Ornamental and weed potential of
Acacia baileyana F. Muell:
Investigations of fertility and leaf colour

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A flowering branch of *Acacia baileyana* F. Muell.

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Abstract

Acacia baileyana F. Muell., is endemic to the Cootamundra region of New South Wales, Australia. It is a widely planted ornamental tree that produces attractive displays of yellow inflorescences. There are two main types, the typical green leaf form and a recently cultivated purple leaf form, variety *purpurea*. Outside its endemic range, the green leaf form has become a weed. The aim of this study was to develop an understanding of the fertility and leaf colour of *A. baileyana* in order to determine its weed potential, and to provide a basis for the commercial development of the species for its foliage, cut flowers and as a indoor flowering pot plant.

The purple colour of the leaves is due to anthocyanin pigments. To characterise the type of anthocyanins in leaf tissue, efficient extraction, separation (reverse-phase HPLC), purification and identification techniques were developed. Rapid purification and identification was achieved using high voltage paper electrophoresis in conjunction with mass spectrometry. The two main anthocyanin compounds of variety *purpurea* identified were delphinidin-3-glucoside and cyanidin-3-glucoside.

Leaf colour is a quantitative trait that was reliably assessed with a grading scale using four colour categories. Each category reflected the anthocyanin content of the tissue. Anthocyanin accumulation in the juvenile leaves was strongly induced at low temperature and at high light intensity. Intense purple leaves were produced only at temperatures below a mean of 21°C maximum and 12°C minimum when plants were grown outside, or at a constant 13°C day and 9°C night temperature when plants were grown in controlled environment rooms. The green foliage trait was found to be dominant over purple leaf colour.

Acacia baileyana required warm temperatures (above 18°C maximum, 13°C minimum) for bud formation and cool temperatures (below 16°C maximum, 9°C minimum) for flowering. Peak flowering was advanced by four months by controlling the time when plants were exposed to warm then cool temperatures. Double the number of plants flowered under these conditions compared with outside grown plants.

Both the green and purple leaf forms had similar reproductive strategies. They are highly self incompatible, have rapid growth and flower by two years of age. Controlled cross pollinations produced an average pod set of 23%, which was much greater than for self pollination (0.36%) and open pollination (less than 0.41%). Under natural conditions, total seed production was high due to the large number of flowers. A novel method using digital image analysis was developed to estimate flower numbers. Maximum flower number for a two-year old plant was 334,808, resulting in 8,007 seeds. For mature trees, flower numbers ranged from 1.25 to 13.2 million, with maximum seed number

of 19,559. Therefore, precocity and high flower numbers may partly explain the weed status of *A. baileyana*. The recessive purple leaf trait may explain why only the green leaf form has been recorded as a weed. However, given the obvious weed potential of the species, any commercialisation of *A. baileyana* should include a strategy to prevent its spread into the surrounding environment.

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Chapter One

General Introduction

1.1 *Acacia*

The genus *Acacia* belongs to the family Leguminosae, sub-family Mimosoidaceae, and contains more than 1350 species occurring on nearly all continents except Europe and Antarctica (Maslin 2001, Maslin et al. 2003). The greatest diversity occurs in Australia, with about 950 species presently recognised (Maslin 1995, Maslin 2001, Maslin et al. 2003). Acacias can be woody trees or shrubs and exhibit a vast range of morphological, biochemical and genetic variations (Maslin 2001). They have adapted to a variety of climates including arid, semi-arid and dry sub-tropical areas, and are tolerant of most soil types (Maslin 2001).

Seedlings produce pinnate and bipinnate leaves that may persist in the mature plant, or alternatively phyllodes may develop (Sedgley 1989). The flowers are small, bisexual and arranged into spherical or cylindrical inflorescences (Sedgley 1996). The colour of the flowers is generally shades of cream or yellow (Sedgley 1989). The most generally used classification of the genus is that of Pedley (1978) which recognised three large subgenera, namely *Acacia, Aculeiferum* and *Phyllodineae*. The mainly Australian subgenus, *Phyllodineae,* is the largest group and is separated into seven sections (Maslin and Pedley 1998). Pedley (1986) has elevated the three subgenera into genera, but this classification is not widely accepted (Maslin 2001, Maslin et al. 2003).

Acacias have been used overseas and in Australia for timber, pulp, firewood, agroforestry, gum, perfume, tannin, essential oils, animal feed, land rehabilitation, soil stabilisation, soil enrichment, human food and as garden plants (Sedgley 1989, Sedgley and Harbard 1994, Maslin 1995). The plants are particularly noticeable during flowering and their attractive appearance has contributed to some acacias being used in ornamental horticulture. Species suitable for the cut flower and foliage industries include those with grey or green bipinnate or phyllodinous leaves and prolific flowering (Sedgley 1996). These species include selections of *A. dealbata, A. retinodes, A. baileyana, A. podalyriaefolia* and their natural hybrids, which have been sold within the European cut flower industry (de Ravel d'Esclapon 1962). The main *Acacia* plantations outside Australia are in southern France, Italy, Israel, Portugal and Japan, where they are known as mimosa (Sedgley 1996, Horlock et al. 2000). Acacias flower in winter and so are popular sources of cut flowers for the Christmas market in the Northern Hemisphere (Parletta and Sedgley 1996). The success of *Acacia* as a cut

flower in Europe indicates the huge potential of this genus as a commercial crop in Australia (Horlock et al. 2000), but only in recent years has interest in Australia turned to cut flower and foliage production (Sedgley 1996). Several species of acacia have been identified as having a potential market in Japan, including *A. baileyana*, *A. baileyana* variety *purpurea* (Plate 1.1A, B, C) and *A. dealbata* (Horlock et al. 2000). They have the desirable characteristics of pale-lemon to yellow-orange, globular flowers, flower from June to December, are not strongly scented, and are readily propagated and cultivated. In addition, *Acacia* species with a compact form and dense flowering may have the potential to be used as indoor flowering pot plants (Sedgley and Parletta 1993).

1.2 *Acacia baileyana*

Acacia baileyana F. Muell (Subgenus *Phyllodineae*, Section Botrycephalae) is an Australian native, commonly known as Cootamundra wattle. The typical form has blue-grey to green foliage and is widely planted as an ornamental tree in Australia, due to its spectacular yellow floral display, bipinnate leaves and fast growth (Plate 1.1D; Boden 1969, Hall and Turnbull 1979). Trees are small, reaching a height of 10 m, are evergreen and live for a maximum of 30 years (Plate 1.1D; Blood 2001). The blue-grey colour of the mature leaves is due to the extremely glaucous bipinnate foliage (Newman 1933, Tame 1992), while the juvenile foliage is green. Individual trees flower for only a few weeks per year (Boden 1969). Ten to 25 flowers are clustered together into ball-shaped flower heads (inflorescences), which are arranged into dense racemes (Plate 1.1A; Elliot and Jones 1984, Simmons 1988). Reproduction is only by seed. Plants initiate flower buds in summer, flower from June to September (Boden 1969), and the mature pods dehisce the following summer (Newman 1934b).

Acacia baileyana was first described in 1888 by Mueller (Newman 1935). It was named after F. M. Bailey who collected the type specimen from a cultivated plant in Brisbane in 1876 (Newman 1935). The origin of the tree was untraceable, as the seed came from a collection supposedly of *A. polybotrya* seeds (Newman 1935). Description of the type specimen from a cultivated plant led to speculation about the origin of *A. baileyana* and investigations of its endemic distribution. Cambage (1902) surveyed the area from Marsden to Narrandera, in New South Wales, and found two *A. baileyana* populations, both in the Cootamundra district. In 1935, Newman found eight populations within a 30-45 km area between Temora and Cootamundra, New South Wales. Newman (1935) suggested that *A. baileyana* may be a relict species that had previously prospered before physiographic or climatic changes occurred, thus restricting it to its current confined habitat. Since Newman's study, land clearing for agriculture and pasture had reduced the occurrence of the species to two distinct locations in New South Wales, 25 km apart (Plate 1.1E; Smith 1993).

The original vegetation of the Cootamundra area was either mixed temperate woodland or open forest (Plate 1.1E; Hall and Turnbull 1979). The natural habitat of *A. baileyana* is characterised by acidic soils (Newman 1933), an altitude range of 300 to 600 m and an average annual rainfall of between 600 and 700 mm (Hall and Turnbull 1979). Plants are most likely to be found on stony, hilly land of south-western slopes, but can also be found in moist gullies and depressions (Hall and Turnbull 1979, Simmons 1988).

1.2.1 Weed status of *Acacia baileyana*

Acacia baileyana is considered to be a serious threat to native vegetation outside its natural habitat, and has been classed as a native environmental weed in Australia (Plate 1.1F; Blood 2001). Plants have had ample time to invade native ecosystems from garden plantings, due to the long cultivation history. The initial escape of *A. baileyana* is likely to have been through human activities, such as from garden plants adjacent to native vegetation, dispersal of seed via the dumping of garden waste and contaminated machinery (Blood 2001, Muyt 2001). The invasive success of *A. baileyana* has been attributed to its rapid growth, early flowering, seed dispersal by ants, frequent fire activity stimulating seed germination and limited susceptibility to damage from pests (Ross 1975, Milton and Moll 1982, Smith 1993, Mulvaney 1991). The typical grey-blue to green leaf form is the main invasive form, but the weed status applies to all forms (Blood 2001).

Plants have escaped from cultivation to become a weed in all states of Australia (Muyt 2001). Weed populations also exist overseas in New Zealand, France, the USA and South Africa (Ross 1975, Hall and Turnbull 1979, Milton and Moll 1982, Robin and Carr 1983, Tame 1992, Whibley and Symon 1992, Blood 2001). The main weed populations in Australia occur in the Blue Mountains and coastal regions south of Sydney, New South Wales; in eucalypt woodlands close to Canberra and Queanbeyan in the Australian Capital Territory; and in Victoria at Lake Tyers east of Melbourne and in the Grampians west of Melbourne. It has become established also in many parts of South Australia, especially the suburbs of Adelaide on the slopes of the Mount Lofty Ranges (Hall and Turnbull 1979). The first naturalised plant collected in Adelaide was found near Mount Lofty in 1943 (Simmons 1988).

Acacia baileyana has invaded heathlands, woodlands, grasslands, dry sclerophyll forests, roadsides, riparian vegetation and pine plantations (Blood 2001, Muyt 2001). It is not suited to very arid areas but can tolerate cool summers and winters, coastal conditions, occasional snowfall, frost and drought (Hall and Turnbull 1979, Simmons 1988, Blood 2001). Invasive plants can form dense thickets that exclude light from beneath them, and also drop leaf and pod material on the soil surface (Muyt 2001). These changes to the native ecosystem can eliminate many indigenous species but allow exotic grasses

to flourish. *Acacia baileyana* is a legume and therefore may have the ability to fix nitrogen. This may lead to increase levels of nitrogen in the soil, which may affect the survival of some indigenous species (Blood 2001).

Acacia baileyana can hybridise with a number of *Acacia* species, including *A. dealbata, A. decurrens, A. podalyriaefolia, A. mearnsii* and *A. leucoclada* (Cheel 1935, Moffett 1965b, Pedley 1987, Whibley and Symon 1992, Blood 2001). The hybrid between *A. baileyana* and *A. dealbata* is an environmental weed (Blood 2001).

1.2.2 Ornamental status of *Acacia baileyana*

Acacia baileyana is a very popular ornamental plant that has been cultivated in Australia and overseas since the turn of the century. In fact, by 1880, before its description, *A. baileyana* had become well established in cultivation throughout Australia and New Zealand (Newman 1935). By 1911, *A. baileyana* was among the ten most frequently planted woody species in Canberra (Mulvaney 1987). *Acacia baileyana* grows fast and is adapted to a wide variety of growing conditions (Elliot and Jones 1984). It has been planted extensively in temperate Australia, New Zealand, America, Africa and southern Europe, mainly for shade, screening, shelter, windbreaks and firewood (Boden 1969, Hall and Turnbull 1979, Elliot and Jones 1984, Simmons 1988).

In Australia, *A. baileyana* is used by florists for its foliage and flower buds (Plate 1.1C). The flowers have a short vase life, less than ten days (Horlock et al. 2000), and therefore are not favoured by florists at present. However, improved pulsing solutions may overcome this problem. An Australian study identified *A. baileyana* as one of five species with potential for the Japanese market, due to its attractive foliage and ball-shaped inflorescences (Horlock et al. 2000). Outside Australia, *A. baileyana* has been grown in plantations for its flowers. In France, *A. baileyana* has been grown on the Riviera specifically for cut flowers known as 'Or Azur' (de Ravel d'Esclapon 1962). *Acacia baileyana* variety *purpurea* is grown in Israel for its cut foliage and the typical green form is grown in Japan for cut flowers (Horlock et al. 2000). In addition, a recent study (Parletta and Sedgley 1998) suggest that *A. baileyana* has potential to be used as an indoor flowering pot plant.

Several forms of *A. baileyana* exist in cultivation, and these differ in juvenile growth colour and growth habit (Cheel 1935, Elliot and Jones 1984), although the mature foliage is blue-grey to green in colour. All varieties produce viable seed, although they may not be true to type (Elliot and Jones 1984). The five main forms are:

- the typical form with green juvenile growth (Elliot and Jones 1984).

- variety *purpurea* (Plate 1.1A, B; Elliot and Jones 1984). This variety has bright purple to burgundy juvenile growth. The colouring appears on the young leaves and leaf stems, peduncles of flowers and in seedling plants (see Plate 6.1). The juvenile growth can vary in the intensity of colour and the length of the coloured growth can range from 1 to 30 cm (Iain Dawson, personal communication). The origin of variety *purpurea* is unknown but it is thought that the form may have originated in the 1970s from seed propagation of the green form (Bill Molyneux, personal communication).

- variety *aurea* (Elliot and Jones 1984, Kodela and Tindale 2001). This variety has golden coloured juvenile growth.

- a form with red juvenile growth (Elliot and Jones 1984).

- a prostrate form with green juvenile growth (Elliot and Jones 1984, Whibley and Symon 1992).

1.3 Thesis Objectives

This thesis will contribute to the understanding of the fertility and leaf colour of *A. baileyana*. The typical green juvenile leaf form (referred to throughout the thesis as the 'green leaf form') and variety *purpurea* (referred to as the 'purple leaf form') have the potential to be further commercialised for their flowers and foliage. The success of such ventures will require an understanding of factors that influence fertility and leaf colour. In addition, an understanding of the weed potential of *A. baileyana* is essential for the appropriate management of plants.

The first objective of this study was to investigate the fertility of the green and purple leaf forms. The breeding system and reproductive efficiency (flower and seed production) of *A. baileyana* weeds in the Adelaide Hills was examined. This will determine if high seed production contributes to the weed status. Plants may also have the potential to be developed as indoor flowering pot plants. The response of flowering to changes in temperature was studied to determine if *A. baileyana* could be manipulated to flower out-of-season. If this proves possible, pot plants could be induced to flower to coincide with key festivals.

Leaf colour plays an important role in determining the aesthetic value and hence success of a floricultural crop. An understanding of the chemistry and genetic control of colour is highly desirable in plant breeding programs that aim to develop improved or novel colour forms. In addition, the registration of a new colour cultivar requires knowledge of the environmental conditions that affect the expression of that colour. The second objective of this study was to examine the chemistry, inheritance and environmental control of purple leaf colour of *A. baileyana*. It was hypothesised that the green colour is dominant over the purple leaf colour. The inheritance studies were also intended to determine why only the green leaf form appears to become a weed.

Plate 1.1 Flowers, foliage and habitat of *A. baileyana*. A: Flowering racemes of the purple and green leaf forms. The purple form is on the left, the green form is on the right. Scale bar = 2.5 cm. B: New growth of the purple leaf form. Scale bar = 1 cm. C: Cut flowers of *A. baileyana* in a bouquet. Scale bar = 2 cm. D: Flowering ornamental tree of *A. baileyana*. The height of the tree is approximately 4 m. E: Endemic population at Ulandra Nature Reserve, New South Wales. The height of the tallest flowering *A. baileyana* trees is approximately 8 m. F: Weed plants invading native vegetation at Millbrook Reservoir, Adelaide Hills, South Australia. The height of the *A. baileyana* trees is approximately 5 m.

Chapter Two

Literature Review

2.1 Fertility

Fertility has been defined as a measure of the performance of a population based on the number of offspring produced per individual (Krebs 1978). Highly fertile plants have the potential to become weeds because they produce many offspring (Adair 1995). Factors that influence seed production include the number of flowers and ovules that are initiated, the success of pollination and fertilisation of the ovules, and their subsequent retention and development (Lloyd 1980, Naylor 1984, Campbell and Halama 1993, Burd 1994, Parra-Tabla et al. 1998, Larson and Barrett 2000). Other factors governing seed production include the availability of resources, environmental conditions and predation levels (Lloyd 1980, Stephenson 1981, Lee and Bazzaz 1982, Fenner 1985, Ayre and Whelan 1989, Campbell and Halama 1993, Parra-Tabla et al. 1998, Trueman and Wallace 1999, Cunningham 2000, Griffin and Barrett 2002). Final fruit numbers are generally lower than those initially set due to fruit abortion during the developmental period (Lloyd 1980, Stephenson 1980, Stephenson 1981, Sutherland and Delph 1984). Abortion of flowers, fruits and seeds can occur at any stage of seed development (Bawa and Webb 1984, Trueman and Wallace 1999).

2.1.1 Factors affecting seed production

2.1.1.1 Number of flowers

The number of flowers with female organs sets the upper limit to the number of fruits that can be produced (Jackson and Sweet 1972). Male flowers are produced in many *Acacia* species and this may reduce the number of potential seeds (Kenrick 2003). For example, in *Acacia tortilis,* 34% of flowers were male (Tybirk 1993), while 71% of flowers were male for *A. nilotica* (Mackey 1997). Thus, as Lloyd (1980) has stated, many flowers may be produced simply to provide pollen.

In acacias, flowering occurs after the period of juvenility has finished (Sedgley and Griffin 1989). Temperature, light, water availability and nutritional status can influence flower development and number (Buttrose et al. 1981, Sedgley 1985, Sedgley 1989, Sedgley and Griffin 1989). Sedgley (1989) suggested that these factors are reflected in the diversity of species that grow in a range of climates. Flower production can also decrease with plant age, as reported in *A. suaveolens* (Morrison

and Myerscough 1989). In contrast, photoperiod does not appear to affect flowering of *Acacia*, which is similar to the situation in other woody perennials (Sedgley 1985).

Temperature can exert strong control over floral initiation and development in *Acacia*, with cool temperatures required for successful flowering in many species (Sedgley 1989, King and Gocal 1999). For example, initiated flowers of *A. pycnantha* developed and opened only when the temperature was below 19°C (Sedgley 1985). Bud formation was not affected by temperature, which may explain why floral buds can be produced all year for this species (Sedgley 1985). Similarly, bud formation of *A. drummondii elegans* can occur at both cool (15°C day / 10°C night) and warm (25°C day / 20°C night) temperatures, but anthesis only occurs at lower temperatures (Parletta and Sedgley 1996). *Acacia glaucoptera*, *A. acinaceae* and *A. myrtifolia* can flower at cool (15°C day / 10°C night) and warm (25°C day / 20°C night) conditions, although there were fewer inflorescences at the higher temperatures (Parletta and Sedgley 1996). Parletta and Sedgley (1996) found that anther development was inhibited by the warm temperature treatment. King and Gocal (1999) suggested that flower initiation and development in *Acacia* may, in fact, be blocked or reversed by high temperatures, and low temperature may merely be a passive condition permitting flowering.

Light intensity can also have a major affect on flowering as low light levels can reduce floral formation (Sedgley and Griffin 1989). The response of flowering in *Acacia pycnantha* to changes in light intensity was studied by comparing plants grown outside and under 70% shadecloth at similar temperatures (Sedgley 1985). The plants under the shadecloth never reached anthesis; thus, high light intensity is required for successful flowering in this species. Sedgley (1989) speculated that the inhibition of anthesis might be due to the shade causing a reduction in assimilates that are available to the developing flowers.

An increase in soil moisture typically has a positive effect on flowering in *Acacia* species, as it may promote floral initiation (Jackson and Sweet 1972, Sedgley and Griffin 1989, King and Gocal 1999, Kenrick 2003). In *A. suaveolens*, the number of inflorescences produced per plant was positively related to rainfall during the initiation of inflorescence buds (Morrison and Myerscough 1989). Some acacias can also flower several times in the year after rain (Preece 1971, Winkworth 1973, Buttrose et al. 1981). These are mainly arid species, such as *A. aneura* (Winkworth 1973), which respond to favourable climatic conditions, although flowering does not always lead to seed set (Preece 1971, Winkworth 1973).

Adverse climatic conditions can also disturb flowering of acacias (Kenrick 1994, cited in Kenrick 2003). For example, Boden (1969) reported that unseasonal weather immediately prior to the flowering period of *A. baileyana* could either delay or induce flowering. A more uniform climate in

Malaysia is thought to increase the flowering period of *Acacia mangium* and *A. auriculiformis* compared with that in Australia (Sedgley et al. 1992).

