to tetraploids was also 1:2. On the other hand, the ratio of diploid and tetraploid stomatal chloroplasts was found to be slightly higher in species such as clover, *Trifolium* spp. (Najcevska and Speckmann, 1968), potato, *Solanum* spp. (Hermsen and De Boer, 1971), and turnip, *Brassica* spp. (Speckmann, *et al.*, 1967). It is

important to note that whilst many species such as wheat and maize show positive correlations between genome size and stomatal chloroplast numbers, this is not always true, as was shown with *Bryum casepitium, Funaria hygrometrica, Physcomitrium piriforme* and *Dumortiera hirsuta* where the number of chloroplasts was found to be constant with increasing levels of ploidy (Mochizuki and Sueoka, 1955)

An important finding adding to the success of the utilization of chloroplasts in the indirect identification of polyploidy was the differential arrangements of the chloroplasts in the black wattle guard cells. Stomatal chloroplasts displayed distinct and regular arrangement configurations in diploids that contrasted with tetraploids. In diploid guard cells, the chloroplasts were significantly less than tetraploids and were polarized towards the ends of the kidney shaped cells. However, in the tetraploids, no such polarization was observed; instead, the chloroplasts were evenly distributed along the periphery of the cells. Due to the paucity of available literature, the only comparative finding was by Bingham (1968) in alfalfa, where the polarization of chloroplasts was observed in photographs of stomatal chloroplasts of diploid, triploid, tetraploid and hexaploid. Stomatal chloroplast arrangements may prove to be a valuable method to perform preliminary identifications of either diploid or tetraploid black wattle.

Other methods of ploidy assessment in black wattle have been attempted with varying levels of success. These methods include the determination of the stomatal frequency, stomatal guard cell length measurements as well as chromosome counting by root tip squashes. In black wattle, stomatal guard cell lengths and frequency were found to change in relation to ploidy level and was thus established as a reliable method of discerning between diploids and tetraploids (Beck *et al.*, 2003a). Whilst this finding is supported by similar results found in other species such as red clover *Trifolium pratens*, ploidy level often needs to be verified by chromosome number determination (Nuesch, 1966). In the case of black wattle, chromosome number determination through root tip squashes is problematic. Whilst Moffett and Nixon (1960) were able to quantify black wattle chromosome numbers in this manner, it was later found to be difficult to reproduce. Black wattle chromosomes display insufficient spreading as a result

of stickiness and clumping, thereby reducing visibility and the accuracy of the assessment. This problem has often been encountered (Chaudhair and Barrow, 1975), thus emphasizing the need for indirect methods of ploidy discernment.

The results of this investigation indicate that stomatal chloroplast frequencies may be used to accurately discern between diploid and tetraploid black wattle trees of different ages, which is of great significance in black wattle breeding programmes since it is relatively quick and inexpensive to execute. That is, provided that the leaves that are used are alive, fresh and young at the time of examination. The benefit of this technology lies in its ability to identify tetraploids at the seedling stage without any laborious cytogenetic analyses.

4.3 POLYPLOIDY AND CHLOROPHYLL CONTENT

Polyploidy usually results in physiological changes often with biochemical implications. These changes tend to affect biochemical processes such as physical diffusion rates of CO_2, biochemical and metabolic functioning such as protein activity and protein production in, for example, the rate of photosynthesis. Cellular photosynthetic rates are often correlated with the amount of DNA in a cell. Therefore, changes in anatomical structure as a result of an increase in chromosome number often influence the biochemistry of the entire plant (Warner and Edwards, 1993).

Increased ploidy is known to affect the content and types of proteins in higher plants. Evidence suggests that an increase in genome size could influence some of the photosynthetic pathways of the cell, thus affecting the production and concentration of various biochemical constituents and organelles within the cell (Warner and Edwards, 1993). Biochemical constituents may include photosynthetic enzymes such as ribulose-1,5-bisphosphate carboxylase (RuBPC) or photosynthetic pigments such as chlorophyll. Hence, an increase in gene dosage may result in an increase in protein production, together with an increase in enzyme activities in a cell. However, this is not always the case, and therefore

requires suitable investigation to verify such occurrences. In *Saccharomyces cerevisiae*, for example, the increased gene dosage was associated with an increased amount of ethanol produced by a cell (Ciferri *et al.,* 1969; Dilorio *et al.*, 1987). Similarly, polyploid maize displayed increased concentrations of chlorophyll and increased photosynthetic enzyme activities due to increased gene dosage (Baer and Shrader, 1985). In alfalfa, *Medicago sativa* the activity and amount of RuBPC as well as the amount of chlorophyll doubled with ploidy level (Warner and Edwards, 1993).

The effects of an increase in the number of nuclear genomes on chlorophyll content have been evaluated in diploid and tetraploid black wattle. It was shown in black wattle that the number of chlorophyll-containing chloroplasts increased from diploids to tetraploids (this work, and in Beck, Fossey and Mathura, 2003b). It was therefore anticipated that the chlorophyll concentration would increase as well, as was found by various authors for a number of other species (Joseph *et al.*, 1981; Meyers *et al.*, 1982; Leech *et al.*, 1985; Warner and Edwards, 1993). In this investigation chlorophyll absorbance was utilised as a tool to quantify increased concentration of chlorophyll on the basis of Beer's Law (Harwood and Moody, 1989), thus providing a possible means to identify polyploids. The data revealed that the overall chlorophyll content in the diploids was 40 % less than that of tetraploids. This separation in chlorophyll content between the two ploidy levels differed significantly ($p < 0.01$) thereby providing an accurate measure of ploidy level in black wattle. Interestingly, in alfalfa, chlorophyll content and other proteins were shown to almost double from diploid to tetraploid plants (Molin *et al.*, 1982), whilst in tall fescue plants chlorophyll concentration increased significantly according to a quadratic function from tetraploid to hexaploid to octaploid to decaploid, with the maximal chlorophyll content in the octaploids (Joseph *et al.*, 1981). A similar result was observed in C_4 grass *Panicum virgatum* where chlorophyll content and other soluble proteins increased from tetraploid to octaploid by 40 – 50 % (Warner *et al.*, 1987). However, in citrus, *Citrus sinensis*, cellular nitrogen and chlorophyll contents were found to increase by only 25 % from diploid to tetraploid citrus (Romero-Aranda *et al.*, 1997).

Interestingly, this tendency of chlorophyll content to increase with an increase in ploidy level is not always apparent. Ploidy determination on the basis of a change in chlorophyll content might be misleading if the nature of that change has not been predetermined. This change in chlorophyll content needs to be identified in relation to ploidy level since not all species display a proportional increase in chlorophyll content with increasing ploidy level. For instance, Warner and Edwards (1989) showed that chlorophyll content remained constant in various levels of ploidy in *Atriplex confertifolia*; diploid, tetraploid, hexaploid, octaploid and decaploid, whereas other proteins were found to increase. Similar evidence was presented for the castor bean, *Ricinus communis,* where the relative chlorophyll content was found to be similar in haploid, diploid and tetraploid plants (Timko and Vasconcelos, 1981).

Another noteworthy tendency of chlorophyll is its propensity for degradation. It was found that the chlorophyll in leaf samples become highly susceptible to degradation as the cells begin to die and decompose, moreover, they become increasingly light and temperature labile (Chabot and Chabot, 1977; Sartory and Grobbelaar, 1984). Thus the factors that exacerbate chlorophyll degradation need to be identified and assessed prior to using chlorophyll absorbance as a diagnostic tool (Herve and Heinonen, 1982). In black wattle, the distance of the collection site from the laboratory and the large variety of trees available were identified as two potential factors that could assist the chlorophyll degradation process in this research. Hence, appropriate leaf storage methods and types of leaf material were examined in black wattle in order to ascertain how best to reduce chlorophyll degradation.

Firstly, the effects of storage methods on chlorophyll content were determined in diploid black wattle. Two generally utilised methods of preservation were investigated, namely that of drying and storage at room temperature as well as freezing of the leaf material. Chlorophyll was extracted from these samples after either one week of storage or one month of storage. In black wattle, absorbance values of chlorophyll extracted from leaf material on the day of collection (day-0) was used as the control. An analysis of variance (ANOVA) revealed that the chlorophyll absorbance values of the different storage treatments were all

significantly lower than the chlorophyll absorbance values of the control ($p < 0.001$). Leaves that were dried for seven days resulted in the least amount of chlorophyll degradation followed by 28 day ice storage, seven day ice storage and lastly 28 day dried storage. These findings were in agreement with that of Jones and Lee (1982) in studies involving several isolated algae-containing water samples whereas Lenz and Fritsche (1980) reported that no significant degradation of chlorophyll occurred in similar water studies even after six months of cold storage. Although freezing is an easy and often effective means of preservation, its success is largely dependant on the species and the type of materials utilised.

Chlorophyll absorbance values within dried and frozen treatments were compared with respect to storage time periods of one week and one month. It was noted that whilst all treatments decreased from the control (day-0), dried samples responded differently to storage periods as compared to frozen samples (LSD = 0.003). Chlorophyll absorbance values of dried material decreased steadily over time from control to seven-day storage to one-month storage, whereas, in the case of frozen material, a similar trend could not be identified. A greater decrease from the control to seven day ice storage was recorded than for the decrease from the control to 28 day ice storage, which indicated a type of 'chlorophyll recovery system' (Strand and Lundmark, 1987).

Secondly, the effect of tree and leaf ages in diploid black wattle was determined. Two types of leaf flushes, old and new flush, were examined in relation to different tree ages; two, four, six, eight and nine year-old; in order to assess whether the choice of material impacts on chlorophyll absorbance values. These chlorophyll absorbance values were compared to young diploid seedling material as a base-value as well as a control. Chlorophyll absorbance of seedling material was used as a base or starting absorbance value since seedlings were the youngest leaf material available. Furthermore, this control absorbance was chosen since seedlings have, in essence, only one type of flush. An analysis of variance (ANOVA), revealed significant differences between tree ages and between leaf ages ($p < 0.001$). An analysis of least significant differences revealed that new flush of all tree age groups were significantly different from the control

(LSD = 0.006). This was mostly true for old flush, except for six year-old old flush which was not significantly different from the control (LSD = 0.006).

The chlorophyll absorbance values of both old and new flush of different age groups produced spectra for which no specific trends could be ascertained. This was probably due to the fact that the identification of old and new flush was based solely upon a visual selection process which, may not have accurately distinguished between the two flush types. Therefore, the data from the two flush types were pooled and revealed a marked increase in chlorophyll absorbance as trees became older. Moreover, this increase was more apparent in new flush than in old flush. However, juvenile characteristics were identified in two year-old black wattle trees, where a marked increase in chlorophyll content was noted, likely to accommodate the increased physiological activities associated with this growth stage. Thus, in black wattle plant material two years and younger would be expected to have a characteristic variable chlorophyll absorbance value. This trend of an increase in chlorophyll content with an increase in tree age was also supported by the ploidy data where chlorophyll content was shown to increase from seedlings to bagged juveniles to field material in both diploid and tetraploid black wattle.

A comparison of the effect of storage and type of leaf material on chlorophyll absorbance of diploid black wattle was compared to that of available data of tetraploid black wattle. Chlorophyll absorbance values revealed that there was no overlapping of absorbance values between diploids and tetraploids for both the storage method-types as well as the types of leaves utilised.

The data generated from this investigation revealed that chlorophyll extraction from black wattle leaves and assessments of chlorophyll absorbance is a potential method to distinguish between diploid and tetraploid black wattle trees of any age group. An advantage of this technology is that it is rapid, reliable, cost effective and accurate and could be of great value to the black wattle industry.

4.4 CONCLUDING REMARKS

In this investigation techniques were developed that allowed for the discernment of diploid and tetraploid black wattle. This investigation revealed that stomatal chloroplast numbers and chlorophyll content increased with an increase in chromosome number from diploid to tetraploid. The difference in stomatal chloroplast frequency was distinct and non-overlapping making it possible to use chloroplast counts and chloroplast arrangement in stomatal guard cells as a diagnostic measure of ploidy in young seedlings.

In the same way, chlorophyll concentration may also be used to distinguish between different ploids. The data revealed that the method of leaf storage, the type of leaf, as well as the age of tree did not influence the discriminating power of this technology.

The discerning power of this technology is of great value to the future research in black wattle breeding programmes. The ploidy level of adult material can now be accurately identified, both easily and cost effectively, making it possible to employ these newly developed techniques in any black wattle breeding programme where different ploids are utilised.

Guidelines for the introduction of these detection techniques into black wattle breeding programmes are as follows (see following page):

GUIDELINES FOR THE APPLICATION OF THESE INDIRECT POLYPLOIDY DETECTION TECHNOLOGIES

- Technology has been established for diploids and tetraploids, therefore when applied to triploids; comparative pilot investigation is suggested for all the detection procedures.
- If this technology is to be transferred to other species, extensive prior analyses will be required to establish whether or not readings overlap between the different ploids.

Chloroplast numbers and chloroplast arrangements

- Leaf material selected for chloroplast number determination should be fresh, alive and viewed within two hours of slide preparation.

Chlorophyll content

- Storage considerations need to be taken into account if material is transported over long distances. This study showed that the length of storage dictated the medium of storage. Should material be stored for over a week, then freezing of samples is preferred, whereas drying of samples is adequate if samples need to be stored for less than a week. In either event, it should be noted that chlorophyll integrity will be compromised if extraction is not immediate, albeit only marginally in black wattle.

A number of questions that require further investigation have been revealed by this research. There are questions such as:

- Chlorophyll contains magnesium as a core metal holding the structure of the molecule together. Therefore, will demetallation and quantification of this ion by means of atomic absorption spectroscopy be a more effective ploidy discerning tool?
- To what extent will different genotypes influence the different discriminating procedures?
- To what extent can the content of other pigments be used to discriminate between different ploids?
- To what extent does light intensity influence chlorophyll content?
- Does this technology have relevance in evaluating ploidy levels in mixaploids?
- Is this technology relevant in evaluating ploidy levels in allopolyploids?
- How effective will flow cytometry be in discriminating between different ploids?

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PUBLICATION

Research Note

Ploidy determination of black wattle (*Acacia mearnsii*) using stomatal chloroplast counts

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SYNOPSIS

The arrangements of chloroplasts and numbers within the stoma were examined as a rapid indirect technique for the identification of ploidy level in black wattle (*Acacia mearnsii de Wild*). Chloroplast counts were made from stomatal guard cells from leaves of known diploid (2n=2x=26) and tetraploid (2n=4x=52) plants grown under nursery conditions. Three-month-old plant material was used and five plants were chosen at random across six lines (3 diploids, 3 tetraploids). For diploids the mean number of chloroplasts per stoma was 9,89 ± 0,170 and 22,75 ± 0,170 for tetraploids. Chloroplasts in diploid guard cells were polarized into the corners, while evenly distributed in the tetraploids. These differences noted between the ploidy levels were significant (P<0,01). The analysis of stomatal chloroplast number and arrangement have proven to be an accurate indirect technique to distinguish between diploid and tetraploid black wattle.

Keywords : black wattle, chloroplasts, ploidy, stoma

INTRODUCTION

Black wattle (*Acacia mearnsii* de Wild), which originated from Australia, was introduced into South Africa in 1864 (Beard, 1957). Following early plantation success, black wattle established its significance in the commercial forestry industry as a high quality tannin, pulp, firewood and charcoal producer. Albeit the usefulness and economic boosting potential of *A. mearnsii* in South Africa (in the absence of its natural competitors) it is also an aggressive colonizer and has been classified as one of the top alien invader species of indigenous vegetation. This species produces between 65 000 to 90 000 seeds per kilogram, which are capable of remaining viable for many years and still maintain 50-80% germination rate and thereby suppressing natural vegetation (Henderson, 1989; 1992; 1998; Joker, 2000; Schumann and Little, 1995; Turnbull *et al.*, 1998). It is well known that invading alien plants have significant impacts on the economy (Kumar and Singh, 1998). The invasion of native woodlands by *A. mearnsii* is a serious problem. Various recommendations to combat wattle have been discussed, such as physical (hand clearing, burning), chemical (xenobiotics, herbicides) and biological (seed-feeding insects) mechanisms with minimal economic strain (and product loss) (Donnelly, 1986; Eccles and Little, 1995; Kay, 1994; Moll and Trinder, 1992; Pieterse and Boucher, 1997).

Another method of limiting the spread of wattle outside plantation boundaries might be to cultivate polyploids. The increased number of genomes in autoploidy gives rise to abnormal variable meiosis due to many different possible chromosome pairing associations. This results in unbalanced chromosome numbers in the gametes and subsequent semi-sterility or even complete sterility, thus restricting the contribution of seeds to the existing seed bank (Anon, 1974; Chaudhari and Barrow, 1975; Ramsey and Schemske, 1998). The polyploidization technique to produce triploids of low fertility has been exploited by the fruit plant breeding industry for years to produce seedless fruit including banana (Ortiz and Vuylsteke, 1998), a *Citrus* hybrid (Cavalcante *et al.*, 2000) and melon (Ezura *et al.*, 1993). Autotetraploids are generally produced by use of spindle inhibitors (commonly colchicine) that prevent microtubule formation and chromosome migration at anaphase (Blakesley *et al.*, 2002). Colchicine-induced tetraploidy in the *Acacia* species has only been reported for *A. mearnsii* (Moffett and Nixon, 1960). Triploids are usually produced by crossing diploids with tetraploids and should also present reduced fertility due to abnormal, variable meiosis and the formation of unbalanced gametes (Anon, 1974; Chaudhari and Barrow, 1975; Ramsey and Schemske, 1998).

Given the various ploidy levels and self-incompatibility in the *Acacia* genus, an effective chromosome counting method is paramount if polyploidization is to be used in the breeding programme (Bennett and Leitch, 1995; Kenrick and Knox, 1989). Traditional ploidy diagnostic methods such as chromosomes counts of Feulgen stained root tip squashes have been used but due to the small sized chromosomes and lack of efficient spreading, the results are unreliable and inaccurate and labour intensive (Mukherjee and Sharma, 1993; WRI, 1951/52). It is therefore necessary to find other indirect, rapid and reliable procedures for ploidy determination in *A. mearnsii*.

Pollen grains, stomata length and frequency as well as number of chloroplasts have proven to be reliable indirect estimates of ploidy (Bingham, 1968; Butterfass, 1960; Najčevska and Speckmann, 1968; Tan and Dunn, 1973). Stomatal lengths have been used previously and reliably in *Trifolium pratense* (Evans, 1955), *Medicago sativa* (Chaudhari and Barrow, 1975; Speckmann *et al.*,1965), *Lolium multiflora, L .perenne, Bromus inermis* (Tan and Dunn, 1973), *A. mearnsii* (Beck *et al.*, 2003), alfalfa, mulberry, *Morus* spp, potato and *Solanum tuberosum* (Chaudhari and Barrow, 1975).

A more accurate, direct technique such as flow cytometry enables deoxyribonucleic acid (DNA) amounts to be estimated from leaf tissue. It is a rapid technique commonly used, however, more empirical research is required before this can be applied to *A. mearnsii* (Dolezel *et al.*, 1992). In this investigation stomatal chloroplast counts were assessed as a reliable and rapid indirect method for detecting ploidy levels in *A. mearnsii*.

MATERIALS AND METHODS

Plant Material

Seeds from six different lines of *A. mearnsii* were collected and germinated under nursery conditions. The lines consisted of three diploids (117, 272, 283) and three colchicine-induced tetraploids (C19/48/19, C19/48/20, C25/48/05). The latter was obtained from experiments done earlier at the Wattle Research Institute (WRI) and confirmed via root tip squashes (WRI, 1950). Five plants from each line were randomly selected for experimentation.

Stomatal Chloroplast Counts

A razor blade was used to strip a thin layer from the abaxial surface of the pinnule and placed on a glass slide and mounted with a drop of stain (1 : 3 Iodine : Potassium in 100ml of distilled water). Cover slips were placed on slides and viewed immediately as slides degrade within 2 hours and visibility is retarded. Fifteen stomatal cells per plant were observed for chloroplast analysis using a light microscope at X 40 magnification.

Statistical Analysis

Data was analysed using GENSTAT® version 4,2 (Lane and Payne, 1996). A one- and two-way analysis of variance (ANOVA) was conducted to determine the variation in chloroplast numbers between plants within each line, between lines within each ploidy level and finally between different levels of ploidy.

RESULTS AND DISCUSSION

The average number of chloroplasts in the cells of all the diploids and all the tetraploids were significantly different ($P<0,01$). For the diploids the average of 9,89 ± 0,222 was clearly half that of the tetraploids, which was 22,43 ± 0,222 (**Table 1**).

The number of chloroplasts in the different guard cells of a plant showed little variation ($P<0,01$), however between plants of the same line, significant differences were noted (**Table 1**). This suggests genotypes not tested previously should be sampled to check if the range of chloroplast numbers lie within

TABLE 1: Average stomatal chloroplast counts for diploid and tetraploid A. mearnsii seedlings. Treatments denoted by the same letters (within each line) are not significantly different (P<0,001) (LSD = 0,6266). Treatments denoted by the same letters (between grand means for each line) are not significantly different (P<0,001) (LSD = 0,2802).