The ratio of male to hermaphrodite flowers in *Acacia* may be influenced by environmental conditions during floral development (Sedgley 1989, Sedgley and Griffin 1989). Kenrick (2003) suggested that the number of male flowers in *Acacia* is a highly variable and plastic character that allows plants to respond to environmental conditions and resources during floral development. She further suggested that this plasticity would allow the quantity of pollen to remain constant, while lowering the expenditure of resources on the female function on the plant.

2.1.1.2 Breeding systems

An understanding of the reproductive biology of *Acacia* is required to determine factors limiting seed and pod yields. Flowering behaviour and breeding system are two processes that affect the quality and quantity of seeds produced. Seed quality and quantity may be improved by mechanisms that enhance cross pollination.

Acacia species possess a multi-stigmatic reproductive unit (inflorescence) and composite pollen grains (polyads) (Newman 1934b, Cookson 1953, Kenrick and Knox 1979, Kenrick and Knox 1982). Australian *Acacia* species have a polyad grain number of 4, 8, 12, or 16, while African species have 16, 32 or 64 pollen grains (Kenrick and Knox 1982). Most (90%) of Australian acacias have 16 grain polyads. In most species the ovule number is less than the pollen grain number of the polyad (Kenrick and Knox 1982). *Acacia baileyana* has a polyad number of 16 and an ovule number of 12; therefore each flower can potentially produce 12 seeds (Newman 1934b).

A polyad can act as a physical barrier to other polyads, as one polyad fits neatly into the stigmatic depression (Moncur et al. 1991). Stigmatic exudate is produced during anthesis in *Acacia,* and may provide a medium for pollen grain germination and pollen tube growth, and may also serve to orientate a polyad into the stigma cup (Kenrick and Knox 1981, Marginson et al. 1985, Kenrick 2003). The optimum temperature for post-pollination stigma secretion of *A. baileyana, A. brownii* and *A. iteaphylla* is 20°C (Marginson et al. 1985). The pollen of *A. baileyana* can remain viable three to seven days after anthesis (Newman 1934b).

The pollen grains of a polyad, and the resulting seeds of a pod, usually reflect the genotype of only one paternal tree (Muona et al. 1991, Sedgley et al. 1992). Thus, the single pollination event involving one polyad per flower results in reduced variability of the progeny. An advantage of this process is that seed set is ensured following a single pollination event (Kenrick and Knox 1982), potentially resulting

in many seeds (Sedgley et al. 1992). Therefore, the polyad makes efficient use of pollinators (Knox and Kenrick 1983). A disadvantage is that the flower essentially has only one chance of successful pollination (Grant et al. 1994), and if polyads have insufficient viable grains to set a pod of ovules, low seed set might result (Kenrick 2003).

In general, acacias produce many flowers. This can lead to a large production of polyads that may increase the likelihood of self pollination (Moncur et al. 1991). Even though there is a greater chance of being self pollinated, *Acacia* species have in fact been shown to be a highly heterozygous outcrossing group (Kenrick and Knox 1985). In addition, *Acacia* species studied so far have low pod set (0.01–0.60%; Milton and Hall 1981, Tybirk 1989, Muona et al. 1991, Grant et al. 1994). Despite this, *Acacia* is one of the most widespread genera in Australia.

Mechanisms to ensure cross pollination will result in greater genetic variability. For example, the dichogamy of female and male reproductive structures can promote outcrossing (Sinha 1971, Kenrick and Knox 1981, Sedgley and Harbard 1993). The Australian species of *Acacia* examined so far are protogynous (Knox et al. 1989, Sedgley 1989), with the stigma receptive to pollen before pollen is released from the anther. In these species, pod set was greatest immediately following flower opening, as the stigma is highly receptive at this time. In contrast, the African species studied, *A. nilotica* (Sinha 1971) and *A. karroo* (Sedgley and Harbard 1993) are protandrous, with the anthers dehiscing before the stigma is receptive.

Self incompatibility can also promote outcrossing in *Acacia*, as genetic incompatibility between parental genotypes can result in ovule or seed abortion (Kenrick et al. 1986, Kenrick and Knox 1989, Sedgley 1989). Seed set may be limited by genetically based pre-zygotic or post-zygotic mortality. Kenrick and Knox (1989) proposed that full and partial self incompatibility in *Acacia* may arise from a gametophytic *S* gene and post-zygotic lethal genes. In *A. retinodes*, a strongly self incompatible species, self pollen tube growth stops at the nucellar tissue of the embryo and does not enter the embryo sac (Bernhardt et al. 1984, Kenrick et al. 1986). Recently, Kenrick (2003) discussed the possibility that self incompatibility in *A. myrtifolia* could be the result of post-zygotic lethal genes. *Acacia myrtifolia, A. paradox, A. terminalis* (Kenrick and Knox 1989), *A. decurrens* and *A. mearnsii* (Moffett 1965a) are partially self compatible, with the ability to self pollinate under certain conditions (Kenrick and Knox 1989). It was found for *A. decurrens* that pods resulting from self pollination had fewer seeds (Philp and Sherry 1946).

Abortion of fruits may allow the maternal parent to selectively remove genetically inferior progeny and regulate the genetic quality of the offspring (Lloyd 1980, Stephenson 1981). For example, *Lotus corniculatus* selectively aborts those fruits with the fewest seeds, and these are likely to have resulted

from self pollination (Stephenson and Winsor 1986). *Lotus corniculatus* retained those fruits from the most intensively pollinated flowers and thus the seeds were of higher quality than those produced without competition (Stephenson and Winsor 1986). Similar studies have not been done in *Acacia*.

The presence of male and hermaphrodite flowers on the same plant can also promote outcrossing (Newman 1934a, Sedgley 1989). The large number of functionally male flowers of *A. nilotica* (Tybirk 1989) may serve to attract pollinators and to increase pollen flow in the population. Hermaphroditic flowers of *A. nilotica* are found at the top of the inflorescence, which allows for convenient pollination (Tybirk 1989).

2.1.1.3 Pollinators

Pollinator movement in *Acacia* is important for outcrossing and seed set. Pollination of *Acacia* flowers may occur through active pollen collection by insects (Bernhardt and Walker 1985) and in specific cases, through passive pollination by birds (Ford and Forde 1976, Kenrick et al. 1987, reviewed in Stone et al. 2003). Native and introduced bees are reported to be the most common group of pollen vectors for acacias (Bernhardt and Walker 1984, reviewed in Stone et al. 2003), but they may encourage self pollination as a result of flower-to-flower travel on the one tree (Grant et al. 1994). The majority of acacias do not have floral nectaries; thus pollen is the only reward for visitors to flowers (Stone et al. 2003). However, acacias possess extrafloral nectaries on the petioles and rachis of the leaves, which can supply nectar to pollinators or visitors (Kenrick 2003, Stone et al. 2003). The nectar resides in a gland making it accessible to a wide range of foragers (Bernhardt 1987), including ants, wasps and birds (Stone et al. 2003). Birds may be attracted to the extrafloral nectaries or to nectar-feeding insects (Ford and Forde 1976), and may be effective at cross pollination as they move between trees. For example, birds are attracted to the extrafloral nectaries of *A. pycnantha* (Vanstone and Paton 1988) and *A. terminalis* (Kenrick et al. 1987).

Seed set may be reduced when there is a shortage of effective pollinators (Bawa and Webb 1984, Ayre and Whelan 1989, Burd 1994, Vaughton and Ramsey 1995). The number of flowers and inflorescences, flowering synchrony and visual impact of the floral display can each potentially influence the frequency of pollinator visits, outcrossing rates and ultimately seed production (Vaughton and Ramsey 1995). For *Acacia*, the inflorescence is the visual attractant to pollinators as the individual flowers are small (Arroyo 1981).

An abundance of flowers not only increases the likelihood of high seed production but it also attracts pollinators (Stephenson 1979), although during mass flowering there is little incentive for the pollinators to move to other plants (Stephenson 1982). Stephenson (1982) suggested that pollinators

are more likely to move from tree to tree during pre or post peak flowering, resulting in more outcrossing. For example, in *Catalpa speciosa*, outcrossing most likely occurs during the final phase of flowering (Stephenson 1982). This phenomenon has not been investigated in *Acacia*.

Flowering needs to coincide with the presence of appropriate pollinators and be synchronised with other members of the plant population. The plant also needs to avoid competition from other species that share its pollinators (Fenner 1985). Plants may compete for pollinator visits by providing highly attractive rewards (reviewed in Stone et al. 2003). A recent study of an *Acacia* community in Tanzania concluded that competition between pollinators is avoided as pollen of different *Acacia* species is released at different times of the day (Stone et al. 2003). Competition for pollinators may also result in inter-specific pollen being deposited onto the stigma preventing pod set, although different polyad sizes between species may prevent this occurring (Kenrick 2003, Stone et al. 2003).

2.1.1.4 Predation

Maturing seeds can provide a rich source of food for a variety of potential predators (Ayre and Whelan 1989). Therefore, a large proportion of seeds may be lost to predators before ripening. For example, predators damaged between 6 and 12% of *Cassia fasciculata* seeds (Lee and Bazzaz 1982). Plants have adapted to provide a variety of mechanical and chemical defence mechanisms against predation. For example, thorns deter predators in some African *Acacia* species. The large number of small flowers or seeds produced by some *Acacia* species may also prove to be too many for the potential predators to destroy (Bawa and Webb 1984).

2.1.1.5 Resources and environmental conditions

Nutritional status of the soil, available water, condition of the plant during seed development, and environmental conditions, can affect the retention of fertilised ovules (Stephenson 1980). Adverse environmental conditions, such as high temperatures, frost, and drought, not only affect flowering in *Acacia*, as previously discussed, but can also reduce fruit and seed set (Kenrick 1994, cited in Kenrick 2003). Tybirk (1989) suggested that total pod and seed production of *A. nilotica* reflects the seasonal conditions.

A main factor in reduced seed production is insufficient resources available to convert each pollinated flower into a fruit (Lloyd 1980, Stephenson 1981, Lee and Bazzaz 1982). For example, the seed production of *A. suaveolens* positively reflected available rainfall (Morrison and Myerscough 1989). This observation is similar to that of Lee and Bazzaz (1982), who found that the addition of water and fertiliser to *Cassia fasciculata* increased the number of fruits per plant. In years with good resources,

plants that initiate extra fruit may retain all or nearly all of them, producing a large number of seeds (Lee and Bazzar 1982). Stephenson (1984) found that resources rather than pollination limited the reproductive output of *Lotus corniculatus*. Seed production was reduced when the plants were partially defoliated, but improved when fertiliser was added. The abortion of flower and juvenile fruit may, therefore, allow plants to match fruit and seed number with the available resources over a wide range of environmental conditions. Thus, by aborting fruits early in their development, plants may conserve resources for the remaining fruits and for future reproduction and vegetative growth (Stephenson 1981).

Each flower and fruit predominantly uses metabolites from adjacent leaves (Lloyd 1980 and references within). Stephenson (1980) determined that each branch of *Catalpa speciosa* supplied most of the assimilates to each infructescence. Therefore, the first flowers to be pollinated have the best chance of forming mature fruits, as they have first use of the nutrient reserves (Stephenson 1981).

2.2 Environmental weeds

Environmental weeds are plants that invade native communities or ecosystems (Humphries et al. 1991, Carr et al. 1992). They can alter the structure, function, species composition and abundance of native communities and are regarded as the principal cause of decline of native vegetation (Humphries et al. 1991, Williams and West 2000). It is estimated that about ten percent of the Australian flora is composed of invasive, naturalised species, introduced since European settlement in the 1800s (Grove 1991), with legumes representing a significant proportion of weeds (Paynter et al. 2003). In south-eastern Australia, legumes are renowned for their invasiveness and are the second most naturalised family of plants (Kloot 1991). This could be due to the general invasive attributes of legumes or simply a consequence of the large family size (Heywood 1989). Horticultural species that have escaped from cultivation form the largest proportion of serious environmental weeds (Humphries et al. 1991, Kloot 1991).

Not all environmental weeds are introduced from overseas. They can also be indigenous species that have spread beyond their natural range (Humphries et al. 1991, Williams and West 2000). A Victorian study has listed approximately 120 Australian plant species that have escaped from cultivated gardens and plantations and have become naturalised in that state (Carr et al. 1992). For example, *Pittosporum undulatum* has extended its range in Victoria as well as invading other states (Gleadow and Ashton 1981). The cultivation of *P. undulatum,* combined with a decline in the frequency of wildfires and the dispersal of seeds by an exotic bird, has favoured regeneration of plants beyond its endemic range (Gleadow and Ashton 1981, Gleadow 1982).

Weeds have attributes for successful arrival, establishment, and dispersal in a new area (Drake and Williamson 1986). Specific characteristics of plants which have a potential for weediness in native ecosystems include: high reproductive capacity, a brief juvenile phase, seeds with prolonged periods of dormancy, quick germination, dense and spreading canopy, efficient short and long distance dispersal, reproductive strategies that facilitate survival in fire prone environments, broad distribution over a range of distinct climate types, rapid growth, capacity to alter soil chemistry, and absence of disease or predators (Adair 1995, Blood 2001, Muyt 2001). Species with high reproductive output in their native habitat have a high invasive potential (Noble 1989). In addition, the success of invasive plants depends on the ability to exploit the resources available in a new habitat. Severely disturbed habitats are likely to contain unused resources and are therefore relatively easy to invade (Hobbs 1989).

Management options for environmental weeds include: biological control for large inaccessible infestations, chemical control, the burning of susceptible species and mechanical destruction of small, accessible infestations (Adair 1995). It is particularly important to develop weed management methods that are integrated with an understanding of the dynamics of the ecosystem in which the weed occurs (Grove 1989).

2.2.1 *Acacia* species as weeds

Some of the potential weed attributes of *Acacia* species are rapid growth, production of large seed crops and effective seed dispersal mechanisms (Ross 1975, Milton and Hall 1981, Milton and Moll 1982, New 1984, Mulvaney 1991, Mackey 1997). Acacias are also shade tolerant to some extent, primary colonisers after disturbances, and have the ability to fix nitrogen via *Rhizobium* bacteria in root nodules that aids establishment under nitrogen deficient conditions (Heywood 1989, Grove 1991). Another attribute of *Acacia* that increases their potential as weeds is their hard seed coat that inhibits germination and imparts longevity. The seeds of *A. melanoxylon* can remain viable in soil for more than 50 years (Farrell and Ashton 1978). The hard seeds require scarification for germination (Myers 1936), but this is enhanced in fire-prone ecosystems (Grove 1991).

The majority of *Acacia* weed species produces dense thickets that exclude grasses and other vegetation (Csurhes and Edwards 1998). For example, the Australian shrubs, *Acacia saligna* and *A. cyclops*, have invaded the lowland ecosystems of the fynbos biome, in the Cape Province, South Africa, forming dense thickets, thereby suppressing the indigenous vegetation and reducing species richness (Holmes 1990). In total, thirteen Australian acacias have naturalised in the Cape Province of South Africa and are replacing indigenous vegetation (Milton and Moll 1982). They generally flower and produce seed prolifically, lack seed predators and have good seed dispersal and longevity (Milton and

Hall 1981). The lack of natural enemies of *A. longifolia* is the major factor contributing to its reproductive success, while the success of *A. saligna* and *A. cyclops* is attributed to their prolific production of hard-coated seeds that accumulate in the soil (Dean et al. 1986). The seed yield of medium sized Australian *Acacia* in the Cape Province is calculated to be in the range of 95,000 to 480,000 per tree. Australian acacias generally produce far larger seed crops in South Africa than in their native habitats (Dean et al. 1986). *Acacia longifolia* in the Cape Province produced 2,500 to 3,500 seeds per m^2 annually, but similar stands in their natural habitat yield only 300 to 400 seeds per m^2 (Weiss and Milton 1984, cited in Dean et al. 1986).

Non-Australian acacia species may also become weeds. The species, *A. nilotica* ssp. *indica*, from Africa and the Indian subcontinent, is one of the worst environmental weeds in Australia. It was introduced as a shade and ornamental tree to central Queensland in the early 1900s (Carter 1994, Paynter et al. 2003). It is a highly invasive shrub and has colonised thousands of hectares of the Mitchell grasslands in north-western Queensland (Humphries et al. 1991). Plants can flower and set seed two to three years after germination (Mackey 1997). One medium sized tree can produce 175,000 seeds in a year (Carter et al. 1989). Seeds are long-lived and are spread by cattle in the region (Mackey 1997).

Indigenous *Acacia* species can also become invasive through human activity or through cultivation (Mulvaney 1991, Humphries et al. 1991). The main Australian *Acacia* weeds are *A. baileyana, A. dealbata, A. decurrens, A. elata, A. iteaphylla, A. longifolia, A. saligna* and *A. sophorae* (Humphries et al. 1991, Robertson 1994). Some species of *Acacia* may also hybridise when found in the same area (Cheel 1935, Philp and Sherry 1949, Moffett 1965b, Leach and Whiffin 1978, Pedley 1987, Nikles and Griffin 1991, Whibley and Symon 1992, Blood 2001) and the hybrids may become environmental weeds (Robin and Carr 1983).

2.3 Leaf colour

The mature leaf colour of *A. baileyana* is blue-grey to green, but forms of *A. baileyana* vary in their juvenile leaf colour (Cheel 1935, Elliot and Jones 1984). The typical and prostrate forms both have green juvenile growth, while variety *purpurea* has purple juvenile growth, and variety *aurea* has golden juvenile growth (Elliot and Jones 1984). In addition, there is a form with red juvenile growth (Elliot and Jones 1984). In all non-green forms, the juvenile colour occurs transiently in the young leaves but disappears as the leaves mature. In other plant species the non-green leaf colour may occur transiently, like *A. baileyana*, or be permanent, or become apparent during autumn (Harborne 1965).

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The main classes of colour pigments in leaves are lipid soluble chlorophylls and carotenoids and the water-soluble flavonoids (Goodwin 1965). Chlorophylls and carotenoids are present in the chloroplasts of leaves. Chlorophylls are photosynthetic pigments that give plants their green colour. Carotenoids are yellow, orange or red pigments in all photosynthesising cells, and act as accessory photosynthetic pigments and anti-oxidants (Scott-Moncrieff 1963, Hall et al. 1976). Normally chlorophyll will mask the appearance of carotenoids, but these pigments can become visible in some plant species when chlorophyll breaks down during autumn, when there is a deficiency in nutrients, or as a result of disease (Harborne 1965).

Flavonoids represent a large class of secondary plant metabolites that contain a 2-phenylbenzopyran nucleus (Mazza and Miniati 1993). These secondary metabolites include the flavones, flavanones, chalcones, aurones, flavonols and anthocyanins. Anthocyanins have been noted in the hypocotyl and cotyledons of *A. baileyana* (Nozzolillo 1973), but the identity of these anthocyanin pigments is not known.

2.3.1 Anthocyanins

Anthocyanins are the most conspicuous of the flavonoids due to their varying colours, from scarlet to blue (Harbourne and Grayer 1988). They are the only flavonoids visible in leaves, and may be permanent, or displayed only in response to developmental or environmental triggers (Harborne 1965). Anthocyanin colouration varies not only as a function of seasonal and developmental factors, but can also differ among individuals of a population, among leaves within a canopy, and even among tissues within a leaf (Gould et al. 2002). They are observed in both juvenile and senescing leaves of many species (Chalker-Scott 2002). Anthocyanins are commonly produced in rapidly expanding leaves of tropical plants, senescing leaves of temperate plants, under surfaces of floating leaves of aquatic plants, abaxial surfaces of leaves of understorey plants, and leaves subjected to various environmental stresses (Woodall and Stewart 1998, Chalker-Scott 1999, Hoch et al. 2001, Chalker-Scott 2002, Gould et al. 2002, Lee 2002a, Neill et al. 2002). Juvenile anthocyanins appear at bud break and generally disappear after leaves are fully expanded (Harborne 1965).

Anthocyanins can accumulate in the vacuoles of the adaxial epidermis, hypodermis, palisade parenchyma, spongy mesophyll, bundle shealth cells, adaxial epidermis, and trichomes (Lee 2002a), although they generally accumulate in peripheral tissues exposed to direct light, such as the upper epidermis (McClure 1975). They are synthesised in the cytoplasm and are then transported to the cell vacuole (Grisebach 1982), typically within the tissues in which they accumulate (McClure 1975). Anthocyanin development is controlled at the level of the individual cell (Steyn et al. 2002).

In some plants, spherical organelles within vacuoles, known as 'anthocyanoplasts', contain the anthocyanin pigments (Peckett and Small 1980). It is possible that as the cell matures, the anthocyanins leak from these organelles. Peckett and Small (1980) suggested that anthocyanoplasts are the site of anthocyanin biosynthesis. They have been detected in 70 species representing 33 families of both dicotyledons and monocotyledons (Peckett and Small 1980).

More recently, Markham et al. (2000) identified coloured bodies, called anthocyanic vacuolar inclusions, in a number of flower petals. These bodies occur predominantly in the adaxial epidermal cells and can enhance both intensity and blueness of flower colour, and have a high affinity for certain anthocyanins.