		Plants within lines					
Ploidy	Lines	1	2	3	4	5	Grand mean
Diploid	117	7,533 a	8,400 b	9,000 b	9,933 c	10,600 d	9,093 a
	272	8,867 a	10,000 b	10,333 b	11,267 c	12,067 d	10,507 c
	283	8,667 a	9,400 b	10,000 bc	10,600 c	11,733 d	10,080 b
Tetraploids	C19/48/19	16,867 a	23,667 b	24,933 c	25,000 c	26,067 d	23,307 f
	C19/48/20	18,267 a	21,000 b	22,267 c	24,467 d	25,733 e	22,347 e
	C25/48/05	18,867 a	20,000 b	20,733 c	23,533 d	25,000 e	21,627 d

the particular ploidy range.

Although the range of measurements of the different plants within a ploidy level did show some significant differences (P<0,01) the range of counts of diploids did not overlap with the counts made for the tetraploids, thus providing a procedure to distinguish between these levels of ploidy.

An important finding was the differential arrangements of the chloroplasts in the guard cells. In the diploids, with significantly less chloroplasts than the tetraploids, the chloroplasts were polarized towards the corners of the cells, while in the tetraploids no polarization was observed with the chloroplasts more or less evenly distributed throughout the cell (Figure 1.).

These findings concur with the literature where this technique has been successfully applied to *Bromis inermis* (Tan and Dunn, 1973), alfalfa (Bingham, 1968), cotton (Chaudhari and Barrow, 1975) and clover (Najĉevska and Speckmann, 1968).

CONCLUSIONS

As far as can be ascertained the results from this study, with respect to the use of the discussed technique, are the first for any tree species. The results show that chloroplast counts together with the differential chloroplast arrangement in the guard cells are able to distinguish between black wattle diploids and tetraploids. The results obtained by Beck *et al.* (2003) for *A. mearnsii,* where stomatal length and frequency measurements were shown to be reliable indirect techniques for ploidy identification, in combination with results obtained from this study, provide a rapid, easy and reliable procedures to distinguish between these two levels of ploidy. .

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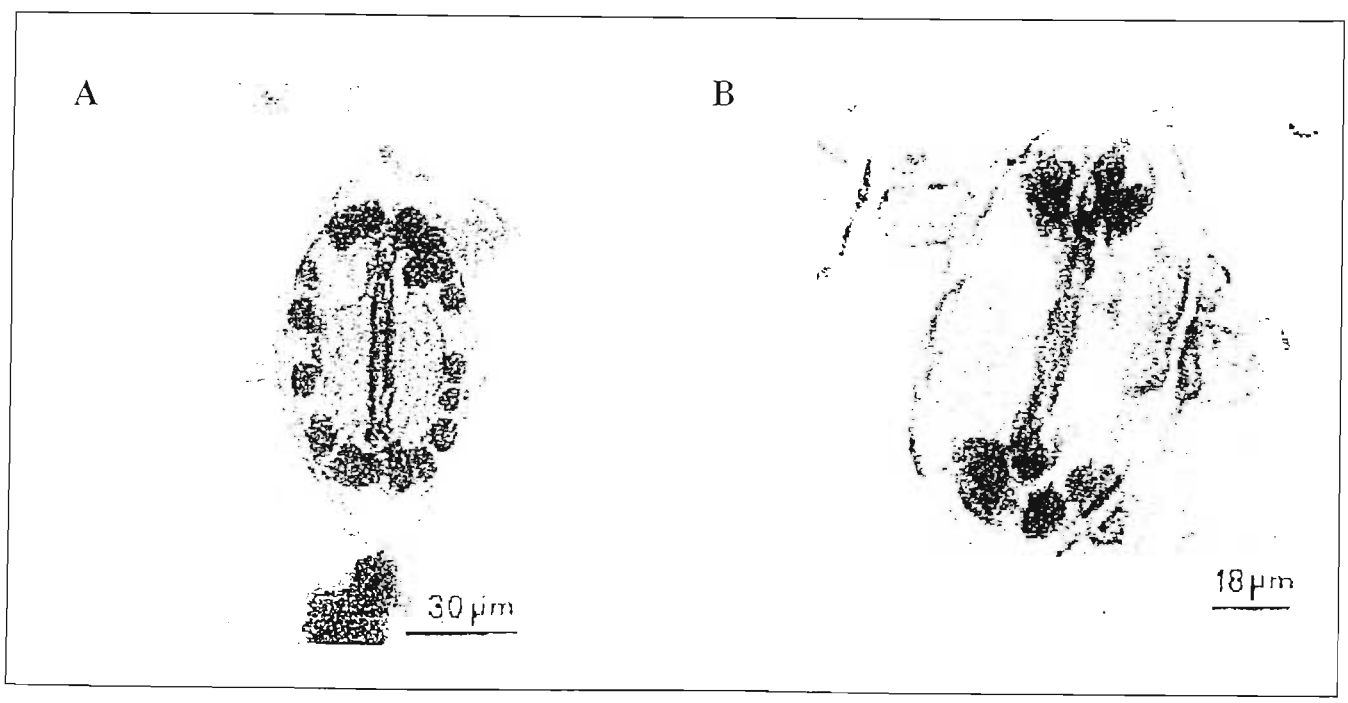

FIGURE 1: Difference in chloroplast number and distribution between tetraploid (A) and diploid (B) guard cells of A. mearnsii

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APPENDIX

There are two sets of experiments in this thesis, that of stomatal-chloroplast analysis and that of chlorophyll content analysis. All supporting information for each of these experiments has been categorized as follows:

A. Solution recipes

This section includes all possible reagents and stains used.

B. Raw data

This section includes raw data sheets for all experiments in this thesis.

C. Datasheets

This section includes examples of raw data as well as statistical tables and programming options.

A SOLUTION RECIPES

1. SOLUTIONS USED IN STOMATAL CHLOROPLAST PREPARATION

1.1 Washing Solution

100 ml distilled water
5 drops Tween-20
5 drops household bleach

Place reagents in 200 ml beaker and stir with spatula.

1.2 Petrie dish preparation

Vermiculite
Filter paper with Petrie dish dimensions
50 ml distilled water

Line Petrie dish with vermiculite and cover with filter paper. Place seeds individually on filter paper and moisten slightly with distilled water. Seal with lid, label and incubate until germinated.

1.3 Staining Solution

100 ml distilled water
1 g iodine crystals
1 g potassium iodide

Place reagents in 200 ml beaker and stir using stirring bar for approximately 15 minutes or until reagents have dissolved. Keep container sealed, foiled and cooled.

2. SOLUTIONS USED IN CHLOROPHYLL EXTRACTION PREPARATION

2.1 Solvent

90 ml laboratory grade acetone
10 ml distilled water

Place reagents in 200 ml beaker and stir with spatula. Cover when not in use. Make fresh samples for each day as acetone is a volatile solvent and readily evaporates thereby altering the solvent-ratios.

2.2 Standard Solution

25 ml volumetric flask
15 ml chlorophyll extract
~10 ml solvent (90% acetone)

Place chlorophyll extract into volumetric flask and fill with solvent to the graduation mark. Homogenize solution by inverting three times. Standard solution should then be kept in the dark on ice until spectroscopy is done (within 15 minutes).

2.3 Cuvette Solution

1 ml standard solution
2 ml solvent (90% acetone)

Place standardized chlorophyll solution into quartz cuvette and fill with solvent to graduation mark using Pasteur pipette. Run spectroscopic analysis immediately with solvent background (blank).

B RAW DATA

1. STOMATAL CHLOROPLAST RAW DATA SHEET.

Diploid lines			Tetraploid lines		
117a	272b	283c	19/19	19/20	25/5
6	8	10	13	16	18
6	8	8	14	16	18
6	8	8	14	17	18
7	8	8	14	17	18
8	8	8	15	18	18
8	8	8	15	18	18
8	9	8	15	18	18
8	9	9	16	18	19
8	9	9	16	18	19
8	9	9	16	18	19
8	9	9	20	20	20
8	10	9	20	20	20
8	10	9	20	20	20
8	10	9	22	20	20
8	10	9	23	20	20
8	10	9	23	20	20
8	10	9	23	20	20
8	10	9	23	20	20
8	10	9	23	20	20
8	10	9	23	20	20
8	10	9	24	20	20
8	10	9	24	20	20
8	10	9	24	21	20
8	10	9	24	22	20
9	10	10	24	22	20
9	10	10	24	22	20
9	10	10	24	22	20
9	10	10	24	22	20
9	10	10	24	22	20
9	10	10	24	22	20
9	10	10	24	22	20
9	10	10	25	22	20
9	10	10	25	22	20
9	10	10	25	22	20
9	10	10	25	22	21
9	10	10	25	22	21
9	10	10	25	22	21
9	10	10	25	22	21
9	10	10	25	22	21
9	10	10	25	22	21
9	11	10	25	22	21

Diploid lines			Tetraploid lines		
117a	272b	283c	19/19	19/20	25/5
9	11	10	25	23	21
9	11	10	25	23	21
9	11	10	25	23	21
9	11	10	25	23	21
9	11	10	25	23	21
10	11	10	25	24	21
10	11	10	25	24	23
10	11	10	25	24	23
10	11	10	25	24	23
10	11	10	25	24	24
10	11	11	25	24	24
10	11	11	25	25	24
10	11	11	25	25	24
10	11	11	25	25	24
10	11	11	25	25	24
10	12	11	25	25	24
10	12	11	25	25	24
10	12	11	25	25	25
10	12	11	25	25	25
10	12	11	25	25	25
10	12	11	25	25	25
10	12	11	25	25	25
10	12	11	25	25	25
10	12	12	25	25	25
10	12	12	26	25	25
11	12	12	26	25	25
11	12	12	26	25	25
11	12	12	26	25	25
11	12	12	26	25	25
11	12	12	27	26	25
11	12	12	27	26	25
11	12	12	27	28	25
11	12	12	27	28	25
11	13	12	28	28	25
Σ = 682	788	756	1748	1676	1622
Σ/N = 9.093333	10.50667	10.08	23.30667	22.34667	21.62667

2. CHLOROPHYLL STORAGE RAW DATA SHEET.

Example of storage raw data set for 2-year diploid dried and extracted after 1-month

Plant repeat 1

λ	Plant 1_1	1_2	1_3	1_4	1_5	1_6	1_7	1_8	1_9	1_10	1_11	1_12	1_13	1_14	1_15
433	0.4799	0.4810	0.4805	0.4430	0.4960	0.4655	0.4682	0.4732	0.4682	0.4766	0.4689	0.4698	0.4705	0.4714	0.4721
456	0.3492	0.3500	0.3496	0.3208	0.3555	0.3375	0.3401	0.3420	0.3379	0.3444	0.3399	0.3400	0.3410	0.3412	0.3422
663	0.2756	0.2762	0.2759	0.2563	0.2832	0.2680	0.2695	0.2718	0.2692	0.2736	0.2698	0.2702	0.2707	0.2711	0.2716

Plant repeat 2

λ	2_1	2_2	2_3	2_4	2_5	2_6	2_7	2_8	2_9	2_10	2_11	2_12	2_13	2_14	2_15
433	0.4730	0.4707	0.4451	0.4981	0.4677	0.4734	0.4725	0.4706	0.4701	0.4738	0.4700	0.4708	0.4761	0.4679	0.4706
456	0.3423	0.3411	0.3229	0.3576	0.3396	0.3432	0.3422	0.3405	0.3397	0.3425	0.3402	0.3408	0.3447	0.3389	0.3401
663	0.2720	0.2706	0.2584	0.2853	0.2701	0.2721	0.2716	0.2706	0.2702	0.2721	0.2703	0.2707	0.2734	0.2692	0.2705

Plant repeat 3

λ	3_1	3_2	3_3	3_4	3_5	3_6	3_7	3_8	3_9	3_10	3_11	3_12	3_13	3_14	3_15
433	0.4740	0.4697	0.4712	0.4706	0.4717	0.4719	0.4673	0.4713	0.4989	0.4604	0.4962	0.4895	0.5100	0.4887	0.4684
456	0.3424	0.3401	0.3412	0.3406	0.3413	0.3417	0.3385	0.3410	0.3709	0.3340	0.3557	0.3614	0.3778	0.3539	0.3381
663	0.2722	0.2701	0.2709	0.2706	0.2712	0.2713	0.2691	0.2713	0.3014	0.2673	0.2834	0.2908	0.3070	0.2809	0.2694

Plant repeat 4

λ	4_1	4_2	4_3	4_4	4_5	4_6	4_7	4_8	4_9	4_10	4_11	4_12	4_13	4_14	4_15
433	0.4902	0.5048	0.4714	0.4711	0.4722	0.4717	0.4711	0.4719	0.4716	0.4707	0.4712	0.4714	0.4716	0.4842	0.4701
456	0.3612	0.3746	0.3410	0.3407	0.3416	0.3414	0.3409	0.3414	0.3412	0.3405	0.3409	0.3411	0.3413	0.3520	0.3411
663	0.2911	0.3045	0.2710	0.2708	0.2713	0.2711	0.2708	0.2712	0.2711	0.2706	0.2709	0.2710	0.2726	0.2812	0.2710

Plant repeat 5

λ	5_1	5_2	5_3	5_4	5_5	5_6	5_7	5_8	5_9	5_10	5_11	5_12	5_13	5_14	5_15
433	0.4732	0.4782	0.4726	0.4755	0.4757	0.4742	0.4749	0.4752	0.4747	0.4750	0.4750	0.4748	0.4749	0.4749	0.4749
456	0.3434	0.3487	0.3424	0.3456	0.3455	0.3444	0.3450	0.3453	0.3447	0.3451	0.3450	0.3449	0.3450	0.3450	0.3449
663	0.2736	0.2783	0.2723	0.2750	0.2752	0.2742	0.2746	0.2750	0.2744	0.2748	0.2747	0.2746	0.2747	0.2747	0.2747

3. CHLOROPHYLL AGE RAW DATA SHEET.

(see following page)

OLD FLUSH

Old Flush 2 year old diploids

WaveL	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	MEAN
433.0	0.5638	0.5421	0.5815	0.5444	0.5851	0.5826	0.6015	0.7131	0.5635	0.5614	0.5915	0.5757	0.5492	0.5860	0.6007	0.4415	0.5223	0.5203	0.5532	0.5490	0.5664
456.0	0.4068	0.3914	0.4182	0.3937	0.4281	0.4193	0.4382	0.5624	0.4065	0.4044	0.4282	0.4187	0.3922	0.4227	0.4437	0.2845	0.3653	0.3633	0.3930	0.3920	0.4086
663.0	0.3221	0.3117	0.3285	0.3140	0.3434	0.3296	0.3485	0.4827	0.3218	0.3197	0.3385	0.3340	0.3075	0.3330	0.3590	0.1998	0.2806	0.2786	0.3058	0.3073	0.3233

Old Flush 4 year old diploids

WaveL	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	
433.0	0.4971	0.4443	0.4423	0.4707	0.4839	0.4827	0.4992	0.4982	0.4987	0.4635	0.4985	0.5082	0.4703	0.4850	0.4847	0.4635	0.4858	0.4775	0.4920	0.4777	0.4812
456.0	0.3566	0.3135	0.3115	0.3351	0.3458	0.3446	0.3587	0.3577	0.3582	0.3291	0.3580	0.3677	0.3346	0.3469	0.3467	0.3291	0.3484	0.3406	0.3527	0.3409	0.3438
663.0	0.2843	0.2463	0.2443	0.2653	0.2748	0.2736	0.2864	0.2854	0.2859	0.2599	0.2857	0.2954	0.2649	0.2759	0.2756	0.2600	0.2777	0.2703	0.2810	0.2705	0.2732

Old Flush 6 year old diploids

WaveL	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	
433.0	0.4982	0.5127	0.5104	0.5043	0.5135	0.5131	0.5074	0.5163	0.5149	0.5128	0.5139	0.5073	0.5132	0.5140	0.5144	0.5108	0.5145	0.5057	0.5093	0.5106	0.5108
456.0	0.3577	0.3722	0.3699	0.3638	0.3730	0.3726	0.3669	0.3758	0.3744	0.3723	0.3734	0.3668	0.3727	0.3735	0.3739	0.3703	0.3740	0.3652	0.3688	0.3701	0.3703
663.0	0.2854	0.2999	0.2976	0.2915	0.3007	0.3003	0.2946	0.3035	0.3021	0.3000	0.3011	0.2945	0.3004	0.3012	0.3016	0.2980	0.3017	0.2929	0.2965	0.2978	0.2980

Old Flush 8 year old diploids

WaveL	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	
433.0	0.5926	0.5937	0.5815	0.5803	0.5788	0.5870	0.5809	0.5279	0.5883	0.5960	0.6015	0.5791	0.5830	0.5915	0.5837	0.5904	0.5903	0.5874	0.6038	0.5541	0.5836
456.0	0.4293	0.4304	0.4182	0.4170	0.4155	0.4237	0.4176	0.4028	0.4250	0.4327	0.4382	0.4158	0.4197	0.4282	0.4204	0.4271	0.4270	0.4241	0.4405	0.3908	0.4222
663.0	0.3396	0.3407	0.3285	0.3273	0.3258	0.3340	0.3279	0.2925	0.3353	0.3430	0.3485	0.3261	0.3300	0.3385	0.3307	0.3374	0.3373	0.3344	0.3508	0.3011	0.3315

Old Flush 9 year old diploids

WaveL	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	
433.0	0.5860	0.5859	0.5836	0.5515	0.5802	0.5549	0.5859	0.5894	0.5532	0.5830	0.5641	0.5776	0.5675	0.5794	0.5919	0.5931	0.5814	0.5887	0.5801	0.5571	0.5767
456.0	0.4290	0.4289	0.4266	0.3945	0.4232	0.3979	0.4289	0.4324	0.3962	0.4260	0.4071	0.4206	0.4105	0.4224	0.4349	0.4361	0.4244	0.4317	0.4231	0.4001	0.4197
663.0	0.3443	0.3442	0.3419	0.3098	0.3385	0.3132	0.3442	0.3477	0.3115	0.3413	0.3224	0.3359	0.3258	0.3377	0.3502	0.3514	0.3397	0.3470	0.3384	0.3154	0.3350

NEW FLUSH

New Flush 2 year old diploids (13, 7,8 tags were blown off, replaced them intuitively)

WaveL	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	MEAN
433.0	0.4799	0.5421	0.5815	0.4430	0.4960	0.4422	0.4820	0.5541	0.4695	0.4621	0.5181	0.5110	0.4758	0.5361	0.4658	0.4708	0.4801	0.5235	0.4615	0.4755	0.4935
455.0	0.3515	0.3941	0.4208	0.3230	0.3578	0.3138	0.3536	0.4061	0.3404	0.3337	0.3799	0.3728	0.3470	0.3930	0.3371	0.3420	0.3468	0.3829	0.3373	0.3444	0.3589
663.0	0.2756	0.3117	0.3285	0.2563	0.2832	0.2442	0.2777	0.3237	0.2698	0.2610	0.3007	0.2937	0.2737	0.3122	0.2654	0.2695	0.2725	0.3029	0.2660	0.2710	0.2830

New Flush 4 year old diploids (12,15 tags were blown off, replaced intuitively)

WaveL	1	2	3	4	5	6	7	8	9	10	11	13	14	16	17	18	19	20	
433.0	0.5611	0.5599	0.5456	0.5527	0.5534	0.5654	0.5412	0.5434	0.5473	0.5495	0.5339	0.5386	0.5542	0.5486	0.5406	0.5751	0.5566	0.5587	0.5514
456.0	0.4041	0.4029	0.3886	0.3957	0.3964	0.4084	0.3842	0.3864	0.3903	0.3925	0.3769	0.3816	0.3972	0.3916	0.3836	0.4181	0.3996	0.4017	0.3944
663.0	0.3194	0.3182	0.3039	0.3110	0.3117	0.3237	0.2995	0.3017	0.3056	0.3078	0.2922	0.2969	0.3125	0.3069	0.2989	0.3334	0.3149	0.3170	0.3097

New Flush 6 year old diploids 13,16,17,19 either tags lost or trees felled

WaveL	1	2	3	4	5	6	7	8	9	10	11	12	14	15	18	20	
433.0	0.5863	0.5828	0.5834	0.5847	0.5984	0.5846	0.5848	0.5868	0.5916	0.5841	0.5882	0.5899	0.5805	0.5969	0.5843	0.5812	0.5868
456.0	0.4293	0.4258	0.4264	0.4277	0.4414	0.4276	0.4278	0.4298	0.4346	0.4271	0.4312	0.4329	0.4235	0.4399	0.4273	0.4242	0.4298
663.0	0.3446	0.3411	0.3417	0.3430	0.3567	0.3429	0.3431	0.3451	0.3499	0.3424	0.3465	0.3482	0.3388	0.3552	0.3426	0.3395	0.3451

New Flush 8 year old diploids 16,17,18,8 either tags lost or trees felled

WaveL	1	2	3	4	5	6	7	9	10	11	12	13	14	15	16	17	
433.0	0.5848	0.5871	0.5837	0.5828	0.5860	0.5871	0.5877	0.5974	0.5899	0.5665	0.5848	0.5614	0.5843	0.5865	0.5848	0.5857	0.5838
456.0	0.4278	0.4301	0.4267	0.4258	0.4290	0.4301	0.4307	0.4404	0.4329	0.4095	0.4278	0.4044	0.4273	0.4295	0.4278	0.4287	0.4268
663.0	0.3431	0.3454	0.3420	0.3411	0.3443	0.3454	0.3460	0.3557	0.3482	0.3248	0.3431	0.3197	0.3426	0.3448	0.3431	0.3440	0.3421