2.3.2 Flavonoid pathway to anthocyanin biosynthesis

The first step to flavonoid biosynthesis is the formation of a 15-carbon nucleus, which is composed of two aromatic rings joined by a 3-carbon unit (chalcone). The various classes of flavonoids are determined by the degree of oxidation of the C-ring. Biosynthesis of anthocyanins proceeds from chalcone to flavanone to dihydroflavonol to anthocyanidin and then to anthocyanin (Mazza and Miniati 1993).

Holton and Cornish (1995) reviewed the flavonoid pathway to anthocyanin biosynthesis. Many structural genes encode anthocyanin biosynthetic enzymes that catalyse the steps from 4-coumaroyl-CoA to the anthocyanidin-3-glucosides (Fig. 2.1). Divisions in the types of anthocyanidins produced occur from naringenin flavanone and dihydrokaempferol. Naringenin flavanone and dihydrokaempferol can be hydroxylated by flavonoid 3'-hydroxylase to produce eriodictyol and dihydroquercetin (precurser to cyanidin), respectively. Naringenin flavanone and dihydrokaempferol can also be hydroxylated by flavonoid 3',5'-hydroxylase to produce pentahydroxyflavanone and dihydromyricetin (precurser to delphinidin), respectively. At least three enzymes are required to convert the colourless dihydroflavonoids (dihydrokaempferol, dihydroquercetin and dihydromyricetin) to anthocyanins. The first of these are dihydroflavonol 4-reductase that reduces dihdroflavonols to leucoanthocyanidins. Further oxidation, dehydration and glycosylation produce the dark-red pelargonidin, red cyanidin and blue delphinidin pigments. Glycosylation, methylation and acylation may modify anthocyanidin 3-glycosides further.

Six anthocyanidins occur most frequently in plants: pelargonidin, cyanidin, paeonidin, delphinidin, petunidin, and malvidin (Stack and Wray 1989, Mazza and Miniati 1993). Each anthocyanidin may

Phenylalanine

$\downarrow$ PAL

C4H | 4CL

4-Coumaroyl-CoA

$\downarrow$ CHS

Naringenin chalcone

$\downarrow$ CHI

F3'H   Eriodictyol ← Naringenin flavanone → Pentahydroxyflavanone   F3'5'H

F3H | F3'H   F3H | F3'5'H   F3H

Dihydroquercetin ← Dihydrokaempferol → Dihydromyricetin

DFR   DFR   DFR

Leucocyanidin   Leucopelargonidin   Leucodelphinidin

LDOX   LDOX   LDOX

Dehydratase   Dehydratase   Dehydratase

Cyanidin   Pelargonidin   Delphinidin

UFGT   UFGT   UFGT

Cyanidin-3-glucoside   Pelargonidin-3-glucoside   Delphinidin-3-glucoside

MT   MT | MT

Paeonidin-3-glucoside   Petunidin-3-glucoside   Malvidin-3-glucoside

Fig. 2.1 A schematic presentation of the anthocyanin biosynthetic pathway. The enzymes involved are boxed, except for dehydratase that is putative. PAL, Phenylalanine ammonia lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3'H, flavanone-3-hydroxylase; F3'5'H, flavanone-3'5'-hydroxylase, DRF, dihydroflavonol 4-reductase; LDOX, leucoanthocyanidin dioxygenase; UFGT, UDP glucose-flavonoid 3-*o*-glucosyl transferase; MT, methyltransferase. Adapted from Boss et al. (1996a) and Holton and Cornish (1995). LDOX is believed to catalyse the first step between leucoanthocyanidins and anthocyanins (Martin and Gerats 1993).

be glycosylated and acylated by different sugars and acids at different positions; thus the actual number of anthocyanins is 15 to 20 times greater than the number of anthocyanidins. The difference between individual anthocyanins is due to the number of hydroxyl groups in the molecule, the degree of methylation of these hydroxyl groups, the nature and number of sugars attached to the molecule and the position of the attachment, and the nature and number of aliphatic or aromatic acids attached to the sugars (Mazza and Miniati 1993). The sugars most commonly bonded to anthocyanidins are glucose, galactose, rhamnose, and arabinose. In many cases, the sugar residues are acylated by p-coumaric, caffeic, ferulic, sinapic, p-hydroxybenzoic, malonic, oxalic, malic, succinic, or acetic acids (Mazza and Miniati 1993). The glycosylation of the anthocyanidins is important in stabilising the molecule and increasing its water solubility. In general, it is the position of the sugar residue, rather than the nature of the sugar, that has a more direct effect on the perceived colour. However, the nature of the sugar can indirectly impact on the colour by affecting subsequent modifications such as methylation, acylation and further glycosylation (Davies and Schwinn 1997). The most common anthocyanin in juvenile and senescing leaves is cyanidin-3-glucoside (Harborne 1965).

2.3.3 Colour stabilising and intensifying effects

In general, pelargonidin-derived pigments produce orange, pink or red colours, cyanidin-derived pigments produce red or mauve colours, and delphinidin-derived pigments produce purple, blue, or blue-black colours. The actual colour of the plant tissue is influenced by a number of internal factors: pH, structure of the pigment, concentration, copigmentation, metal complexing of the anthocyanin pigment, and the presence of enzymes, oxygen, ascorbic acid, sugars and their degradation products (Osawa 1982, Brouillard 1983, Mazza and Miniati 1993, Davies and Schwinn 1997). Hydroxyl groups, methoxyl groups, sugars and acylated sugars have a marked effect on colour intensity and stability of anthocyanins (Mazza and Miniati 1993).

Increased concentration of anthocyanin pigments in plant tissues intensifies their colour and may enhance colour stability through the phenomena of intermolecular copigmentation and self-association (Mazza and Miniati 1993). Anthocyanins can form chemical bonds either between themselves (intramolecular copigmentation and self-association) or with other phenolic molecules (intermolecular copigmentation) resulting in an increase in colour intensity. Intermolecular copigments include a large variety of compounds (flavonoids, glycosides, polyphenols, alkaloids, amino acids, and organic acids) (Brouillard 1988, Mazza and Miniati 1993). The bonds stabilise the pigment and result in a shift in wavelength of maximum absorbance to higher wavelengths, known as a 'bathochromic shift'. Therefore, copigmentation results in the blueing of red shades.

The pH of the vacuole has a major effect on the secondary structure of the anthocyanins and thus on the resultant colour. A decrease in pH causes a shift towards the red cation form of the anthocyanin, while an increase in the pH causes a shift towards the bluer quinonoidal types (Brouillard and Dangles 1993). Changes in the pH of the vacuole and the presence of metal ions can influence the hydrogen bonding and the degree of compression of the stacked rings (copigments), affecting the perceived colour (Griesbach 1996).

2.3.4 Environmental and abiotic factors regulating anthocyanin accumulation

Flavonoid synthesis can be induced in response to a wide range of stresses including cold, heat, salt, drought, wounding, flooding, wind, and nitrogen and phosphorus deficiency (Chalker-Scott 1999 and 2002). This indicates that flavonoid synthesis may be part of a general stress response in plants. For anthocyanin synthesis, induction occurs in specific tissues at defined times, in response to visible and UVB radiation, cold, nutritional deficiency (especially nitrogen and phosphorus) and water stress (Vince-Prue 1975, Mancinell 1983, Christie et al. 1994, Lopez-Cantarero et al. 1994, Dixon and Paiva 1995, Chalker-Scott 1999, Stone et al. 2001, Steyn et al. 2002). Damage to leaves by insects may induce the production of anthocyanins around the lesions (Stone et al. 2001).

Low temperatures have been shown to induce anthocyanin synthesis in the leaves of a number of species, perhaps in association with high light intensity (Chalker-Scott 1999, Chalker-Scott 2002, Lee 2002a). Christie et al. (1994) showed that maize seedlings exposed to low temperature exhibit rapid increases in anthocyanin production. Low temperatures also induced the accumulation of anthocyanins in Japanese parsley (Hasegawa et al. 2001) and in *Cotinus coggygria* (Oren-Shamir and Levi-Nissim 1997). For Japanese parsley, dihydroflavonol 4-reductase (refer to Fig 2.1) was induced at low temperatures resulting in the accumulation of cyanidin (Hasegawa et al. 2001).

Light has been reported to be one of the most important environmental factors regulating the accumulation of anthocyanins (Grisebach 1982, Mol et al. 1996). In general, induction of anthocyanins requires high light intensities, with the concentration of anthocyanin in plants and individual leaves varying in relation to light exposure levels (Mancinelli 1983). Endogenous signals, developmental stage, environmental factors and previous light exposure can modify the effect of light on anthocyanin synthesis (Mancinelli 1983). Continuous UV, blue, red and far-red light or a combination of these wavelengths, are effective in increasing the enzymatic activity of the flavonoid biosynthetic pathway (Mancinelli 1983, Beggs et al. 1987, Beggs and Wellman 1994). In particular, a light response has been found in the UVB (290-320 nm), UVA (320-400 nm), blue (400-480 nm), red (600-690 nm) and far-red (710-760 nm) regions of the spectrum, through mediation by phytochrome,

cryptochrome or the putative UV-receptor (Mancinelli 1983, Mol et al. 1996, Steyn et al. 2002). It has also been suggested that anthocyanins may be induced in many tissues in response to intense white light (Martin and Gerats 1993). For example, white light was shown to induce anthocyanin pigmentation in eggplant (*Solanum melangena*) (Toguri et al. 1993). Chalker-Scott (1999) hypothesised that UVB is the only photoinducer of anthocyanins, with phytochrome modulating their relative amounts.

Anthocyanin concentration may also be influenced by daylength. For example, *Pinus contorta* seedlings have reduced anthocyanin accumulation when exposed to short days several months earlier (Camm et al. 1993). In some plants, small quantities of anthocyanins are formed during a dark incubation period interrupted by a few minutes exposure to light (Mancinelli 1983). The response induced by short periods of irradiation shows the typical characteristics of a phytochrome-mediated response: red/far red photoreversibility. In other plants, however, a single, short irradiation has no apparent effect on the production of anthocyanin, but an effect occurs after prolonged exposure (Mancinelli 1983). It should be noted that the phytochrome control of flowering and of flavonoid accumulation may act independently (McClure 1975).

2.3.5 Function of leaf anthocyanins

The physiological significance of anthocyanins in leaves is poorly understood, although they are believed to function as absorbers of harmful levels or wavelengths of light and as osmotic adjusters (Chalker-Scott 1999).

As stated previously, anthocyanins can be present at all developmental stages in leaves or appear only at specific stages of plant growth, for instance in seedlings or young leaves, and/or they may accumulate as a result of stress (Hrazdina 1982, Chalker-Scott 2002). The transitory nature of foliar anthocyanin accumulation could allow plants to respond quickly and temporarily to environmental variations rather than through more permanent anatomical and morphological modifications (Chalker-Scott 2002). Gould et al. (2002) proposed that any single explanation for the presence of anthocyanins in leaves must accommodate both the variability in pigmentation patterns over time and space, and the diverse range of triggers.

Chalker-Scott (1999, 2002) postulated that the plant must benefit from anthocyanins, as energy is needed for their production and degradation. Anthocyanins may, in fact, have developed various functions over evolutionary time. Gould et al. (2002) suggested that anthocyanins confer a phytoprotective role, rather than being the default end-product of a saturated flavonoid metabolism. However, the often proposed function of anthocyanins as protectants from ultra violet (UV) light is

under debate, as they absorb poorly in the UVA and UVB regions (Jorgensen 1994). Anthocyanins are less effective UV-protectants than other flavonoids, and in many leaves anthocyanins are not optimally located for UVB screening (Lee and Gould 2002).

A more recently proposed phytoprotectant role is that anthocyanins intercept high-energy quanta that would otherwise be absorbed by chlorophyll b (Gould et al. 2002). The formation of anthocyanins in peripheral cell layers in seedlings may therefore act as an internal regulatory agent, controlling the amount of light reaching the photosynthetic cells (Drumm-Herrel and Mohr 1985, Beggs et al. 1987, Stafford 1991) in proportion to the anthocyanin concentration (Neill and Gould 1999). By absorbing quanta that would otherwise be intercepted by chloroplasts, anthocyanins may reduce both the requirements for non-photochemical quenching and the structural damage associated with chronic photoinhibition under high light intensity and low temperature (Lee and Gould 2002). For example, Gould et al. (2000) believed that anthocyanins in leaves of *Quintinia serrata* may serve to protect shade-adapted chloroplasts from brief exposure to high intensity sunlight.

Recent evidence favours the photoprotective role of anthocyanins in senescing and juvenile leaves (Drumm-Herrel and Mohr 1985, Steyn et al. 2002). Field et al. (2001) and Hoch et al. (2001) proposed that autumnal anthocyanins protect senescing foliage from photoinhibitory irradiances, allowing the resorption of foliar nutrients to occur. Evidence also supports a photoprotective function in plants experiencing environmental stress (Field et al. 2001, Hoch et al. 2001, Steyn et al. 2002). For example, young expanding leaves are more susceptible to photoinhibition and photobleaching of photosynthetic pigments than mature leaves (reviewed in Steyn et al. 2002). The photosensitivity of young leaves is restricted to an early phase of development prior to intense greening, and the leaves may form protective pigments only when necessary, and only to the extent required for protection (Drumm-Herrel and Mohr 1985). Thus, anthocyanin production in young leaves could be a means of photoprotection during the early phase of development of the photosynthetic apparatus (Drumm-Herrel and Mohr 1985), although the total flavonoid content is probably more important for protection from photoinhibition than levels of individual anthocyanins, flavones and flavonols (Gould et al. 2000). This may explain the widespread control of juvenile anthocyanin synthesis by light (Drumm-Herrel and Mohr 1985). Photoprotection is probably not a universal function of anthocyanins in all leaf tissue, as this feature is unlikely to be important under the reduced light environment within a tropical forest (Steyn et al. 2002).

The presence of anthocyanins in leaves could also confer a significant antioxidant advantage. Anthocyanins may function as general protectors against oxidative damage by scavenging reactive oxygen species (Gould et al. 2002). These reactive oxygen species are produced as a result of biotic

and abiotic stimuli that induce anthocyanin synthesis, including drought stress, extremes of temperature, heavy metal toxicity, salinity, UV radiation, ozone exposure, herbicides and pathogen attack (Gould et al. 2002, Lee and Gould 2002, Steyn et al. 2002). For example, the red leaves of *Elatostema rugosum* were more effective scavengers of free radicals and reactive oxygen species than green leaves, and thus were better at combating oxidative stress (Neill et al. 2002). A problem with this hypothesis for the antioxidant role of anthocyanins is that anthocyanins are not optimally located within the plant cell for reactive oxygen scavenging (Gould et al. 2002).

Many evergreen perennials synthesise foliar anthocyanins during the winter months, but species lose their red colouration in the warmer months (Chalker-Scott 2002). Anthocyanins may confer resistance to water and cold stress by osmotic adjustments of the vacuolar sap, thus inducing drought resistance and cold tolerance (Chalker-Scott 1999, Chalker-Scott 2002). Chalker-Scott (2002) hypothesised that low temperature exposure could induce a small but significant increase in stress resistance by osmotic regulators, with anthocyanins in the vacuole depressing the freezing point.

In addition to photoprotection, antioxidant activity and osmoregulation, other minor functions of foliar anthocyanins have been proposed, including attracting pollinators and fruit dispersing birds, deterring herbivores from feeding on the foliage, increasing leaf temperature, drought resistance, cold tolerance, chelating heavy metals, and defending the plant against plant pathogens (Mazza and Miniati 1993, Forkmann 1994, Holton and Cornish 1995, Chalker-Scott 1999, Hoch et al. 2001, Chalker-Scott 2002, Lee 2002b, Lee and Gould 2002). Both Chalker-Scott (1999) and Lee and Gould (2002) believe that anti-pathogen and anti-herbivore roles of anthocyanins are speculative. In fact, leaf-damaging insects and fungal pathogens may affect the functioning of the photosynthesis apparatus, thereby resulting in photoinhibition and increased levels of anthocyanins (Stone et al. 2001, Lee and Gould 2002).

2.3.6 Genes involved in biosynthesis of anthocyanins

The production of anthocyanins in a particular tissue is determined by the activity of the structural genes that, in turn, are controlled by the expression of specific regulatory genes (Davies and Schwinn 1997). Structural genes include those that are responsible for synthesis of the anthocyanidins, as well as hydroxylation, methylation, glycosylation and acylation of the anthocyanidin molecules. Other genes can influence colouration by controlling copigmentation, the pH of the vacuole and the interaction of flavonoids with metal ions and distribution of pigmented cells (Forkmann 1994). The anthocyanin synthesis pathway is highly regulated in response to developmental and environmental cues, mainly at the level of transcription (Winkel-Shirley 2002).

There is strong evidence to suggest that flavonoid enzymes can be assembled into a membrane-bound macromolecular complex, which may provide an important mechanism for controlling pathway flux and determining the relative levels of specific end-products (Winkel-Shirley 2002). The genetics of flavonoid biosynthesis have been studied extensively in maize (*Zea mays*), snapdragon (*Antirrhinum majus*) and petunia (*Petunia hybrida*) (reviewed in Holton and Cornish 1995). Mutants have been used to identify genes that are involved in anthocyanin production.

Studies using mutants have helped to identify several of the structural genes involved in the basic biosynthetic reactions leading to the different flavonoid classes (Fig. 2.1). Mutations include blocks in the early steps of biosynthesis leading to uncoloured tissues or blocks in later steps, which then result in changes in the nature of particular anthocyanins formed (Forkmann 1994). In most cases the structural genes are replicated (Mol et al. 1996). For example, most angiosperms possess multiple chalcone synthase genes.

The distribution of anthocyanins within organs and tissues is generally determined by tissue-specific expression of regulatory genes (Steyn et al. 2002). These genes control the expression of structural genes in response to environmental and developmental triggers (Forkmann 1994, Holton and Cornish 1995, Mol et al. 1996). They determine the location, timing, and amount of anthocyanin expression and can switch the whole pathway or parts of the pathway on or off. These regulatory genes can also influence flavonoid concentration by enhancing or reducing pigment levels by enzymatic or chemical degradation, or by preventing pigment accumulation altogether. They also control the pattern of pigment expression within leaves (Forkmann 1994). Mutations of regulatory genes that cause an increase or decrease in or a modified distribution of flavonoid production within the flower or plant have also been described (Martin and Gerats 1993).

In most species, anthocyanin regulatory genes are replicated (Mol et al. 1996). There are probably distinct regulatory mechanisms for the genes required in the early biosynthetic steps leading to flavonols and the precursors of proanthocyanidins and anthocyanins, and for those catalysing later steps (reviewed in Mol et al. 1996). In addition to regulation of the pathways in response to specific internal and external cues, flavonoid biosynthesis also seems to be sensitive to the rate of flux through the system and the accumulation of phenylpropanoid intermediates. The pattern of anthocyanin accumulation is controlled mainly by the tissue specificity of the expression of the transcription factors that directly interact with the promoters of the target anthocyanin genes. Regulatory alleles with different expression levels and sensitivity to light have been described (Mol et al. 1996).

Regulatory genes have been well documented in maize. The regulatory genes consist of the duplicate genes *R* (which includes *R, S, Lc,* and *Sn)* and *B* loci (Dooner et al. 1991). The *Lc* gene of the *R*

family regulates the genes for leaf colour. Accumulation of anthocyanins also requires the presence of a member of the *C1/Pl* gene family. Each gene determines pigmentation of different parts of the plant. Both *R (Lc)* and *C1* are necessary to produce leaf pigmentation (Quattrocchio et al. 1993).

2.3.7 Inheritance of leaf colour

Many of the structural genes, which have been discussed previously (section 2.3.2), are common to different species (Goodrich et al. 1992, Forkmann 1994, Davies and Schwinn 1997). Single genes have been correlated with particular structural alterations in the anthocyanin molecule or with the presence / absence of particular flavonoid types (Forkmann 1994). For example, in blueberry plants the lack of red pigment in the leaves, buds and fruit is controlled by a recessive allele (Lyrene 1988). Red leaf colour in peach is controlled by a single locus, designated *Gr*. The *Gr* allele is incompletely dominant and the *GrGr, Grgr* and *grgr* genotypes are red, light-red and green leaved, respectively (Chaparro et al. 1995). Wilcox (1982) hypothesised that seedling anthocyanins in *Eucalyptus fastigata* are controlled by a single segregating locus, with purple colouration a dominant trait.

The production of coloured pigments in maize, snapdragon and petunia is dependent on the particular regulatory gene being dominant (Dooner et al. 1991). The known exceptions to this dominant condition are the negative regulators of pigmentation—the *C1-I* allele in maize and the *Elute* allele in snapdragon (Dooner et al. 1991). Seeds of maize containing the dominant allele of *C1*, together with the dominant alleles of other genes in the anthocyanin pathway, are deeply pigmented. On the other hand, seeds that have the same genetic constitution but are homozygous for the recessive allele *c1*, are colourless (Cone et al. 1986).

The inheritance of specific colours in *Petunia hybrida* flowers can be explained through the combined inheritance of anthocyanin pigmentation and vacuolar pH. Anthocyanin pigmentation is controlled by multiple independent genes (*Hf* and *Mf*) that follow simple Mendelian genetics (Griesbach 1996). The inheritance of vacuolar pH is more complex, as it is controlled by two independent codominant genes (*Ph1* and *Ph2*) and is influenced by the cellular environment. Linkage of the various pH and anthocyanin genes represents the expression of all of the potential gene combinations (Griesbach 1996).

2.4 Conclusions

Acacia baileyana has been cultivated for more than 120 years. Four novel forms have recently been described, and these differ in leaf colour and growth habit to the common green leaf form. Interestingly, it is only the green leaf form that has been recorded as a weed in Australia, as well as in

several other countries. Even though the foliage and flowers of *A. baileyana* are already used to a small extent by florists within Australia, little has been done to increase the success of the species as a horticultural commodity. *Acacia baileyana* has been identified as a potential species to be further developed for its cut foliage and cut flowers (Horlock et al. 2000), and as a indoor flowering pot plant (Parletta and Sedgley 1998). This potential will only be realised if improved cultivars or new hybrids are developed and if the mechanisms controlling flowering and expression of leaf colour can be manipulated.

The development of novel cultivars and hybrids require knowledge of the breeding system of the species. Descriptive aspects of the floral biology and seed production of *A. baileyana* have been reported by Newman (1933, 1934a, 1934b, 1935) in the 1930s. More recent studies on other *Acacia* species have looked at polyad and ovule numbers (Kenrick and Knox 1982), stigma receptivity (Sinha 1971, Knox et al. 1989, Sedgley 1989, Sedgley and Harbard 1993), and self incompatibility (Moffett 1965a, Bernhardt et al. 1984, Kenrick et al. 1986, Kenrick and Knox 1989). Because the genus *Acacia* contains at least 1350 species (Maslin 2001, Maslin et al. 2003), variations in breeding systems are likely to exist. For example, variations occur in the level of self incompatibility (Moffett 1965a, Bernhardt et al. 1984, Kenrick et al. 1986, Kenrick and Knox 1989). It is therefore, important to undertake studies on the breeding strategy of *A. baileyana*, for the production of new cultivars.

Understanding aspects of floral biology are essential for the manipulation of flowering. This includes studies on the environmental requirements of flowering, so that flowering may be manipulated to coincide with key festivals. Recent studies on a few species of *Acacia* (Sedgley 1985, Parletta and Sedgley 1996) showed that flowering was affected by changes in temperature and light. *Acacia baileyana* may have a similar response, but this requires demonstration.

An understanding of leaf colour pigments of *A. baileyana* is necessary for manipulating colour, as well as breeding new colour cultivars. Variations in leaf colour of a horticultural product may increase the market range. Even though there are four different juvenile leaf colour forms of *A. baileyana* (green, purple, red and golden), nothing is known about the factors that affect the expression of colour, the types of pigments present, or the inheritance of colour.

Development of *A. baileyana* as a horticultural commodity cannot be considered without taking into account its weed status. Indeed, many investigations on *A. baileyana* to date have concerned this aspect (Ross 1975, Milton and Moll 1982, Mulvaney 1991, Smith 1993). These studies have suggested that *A. baileyana* is a weed due to its rapid growth, early flowering, seed dispersal by ants, frequent fire activity stimulating seed germination and limited susceptibility to damage from pests.

However, no one has yet considered total seed production in the species. Success as a weed may result in part from heavy seed production.

Studies on leaf colour, flowering and breeding system is essential for future commercial development of *A. baileyana*. An understanding of its weed status is also important for the appropriate management of cultivated plants.

The thesis is organised under the following chapters to investigate the fertility and leaf colour, and to determine the ornamental and weed potential of *A. baileyana*.

Chapter 3 – Environmental control of flowering

Chapter 4 – Breeding system and reproductive efficiency

Chapter 5 – Flower and seed production

Chapter 6 – HPLC methods for anthocyanin identification

Chapter 7 – Purification and identification of anthocyanins

Chapter 8 – Colour categories to describe leaf colour

Chapter 9 – Expression and inheritance of purple leaf colour

Environmental control of bud formation and flowering of clonal *Acacia baileyana*[1]

3.1 Introduction

Acacias with attractive flowers and foliage may be suitable as cut stems and as indoor flowering pot plants if vegetative growth and flowering can be controlled. An ideal flowering pot plant is one that is compact, and will flower in excess of 14 days under typical room conditions, at a temperature of 20°C under low light (Lamont 1987). Dwarfing of some plant species has been achieved using chemical growth regulators, removal of the terminal bud, or by growing plants under high night temperatures (Larson 1985, Armitage 1988, Moe 1990, Moe et al. 1991). Some species of *Acacia*, including *A. baileyana*, will produce a dwarf form in response to the growth retardant paclobutrazol, but this does not stimulate flowering as has been found for some other plants (Parletta and Sedgley 1996). Studies have been conducted to induce flowering of pot plants through environmental and chemical control (Bodson 1983, Armitage 1988, Bunker 1995, Podwyszyńska et al. 2000). Flowering cut stems of a range of genera have also been produced following environmental manipulation (Zieslin and Mor 1990, Kofranek 1992, Shillo and Ronen 1997).

The ability to advance or delay the flowering period of cut stems and pot plants is an advantage in targeting key markets. In western cultures, flowering needs to be synchronised for celebrations, including Easter, Mother's Day and Christmas. An understanding of the control of the flowering process is required in order to manipulate the time of flowering. Under natural conditions, *A. baileyana* racemes appear in late December and flower from late June to early September (Boden 1969). Water availability, nutritional status, temperature and light all influence the flowering of acacias (Buttrose et al. 1981, Sedgley 1985, Sedgley 1989, Sedgley and Griffin 1989).

While there appears to be little or no effect of photoperiod on flowering of *Acacia* (Sedgley 1985), it

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[1] Morgan A, Sedgley M (2002) Environmental control of bud formation and flowering of clonal *Acacia baileyana* F. Muell. for ornamental horticulture. *Australian Journal of Experimental Agriculture* **42**, 211–216 (Appendix 3)

has been suggested that lowering the temperature at anthesis may be used to induce out-of-season flowering, as a number of acacias only flower when grown at low temperatures (Sedgley 1985, Parletta and Sedgley 1996). It was found for *A. pycnantha* that high temperature and low light intensity inhibited flower development, although bud formation was not affected by temperature (Sedgley 1985). Initiated buds developed and opened only when the temperature was lower than 19°C, allowing meiosis to occur. Similar results were obtained for *A. drummondii* ssp. *elegans*; floral initiation occurred at both 15°C day / 10°C night (15/10°C) and 25°C day / 20°C night (25/20°C) temperatures, but anthesis occurred only under the cooler conditions (Parletta and Sedgley 1996). Flower buds at 25/20°C had small, under-developed anthers. *Acacia buxifolia* flowered only at 15/10°C, but *A. glaucoptera, A. acinacea* and *A. myrtifolia* flowered at both 15/10°C and 25/20°C, although the number of flowers was lower at 25/20°C. Following transfer from 15/10°C to 25/20°C, flowering was reduced, whereas flowering increased following transfer in the opposite direction.

Studies on acacias to date have been conducted with seed-propagated material. Seedlings go through a non-flowering juvenile phase that generally varies between one to ten years, depending on species and environment (Sedgley and Griffin 1989). Some seed-propagated acacias grown in controlled environments can flower within 17 months (Parletta and Sedgley 1996). Seed propagated *Acacia baileyana* grown outside can flower within two years, but variability is high, leading to inconsistency in yield and quality (Sedgley, personal communication).

The aim of this research was to induce out-of-season flowering of *A. baileyana* by manipulating temperature and light conditions. The timing of inflorescence bud formation and flowering was recorded for the green and purple leaf forms, which had been propagated from cuttings, and grown under five temperature / light regimes.

3.2 Materials and methods

3.2.1 Plant material

Terminal semi-hardwood cuttings were used to obtain clonal material. Over 3000 cuttings were collected from four purple leaf form and four green leaf form adult plants growing in gardens in the Adelaide Hills, South Australia. Four defined colour categories were used to classify the colour of each leaf sample. The colour categories were established by examining (by eye) the youngest, fully-expanded leaves of *A. baileyana*, and, based on the range of visible colour observed, the following were defined: category 1 (pure green), category 2 (between a trace and approximately half maximal purple colouration), category 3 (more purple colour than those in category 2, but not totally purple)

and category 4 (intense purple) (Plate 3.1A). Between three and five leaves were assessed per plant. Refer to Chapter 8 for validation of the four colour categories. The mother plants were seed-propagated and therefore genetically heterogeneous, and grown in a similar climate and soil type. Plate 3.1B is an example of flowering stems of the purple (colour category 4) and green (colour category 2) leaf forms.

3.2.2 Propagation of stem cuttings

The cutting material was collected on 24 and 25 February 1997 (Table 3.1) and processed using the method of Schwarz et al. (1999). The fresh tips, pods and lower two thirds of leaves were removed from the cuttings, and trimmed to 12 cm. The cuttings were dipped into 10% sodium hypochlorite for 1 min and rinsed in reverse-osmosis water. The lower 10 mm of cuttings were dipped for 5 s in 5000 mg/L indolebutyric acid (IBA) in 50% ethanol and air-dried for 30 s. Ten cuttings were planted per pot (Glocke and Sedgley 1995), each 15 cm in diameter (2 L in volume) and containing a 1:1:1 mix of sterilised peat: perlite: sand.

Table 3.1 Timing of experimental treatments. LH, low to high temperature (from 13/9°C to 23/19°C); HL, high to low temperature (from 23/19°C to 13/9°C); MM, constant intermediate temperature (18/13°C).

Experimental stage	Date
Cutting material collected and propagated	24 and 25 February 1997
Rooted cuttings potted up	27 May 1997
Cuttings placed into environmental treatments	1 August 1997
Cuttings exchanged between temperature regimes for treatments LH and HL	6 January 1998
Cuttings from treatments LH, HL and MM taken outside	15 June 1998
Experiment completed	30 September 1998

The pots were placed in a mist propagator with 30% shade, at a base temperature of 20–25°C, located in a glasshouse, for 13 weeks. Misting was for 6 s every 20 min for 12 h during the day with no misting during the night. Cuttings were irrigated about three times per week. The glasshouse temperature was 25–28°C during the day and 12–15°C during the night. The cuttings were harvested on 27 May 1997, and the number of plants with roots was recorded (Table 3.2). Individual cuttings with roots were transplanted into 10 cm diameter pots containing native plant soil mix [2:1, composted

Plate 3.1 Colour of *A. baileyana* juvenile leaves. A: The four colour categories and Royal Horticultural Society colour charts for leaves of *A. baileyana*. Category 1 is pure green, category 2 is between a trace and approximately half maximal purple colouration, category 3 is more purple colour than those in category 2, but not totally purple and category 4 is intense purple. The left colour chart is green group 138, while the right colour chart is purple group 79. Scale bar = 2 cm. B: Flowering stems of the purple leaf form (left, colour category 4), and the green leaf form (right, colour category 2). Scale bar = 2.5 cm.

Table 3.2 Success of cutting propagation of several genotypes of *A. baileyana*. Cutting material propagated on 24 and 25 February 1997.

Genotype	Colour	Colour category	Number of cuttings	Cuttings with roots (27/5/97)		Cuttings with new shoots and leaves (27/5/97)		Cuttings with new shoots and leaves (1/8/97)	
				No.	%	No.	%	No.	%
1	Purple	4	330	137	42	99	30	96	29
2	Purple	4	370	128	35	67	18	52	14
3	Purple	4	440	196	45	107	24	97	22
4	Purple	4	350	78	22	21	6	12	3
Total (average)	Purple		1490	539	(36)	294	(20)	257	(17)
5	Green	2	260	158	61	153	59	18	7
6	Green	2	360	76	21	29	8	18	5
7	Green	2	380	76	20	48	13	23	6
8	Green	2	600	66	11	23	4	5	1
Total (average)	Green		1600	376	(28)	253	(21)	64	(5)
Total (average)	Purple + green		3090	915	(32)	547	(20)	321	(11)
P-value									0.071

pinebark: coarse white sand with 0.8 g/L iron sulphate, 1 g/L dolomite, 0.5 g/L ground lime, 0.5 g/L gypsum, and 3 g/L low phosphorus Osmocote®, at pH 6] and grown in a glasshouse. The number of cuttings with new shoots and leaves was noted on 27 May and 1 August 1997. The percentage of cuttings with new shoots and leaves at 1 August 1997 was analysed by ANOVA assuming a completely randomised design.

3.2.3 Experimental treatments

On 1 August 1997, rooted cuttings sourced from the six most successful mother plants were allocated to five environmental treatments (Table 3.2). For the green leaf form, 18 cuttings from each of two mother plants (Genotype 5 and 6) and 23 cuttings from another mother plant (Genotype 7) were available for the experiment (Table 3.2). For the purple leaf form, 96 (Genotype 1), 52 (Genotype 2) and 97 (Genotype 3) cuttings were available for the experiment.

Approximately 60 of the cutting-propagated plants from the six genotypes were used in each of the following treatments: L, low temperature growth cabinet (13.0°C/9.0°C; 13/9°C); M, medium temperature growth cabinet (18.3°C/13.3°C; 18/13°C); H, high temperature growth cabinet (23.3°C/19.3°C; 23/19°C); outside at the Waite Campus, The University of Adelaide, South Australia; and shadehouse at the Waite Campus. These growth cabinet temperatures were similar to those used by Parletta and Sedgley (1996) for the production of *Acacia* pot plants.

Plants within each treatment were arranged in a completely randomised design. Plants from the growth cabinets were transferred between cabinets on January 1998, after about five months treatment. The transfer was from either a low (L) to high (H) temperature (from 13/9°C to 23/19°C; LH), from a high (H) to low (L) temperature (from 23/19°C to 13/9°C; HL) or the plants were kept at a constant intermediate (M) temperature (18/13°C; MM; Table 3.3). The time of transfer coincided with a medium bud size for the HL treatment. Plants were transferred from high to low temperatures, as this has been found to increase flowering for some *Acacia* species (Parletta and Sedgley 1996). Plants were transferred from the cabinets to the outside environment on 15 June 1998, and were maintained there until 30 September after the end of the natural flowering period (Table 3.1).

The 12 h per day light source for the growth cabinets was metal halide lamps (spectrum 280–800 nm). The mean photosynthetic photon flux density for the three growth cabinets was 189 ± 38 μmol m^{-2}s^{-1}, measured by a LICOR quantum sensor (Model LI-185A, LICOR Instruments, Nebraska). Temperature and light conditions for the outside treatment are shown in Table 1 in Appendix 2 for 1997 and 1998. The outside temperature, daylength hours and solar radiation were measured by a weather station located at the Waite Campus, The University of Adelaide. The mean monthly solar

radiation from August 1997 to September 1998 was 15 MJ m^{-2}, which was equivalent to a photosynthetic photon flux density, assuming a flat spectral distribution curve of 400–700 nm, of 704 μmol m^{-2}s^{-1}. The shadehouse, consisting of black shadecloth, excluded 75% of the incident sunlight. The temperature and daylength during the period of anthesis for the outside treatment was determined from the nearby weather station. The shadehouse was considered to have similar temperature and daylength conditions to outside. Plants were fertilised with full strength Thrive® once per month.

Table 3.3 Temperature and daylength for the five treatments.

Treatment	Mean max./ min. temperature (°C)			Average daylength (h)	
	1 Aug. – 6 Jan.	7 Jan. – 15 June	16 June – 30 Sept.	1 Aug. – 15 June	16 June – 30 Sept.
LH	13/9[w]	23/19	16/9	12	11
HL	23/19[x]	13/9	16/9	12	11
MM	18/13[y]	18/13	16/9	12	11
Outside	21/12	22/13	16/9	12.5	11
Shadehouse[z]	21/12	22/13	16/9	12.5	11

[w] 13/9; 13.0 ± 0.2°C day and 9.0 ± 0.2°C night
[x] 23/19; 23.3 ± 0.3°C day and 19.3 ± 0.5°C night
[y] 18/13; 18.3 ± 0.3°C day and 13.3 ± 0.3°C night
[z] Outside temperature and daylength

3.2.4 Measurements

All plants were monitored for leaf colour, presence of inflorescence buds and flowers. Bud formation and flowering were recorded following transfer of plants between environments on 6 January 1998 until October 1998, every two weeks. The presence of buds and flowers were noted for each plant to investigate the timing of bud formation and flowering. The size of the inflorescence buds was also recorded, as small (buds tight together and green in colour), medium (green in colour) or large (green to yellow in colour) (Plate 3.2C). Percentage flowering was recorded as the number of plants with flowers. The proportions of plants flowering in each treatment was analysed using ANOVA. The date of peak flowering was determined as the maximum number of plants flowering at one recording time, while the period of anthesis was the period that the plants flowered.

Plate 3.2 Photographs taken on 23 March 1998, showing stages of bud formation and flowering in the different environmental conditions. A: From left to right, plants grown at 23/19°C (H), 18/13°C (M), 13/9°C (L). Scale bar = 4 cm. B: Plants from shadehouse (left) and outside (right). Scale bar = 3 cm. C: From left to right, racemes from treatments: HL (from high to low temperatures; flowering inflorescences), MM (constant intermediate temperature; two racemes; large inflorescences), LH (from low to high temperature; small inflorescences), shadehouse (medium inflorescences) and outside (medium inflorescences). Scale bar = 1.3 cm. Note that some inflorescences have dropped from the MM treatment.
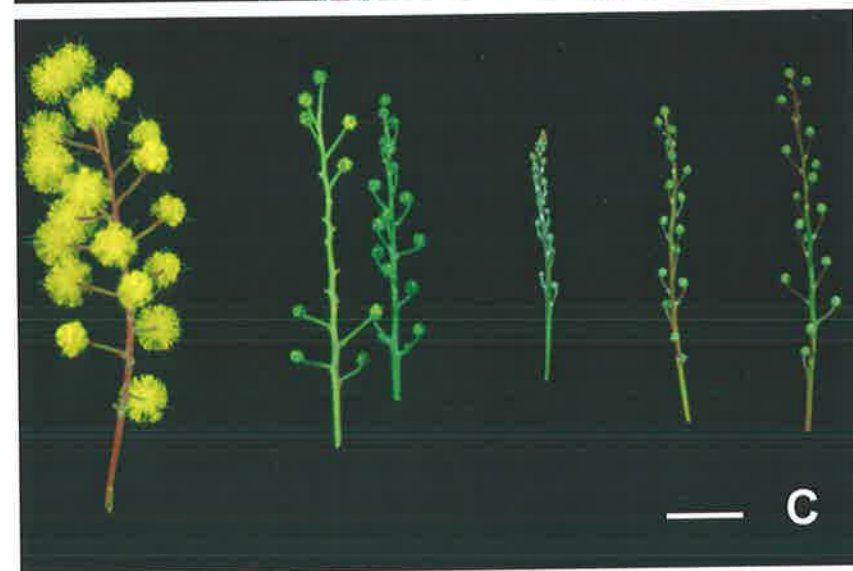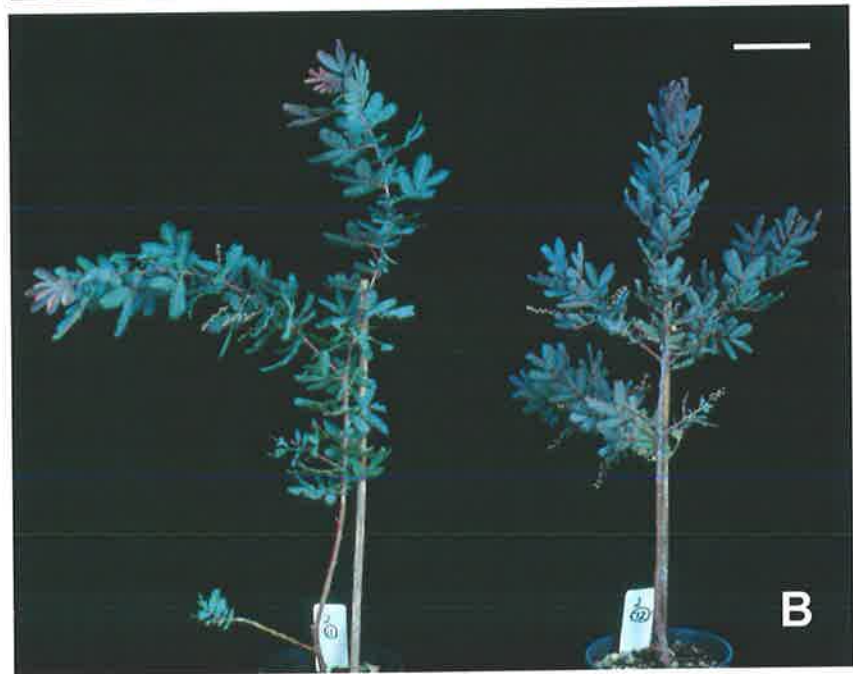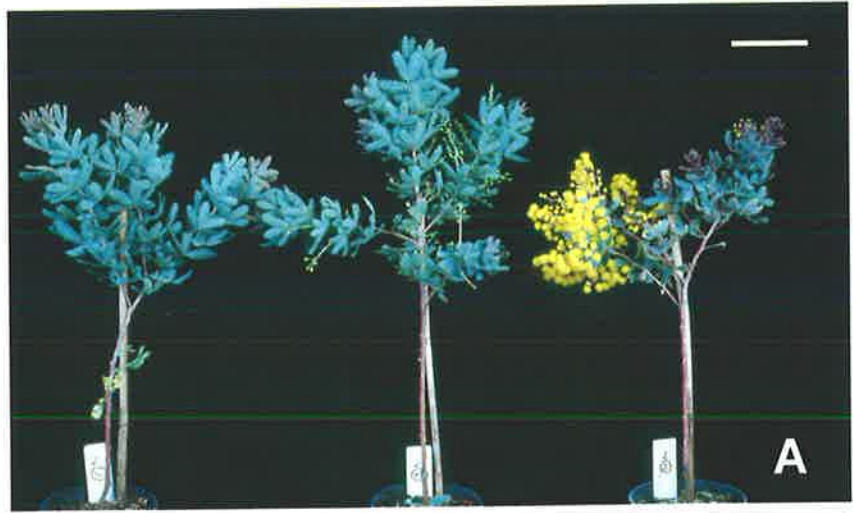
3.3 Results

3.3.1 Success of cutting propagation

Only 20% of cutting material initiated new shoots and leaves in May, even though 32% of the material produced roots (Table 3.2). The percentage of cuttings with new shoots and leaves fell to 11% after a further two months through shoot death. Three of the four purple genotypes produced between 14 and 29% cutting survival. The green leaf genotypes had less than 7% survival, and high mortality from June to August, when survival fell from 21 to 5%. There was no significant difference at the 5% level ($P = 0.071$) between the proportion of purple and green cuttings producing new shoots and leaves in August. Only three purple and three green leaf forms were used in the experiment, as genotype 4 (a purple form) and genotype 8 (a green form) had poor rooting success (Table 3.2).