New Flush 9 year old diploids

WaveL	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	
433.0	0.5926	0.6160	0.6147	0.6048	0.6149	0.6181	0.6054	0.6164	0.6171	0.6155	0.6159	0.6170	0.6294	0.6183	0.6159	0.6169	0.6024	0.6042	0.6027	0.5931	0.6116
456.0	0.4293	0.4527	0.4514	0.4415	0.4516	0.4574	0.4447	0.4557	0.4564	0.4548	0.4552	0.4563	0.4687	0.4576	0.4552	0.4562	0.4417	0.4435	0.4420	0.4324	0.4502
663.0	0.3396	0.3630	0.3617	0.3518	0.3619	0.3678	0.3551	0.3661	0.3668	0.3652	0.3656	0.3667	0.3791	0.3680	0.3656	0.3666	0.3521	0.3539	0.3524	0.3428	0.3606

4. CHLOROPHYLL PLOIDY RAW DATA SHEET.

DIPLOID DATA

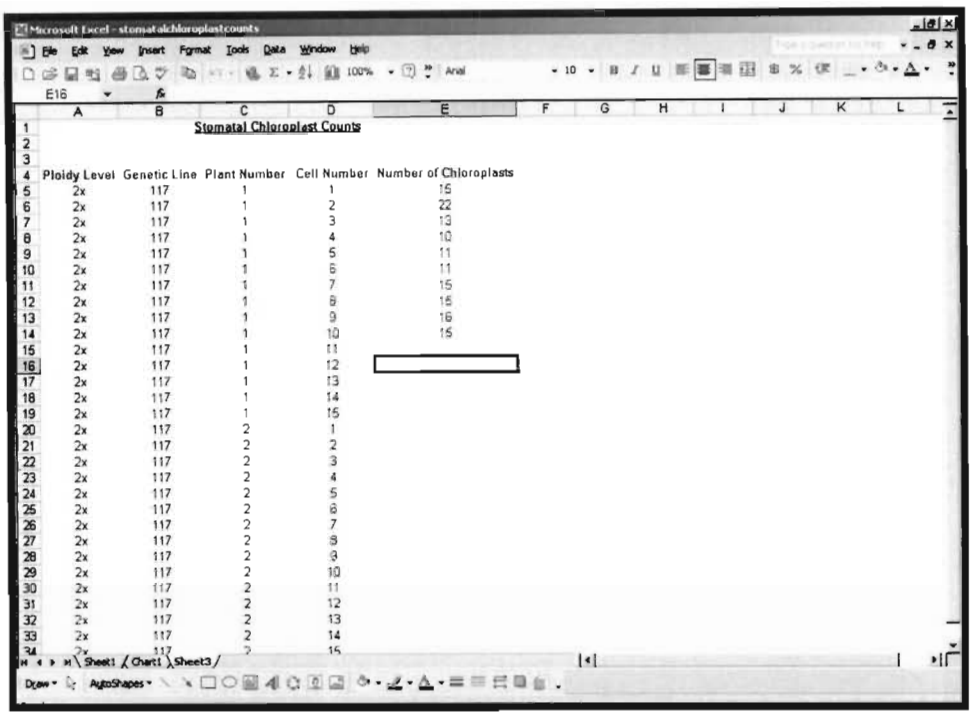
	Seedlings				Bags				Field			
λ	seedlings Line a	seedlings Line b	seedlings Line c	Seedling Total Ave	bags Line a	bags Line b	bags Line c	bags Total Ave	field Line a	field Line b	field Line c	field Total Ave
433	0.4778	0.4810	0.4805	0.4797	0.4871	0.5089	0.4878	0.4948	0.5184	0.5268	0.5256	0.5235
456	0.3504	0.3535	0.3531	0.3523	0.3564	0.3782	0.3570	0.3641	0.3841	0.3910	0.3906	0.3885
663	0.2748	0.2777	0.2774	0.2766	0.2828	0.3046	0.2834	0.2905	0.3084	0.3145	0.3139	0.3123

TETRAPLOID DATA

	Seedlings				Bags				Field			
λ	Seedling Line a	Seedling Line b	Seedling Line c	Seedling Total Ave	Bags Line a	Bags Line b	Bags Line c	Bags Total Ave	Field Line a	Field Line b	Field Line c	Field Total Ave
433	0.8095	0.8177	0.8184	0.8151	0.8185	0.8364	0.8400	0.8314	0.8800	0.8783	0.8696	0.8762
456	0.5921	0.5992	0.5996	0.5969	0.6005	0.6163	0.6196	0.6119	0.6572	0.6555	0.6514	0.6548
663	0.4697	0.4762	0.4764	0.4740	0.4778	0.4924	0.4954	0.4883	0.5317	0.5300	0.5239	0.5287

C DATASHEETS

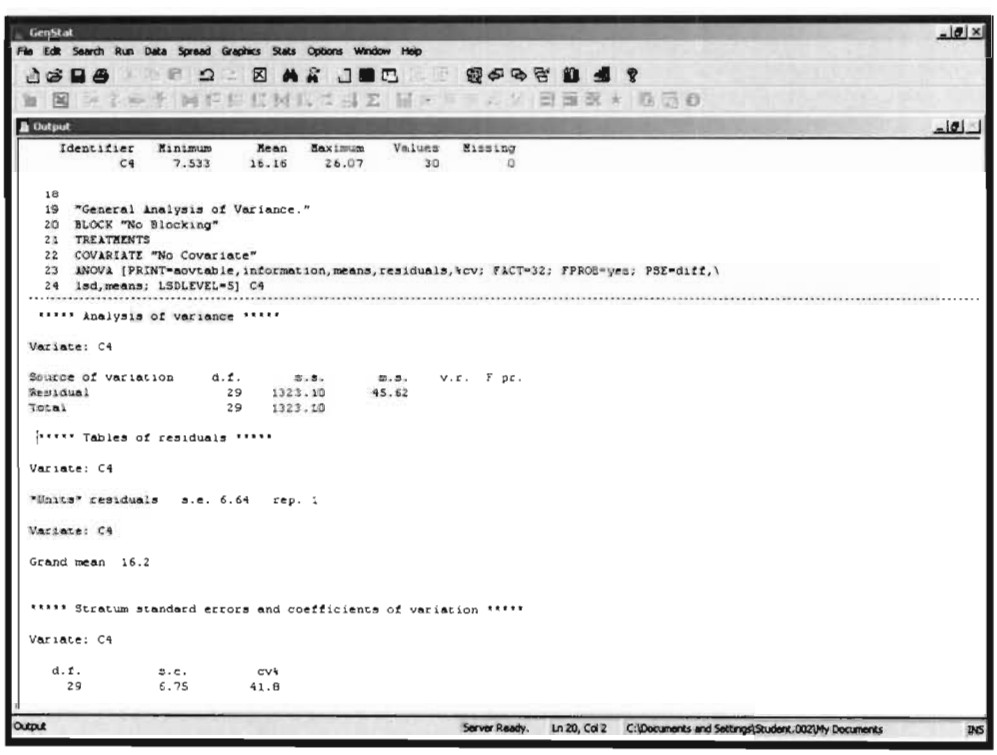
1. EXAMPLE OF MICROSOFT® EXCEL SPREADSHEETS USED FOR RECORDING RAW DATA SETS.



Stomatal Chloroplast Counts

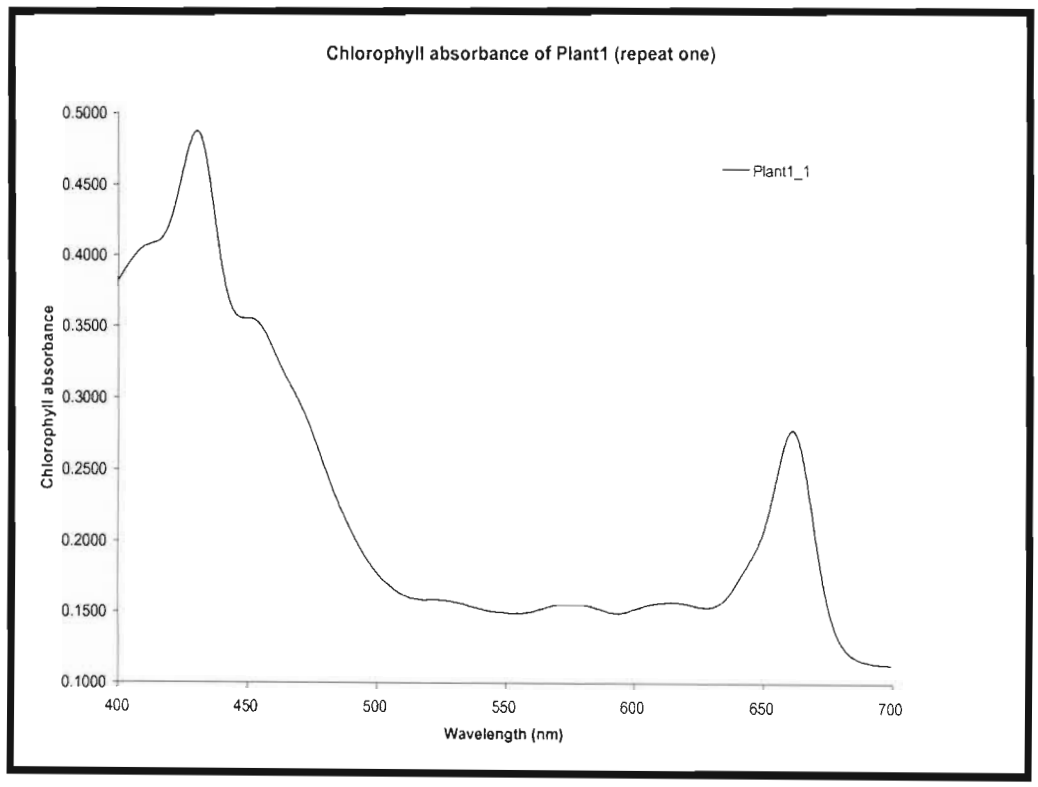
Ploidy Level	Genetic Line	Plant Number	Cell Number	Number of Chloroplasts
2x	117	1	1	16
2x	117	1	2	22
2x	117	1	3	13
2x	117	1	4	10
2x	117	1	5	11
2x	117	1	6	11
2x	117	1	7	15
2x	117	1	8	16
2x	117	1	9	16
2x	117	1	10	16
2x	117	1	11	
2x	117	1	12	
2x	117	1	13	
2x	117	1	14	
2x	117	1	15	
2x	117	2	1	
2x	117	2	2	
2x	117	2	3	
2x	117	2	4	
2x	117	2	5	
2x	117	2	6	
2x	117	2	7	
2x	117	2	8	
2x	117	2	9	
2x	117	2	10	
2x	117	2	11	
2x	117	2	12	
2x	117	2	13	
2x	117	2	14	
2x	117	2	15	