3.3.2 Bud formation

Plants in treatment LH formed buds only when transferred to the high temperature of 23/19°C (Table 3.4). Plants grown at other temperatures formed buds before 6 January 1998 (Table 3.4, Table 3.5). The mean maximum and minimum temperatures at bud formation were similar in all treatments, except in MM, in which they were lower (Table 3.4). Temperatures at or above 18/13°C appeared to be required for bud formation. Daylength hours at bud formation were longer for plants outside and in the shadehouse than for the growth cabinet plants.

3.3.3 Floral development

The duration and timing of inflorescence development was influenced by the five environmental treatments (Table 3.5). All genotypes within a treatment were at the same stage of bud formation. A difference in floral development was observed for four out of the five treatments, with outside and shadehouse plants having similar pattern of floral development (Table 3.5). Plants grown in the MM and the LH treatments flowered over the same period (August to September) even though bud formation occurred at different times (Table 3.4). Some inflorescences dropped from plants held in the MM and the LH treatments (Table 3.5, Plate 3.2C).

3.3.4 Anthesis

Plants that had been grown outside and in the shadehouse flowered from June to late August, while plants in the HL treatment flowered four months earlier, from March to May (Table 3.4, Table 3.5, Plate 3.2). In contrast, the plants in the LH and MM treatments flowered only when placed outside

Table 3.4 Effect of environmental conditions on the timing of bud formation and flowering of *A. baileyana*. Plants were taken outside on 15 June 1998. LH, low to high temperature (from 13/9°C to 23/9°C); HL, high to low temperature (from 23/19°C to 13/9°C); MM, constant intermediate temperature (18/13°C). Values followed by the same letter are not significantly different at *P* = 0.005.

Treatments	LH	HL	MM	Outside	Shadehouse
Bud formation					
Date of bud formation	24 Feb.	Before 6 Jan.	Before 6 Jan.	Before 6 Jan.	Before 6 Jan.
Temperature at bud formation (mean max./min. °C)	23/19	23/19	18/13	25/14	25/14[x]
Daylength at bud formation (h)	12	12	12	14.5	14.5
Flowering period					
Date of peak flowering	10 Sept.	1 Apr.	2 Aug.	29 July	29 July
Advancement (+) or delay (-) in flowering as compared to outside (days)	-44	+120	-5	0	0
Period of anthesis (days)	2 Aug.–16 Sept. (46)	12 Mar.–7 May (57)	2 Aug.–16 Sept. (46)	26 June–26 Aug. (62)	15 June–26 Aug. (73)
Temperature at anthesis (mean max./min. °C)	16/9	13/9	16/9	15/8	15/8[x]
Daylength at anthesis (h)	11	12	11	10	10
Percentage of plants that flowered [y]	42 ± 0.10 a,b	55 ± 0.08 a	19 ± 0.07 c	27 ± 0.07 b,c	15 ± 0.08 c

[x] Outside temperature
[y] Values are mean ± s.e.

from August to September. The mean maximum and minimum temperature at anthesis were similar for all treatments, with plants flowering when the temperatures were at or below 16/9°C. The daylength hours varied from 10 to 11 in most of the treatments, except for plants in HL treatment, which had 12 h of daylight.

There was no significant difference between the green and purple leaf form cuttings in the proportion that flowered ($P = 0.35$). However, there was a significant difference ($P = 0.005$) between treatments in the proportion of cuttings that flowered (Table 3.4). The most successful environment for flowering was the HL treatment, where 55% flowered, and then LH (42% flowered), whereas only 27% of plants flowered in the outside treatment (Table 3.4). Fewer plants flowered in the shadehouse and MM treatments.

Table 3.5 Effect of environmental conditions on the timing of inflorescence bud development and inflorescence growth in *A. baileyana*. LH, low to high temperature (from 13/9°C to 23/19°C); HL, high to low temperature (from 23/19°C to 13/9°C); M, constant intermediate temperature (18/13°C). L, large inflorescences, green to yellow in colour; M, medium inflorescences, green in colour; S, small inflorescences, buds tight together and green in colour; F, flowering.

Treatments	Jan.	Feb.	Mar.	Apr.	May	June[x]	July	Aug.	Sept.
HL	L	L	F	F	F	–	–	–	–
LH	–	S	S	M	M[y]	M	L	F	F
MM	S, L	S, L	S, M, L[y]	S, M, L[y]	S, M[y]	S, M	L	F	F
Outside	S	M	M	M	L	F	F	F	–
Shadehouse	S	M	M	M	L	F	F	F	–

[x] Plants were taken from growth cabinets to outside on 15 June, 1998.
[y] Buds dropped from the larger inflorescences.

3.4 Discussion

Peak flowering of one-year-old *A. baileyana* cuttings was advanced by four months, by exposing plants to warm then cool temperatures. Twice the number of plants flowered under this temperature regime compared with outside plants. This is a substantial change to flowering time and success, with important implications for cut stem and flowering pot plant production. If the transition from the hot to cool conditions was a month later, the peak flowering may occur closer to Mother's Day, in early May. The potential therefore exists to synchronise flowering with a major celebration.

It has been proposed that the flowering of acacias involves a number of steps that are sensitive to varying environmental conditions (Sedgley 1986). The results of the current study support this

hypothesis. *Acacia baileyana* grown outside formed flower buds in summer when vegetative growth was the greatest and then flowered in late June. Plants required warm temperatures for bud formation (at or above a mean maximum of 18°C and a minimum of 13°C), and cool temperatures for flowering (at or below a mean maximum of 16°C and a minimum of 9°C). Bud formation was inhibited at the low temperature of 13/9°C. Only plants from the HL treatment flowered in the growth cabinet, while plants growing at MM and LH treatments flowered only when taken outside to a cooler temperature. Thus, buds formed under high temperature conditions proceeded to anthesis only when transferred to a cooler environment. Therefore, there is a different temperature requirement for bud formation than for flowering. Bud formation in *A. pycnantha* (Sedgley 1985) and *A. drummondii* ssp. *elegans* (Parletta and Sedgley 1996) on the other hand, did not appear to be influenced by temperature, but cool temperatures were required for flowering. Therefore, high temperatures may entirely or partially block flowering of *Acacia*. King and Gocal (1999) suggested that low temperature may be the passive condition permitting flowering.

Temperature during bud development was also critical for flowering, as large buds dropped when the temperature was higher than 18/13°C. For example, new buds from the MM treatment developed constantly but only 19% of these plants flowered outside. This low percentage of flowering was due to the older buds dropping. Only the younger buds developed and flowered when placed outside in the cooler environment. Some buds also aborted in the LH treatment, which would explain the lower percentage of flowering compared to the HL treatment. Bud drop may have occurred for plants in HL treatment but this was not recorded, as measurements were made after 6 January 1998. In *A. pycnantha*, meiosis appeared to be a very sensitive stage and was inhibited by temperatures over 19°C, causing buds to drop (Sedgley 1985, Sedgley 1989). This may also occur in *A. baileyana,* with temperatures at about 18/13°C and higher inhibiting meiosis. It would therefore be important to change between warm and cool conditions at the medium bud stage, before buds start to drop, to obtain the highest possible number of flowering plants.

There was no difference in flowering success between the two leaf colour forms, although the success of flowering was highly variable between treatments, ranging 15–55%. This difference could not be solely attributed to temperature at bud formation and anthesis, and may also be due to differences in light intensity and quality. Temperatures at bud formation and anthesis were very similar in plants grown outside and in the shadehouse; however, about twice the number of outside plants flowered. The decrease in flowering in the shadehouse is probably due to the 75% reduction in light intensity under the shadecloth. Shading may reduce photosynthesis, so that fewer assimilates are available for flower production (Sedgley 1989). Sedgley (1985) noted that a similar level of light inhibited floral development at an early stage in *A. pycnantha*. Differences in the number of plants flowering between

the HL and outside treatments could be due to differences in light quality and intensity, although fluctuations in temperature for the outside plants may be a factor. Photoperiod does not seem to affect the flowering of most woody plants, but may affect flowering in conjunction with temperature. This has been shown for *Chamelaucium uncinatum* (Geraldton wax) (Shillo and Ronen 1997). Future studies could, therefore, investigate the combined affect of temperature and photoperiod on the flowering of *A. baileyana.*

The temperature at which *A. baileyana* plants are grown will have an effect on flowering success. Newman (1934a) noted that *A. baileyana* in Australia set abundant fruit on an inland plateau at 600 m, but on the coast it flowered earlier and set no fruits. Similarly in South Africa, this species aborted its buds in the south-western Cape but flowered and set fruits at Pretoria at 1500 m altitude (Milton and Hall 1981). This difference in flowering at the different altitudes may be due to different temperature conditions affecting flower development.

The cutting propagation method needs to be improved if flowering pot plants are to be produced commercially. Only 32% of cuttings had roots after three months, which can be compared with 39% of *A. baileyana* cuttings having roots after two months in a study by Schwarz et al. (1999). Taking cuttings at a different time of the year may increase cutting success. Schwarz et al. (1999) compared the effect of genotype and IBA on adventitious root formation in stem cuttings of *A. baileyana* in autumn (late February) and spring (late August). They found the autumn cuttings rooted significantly more frequently than the spring cuttings, at 5000 ppm IBA. To improve commercial cutting production, superior, high rooting mother plants should be selected. These mother plants should be kept at a juvenile, vegetative stage, under favourable conditions, to increase rooting success.

Flowering may be induced at various times of the year by controlling the time when the plants are placed at different temperature regimes. Future research could aim to produce good quality flowering cut stems and indoor flowering pot plants of *A. baileyana* for Mother's Day and for other times of the year, by controlling the time of the transfer from warm to cool conditions. The timing of the transition between these temperature conditions is important to prevent inflorescence drop. To further develop *A. baileyana* as cut stems or indoor flowering pot plants, mother plant selection of cutting material with good rooting success is required. In addition, propagation and survival of cuttings needs to be addressed to improve production and cost-efficiency.

Breeding system, reproductive efficiency and weed potential of *Acacia baileyana*[1]

4.1 Introduction

Acacia baileyana is a environmental weed which has invaded bush areas in south-eastern Australia from ornamental plantings (Hall and Turnbull 1979, Blood 2001). The environmental weed classification applies to both the green and purple leaf forms, although only the green form has been observed as a weed. There are several possible reasons why the purple form is not a weed at present. First, the purple leaf form has been cultivated only since about the 1970s (Bill Molyneux, personal communication); thus, it may not have had the time to escape into natural bush. Second, this variety may be a less invasive form, producing low numbers of viable seeds. Third, seeds from the purple form are not always true to type, as offspring can be green in colour. The purple colour may be due to a recessive gene(s), thus the offspring from an invading purple leaf plant, would have a higher likelihood of being the green leaf form.

The proliferation of a woody species into a new environment is dependent on the plant producing enough seeds, and hence seedlings, to invade the area. Weeds usually produce high numbers of viable seeds (Adair 1995); thus, screening of plants for introduction to a new area should include measurements of seed production (Carter 1994). Seed number may be limited by the amount and availability of suitable pollen, the availability of resources, predation of reproductive structures and adverse environmental conditions (discussed in Vaughton and Ramsey 1995), although the number of female flowers will set the upper limit to the number of fruits that can be produced (Lloyd 1980, Naylor 1984). In addition, as shown in Chapter 3, environmental factors will influence flowering success of *A. baileyana*. Warm temperatures were required for bud formation and cool temperatures were necessary for anthesis, with high light intensity increasing flowering success.

Seed quantity and quality are also affected by the breeding system. Self compatibility may increase the production of seeds from trees, although these seeds may be of inferior quality to outcrossed seeds.

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[1] Morgan A, Carthew SM, Sedgley M (2002) Breeding system, reproductive efficiency and weed potential of *Acacia baileyana*. *Australian Journal of Botany* **50**, 357–364 (Appendix 3)

Self incompatibility has been recorded in some species of *Acacia* and is considered an important outbreeding mechanism (Kenrick and Knox 1989). It has been shown for *A. retinodes* that pollen tubes were inhibited in the nucellus of the ovule and not in the stigma or style (Kenrick et al. 1986). Newman (1934b) suggested that *A. baileyana* was capable of self pollination, although this was not quantified.

This chapter examines the weed potential of *A. baileyana* in terms of its breeding system and seed production, to determine its reproductive efficiency. The two leaf colour forms are compared to investigate the potential for the purple form to become a weed.

4.2 Materials and methods

4.2.1 Plant material

Plants used in the study were grown at the locations listed in Table 4.1. Leaf colour was assessed using an allocated visual colour category (refer to Chapter 3 for details). Green leaf plants were either colour category 1 or 2, while purple leaf plants were typically colour category 3 and 4. Three of the locations are shown in Plate 4.1. Mature flowering specimens came from the Adelaide Hills.

A plantation of one-year-old seedlings was established in June 1996, at the Waite Campus of The University of Adelaide, to compare the growth, flowering and seed production of the green and purple forms of *A. baileyana* (Table 4.1). Twenty-five purple leaf and 95 green leaf plants were planted at a 3 x 3 m spacing at random locations. The plants had been germinated from seed the previous year. The purple form was grown from two seed lots obtained from Nindethana Seed Service (Albany, Western Australia), while seed for the green form was sourced from both Nindethana and CSIRO Forestry. Seeds were surface sterilised in 70% ethanol for 2 min, placed in 4% bleach for 5 min, then washed with three rinses of reverse osmosis water. They were placed into 'just off the boil' hot water overnight, and sown into black plastic tubes (10 cm diameter), containing by volume 1:1:1 sand: perlite: peat moss plus nutrients, and kept in a 25°C glasshouse. Plants were hardened off in a shadehouse (75% of the incident sunlight excluded) before planting. The plantation was irrigated as required and fertilised biannually with 0.5 kg per plant of Pivot-Gro-well Blood and Bone Blend® fertiliser.

Table 4.1 Details of the location, status, leaf colour and number of *A. baileyana* plants used in the study to investigate their breeding systems and reproductive efficiency. For the breeding system and natural reproductive output studies, the number of plants refers to the number of pollen-recipient trees used.

Study	Experiment number	Plant status	Leaf colour (number of plants)	Location	Flowering season	Measurements and pollination treatments conducted	Location of measurements and pollinations treatments
Growth and timing of flowering	1	Cultivated	purple (25) and green (95)	Waite Campus	1996, 1997, 1998	height, presence of flowers	whole tree
Breeding systems	2	Cultivated	purple (1) and green (2)	Adelaide Hills	1996	self, cross, control, open	whole branches
	3	Weed	green (14)	Adelaide Hills	1996	bag control, open	whole branches
Natural reproductive output	4	Weed and cultivated	purple (6) and green (27)	Adelaide Hills	1997	open	whole branches
	5	Cultivated	purple (16) and green (21)	Waite Campus	1997	open	whole tree
	6	Cultivated	purple (10) and green (10)	Waite Campus	1998	open	whole branches

Plate 4.1 Some of the *A. baileyana* plants used in the growth and pollination studies. A: Weeds growing at Mount Bold Reservoir, Adelaide Hills.   The height of the tallest trees is approximately 3 m. B: Weeds growing at Millbrook Reservoir, Adelaide Hills. The height of the tallest trees is approximately 4 m. C: One-year-old plantation at the Waite Campus. The height of the young plants is 50 cm. D: Three-year-old trees in the same plantation. The height of the tallest trees is approximately 3 m.

4.2.2 Growth and timing of first flowering

Time to reproductive maturity was determined by recording the timing of first flowering of the purple and green leaf forms in the Waite Campus plantation (Experiment 1, Table 4.1). Height of the purple and green leaf plants in the plantation was measured every three months to determine growth rates. The presence of flowers on each tree was recorded every two weeks during the flowering season, from June to August, in 1996, 1997 and 1998.

4.2.3 Investigation of breeding systems using controlled pollinations

The breeding system was investigated using controlled pollinations on cultivated plants from the Adelaide Hills.

Controlled and open pollinations were conducted on three cultivated *A. baileyana* plants growing in gardens in the Adelaide Hills (Experiment 2, Table 4.1). Two plants were the green leaf form and the other plant was the purple leaf form. Treatments were applied to these three pollen-recipient trees from four pollen-source plants between June and August 1996 (Table 4.2). Controlled pollination treatments included self, intra-variety and inter-variety crossing and a control.

Table 4.2 Controlled (self, cross and control) and open pollination treatments for three pollen-recipient *A. baileyana* trees in the Adelaide Hills.

Pollen recipient		Pollen source		Pollination treatment
Plant code	Colour category	Plant code	Colour category	
P5	4	P5	4	self
P5	4	P3	4	intra-variety cross
P5	4	G5	2	inter-variety cross
P5	4	G3	2	inter-variety cross
P5	4	none		control
P5	4	unknown		open
G2	2	G2	2	self
G2	2	G5	2	intra-variety cross
G2	2	P3	4	inter-variety cross
G2	2	P5	4	inter-variety cross
G2	2	none		control
G2	2	unknown		open
G5	2	G5	2	self
G5	2	G3	2	intra-variety cross
G5	2	P3	4	inter-variety cross
G5	2	P5	4	inter-variety cross
G5	2	none		control
G5	2	unknown		open

For each of the three pollen-recipient plants, six branches per treatment were bagged prior to anthesis, at the yellow bud stage (Plate 4.2A), with a polyester bag containing a clear PVC viewing window (Plate 4.2E). Several branches on five other trees were also bagged at the yellow bud stage as a source of pollen. At anthesis on the pollen-recipient trees, all leaves, buds, inflorescences and flowers not at the female stage (style fully extended) on the six branches were removed with fine forceps (Plate 4.2B), to prevent moisture build-up in the bags. As the flowers are protogynous (stigma is receptive before the pollen is released) the anthers were not removed. Branches used as the source of pollen were placed into paper bags once the anthers had dehisced. The fresh pollen was immediately collected from these branches by placing a pen wrapped with thin plastic into the bag containing male-stage flowers. The pollen adhered to the plastic due to electrostatic forces. Female-stage flowers were pollinated by placing the pollen onto the stigmatic surface. Plate 4.2C depicts a polyad that has been successfully placed onto the stigma, and Plate 4.2D shows pollen tubes penetrating the ovule. Pollinated flowers were counted for the cross, self and control treatments. Over 100 flowers per branch were pollinated. A hand-held lightscope was used to check that a polyad had been placed onto the stigma. After pollination, the flowers were re-bagged (Plate 4.2E, F). Once the pods started to develop, the bags were removed (Plate 4.3A). In November, before pods started to drop, they were bagged with net bags to collect seeds (Plate 4.3D, E). Plate 4.3F depicts a branch with open pods releasing seeds.

For the self-treatment study, pollen from the same tree was applied. For intra-variety crossing, pollen from another tree of the same form (green or purple) was applied, while for inter-variety crossing, pollen from a tree of the other form (green or purple) was applied. The control treatment involved processing flowers as for a pollination treatment but no pollen was applied. For all of these four treatments, branches were bagged after pollination.

The controlled pollinations were compared to open, natural pollinations (Table 4.2). For open pollination, six branches per tree were tagged when all the buds were at the yellow bud stage. To estimate the number of flowers, the number of inflorescences per branch was counted and multiplied by 14.2 (the average number of flowers per inflorescence for 10 trees at Millbrook Reservoir weed site, see Table 5.1, Chapter 5). The branches were bagged in November to collect any pods and seeds that dropped.

For all treatments, the pods were counted at weeks 8 (initial set), 11, 17 and 24 (final set) after the six branches were initially bagged or tagged. The pods and seeds were harvested in January 1997, at week 24, and counted separately for each branch.

Plate 4.2 Hand pollination of *A. baileyana*. A: Inflorescences at different stages of flower opening. The left raceme consists of inflorescences at the yellow bud stage. The middle raceme consists mainly of inflorescences at the female stage, with the stigma fully extended. The right raceme consists mainly of male stage flowers, with the anthers extended. Scale bar = 1 cm. B: Removal of flowers not at the female stage. Scale bar = 1 cm. C: A polyad on the stigma, as indicated by the arrow. Scale bar = 32 μm. D: Pollen tubes penetrating the ovules. Scale bar = 320 μm. E: A pollinated branch covered with a polyester bag. Scale bar = 8 cm. F: A tree with the pollination treatments covered with polyester bags. Scale bar = 40 cm. C and D are fluorescence micrographs of aniline blue stained material produced by Dr Merran Matthews (1998).
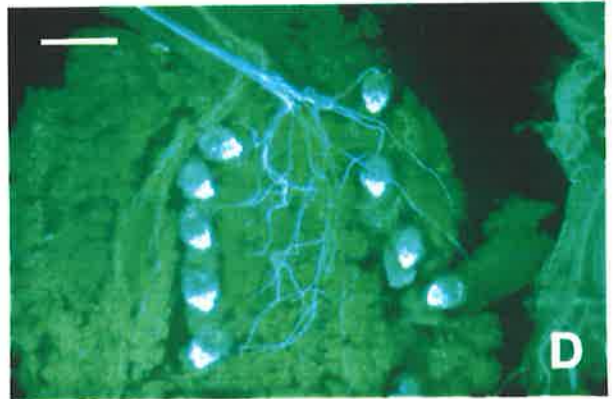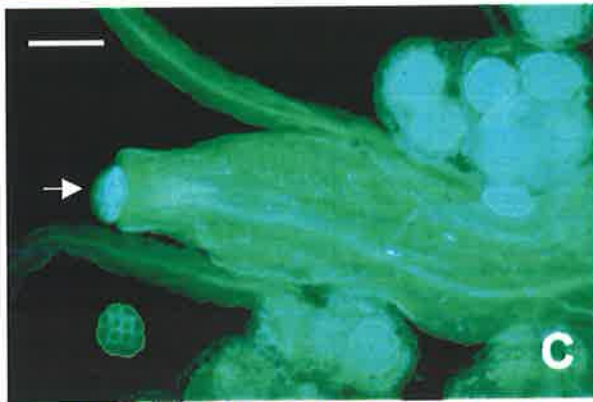
Plate 4.3 Stages of pod development in *A. baileyana*. A: Young pods. Scale bar = 3 cm. B: Pods formed from cross pollination. Scale bar = 3.5 cm. C: Pods formed from self pollination. Scale bar = 4.5 cm. D: Pods covered with netting bags to collect any pods and seeds that drop. Scale bar = 4.5 cm. E: Pods starting to open within the netting bags. Scale bar = 4 cm. F: Open pods on a branch. Scale bar = 2.7 cm.

For each branch, percentage pod set was determined by:

(number of pods x 100) ÷ (number of flowers).

For each branch, the percentage of aborted pods was determined by:

(initial % pod set –final % pod set) x 100 ÷ (initial % pod set).

Seeds per pod were determined by dividing the number of seeds by the number of pods for each of the six branches. The number of indents in the pods was also counted. The indents represent the initial number of seeds that were formed. For each branch the percentage of seeds aborted or not fertilised (unformed seeds) was determined by:

(number seed indents – number of seeds) x 100 ÷ (number of seed indents). It was assumed that seeds had aborted as seed traces were present in the pods.

Data for each treatment for all three pollen recipient trees were averaged as there were no between-tree differences. Cross pollinated treatments were combined for purposes of analysis.

Seeds were germinated from self ($n = 31$), control ($n = 22$), and cross treatments ($n = 1080$). Seeds from the open pollinations were not germinated. Seeds were germinated as previously described in section 4.2.1. After the hot water treatment, the seeds were sown into vermiculite in trays and then the trays placed into a growth cabinet at 25°C day / night. The number of seeds that germinated was recorded two months later.

4.2.4 Natural reproductive output

Natural reproductive output from open pollinations was compared for weed and cultivated plants from the Adelaide Hills and purple and green leaf forms from the Waite Campus plantation. The method used to determine reproductive output was as follows. Six branches per tree were tagged when all the buds were at the yellow bud stage. To estimate the number of flowers, the number of inflorescences per branch was counted and multiplied by 14.2 (the average number of flowers per inflorescence for 10 trees at Millbrook Reservoir weed site, see Table 5.1, Chapter 5). The branches were bagged in November to collect any pods and seeds that dropped. Pods and seeds were harvested and counted 24 weeks after the branches were initially tagged. The mean percentage final pod set, mean number of seeds per pod, and mean percentage of seeds aborted at harvest were calculated, as described previously for the controlled pollinations (section 4.2.3).

4.2.4.1 Weed plants

Open pollination was compared to a bagged control ('bag control') for weed plants, in July 1996. Weed plants were from three sites in the Adelaide Hills, South Australia: Mount Bold Reservoir (five

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plants), Millbrook Reservoir (five plants) and near the town of Stirling (four plants) (Experiment 3, Table 4.1; Plate 4.1). The open pollination treatments were applied to branches following the method described above (section 4.2.4). Six extra branches per tree were covered with polyester bags at the yellow bud stage, to represent the bag control treatment. Flower numbers, for both open and bag control treatments, were estimated as described above (section 4.2.4). The bags were removed once pods had started to develop.

4.2.4.2 Comparison of weed and cultivated plants

Pod and seed set from open pollinations were compared between cultivated and weed plants in July 1997, following the method described above (section 4.2.4). Plants consisted of 10 weed trees from Millbrook Reservoir, 11 weed trees from Mt Bold Reservoir and six green and six purple leaf cultivated plants from the Adelaide Hills (Experiment 4, Table 4.1).

4.2.4.3 Comparison of purple and green leaf forms

Pod and seed set from open pollinations were compared between purple and green leaf forms from the Waite Campus plantation for the 1997 and 1998 flowering seasons. Total flower, pod and seed production per tree were estimated from the 1997 flowering season. Counts were estimated from 16 purple leaf and 21 green leaf plants in the plantation. Flower counts were estimated by counting the total number of racemes per plant, and for 10% of these racemes, counting the number of inflorescences. The number of flowers was estimated as described in section 4.2.4. All pods and seeds per tree were harvested and counted in December 1997, before they had started to drop. Percentage pod set at harvest was determined by dividing the total number of pods by the total number of flowers for each tree, multiplied by 100. Seeds per pod at harvest were calculated by dividing the total number of seeds by the total number of pods for each tree.

Open pollinations from 10 purple and 10 green leaf plants in the plantation were compared for the 1998 flowering season. Counts were made from branches, as the trees were too large for total counts, following the method described above (section 4.2.4).

4.2.5 Statistical analyses

For proportions of pod set, pods aborted, seeds per pod, and seeds aborted, a model was fitted by restricted (or residual) maximum likelihood (REML) (Patterson and Thompson 1971). This approach was used in preference to analysis of variance (ANOVA) as it is capable of handling unbalanced data. When the data are balanced ANOVA and REML give equivalent results. Furthermore, REML, in the same way as ANOVA, allows the design of the experiment to be taken into consideration by allowing

more than one source of variation to be accounted for, such as between trees and within trees (branches). Such terms are included as random effects in the REML formulation and correspond to the block structure in ANOVA. The treatments imposed in the experiment, such as pollen source, are included in the REML model as fixed effects and are tested by a Wald-test, which approximates chi-squared under the hypothesis of no effect (Cox and Hindley 1974). For pod set, the results should be interpreted with caution, as the data are not normally distributed with constant variance as required for REML analysis. Transformations were tried but the outcome result did not change. Therefore, for simplicity, the data were not transformed. Approximate l.s.d. values were calculated to look at specific differences between treatments. Standard errors were also determined to look at variability between the individual branches or trees. All analyses were performed with the statistical package GenStat (Harding et al. 2000).

4.3 Results

4.3.1 Growth and timing of first flowering

There was little difference in growth between the purple and green forms, with plants reaching 3 m in height at three years of age (i.e. June 1998; Fig. 4.1). The main period of growth was during summer. The rate of growth was less from June to September. Most plants flowered at two years of age, with all plants flowering at three years (Table 4.3).
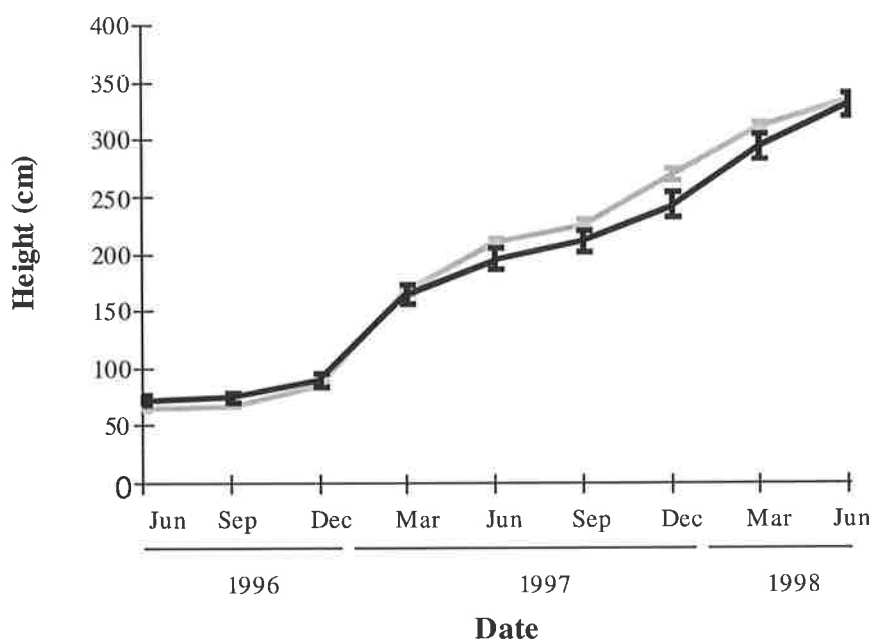
Fig. 4.1 Growth of green ($n = 95$, grey line) and purple ($n = 25$, black line) forms of *A. baileyana* in the Waite Campus plantation. The plants were 12 months old when planted in June 1996. Bars indicate standard error of the mean height for each interval.

Table 4.3 Percentage of the green and purple leaf forms of *A. baileyana* flowering in 1996, 1997 and 1998, in the Waite Campus plantation.

Year	Age (years)	Purple leaf trees flowering (%) ($n = 25$)	Green leaf trees flowering (%) ($n = 95$)
1996	1	1	0
1997	2	72	83
1998	3	100	100

4.3.2 Investigations of breeding systems using controlled pollinations

There were no differences between inter- and intra-variety crosses (data not shown) and so these data were pooled. Initial and final pod set were significantly higher in cross pollinated plants than for the other pollination treatments (Table 4.4, Plate 4.3B, C). Open pollinated flowers showed similar pod set to the self and bagged treatments. Pod abortion occurred up to about week 17 for all treatments (Fig. 4.2). There were no differences between the treatments in proportions of aborted pods. There were significantly more seeds per pod in cross pollinated plants than in all other treatments, with open pollinated plants having the second highest number. The self and control treatment had similar low number of seeds per pod. Fewer seeds aborted for the cross pollination compared to the other treatments. Seed germination for the self, control and cross-pollination treatments were similar and high, over 90% (Table 4.4), with no difference in appearance between seedling types.

4.3.3 Natural reproductive output

4.3.3.1 Weed plants

For the weed plants, final pod set and seeds per pod were higher for open pollinations than for the bag control treatment, and there was no difference in percentage seeds aborted (Table 4.5).

4.3.3.2 Comparison of weed and cultivated plants

There was no difference between the final pod set and number of seeds per pod for weed and cultivated plants (Table 4.6). Seed abortion for weed plants growing at Millbrook Reservoir was higher than that at Mount Bold Reservoir. There was no difference between the purple and green cultivated plants. There was significant ($P < 0.001$) variability between plants within a site.

Table 4.4 Pod set, seed production and seed germination from controlled and open pollination treatments of *A. baileyana*. Number of branches is given in parentheses. For mean number of pods and seeds aborted and mean number of seeds per pod, only the data from the branches that had pods were included. Treatment means followed by the same letter in the same column are not significantly different at the 5% level.

Pollination treatment	Total number of flowers used in treatment	Mean pod set ± s.e. (%)		Mean pods aborted ± s.e. (%)	Mean no. of seeds per pod ± s.e.	Mean no. of seeds aborted ± s.e. (%)	Seeds germinated (%)
		Initial (8 weeks)	Final (24 weeks)				
Cross	9227 (52)	37.80 ± 2.58 b (52)	23.09 ± 1.64 b (52)	37.41± 2.14 (52)	7.22 ± 0.24 c (52)	9.75 ± 1.04 b (52)	94 (1080)
Self	3820 (18)	0.54 ± 0.15 a (18)	0.36 ± 0.11 a (18)	30.14 ± 13.03 (9)	2.02 ± 0.69 a (8)	42.07 ± 14.63 a (8)	90 (31)
Control	3037 (17)	0.82 ± 0.23 a (17)	0.38 ± 0.11 a (17)	52.05 ± 12.57 (12)	2.10 ± 0.75 a (8)	31.62 ± 10.53 a (8)	91 (22)
Open	54698 (18)	0.81 ± 0.18 a (18)	0.41 ± 0.11 a (18)	46.31 ± 5.93 (16)	5.10 ± 0.66 b (15)	28.38 ± 5.59 a (15)	–
P-values		< 0.001	< 0.001	0.11	< 0.001	< 0.001	
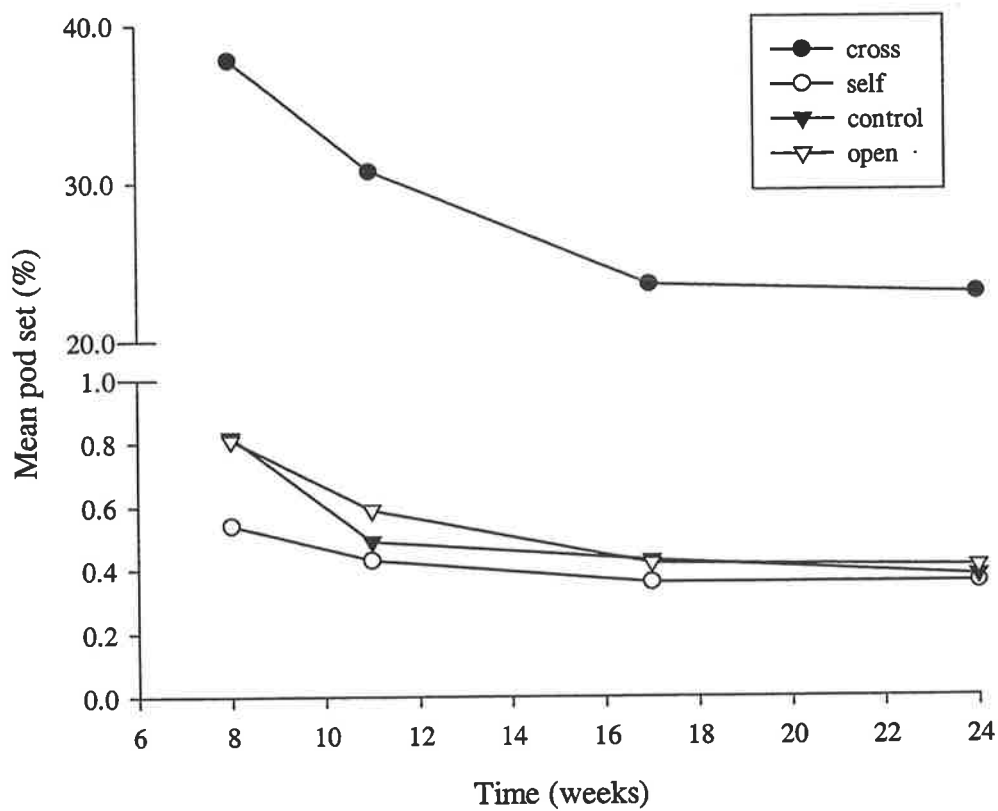
Fig. 4.2 The progression of pod set over time for cultivated *A. baileyana* plants used in the breeding system studies. Pod set is presented for cross, self, control and open pollination treatments. Time (weeks) is the period from when the branches were bagged at the yellow bud stage (week 0) to harvest (week 24).

Table 4.5 Pod set and seed production from open and bag control pollination treatments for 14 *A. baileyana* weed plants from the Adelaide Hills, during 1996. Number of branches is given in parentheses. For mean number of seeds per pod and mean number of seeds aborted, only the data from the branches that had pods were included.

Pollination treatment	Total no. flowers used in treatment	Mean final pod set ± s.e. (%)	Mean no. of seeds per pod ± s.e.	Mean no. of seeds aborted ± s.e. (%)
Open	313948 (83)	0.13 ± 0.03 (83)	4.47 ± 0.34 (41)	33.06 ± 3.30 (41)
Bag control	253200 (71)	0.04 ± 0.02 (71)	2.49 ± 0.25 (22)	31.52 ± 5.49 (22)
P-values		< 0.001	< 0.001	0.82

Table 4.6 Pod set and seed production from open pollinated weed and cultivated *A. baileyana* plants from the Adelaide Hills, during 1997.
Number of branches is given in parentheses. For mean number of seeds per pod and mean number of seeds aborted, only the data from the branches that had pods were included. Treatment means followed by the same letter in the same column are not significantly different at the 5% level.

Plant status	Leaf colour	No. trees	Total no. flowers used in treatments	Mean final pod set ± s.e. (%)	Mean no. of seeds per pod ± s.e.	Mean no. of seeds aborted ± s.e. (%)
Weed (Millbrook Reservoir)	green	10	614761 (60)	0.12 ± 0.02 (60)	3.99 ± 0.30 (44)	37.50 ± 4.98 a (44)
Weed (Mt Bold Reservoir)	green	11	601171 (66)	0.07 ± 0.02 (60)	5.34 ± 0.38 (29)	19.48 ± 3.94 b (29)
Cultivated	purple	6	274884 (36)	0.11 ± 0.03 (36)	2.73 ± 0.43 (21)	29.10 ± 6.93 c (21)
Cultivated	green	6	300642 (36)	0.13 ± 0.03 (36)	3.02 ± 0.31 (26)	29.35 ± 5.27 c (26)
P-values				0.61	0.10	0.03

4.3.3.3 Comparison of purple and green leaf forms

In 1997, no significant differences were observed between the green and purple cultivated forms in final pod set, seeds per pod, and mean total production of flowers, pods and seeds (Table 4.7). The data collected from each tree was highly variable, as evident in the large standard errors and range, and this may mask any real difference between the purple and green forms. For all measurements except mean number of seeds per pod, the mean values were much higher for the green form. Mean total number of flowers for the green form was 130,302 (maximum 334,808) and for the purple form, 75,680 flowers (Maximum 319,259). The highest number of seeds produced per tree was 8008 (mean of 1798) for the green form and 5332 (mean of 833) for the purple form.

Branch counts conducted in the following season showed that the green cultivated plants had a slightly higher reproductive efficiency than the purple cultivated plants (Table 4.8). The green form had a marginally higher final pod set and number of seeds per pod than the purple plants, while seed abortion was lower. Pod abortion occurred up to week 16 for both forms (Fig. 4.3). These data were highly variable, as shown by the standard error and range, with about 60 branch measurements recorded.

4.4 Discussion

4.4.1 Breeding systems

This study shows that A. baileyana is highly outcrossing, with an average index of self incompatibility (i.e. self pod set ÷ cross pod set; Zapata and Arroyo 1978) of 0.02. Thus, A. baileyana is highly self incompatible, although plants do have a very low ability to self pollinate. High levels of out crossing have also been reported in other Acacia species studied (Philp and Sherry 1946, Moffett 1956, Kenrick and Knox 1989). Kenrick and Knox (1989) compared levels of self incompatibility in seven species of Acacia. There was variation in the self incompatibility index, ranging from 0 to 11, with only one species being self compatible. The other six species were either highly self incompatible or partially self compatible.

In the present study, mean final pod set for cross pollination was 23%. This was much higher than the 0.36% obtained from self pollination. Low levels of pod set were mainly due to high levels of pod abortion. There was no difference in pod abortion between the cross, self, control and open treatments. The timing of pod abortion, within the first four months after pollination, may conserve many resources for the remaining pod development (Stephenson 1981). The similar pod abortion between treatments may be the result of dominating environmental factors that affected all treatments equally. Examples of such factors could be predation, as fungal galls and gall midges were noticed

Table 4.7 Flower production, pod set and seed production from open pollinated purple and green leaf forms of *A. baileyana*, during 1997. Total counts were conducted on two-year-old trees from the Waite Campus plantation. Total number of flowers, pods and seeds were estimated from hand counts. Number of trees is given in parentheses. For number of seeds per pod and number seeds per tree, only the data from the trees that had pods were included.