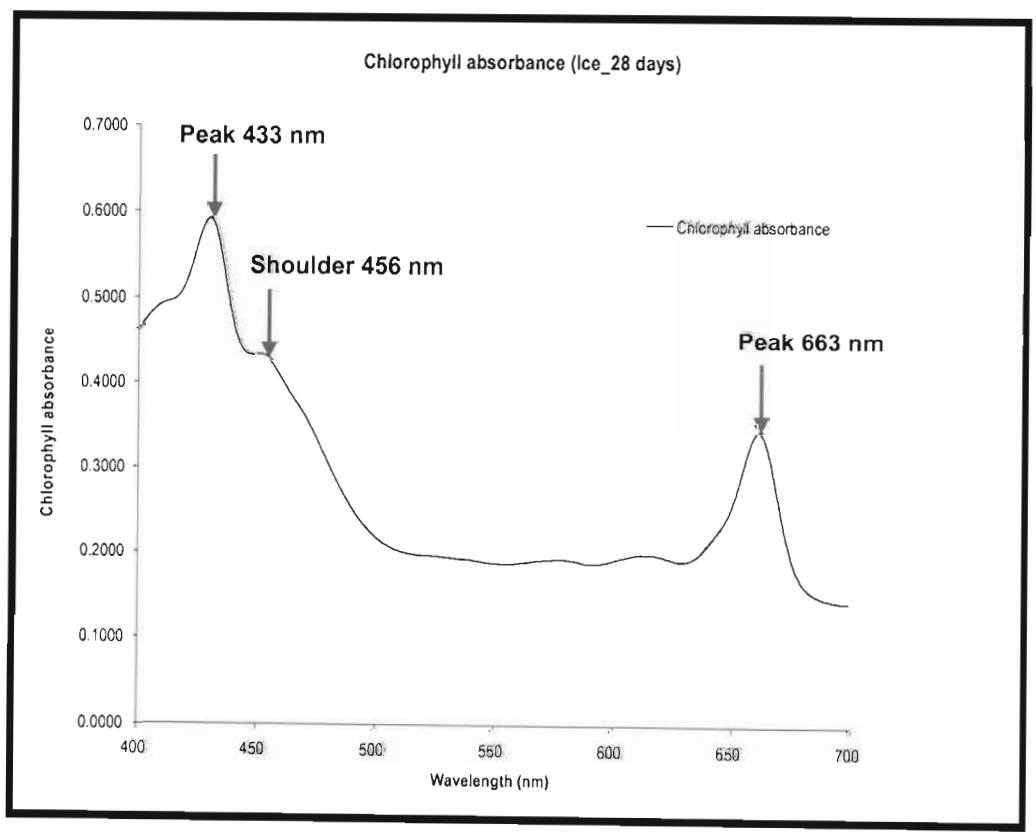
2. EXAMPLE OF GENSTAT® STATISTICAL TABLES AND PROGRAMMING USED ON RAW DATA.



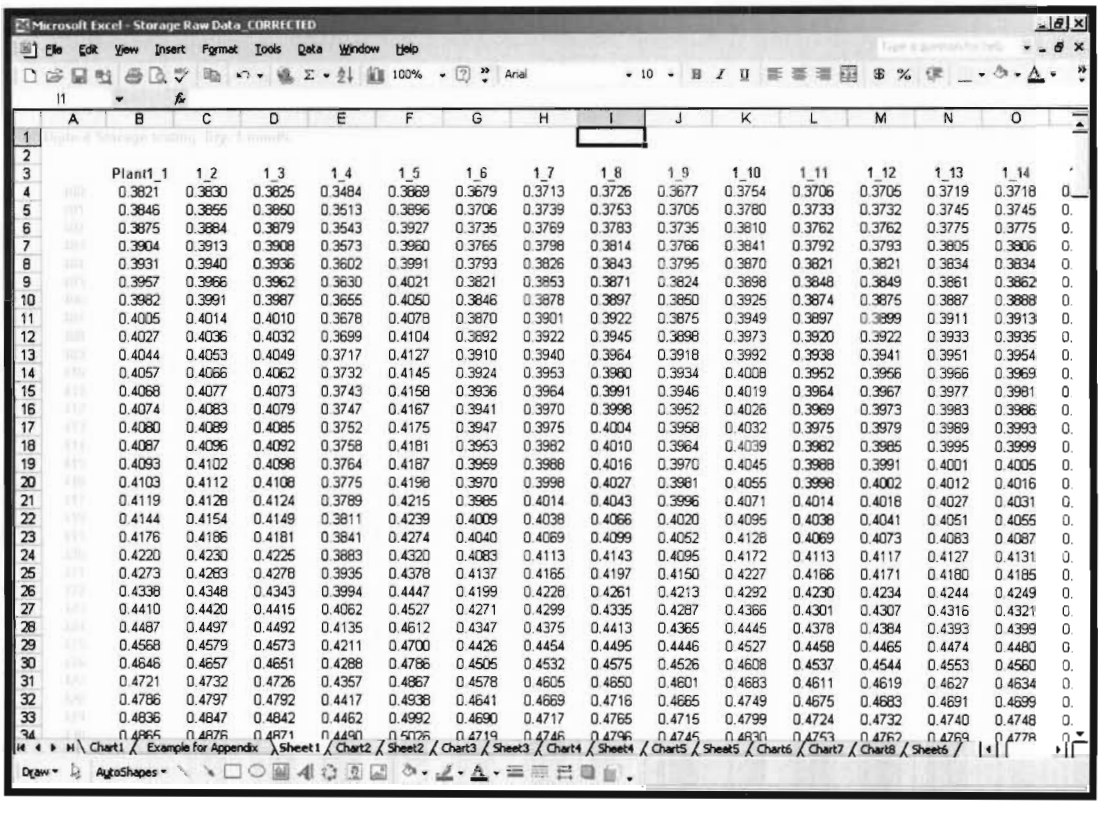
```
    Identifier   Minimum      Mean   Maximum    Values   Missing
            C4     7.533     16.16     26.07        30         0

 18
 19  "General Analysis of Variance."
 20  BLOCK "No Blocking"
 21  TREATMENTS
 22  COVARIATE "No Covariate"
 23  ANOVA [PRINT=aovtable,information,means,residuals,%cv; FACT=32; FPROB=yes; PSE=diff,\
 24  lsd,means; LSDLEVEL=5] C4
------------------------------------------------------------------------------

 ***** Analysis of variance *****

Variate: C4

Source of variation     d.f.       s.s.       m.s.    v.r.  F pr.
Residual                  29    1323.10      45.62
Total                     29    1323.10

 ***** Tables of residuals *****

Variate: C4

"Units" residuals   s.e. 6.64   rep. 1

Variate: C4

Grand mean  16.2


***** Stratum standard errors and coefficients of variation *****

Variate: C4

   d.f.          s.e.          cv%
     29          6.75         41.8
```