	Total no. flowers per tree	Final pod set (%)	Total no. of pods per tree	Total no. of seeds per pod	Total no. of seeds per tree
Purple form (16 trees)					
Mean ± s.e.	75,680 ± 23,945 (16)	0.07 ± 0.04 (16)	68 ± 55 (16)	6.56 ± 0.50 (8)	833 ± 645 (8)
Range	114–319,259	0–0.55	0–880	4.3–9.1	20–5,332
Green form (21 trees)					
Mean ± s.e.	130,302 ± 25,532 (21)	0.14 ± 0.03 (21)	261 ± 87 (21)	5.53 ± 0.31 (16)	1,798 ± 570 (16)
Range	241–334,808	0–0.52	0–1,345	3.2–8.3	25–8,007
P-values	0.14	0.18	0.09	0.08	0.31

Table 4.8 Pod set and seed production from open pollinated purple and green leaf forms of *A. baileyana*, during 1998. Counts were conducted on branches from three-year-old trees from the Waite Campus plantation. Number of branches is given in parentheses. For mean number of seeds per pod and mean number of seeds aborted, only the data from the branches that had pods were included.

	Total no. flowers used in treatment	Mean final pod set (%)	Mean no. of seeds per pod	Mean no. seeds aborted (%)
Purple form (10 plants)				
Mean ± s.e.	719486 (60)	0.07 ± 0.01 (60)	3.27 ± 0.32 (37)	53.46 ± 13.80 (37)
Range		0–0.49	0–10	0–100
Green form (10 plants)				
Mean ± s.e.	885853 (59)	0.11 ± 0.02 (59)	4.11 ± 0.30 (51)	27.16 ± 4.45 (51)
Range		0–0.67	0–9	0–100
P-values		0.04	0.03	< 0.001
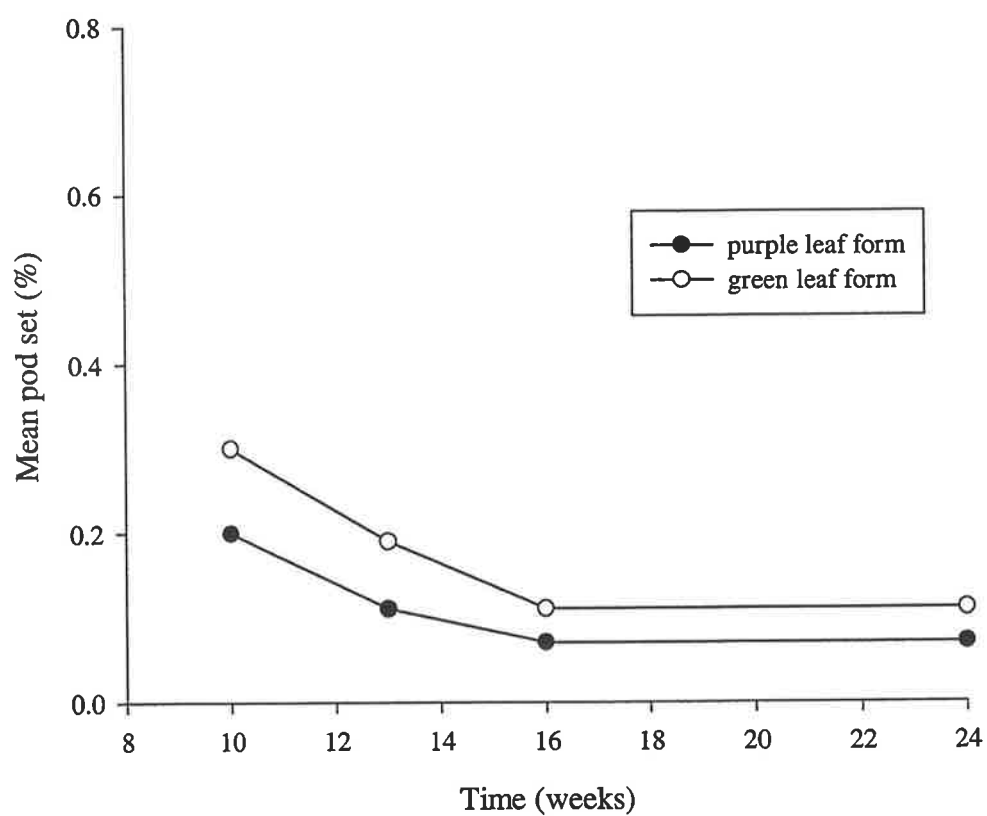
Fig. 4.3 The progression of pod set over time, for open pollinated purple and green leaf forms of
***A. baileyana*, growing in the Waite Campus plantation, in 1998.** Time (weeks) is from when
branches were bagged at the yellow bud stage (week 0) to harvest (week 24).

(Plate 4.4C, D), adverse climatic conditions during this period, or limitation in nutrient resources. Alternatively, similar pod abortion rates for cross and self treatments may simply be co-incidental. Inflorescences comprise many flowers arranged in a sphere, so in the case of cross pollinated treatments, the inflorescence may be able to support only a limited number of pods. Thus, there may be competition for limited resources within an inflorescence (Wyatt 1982). In contrast, pod abortion for the self treatment may be due to self incompatibility.

Acacia baileyana can potentially produce 12 seeds per pod as it normally has 12 ovules (Kenrick and Knox 1982). For the cross pollination treatment in the present study, a mean of 7.2 seeds per pod (range 3 to 11) was recorded. The mean number of seeds per pod was much lower for self pollination in this study, being an average of 2.0 seeds per pod. Plate 4.4A represents examples of pods of different size, containing either 11, six or three seeds. Low numbers of seeds per pod for self pollination have also been recorded for *A. mearnsii* (Moffett 1956) and for *A. decurrens* (Philp and Sherry 1946). Low seed set for self pollination compared to the cross treatment could be due to self incompatibility occurring at the pollination or fertilisation level. Post-zygotic mechanisms are also likely to be present, as there was higher abortion of immature seeds for the self treatments (Plate 4.4B). There was no difference in seed germination between the self and cross treatments. The seedlings from the self pollinations were healthy and non-chlorotic, as was found for self pollinated seedlings of *A. decurrens* (Philp and Sherry 1946). Therefore, selection for superior self seeds seems to have already occurred at the post-zygotic level, although it is possible that differences may manifest in post-seedling growth.

4.4.2 Natural reproductive output

The low final pod set (average 0.07–0.41%) for open pollination was within the range reported for other acacias (0.01–0.60%; Milton and Hall 1981, Tybirk 1989, Moncur et al. 1991, Tybirk 1993, Grant et al. 1994). Pod set in this group of acacias is low compared to other legume trees. For example, Bawa and Webb (1984) determined pod set to be between 1 and 10% for five tree species belonging to Caesalpinoideae and Papilionoideae of the Leguminosae family, with all reported to be self incompatible. The pod set results obtained here for open pollinations are only an estimate, as an average of 14.2 flowers per inflorescence was used to determine flower number. It would have been very time consuming to count individual flowers. This method may give an inaccurate estimate of pod set as flowers per inflorescence were found to be significantly different between the trees at Millbrook Reservoir (see Chapter 5).

Open pollination pod set was lower than for cross pollination but similar to self pollination in the controlled pollination study. However, it is difficult to compare between the two treatments, as

Plate 4.4 Characteristics of *A. baileyana* pods. A: Different pod sizes. Scale bar = 1.2cm. B: Viable (large) and non-viable (small) seeds. Scale bar = 0.5cm. C: Gall midges (Diptera: Cecidomyiidae) damaging inflorescences. Scale bar = 2.3cm. D: Fungal galls damaging inflorescences. Scale bar = 2.3cm.

A

B

C

D

movement of pollen by pollinators was required for open pollination, while pollen was applied for cross and self pollination. Open pollination of 14 weed plants resulted in higher pod and seed set than in the bag control treatment.

Average number of seeds per pod varied from 2.7 to 6.6 between plants for the open pollination treatment. This variation in number of seeds per pod for open pollination was higher than for the bag control treatment and was intermediate between self and cross pollination from the controlled pollination study. There were also variations in number of seeds aborted, from 20 to 54%, which is similar to level of seed abortion in the control and self pollination treatments.

There are three possible reasons for the low and variable pod set and seeds per pod for open pollination recorded in this study. First, the available pollinators may not be very active in winter, or there may be a deficiency of native pollinators due to cultivation and urbanisation. This would result in a lack of polyads reaching the stigma. Observations during the course of this research would suggest that honey bees are the most likely pollinators. The effectiveness and efficiency of pollinators could be determined by examining stigmas and styles for the presence of polyads and pollen tube growth.

Second, open pollinated pods and seeds may be primarily the result of ineffective self pollination events, occurring at different levels for each plant. Self pollination may be favoured because of the high flower density and thus abundance of polyads. Plants produce many flowers to attract pollinators. This floral reward may be so great due to the mass flowering that the pollinators have less incentive to move to other trees. Once a 'self'-polyad is on the stigma, it can act as a physical barrier to other polyads and clog the stigma (Moncur et al. 1991). The number of viable pollen grains deposited on a receptive stigma is not a factor for A. baileyana, since only one polyad is required to successfully fertilise all the ovules. Cross pollination may occur mainly at the initial and final phases of flowering, as greater pollinator movement may occur between trees because of lower flower numbers at these times (Stephenson 1982). It would be interesting to investigate the genetic composition of seeds derived from open pollination at different times during the season.

Third, pod and seed production may be limited by adverse environmental conditions, including predation or lack of available nutrients for growth and development. A lack of nutrients may influence pod production at either the individual flower or inflorescence level. However, resource limitation is probably not a major factor, as pod set can reach 23% with controlled cross pollination, and the trees in the Waite Campus plantation were adequately supplied with water and fertiliser.

4.4.3 Flower and seed production

Maximum flower production for purple and green leaf forms from the plantation trees at two-years of age was over 300,000. The more flower produced, the greater is the potential for producing seeds for the next generation. Even though pod set was low, total seed production on trees was high due to the high flower production. At two years of age a plant may produce over 8,000 seeds (range 20–8,007). Seed production can, therefore, be high for young plants.

4.4.4 Differences between plants

Little difference in rate of growth was observed between the purple and green leaf forms of *A. baileyana*. The main growth period occurred in summer after seeds had dropped, with minimal growth occurring in winter during the flowering period. Resources are most likely being utilised for seed production during winter and spring.

The purple and green leaf forms had similar pod and seed set. The cultivated purple and green plants from the Adelaide Hills were similar in their reproductive output, but there were indications that the green form was more productive in the Waite Campus plantation. This difference may be due more to between-plant differences, rather than intrinsic differences between the two forms.

In general, there were also no consistent differences in reproductive output between weed and cultivated plants, nor between years. Both types of plants may be growing in suitable and low competitive conditions. The absence of obvious differences between weed and cultivated plants may be due to the large variability between plants within each site and in some cases between branches within a tree. Branch differences could be due to their location; for example, pollinators may be attracted to the warmer side of the tree. In addition, branches may be affected by the prevailing weather conditions. The only unusual difference between open pod set was the high value of 0.41%, for the trees used in the controlled pollinations. These trees may have attracted more effective pollinators.

4.4.5 Weed status

Both the purple and green leaf forms of *A. baileyana* can grow very rapidly and flower by two years of age. These findings support Mulvaney's (1991) classification of *A. baileyana* as an environmental weed. The purple form is as reproductively efficient as the green form, but is not currently present in weed populations, which may be due to the lack of opportunity it has had to establish itself since first being cultivated commercially in the 1970s. The purple form could, however, have invaded some bush areas but as the offspring are the green leaf form, its weed status may not have been noticed.

This study determined that *A. baileyana* possesses two of the 12 attributes that increase the likelihood that a species can successfully invade native bush (Adair 1995, Williams and West 2000): high input of viable seeds and short (<2 years) development time to reproductive maturity. This is similar to *Pittosporum undulatum*, another native environmental weed, which has early seed production and fast growth (Gleadow and Ashton 1981). Two other invasive attributes previously described for *A. baileyana* are seed longevity (Newman 1934b) and mass seed germination after fire. An Australian species, *A. longifolia*, a weed in South Africa, also has invasive attributes similar to those of *A. baileyana*, including high annual production of long-lived seeds that accumulate in the soil (Milton and Hall 1981, Pieterse and Cairns 1988).

4.4.6 Efficiency of reproduction

The efficiency of natural reproduction for *A. baileyana* is dependent on how 'reproduction' is defined. In terms of pod set, the efficiency was low, but in terms of flower production or total seeds produced, reproductive efficiency is high. In fact, precocity and high flower numbers, which result in high seed production, appear to be important factors for the weed status of *A. baileyana* in the Adelaide Hills. Seed production, germination and seedling growth conditions may be ideal in other regions of Australia and overseas. An increase in fire frequency in the Adelaide Hills could increase the spread of this weed, due to mass germination. Plantations of either form of *A. baileyana* and hybrids for horticultural production must be carefully managed to prevent their spread. Ideally, plantations should not be established near native bush. Regular harvest for the cut stem industry will also limit seed escape. Management options for acacia weeds include cutting out plants, chemical or biological control, or fire control by either excluding fire or burning followed by plant removal (Milton and Hall 1981, Blood 2001, Muyt 2001). As only a few weed populations of *A. baileyana* exist in South Australia they should be removed in the near future before a greater spread occurs. It is recommended that *A. baileyana* in and around bushland should be replaced with indigenous acacias and any hybrid plants destroyed to prevent further genetic pollution (Muyt 2001).

An understanding of the reproductive output of older trees from a weed site is needed to determine their seed production and, therefore, weed potential. This will be examined in the following chapter (Chapter 5). Future studies should include comparing the weed populations to the endemic populations, to determine if the weed plants are more reproductively efficient.

Using digital image analysis to estimate flower numbers of *Acacia baileyana* and hence determine seed production and weed potential[1]

5.1 Introduction

Acacia baileyana has escaped from cultivation to become an environmental weed in Australia (Mulvaney 1991) and overseas (Ross 1975, Hall and Turnbull 1979, Milton and Moll 1982, Robin and Carr 1983, Tame 1992, Whibley and Symon 1992, Blood 2001). One of the attributes of a weed is the production of a high number of viable seeds (Adair 1995) and the success of *A. baileyana* in South Africa as a weed has been attributed to its large seed production (Milton and Moll 1982).

Most acacias produce large numbers of flowers but only a small proportion develop into fruit (Plate 5.1C, D) (New 1984). Information on flowering is very useful to predict the extent of fruit set. Most studies on *Acacia* have reported flower and pod production from only a subset of branches (Grant et al. 1994, Kenrick and Knox 1989, Pieterse and Cairns 1988), inflorescences (Sedgley et al. 1992, Morrison and Myerscough 1989), or racemes (Kenrick et al. 1987). For example, in a study on Australian weeds of *A. saligna* and *A. cyclops* in South Africa, pod set was determined from branch counts, with the total number of seeds estimated by weighing all the pods on the trees (Milton and Hall 1981). Moffett (1956) stated that counting flowers and pod set in *Acacia* is not feasible. However, without this information, total productive output cannot be determined. Some authors have determined total pod production by counting a proportion of the tree and extrapolating (Tybirk 1989, Tybirk 1993), but very few investigators have endeavoured to count total flower numbers on trees. Baranelli et al. (1995) estimated the total number of inflorescences for trees of *A. caven* by counting the number of inflorescences in a quarter of the crown and scaling up for the whole tree. Morrison and Myerscough (1989) counted the total number of inflorescences per plant, and the number of flowers

per inflorescence for *A. suaveolens*, but this was feasible because there were fewer than 88 inflorescences per plant.

In the previous chapter, total flower and pod numbers was estimated on two-year-old *A. baileyana* plants growing in the Waite Campus plantation. Estimates were made by counting total numbers of racemes. This was possible as the trees were small. Flower numbers ranged from 114–334,808. To determine total flower numbers of larger trees, counting the total number of racemes would be very time consuming (Plate 5.1A, C). Therefore, a non-destructive, digital photographic image method was investigated as a technique for estimating the total numbers of flower on mature *A. baileyana* trees. The ability to easily estimate flower number will increase our understanding of the weed potential of the species. The very high production of flowers is probably the basis of its success as a weed.

A digital image method was chosen because the blue-grey to green foliage contrasts with racemes of bright yellow inflorescences (Plate 5.1A, C). However, the digital image method could not be used to estimate total pod numbers as pods and leaves were similar in colour (Plate 5.1D). In addition, flower, pod and seed numbers, pod set and seeds per pod were determined from counts on branches. Using the total estimated number of flowers determined from digital image analysis and the flower and seed counts from the branches, total seed production per tree was estimated.

Digital photographic image analysis has been used previously to estimate leaf area (Baker et al. 1996, Bignami and Rossini 1996), plant dimensions (Bignami and Rossini 1996), and canopy volume (Sydnor et al. 1975, Miller and Lightner 1987, Bignami and Rossini 1996). The benefit of a photographic method for determining plant characteristics, such as flower number, is that it is non-destructive and thus allows post-flowering events, such as fruit set, to be quantified. Repeated measurements may also be done without affecting plant growth and production, and several parameters can be calculated from the one image.

5.2 Materials and methods

5.2.1 Tree selection

The study was conducted in the water catchment area of the Millbrook Reservoir, near Adelaide, South Australia, where *A. baileyana* has invaded the natural bush. Ten solitary trees (i.e. non-overlapping canopies) with dense flowering canopies were selected when the trees were at peak flowering. Flowering trees of different size were chosen to obtain a range of canopy volumes.

Plate 5.1 Digital image analysis of area of yellow flowers and canopy area in *A. baileyana*. A: An outline of the tree canopy is drawn to exclude neighbouring trees. Scale bar = 100 cm. B: Resulting surface area of flowers. Scale bar = 100 cm. C: A tree covered in flowers. Scale bar = 100 cm. D: Same tree as for C, but during pod set. Scale bar = 100 cm.

100 cm

A

B

C

D
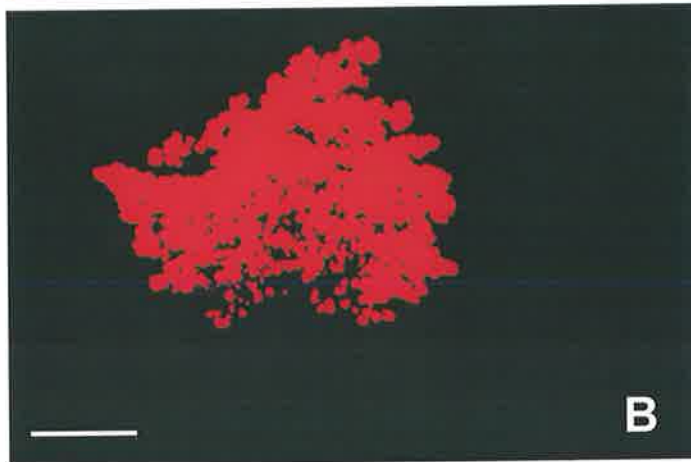
5.2.2 Digital images

The ten trees were photographed from four approximately equidistant points around the perimeter of each canopy. Photographs were taken using an EOS Canon 500 camera, with an EF 35-80 mm zoom lens, and Kodak, 35 mm 400 ASA print film. A metre rule was included in each photograph as a scale. To obtain a digital image, each photograph (10.2 x 15.2 cm) was scanned using a Hewlett Packard ScanJet IIcx/T colour scanner to produce an image of size 700 x 500 pixels. The brightness and contrast for each image was standardised visually to account for trees photographed under different light conditions.

A schematic diagram indicating the sequence of measurements made from the digital images is shown in Figure 5.1. This figure also shows the sequence for analysis of the destructive samples (see below).

5.2.3 Proportion of yellow flowers

Each of the four images per tree was analysed digitally for surface area (SA) of vegetation and area of yellow flowers (AY), by the computer image analysis program *Video Pro* (Leading Edge, Australia) (Plate 5.1A, B). Inflorescences at flowering and bud stage were yellow, while dying inflorescences were dark-yellow. From the four images analysed for each tree, average SA and AY were calculated. Proportion of yellow (PY) for the tree was determined by PY = average AY ÷ average SA.

5.2.4 Canopy volume

For each of the four images from each tree, the radius of a circle of area equal to the image canopy SA was calculated. The geometric mean of the four radii was calculated ($r = (r_1 r_2 r_3 r_4)^{1/4}$), and the volume of the tree canopy was estimated as the volume of a sphere of radius r, namely $4/3 \pi r^3$. Geometric mean was chosen because mathematical analysis gave a volume closer to the true volume for ellipsoidal canopies. Arithmetic means of four radii would give similar but slightly higher values for radii and hence volume.

5.2.5 Flower counts

A destructive sample was taken on four sides of each tree at the same time as photography to estimate number of flowers. A 1 m x 0.5 m quadrant was positioned at the mid point of each side of the canopy and all inflorescences within this quadrant from the edge of the canopy to the centre of the tree were harvested. The distance from the edge of the canopy to the centre of the tree was measured to calculate the volume of the box-shaped sample.

In *A. baileyana*, 10 to 25 flowers are clustered together into flower heads (inflorescences) arranged as

Fig. 5.1 A flow diagram of the digital image analysis and destructive sampling techniques used to estimate and predict total number of flowers on trees.