3. EXAMPLES OF CHLOROPHYLL ABSORBANCE SPECTRA RECORDED FROM SPECTROMETER AND REPRODUCED IN MICROSOFT® EXCEL





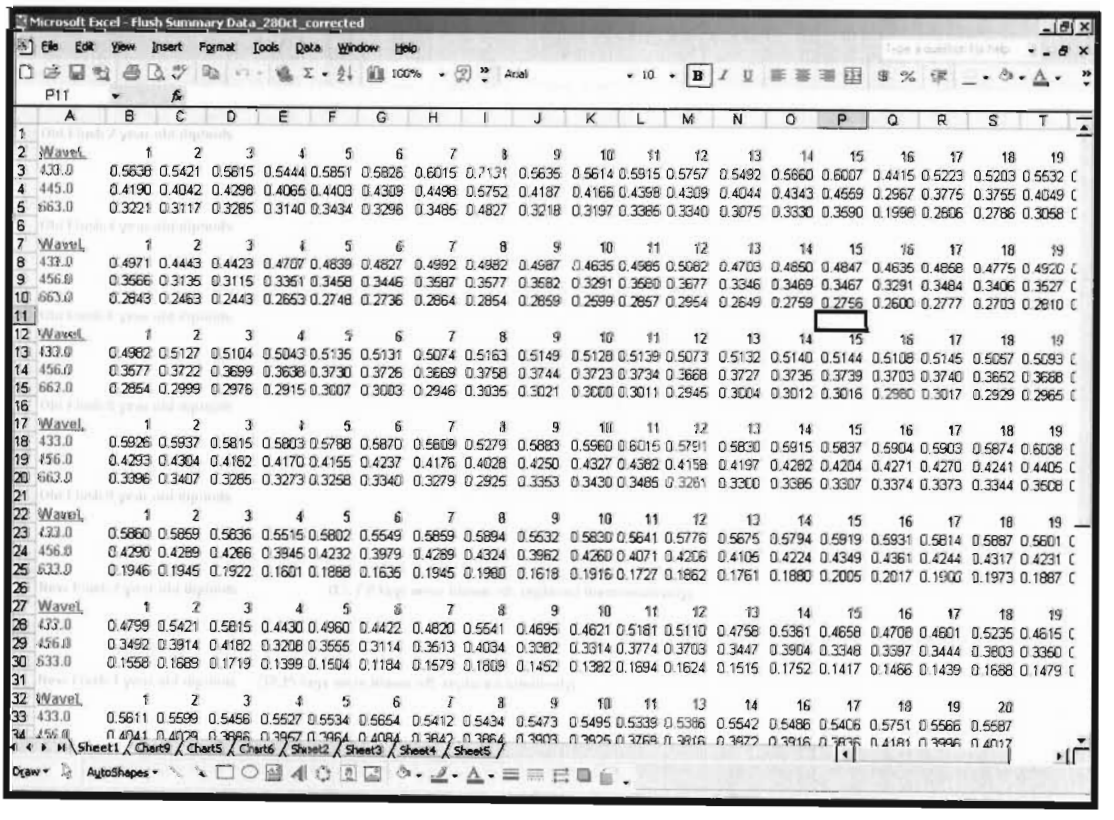
4. EXAMPLE OF CHLOROPHYLL ABSORBANCE DATASHEET RECORDED FROM SPECTRA



Microsoft Excel - Storage Raw Data_CORRECTED

	Plant1_1	1_2	1_3	1_4	1_5	1_6	1_7	1_8	1_9	1_10	1_11	1_12	1_13	1_14
4	0.3821	0.3830	0.3825	0.3484	0.3669	0.3679	0.3713	0.3726	0.3677	0.3754	0.3706	0.3705	0.3719	0.3718
5	0.3846	0.3855	0.3850	0.3513	0.3696	0.3706	0.3739	0.3753	0.3705	0.3780	0.3733	0.3732	0.3745	0.3745
6	0.3875	0.3884	0.3879	0.3543	0.3927	0.3735	0.3769	0.3783	0.3735	0.3810	0.3762	0.3762	0.3775	0.3775
7	0.3904	0.3913	0.3908	0.3573	0.3960	0.3765	0.3798	0.3814	0.3766	0.3841	0.3792	0.3793	0.3805	0.3806
8	0.3931	0.3940	0.3936	0.3602	0.3991	0.3793	0.3826	0.3843	0.3795	0.3870	0.3821	0.3821	0.3834	0.3834
9	0.3957	0.3966	0.3962	0.3630	0.4021	0.3821	0.3853	0.3871	0.3824	0.3898	0.3848	0.3849	0.3861	0.3862
10	0.3982	0.3991	0.3987	0.3655	0.4050	0.3846	0.3878	0.3897	0.3850	0.3925	0.3874	0.3875	0.3887	0.3888
11	0.4005	0.4014	0.4010	0.3678	0.4078	0.3870	0.3901	0.3922	0.3875	0.3949	0.3897	0.3899	0.3911	0.3913
12	0.4027	0.4036	0.4032	0.3699	0.4104	0.3892	0.3922	0.3945	0.3898	0.3973	0.3920	0.3922	0.3933	0.3935
13	0.4044	0.4053	0.4049	0.3717	0.4127	0.3910	0.3940	0.3964	0.3918	0.3992	0.3938	0.3941	0.3951	0.3954
14	0.4057	0.4066	0.4062	0.3732	0.4145	0.3924	0.3953	0.3980	0.3934	0.4008	0.3952	0.3956	0.3966	0.3969
15	0.4068	0.4077	0.4073	0.3743	0.4158	0.3936	0.3964	0.3991	0.3946	0.4019	0.3964	0.3967	0.3977	0.3981
16	0.4074	0.4083	0.4079	0.3747	0.4167	0.3941	0.3970	0.3998	0.3952	0.4026	0.3969	0.3973	0.3983	0.3986
17	0.4080	0.4089	0.4085	0.3752	0.4175	0.3947	0.3975	0.4004	0.3958	0.4032	0.3975	0.3979	0.3989	0.3993
18	0.4087	0.4096	0.4092	0.3758	0.4181	0.3953	0.3982	0.4010	0.3964	0.4039	0.3982	0.3985	0.3995	0.3999
19	0.4093	0.4102	0.4098	0.3764	0.4187	0.3959	0.3988	0.4016	0.3970	0.4045	0.3988	0.3991	0.4001	0.4005
20	0.4103	0.4112	0.4108	0.3775	0.4198	0.3970	0.3998	0.4027	0.3981	0.4055	0.3998	0.4002	0.4012	0.4016
21	0.4119	0.4128	0.4124	0.3789	0.4215	0.3985	0.4014	0.4043	0.3996	0.4071	0.4014	0.4018	0.4027	0.4031
22	0.4144	0.4154	0.4149	0.3811	0.4239	0.4009	0.4038	0.4066	0.4020	0.4095	0.4038	0.4041	0.4051	0.4055
23	0.4176	0.4186	0.4181	0.3841	0.4274	0.4040	0.4069	0.4099	0.4052	0.4126	0.4069	0.4073	0.4083	0.4087
24	0.4220	0.4230	0.4225	0.3883	0.4320	0.4083	0.4113	0.4143	0.4095	0.4172	0.4113	0.4117	0.4127	0.4131
25	0.4273	0.4283	0.4278	0.3935	0.4378	0.4137	0.4165	0.4197	0.4150	0.4227	0.4166	0.4171	0.4180	0.4185
26	0.4338	0.4348	0.4343	0.3994	0.4447	0.4199	0.4228	0.4261	0.4213	0.4292	0.4230	0.4234	0.4244	0.4249
27	0.4410	0.4420	0.4415	0.4062	0.4527	0.4271	0.4299	0.4335	0.4287	0.4366	0.4301	0.4307	0.4316	0.4321
28	0.4487	0.4497	0.4492	0.4135	0.4612	0.4347	0.4375	0.4413	0.4365	0.4445	0.4378	0.4384	0.4393	0.4399
29	0.4568	0.4579	0.4573	0.4211	0.4700	0.4426	0.4454	0.4495	0.4446	0.4527	0.4458	0.4465	0.4474	0.4480
30	0.4646	0.4657	0.4651	0.4288	0.4786	0.4505	0.4532	0.4575	0.4526	0.4608	0.4537	0.4544	0.4553	0.4560
31	0.4721	0.4732	0.4726	0.4357	0.4867	0.4578	0.4605	0.4650	0.4601	0.4683	0.4611	0.4619	0.4627	0.4634
32	0.4786	0.4797	0.4792	0.4417	0.4938	0.4641	0.4669	0.4716	0.4665	0.4749	0.4675	0.4683	0.4691	0.4699
33	0.4836	0.4847	0.4842	0.4462	0.4992	0.4690	0.4717	0.4765	0.4715	0.4799	0.4724	0.4732	0.4740	0.4748
34	0.4865	0.4876	0.4871	0.4490	0.5026	0.4719	0.4746	0.4796	0.4745	0.4830	0.4753	0.4762	0.4769	0.4778

5. EXAMPLE OF CHLOROPHYLL ABSORBANCE CONDENSED DATASHEET EXTRACTED FROM SPECTRAL DATA SETS



Microsoft Excel - Flush Summary Data_28Oct_corrected

	Wavel.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
3	433.0	0.5638	0.5421	0.5815	0.5444	0.5851	0.5826	0.6015	0.7131	0.5635	0.5614	0.5915	0.5757	0.5492	0.5660	0.6007	0.4415	0.5223	0.5203	0.5532
4	445.0	0.4190	0.4042	0.4298	0.4065	0.4403	0.4309	0.4498	0.5752	0.4187	0.4166	0.4398	0.4309	0.4044	0.4343	0.4569	0.2967	0.3775	0.3755	0.4049
5	663.0	0.3221	0.3117	0.3285	0.3140	0.3434	0.3296	0.3485	0.4827	0.3218	0.3197	0.3386	0.3340	0.3075	0.3330	0.3690	0.1998	0.2606	0.2786	0.3058
6																				
7	Wavel.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
8	433.0	0.4971	0.4443	0.4423	0.4707	0.4839	0.4827	0.4992	0.4982	0.4987	0.4635	0.4585	0.5062	0.4703	0.4850	0.4847	0.4635	0.4868	0.4775	0.4920
9	456.0	0.3566	0.3135	0.3115	0.3351	0.3458	0.3446	0.3587	0.3577	0.3582	0.3291	0.3580	0.3677	0.3346	0.3469	0.3467	0.3291	0.3484	0.3406	0.3527
10	663.0	0.2843	0.2463	0.2443	0.2653	0.2748	0.2736	0.2864	0.2854	0.2859	0.2599	0.2857	0.2954	0.2649	0.2759	0.2756	0.2600	0.2777	0.2703	0.2810
11																				
12	Wavel.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
13	433.0	0.4982	0.5127	0.5104	0.5043	0.5135	0.5131	0.5074	0.5163	0.5149	0.5128	0.5139	0.5073	0.5132	0.5140	0.5144	0.5108	0.5145	0.5057	0.5093
14	456.0	0.3577	0.3722	0.3699	0.3638	0.3730	0.3726	0.3669	0.3758	0.3744	0.3723	0.3734	0.3668	0.3727	0.3735	0.3739	0.3703	0.3740	0.3652	0.3688
15	663.0	0.2854	0.2999	0.2976	0.2915	0.3007	0.3003	0.2946	0.3035	0.3021	0.3000	0.3011	0.2945	0.3004	0.3012	0.3016	0.2980	0.3017	0.2929	0.2965
16																				
17	Wavel.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
18	433.0	0.5926	0.5937	0.5815	0.5803	0.5788	0.5870	0.5809	0.5279	0.5883	0.5960	0.6015	0.5791	0.5830	0.5915	0.5837	0.5904	0.5903	0.5874	0.6038
19	456.0	0.4293	0.4304	0.4162	0.4170	0.4155	0.4237	0.4176	0.4028	0.4250	0.4327	0.4362	0.4158	0.4197	0.4262	0.4204	0.4271	0.4270	0.4241	0.4405
20	663.0	0.3396	0.3407	0.3285	0.3273	0.3258	0.3340	0.3279	0.2925	0.3353	0.3430	0.3485	0.3281	0.3300	0.3385	0.3307	0.3374	0.3373	0.3344	0.3508
21																				
22	Wavel.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
23	433.0	0.5860	0.5859	0.5836	0.5515	0.5802	0.5549	0.5859	0.5894	0.5532	0.5830	0.5641	0.5776	0.5675	0.5794	0.5919	0.5931	0.5814	0.5887	0.5601
24	456.0	0.4290	0.4289	0.4266	0.3945	0.4232	0.3979	0.4289	0.4324	0.3962	0.4260	0.4071	0.4206	0.4105	0.4224	0.4349	0.4361	0.4244	0.4317	0.4231
25	663.0	0.1946	0.1945	0.1922	0.1601	0.1868	0.1635	0.1945	0.1980	0.1618	0.1916	0.1727	0.1862	0.1761	0.1880	0.2005	0.2017	0.1900	0.1973	0.1887
26																				
27	Wavel.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
28	433.0	0.4799	0.5421	0.5815	0.4430	0.4960	0.4422	0.4820	0.5541	0.4695	0.4621	0.5181	0.5110	0.4758	0.5361	0.4658	0.4708	0.4601	0.5235	0.4615
29	456.0	0.3492	0.3914	0.4182	0.3208	0.3555	0.3114	0.3613	0.4034	0.3382	0.3314	0.3774	0.3703	0.3447	0.3904	0.3348	0.3397	0.3444	0.3803	0.3350
30	633.0	0.1558	0.1689	0.1719	0.1399	0.1504	0.1184	0.1579	0.1809	0.1452	0.1382	0.1694	0.1624	0.1515	0.1752	0.1417	0.1486	0.1439	0.1688	0.1479
31																				
32	Wavel.	1	2	3	4	5	6	7	8	9	10	11	13	14	16	17	18	19	20	
33	433.0	0.5611	0.5599	0.5456	0.5527	0.5534	0.5654	0.5412	0.5434	0.5473	0.5495	0.5339	0.5386	0.5542	0.5486	0.5406	0.5751	0.5566	0.5587	
34	456.0	0.4041	0.4028	0.3886	0.3957	0.3964	0.4084	0.3842	0.3864	0.3903	0.3925	0.3769	0.3816	0.3972	0.3916	0.3836	0.4181	0.3996	0.4017	

6. CHLOROPHYLL ABSORBANCE GENSTAT® ANALYSES FOR STORAGE DATA, AGE DATA and PLOIDY DATA

STORAGE DATA GENSTAT OUTPUT

```
 641
 642  "General Analysis of Variance."
 643  BLOCK plant
 644  TREATMENTS treatment*wavelength
 645  COVARIATE "No Covariate"
 646  ANOVA [PRINT=aovtable,information,means,%cv; FACT=32; FPROB=yes;
PSE=diff,lsd; LSDLEVEL=5]\
 647   absorbance


***** Analysis of variance *****

Variate: absorbance

Source of variation      d.f.       s.s.       m.s.     v.r.  F pr.

plant stratum               4  0.0870574  0.0217643    71.76

plant.*Units* stratum
treatment                   4  0.5655926  0.1413982   466.21  <.001
wavelength                  2  8.5814867  4.2907433 1.415E+04<.001
treatment.wavelength        8  0.0251203  0.0031400    10.35  <.001
Residual                 1106  0.3354410  0.0003033

Total                    1124  9.5946980


* MESSAGE: the following units have large residuals.

plant 2     *units* 102        0.06248   s.e. 0.01727
plant 2     *units* 103        0.06398   s.e. 0.01727
plant 2     *units* 104        0.08158   s.e. 0.01727
plant 2     *units* 105        0.08308   s.e. 0.01727
plant 2     *units* 120        0.05756   s.e. 0.01727
plant 2     *units* 147        0.06191   s.e. 0.01727
plant 2     *units* 148        0.06341   s.e. 0.01727
plant 2     *units* 149        0.08091   s.e. 0.01727
plant 2     *units* 150        0.08241   s.e. 0.01727


***** Tables of means *****

Variate: absorbance

Grand mean  0.38124

 treatment    control dry_month  dry_week ice_month  ice_week
              0.42522   0.36433   0.37757   0.37137   0.36773

 wavelength    433.00    456.00    663.00
               0.49752   0.35919   0.28702

 treatment wavelength    433.00    456.00    663.00
```

control	0.55226	0.40155	0.32185
dry_month	0.47459	0.34429	0.27411
dry_week	0.48740	0.35633	0.28900
ice_month	0.48849	0.34871	0.27690
ice_week	0.48486	0.34507	0.27326

*** Standard errors of differences of means ***

Table	treatment	wavelength	treatment wavelength
rep.	225	375	75
d.f.	1106	1106	1106
s.e.d.	0.001642	0.001272	0.002844

*** Least significant differences of means (5% level) ***

Table	treatment	wavelength	treatment wavelength
rep.	225	375	75
d.f.	1106	1106	1106
l.s.d.	0.003222	0.002495	0.005580

***** Stratum standard errors and coefficients of variation *****

Variate: absorbance

Stratum	d.f.	s.e.	cv%
plant	4	0.009835	2.6
plant.*Units*	1106	0.017415	4.6

AGE DATA GENSTAT OUTPUT

FLUSH DATA - INCLUDING A DIPLOID CONTROL

```
 605
 606  "General Analysis of Variance."
 607  BLOCK rep
 608  TREATMENTS age*waveL
 609  COVARIATE "No Covariate"
 610  ANOVA [PRINT=aovtable,information,means,%cv; FACT=32; FPROB=yes;
PSE=diff,lsd; LSDLEVEL=5]\
 611   chloro
```

***** Analysis of variance *****

Variate: chloro

Source of variation	d.f.(m.v.)	s.s.	m.s.	v.r.	F pr.
rep stratum	19	0.0380775	0.0020041	5.44	
rep.*Units* stratum					
age	12	1.0243534	0.0853628	231.63	<.001
waveL	2	7.5963554	3.7981777	1.031E+04	<.001
age.waveL	24	0.3544664	0.0147694	40.08	<.001
Residual	602(120)	0.2218588	0.0003685		
Total	659(120)	8.0554234			

* MESSAGE: the following units have large residuals.

rep 8	0.01667	s.e. 0.00699	
rep 16	-0.01654	s.e. 0.00699	
rep 3	*units* 16	0.08437	s.e. 0.01687
rep 3	*units* 17	0.05830	s.e. 0.01687
rep 8	*units* 1	0.13001	s.e. 0.01687
rep 8	*units* 2	0.13778	s.e. 0.01687
rep 8	*units* 3	0.14273	s.e. 0.01687
rep 8	*units* 10	-0.07236	s.e. 0.01687
rep 8	*units* 12	-0.05564	s.e. 0.01687
rep 16	*units* 1	-0.10838	s.e. 0.01687
rep 16	*units* 2	-0.10751	s.e. 0.01687
rep 16	*units* 3	-0.10696	s.e. 0.01687

***** Tables of means *****

Variate: chloro

Grand mean 0.41308

age	diploid_a	diploid_b	diploid_c	NF_2year	NF_4year	NF_6year	NF_8year
	0.38211	0.39278	0.38535	0.37847	0.36929	0.45189	0.45046

age	NF_9year	OF_2year	OF_4year	OF_6year	OF_8year	OF_9year
	0.47412	0.43683	0.36606	0.39310	0.44575	0.44383

waveL	433.00	456.00	663.00
	0.54189	0.39520	0.30215

age	waveL	433.00	456.00	663.00
diploid_a		0.49525	0.36283	0.28825
diploid_b		0.50640	0.37342	0.29853
diploid_c		0.49878	0.36611	0.29115
NF_2year		0.49353	0.35890	0.28297
NF_4year		0.55209	0.39509	0.16069
NF_6year		0.58479	0.42779	0.34309
NF_8year		0.58336	0.42636	0.34166
NF_9year		0.61157	0.45021	0.36059
OF_2year		0.56642	0.42076	0.32331
OF_4year		0.48119	0.34382	0.27316
OF_6year		0.51087	0.37037	0.29807
OF_8year		0.58359	0.42220	0.33147
OF_9year		0.57673	0.41973	0.33503

*** Standard errors of differences of means ***

Table	age	waveL	age waveL
rep.	60	260	20
d.f.	602	602	602
s.e.d.	0.003505	0.001684	0.006071

(Not adjusted for missing values)

*** Least significant differences of means (5% level) ***

Table	age	waveL	age waveL
rep.	60	260	20
d.f.	602	602	602
l.s.d.	0.006883	0.003307	0.011922

(Not adjusted for missing values)

***** Stratum standard errors and coefficients of variation *****

Variate: chloro

Stratum	d.f.	s.e.	cv%
rep	19	0.007168	1.7
rep.*Units*	602	0.019197	4.6

PLOIDY / CHLOROPHYLL ANALYSIS DATA

```
201  "General Analysis of Variance."
 202  BLOCK rep
 203  TREATMENTS Treatment*wavelength*line
 204  COVARIATE "No Covariate"
 205  ANOVA [PRINT=aovtable,information,means,%cv; FACT=32; FPROB=yes;
PSE=diff,lsd; LSDLEVEL=5]\
 206   absorbance

***** Analysis of variance *****

Variate: absorbance

Source of variation      d.f.        s.s.        m.s.     v.r.  F pr.

rep stratum                 9   0.785E-02   0.873E-03     9.94

rep.*Units* stratum
Treatment                   5   1.077E+01   2.154E+00 2.454E+04<.001
wavelength                  2   7.965E+00   3.982E+00 4.536E+04<.001
line                        2   0.722E-02   0.361E-02    41.12  <.001
Treatment.wavelength       10   0.436E+00   0.436E-01   496.24  <.001
Treatment.line             10   0.118E-01   0.118E-02    13.45  <.001
wavelength.line             4   0.333E-04   0.833E-05     0.09  0.984
Treatment.wavelength.line
                           20   0.114E-03   0.571E-05     0.06  1.000
Residual                  477   0.419E-01   0.878E-04

Total                     539   1.924E+01


* MESSAGE: the following units have large residuals.

rep 1        -0.00772   s.e. 0.00381

rep 1     *units* 19       -0.03069   s.e. 0.00881
rep 6     *units* 46        0.02822   s.e. 0.00881
rep 6     *units* 49        0.02822   s.e. 0.00881
rep 6     *units* 52        0.02822   s.e. 0.00881
rep 10    *units* 39        0.05573   s.e. 0.00881
rep 10    *units* 42        0.05824   s.e. 0.00881
rep 10    *units* 45        0.05965   s.e. 0.00881


***** Tables of means *****

Variate: absorbance

Grand mean  0.51418

 Treatment    2x_bag 2x_field  2x_seed   4x_bag 4x_field  4x_seed
             0.34300  0.40816  0.37463  0.64410  0.68640  0.62878

 wavelength   433.00   456.00   663.00
             0.67147  0.49527  0.37579

     line         a        b        c
            0.50960  0.51854  0.51440

 Treatment wavelength   433.00   456.00   663.00
```

2x_bag	0.49462	0.36390	0.17047
2x_field	0.52360	0.38857	0.31230
2x_seed	0.48776	0.35535	0.28079
4x_bag	0.83162	0.61213	0.48854
4x_field	0.87597	0.65469	0.52855
4x_seed	0.81523	0.59700	0.47411

Treatment	line	a	b	c
2x_bag		0.33551	0.35734	0.33614
2x_field		0.40365	0.41076	0.41007
2x_seed		0.37272	0.37579	0.37538
4x_bag		0.63224	0.64838	0.65167
4x_field		0.68964	0.68794	0.68163
4x_seed		0.62380	0.63105	0.63150

wavelength	line	a	b	c
433.00		0.66656	0.67619	0.67165
456.00		0.49063	0.49946	0.49573
663.00		0.37160	0.37998	0.37581

Treatment	wavelength	line	a	b	c
2x_bag	433.00		0.48715	0.50894	0.48778
	456.00		0.35642	0.37824	0.35705
	663.00		0.16297	0.18484	0.16360
2x_field	433.00		0.51842	0.52678	0.52561
	456.00		0.38409	0.39099	0.39064
	663.00		0.30844	0.31452	0.31394
2x_seed	433.00		0.48575	0.48903	0.48851
	456.00		0.35346	0.35649	0.35610
	663.00		0.27896	0.28186	0.28154
4x_bag	433.00		0.81846	0.83635	0.84003
	456.00		0.60050	0.61634	0.61956
	663.00		0.47776	0.49245	0.49541
4x_field	433.00		0.88003	0.87832	0.86956
	456.00		0.65719	0.65548	0.65139
	663.00		0.53171	0.53000	0.52393
4x_seed	433.00		0.80952	0.81774	0.81843
	456.00		0.59214	0.59922	0.59963
	663.00		0.46974	0.47617	0.47643

*** Standard errors of differences of means ***

Table	Treatment	wavelength	line	Treatment wavelength
rep.	90	180	180	30
d.f.	477	477	477	477
s.e.d.	0.001397	0.000988	0.000988	0.002419

Table	Treatment line	wavelength line	Treatment wavelength line
rep.	30	60	10
d.f.	477	477	477
s.e.d.	0.002419	0.001711	0.004190

*** Least significant differences of means (5% level) ***

Table	Treatment	wavelength	line	Treatment wavelength
rep.	90	180	180	30

d.f.	477	477	477	477
l.s.d.	0.002745	0.001941	0.001941	0.004754

Table	Treatment line	wavelength line	Treatment wavelength line
rep.	30	60	10
d.f.	477	477	477
l.s.d.	0.004754	0.003361	0.008234

***** Stratum standard errors and coefficients of variation *****

Variate: absorbance

Stratum	d.f.	s.e.	cv%
rep	9	0.004020	0.8
rep.*Units*	477	0.009370	1.8