From 4 destructive samples per tree				From 4 digital images per tree		

```
From 4 destructive samples per tree                    Av. no.                    From 4 digital images per tree
                                                     flowers per
                                                    inflorescence
   ┌──────────┐  ┌──────────┐  ┌──────────────┐    (NFI), for all    ┌──────────┐  ┌──────────┐  ┌──────────┐
   │   Av.    │  │   No.    │  │     No.      │    inflorescences    │   Av.    │  │   Av.    │  │Geometric │
   │ sample   │  │ racemes  │  │inflorescences│     from c. 50       │area yellow│  │ surface  │  │  mean    │
   │ volume   │  │   in     │  │ per raceme   │     racemes per      │  (AY)    │  │ area (SA)│  │  radius  │
   │  (SV)    │  │ sample   │  │(NIR), for 150│        tree          └──────────┘  └──────────┘  └──────────┘
   └──────────┘  │  (NR)    │  │ racemes from │                     
                 └──────────┘  │ each sample  │                     
                               └──────────────┘                     
```

Estimated no. flowers per sample
(NFS) = av. NI x av. NFI

No. of inflorescences in sample
(NI) = NR x NIR

Proportion yellow
$$(PY) = \frac{av.\ AY}{av.\ SA}$$

Canopy
volume
(CV)

Estimated density of flowers
$$(DF) = \frac{NFS}{av.\ SV}$$

Statistical modelling to predict
density of flowers from PY.
Predicted $DF = e^{\alpha} \times PY^{\beta}$

Estimated total number
of flowers on tree (ENF)
= DF x CV

Predicted total number of flowers on
tree (PNF) = predicted DF x CV

spheres (Simmons 1988). The inflorescences are arranged in dense racemes (Elliot and Jones 1984). All the racemes in each of the four destructive samples per tree were counted. To determine the mean number of inflorescences per raceme, 150 racemes were selected haphazardly from each sample and the number of inflorescences on each counted. The average number of flowers per inflorescence was estimated separately for each tree, based on all inflorescences collected from an average of five racemes (range 3–19) at 9 to 15 sites per tree, totalling about 50 (range 48–94) racemes per tree. The total number of inflorescences sampled per tree ranged from 398 to 1,027. For each tree, an unweighted mean number of flowers per inflorescence was calculated from all the inflorescences. The average number of flowers per sample was estimated by multiplying the average number of inflorescences per sample by average number of flowers per inflorescence.

For each tree, the estimated average number of flowers from the four destructive samples was divided by the average volume of the four samples to give an estimate of the density of flowers for the tree per cubic metre. The total number of flowers per tree was then estimated by multiplying the estimated flower density by the estimated canopy volume.

5.2.6 Total pod and seed production

Six branches per tree were tagged, and the number of inflorescences was counted *in situ*. Net bags were placed over the branches in November to collect any dropped pods. In January the pods were collected and counted, and opened to record the number of seeds. Percentage pod set was determined for the 10 trees by dividing the number of pods by the number of flowers for each of the six branches. The number of flowers per branch was estimated by multiplying the number of inflorescences by the number of flowers per inflorescence. Average percentage pod set per tree was calculated by averaging the individual pod set for each branch. Average seeds per pod for each tree was calculated by averaging the individual seeds per pod for each branch.

To estimate the total number of pods per tree, the total estimated flower number was multiplied by the number of pods from the branch counts, and divided by the number of flowers from the branches. To estimate the total number of seeds per tree, the total estimated flower number was multiplied by the number of seeds from the branch counts, divided by the number of flowers from the branches.

5.2.7 Relationship between density of flowers and proportion of yellow

The number of flowers is positively related to both tree canopy size and density of flowers. To obtain a relationship to predict the density of flowers from digital images, the flower density from samples was related to proportion of yellow (PY). The following model was assumed initially:

$$\text{number of flowers} = \text{constant} \times PY^{\beta} \times \text{size}^{\delta},$$

where the number of flowers is proportional to some power of PY and to some measure of size of the canopy. Size measurements that could be used are radius, surface area, or volume; the power δ will adjust according to the best units of measurement.

Number of flowers in this model can be replaced by the density of flowers in the canopy (estimated from the destructive samples) multiplied by the volume of the canopy, giving:

$$\text{density} \times \text{volume} = \text{constant} \times PY^{\beta} \times \text{size}^{\delta}$$

Since volume is a function of size, the equation can be simplified to:

$$\text{density} = \text{constant} \times PY^{\beta} \times \text{size}^{\gamma},$$

with a different constant and size now having a different power. This was logarithmically transformed to stabilise the variance of flower density, as larger densities tend to have larger variance. Multiple linear regression of the logarithm of density of flowers on the logarithm of the PY and the logarithm of a size measure provided estimates of the parameters α, β and γ. Hence,

$$\text{predicted density} = e^{\alpha} \times PY^{\beta} \times \text{size}^{\gamma},$$

substituting regression estimates for parameters α, β and γ. Multiple linear regression was undertaken using the statistical package GenStat (Harding et al. 2000).

5.2.8 Predicted total flower number

The fitted model enabled prediction of density of flowers based only on information from the digital images, namely PY and size. The total number of flowers on a tree could then be predicted as the product of the predicted density and the estimated canopy volume. Therefore, predicted total number of flowers per tree = predicted density x canopy volume.

5.2.9 Standard error

Standard errors of PY, canopy radius, canopy volume, predicted total number of flowers, average number of flowers per sample, density of flowers, and estimated total number of flowers were calculated using the delta method for variances of derived variables (Kotz et al. 1988). Variance of the average number of flowers per inflorescence was estimated as the 'between sites' mean square, from an analysis of variance, allowing for site effects, divided by the number of inflorescences. Standard error was obtained as the square root of the variance. Standard errors for all other means in

Tables 1, 2 and 3 were estimated as the square root of the sample variance divided by $n = 4$.

5.2.10 Differences between trees

Tests were performed for differences between trees for five variables: PY, average number of inflorescences per raceme, average number of flowers per inflorescence, pod set, and seeds per pod. In each case, a random effects analysis of variance was performed, testing whether the variance between trees was significantly different from zero. Variance components were also included, where relevant, for samples or sites within trees, racemes within sites, and inflorescences within racemes. These analyses were performed using the statistical package GenStat (Harding et al. 2000).

5.3 Results

Density of flowers (DF) per m^3 estimated from the destructive samples ranged between 124,064 and 462,578 per m^3 (Table 5.1). There was a large within-tree variation in flower density, as shown by the large standard errors. The estimated total flower number per tree ranged from 1.25 million to over 13 million, with an average of 5.5 million. The number of racemes in the destructive samples varied by more than three-fold between trees, but the standard error was large between samples within individual trees. The number of inflorescences per raceme varied significantly between trees, with an average of 11.1 ($P = 0.002$). The mean number of flowers per inflorescence also varied significantly between trees, ranging from 12.0 to 16.7, with an mean of 14.2 ($P < 0.001$, Table 5.1).

Canopy surface area ranged from 5.1 to 23 m^2 for the 10 trees, while the tree radius ranged from 1.3 to 2.7 m (Table 5.2). There was approximately a 10-fold difference between the trees in canopy volume (range 8.5–82 m^3), and a 5-fold difference in area of yellow flowers (range 2.0–9.9 m^2). There was significant variability in the proportion of yellow per tree ($P < 0.001$), from 0.37 to 0.65 (Table 5.2).

There was significant variability in average percentage pod set between the 10 trees ($P < 0.001$), ranging from zero to 0.36%, with an average of 0.12% (Table 5.3). However, pod set analysis should be interpreted with caution, as the data did not exhibit constant variance. The average seeds per pod for each tree was significantly different between the 10 trees (range 2.6–6.0, $P = 0.051$), with an overall average for all the trees of 4.0. For total estimated number of pods per tree, there was a four-fold difference between the nine trees that produced pods, and for the total estimated seed numbers, there was nearly a six-fold difference. The range in total pod numbers for the 10 trees was zero to 8,135 (average 4,317). For the trees that had successful pod formation, the range in seed numbers was from 6,274 to 36,387 (average 19,559).

From the multiple linear regression to predict ln DF, neither ln PY ($P = 0.127$) nor ln size ($P = 0.682$) were significant. Due to the high P-value for ln size, ln size was removed from the model, giving the simplified model:

ln DF =	13.302	+	1.310 ln PY
s.e. =	0.45		0.57
$P =$	< 0.001		0.052

The regression for ln density on ln PY, gave an $R^2 = 0.40$ (Fig. 5.2), and $P = 0.052$.

Therefore, predicted density of flowers $= e^{13.302} \times PY^{1.310}$

$= 598,440 \times PY^{1.310}$

and predicted total number of flowers $=$ predicted density of flowers x canopy volume

$= 598,440 \times PY^{1.310}$ x canopy volume

Using this equation the calculated predicted number of flowers was found to be similar to the number estimated by destructive sampling (Table 5.1, Table 5.2, Fig. 5.3). Predicted number of flowers ranged from 1.5 to 16.3 million, with an average of 5.6 million (Table 5.2).

5.4 Discussion

Digital image analysis provided a non-destructive method to predict flower numbers of *A. baileyana*. Although the regression equation had an R^2 of only 0.40 and a P-value of 0.052, the image analysis technique shows potential, avoiding the need for costly and time-consuming destructive sampling. No direct comparison was made between the estimated number of flowers from destructive sampling and variables derived from digital images, as both depend on canopy volume. Instead, a relationship was derived between the components of these variables excluding canopy volume, namely estimated flower density (from destructive samples) and estimated proportion yellow (from digital images). This relationship was then used to determine predicted flower numbers. The predicted (from image digital analysis) and estimated (from destructive sampling) flower numbers were similar for the 10 trees, as shown by the means and standard errors.

One potential limitation in a model such as this is the calculation of canopy volume, as it is a vital component in the estimation of flower number. In this study, the canopy volume was assumed to be

Table 5.1 *Acacia baileyana* flower data (means ± standard errors) derived from destructive sampling.

Tree	Destructive sample volume m^3 ($n = 4$)	No. racemes x 10^3 per destructive sample ($n = 4$)	No. inflorescences per raceme ($n = 4$ means, each from 150 racemes)	No. flowers per inflorescence ($n = c.50$)	Density of flowers x 10^5 (flower no. per m^3) ($n = 4$)	Estimated total no. flowers x 10^6 per tree (ENF)
1	1.17 ± 0.07	2.74 ± 1.12	10.1 ± 0.45	12.3 ± 0.37	2.80 ± 1.06	2.48 ± 1.04
2	1.08 ± 0.07	2.32 ± 0.48	9.1 ± 0.58	12.0 ± 0.31	2.42 ± 0.45	2.41 ± 0.52
3	1.94 ± 0.27	2.90 ± 0.93	11.1 ± 1.35	13.5 ± 0.41	2.25 ± 0.92	13.17 ± 5.89
4	1.36 ± 0.08	1.31 ± 0.27	9.5 ± 1.52	13.9 ± 0.67	1.38 ± 0.49	3.09 ± 1.12
5	0.92 ± 0.07	2.13 ± 0.32	10.5 ± 0.78	14.2 ± 0.45	3.46 ± 0.51	2.96 ± 0.53
6	1.17 ± 0.09	2.24 ± 0.35	14.8 ± 1.14	16.2 ± 0.44	4.63 ± 0.73	8.07 ± 1.46
7	1.26 ± 0.03	1.65 ± 0.41	12.4 ± 1.01	16.7 ± 0.26	2.87 ± 0.80	3.37 ± 1.11
8	1.71 ± 0.15	1.85 ± 0.23	10.7 ± 0.46	13.8 ± 0.75	1.60 ± 0.17	13.14 ± 2.07
9	0.88 ± 0.07	0.86 ± 0.12	8.5 ± 0.19	15.0 ± 0.28	1.24 ± 0.21	1.25 ± 0.26
10	1.66 ± 0.05	1.00 ± 0.29	14.2 ± 1.67	14.5 ± 0.64	1.34 ± 0.46	5.49 ± 1.91
Mean	1.3	1.90	11.1	14.2	2.24	5.5
P-value			0.002 [x]	< 0.001 [y]		

[x] Indicates a significant difference in number of inflorescences per raceme between trees.
[y] Indicates a significant difference in number of flowers per inflorescence between trees.

Table 5.2 *Acacia baileyana* **canopy data (means ± standard errors) and predicted number of flowers determined by digital image analysis.**

Tree	Canopy surface area (SA) m^2 ($n = 4$)	Canopy radius m ($n = 4$)	Canopy volume (CV) m^3	Area of yellow flowers (AY) m^2 ($n = 4$)	Proportion of yellow (PY)	Predicted total no. of flowers x 10^6 per tree (PNF)
1	5.3 ± 0.56	1.3 ± 0.08	8.9 ± 1.57	2.0 ± 0.39	0.38 ± 0.04	1.48 ± 0.95
2	5.7 ± 0.40	1.3 ± 0.05	10.0 ± 1.08	3.7 ± 0.33	0.65 ± 0.02	3.38 ± 1.40
3	18.6 ± 2.31	2.4 ± 0.15	58.5 ± 10.79	8.1 ± 1.13	0.43 ± 0.05	11.70 ± 6.78
4	9.6 ± 0.51	1.7 ± 0.05	22.3 ± 1.74	3.6 ± 0.26	0.37 ± 0.02	3.63 ± 2.26
5	5.1 ± 0.32	1.3 ± 0.04	8.5 ± 0.86	3.3 ± 0.45	0.65 ± 0.05	2.88 ± 1.22
6	8.2 ± 0.49	1.6 ± 0.05	17.5 ±1.57	4.5 ± 0.54	0.54 ± 0.04	4.72 ± 2.15
7	6.4 ± 0.73	1.4 ± 0.08	11.7 ± 2.04	3.1 ± 0.70	0.49 ± 0.06	2.75 ± 1.45
8	23.0 ± 1.78	2.7 ± 0.10	82.0 ± 9.58	9.9 ± 0.88	0.43 ± 0.03	16.26 ± 9.03
9	5.7 ± 0.48	1.3 ± 0.05	10.1 ± 1.22	2.5 ± 0.37	0.44 ± 0.05	2.08 ± 1.15
10	14.4 ± 0.32	2.1 ± 0.02	41.1 ± 1.34	5.3 ± 0.38	0.37 ± 0.02	6.60 ± 4.12
Mean	10.2	1.7	27.1	4.6	0.48	5.6
P-value					< 0.001 [x]	

[x] Indicates a significant difference in PY between trees.

Table 5.3 Total pod and seed numbers estimated for 10 *Acacia baileyana* plants, extrapolated from pod and seed numbers per branch, with total flower counts and number of flowers per branch as the reference. Pod set and number of seeds per pod (mean ± standard error) were also determined per branch. Means per branch were derived from six branch samples.

| Tree | Mean per branch | | | | | | From estimated number of flowers | |
	No. inflorescences	No. flowers	No. pods	No. seeds	Pod set ± s.e (%) (n = 6)	No. of seeds per pod ± s.e (n)	Estimated no. pods per tree	Estimated no. seeds per tree
1	877	10789	9	38	0.08 ± 0.02	4.4 ± 1.0 (6)	2259	8616
2	601	7216	6	34	0.08 ± 0.04	5.7 ± 1.0 (3)	2007	11373
3	1100	14843	9	41	0.07 ± 0.03	3.3 ± 1.2 (4)	8135	36387
4	537	7469	19	61	0.23 ± 0.06	2.9 ± 0.7 (5)	8002	25111
5	575	8165	6	17	0.08 ± 0.04	2.6 ± 0.3 (4)	2292	6274
6	727	11783	8	29	0.07 ± 0.05	2.9 ± 1.1 (5)	5595	19868
7	654	10913	17	52	0.16 ± 0.06	3.6 ± 0.5 (5)	5297	16148
8	787	10863	0	na	0	na	0	na
9	727	10898	42	269	0.36 ± 0.05	6.0 ± 0.7 (6)	4778	30953
10	631	9145	8	36	0.09 ± 0.02	4.2 ± 0.5 (6)	4801	21306
Mean	4329	61250	75	384	0.12	4.0	4317	19559
P-value					< 0.001 [y]	0.051 [z]		

[y] Indicates a significant difference in percentage pod set between trees.
[z] Indicates a significant difference in seeds per pod between trees.
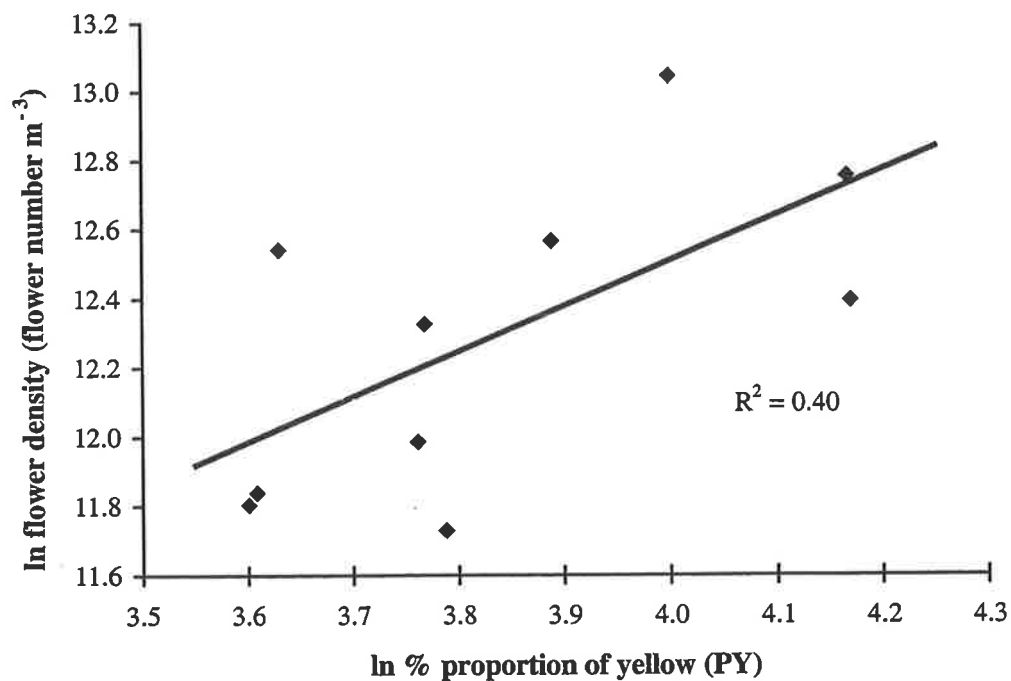
Fig. 5.2 Relationship between estimated density of flowers from destructive sampling and proportion of yellow (PY) in photographic images, with fitted regression line, ln density = 13.302 + 1.310 ln PY.
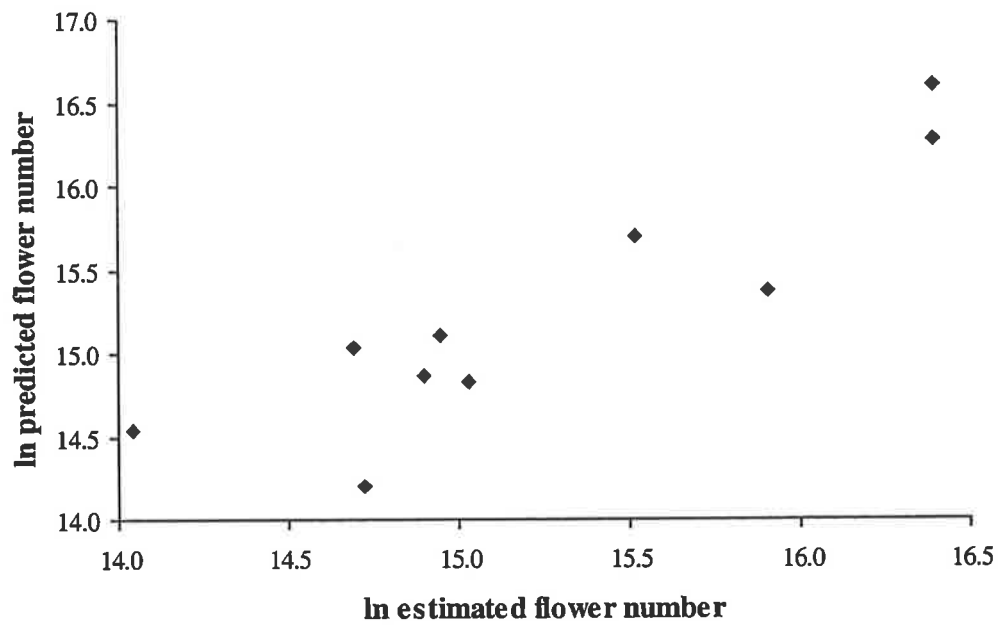
Fig. 5.3 Relationship between flower numbers predicted by digital image analysis and flower numbers estimated from destructive sampling.

that of a sphere with a radius equal to an average radius calculated from surface areas from the photographs. Several methods have previously been used to determine canopy volume of plants. Sydnor et al. (1975) used photographic methods to determine the canopy volume of *Chrysanthemum morifolium*, assuming that the plants were cylindrical in shape. Bignami and Rossini (1996) determined canopy volume of young hazelnut plants, using image analysis methods, assuming volume of an inverted cone. In this latter study, plant size parameters (height, width, canopy cross-sectional area and volume) were correctly predicted by image processing. Miller and Lightner (1987) determined canopy volume of apple trees using digital image analysis and a mathematical formula that correlated volume with trunk circumference and cross-sectional area. In the current study, a sphere was used to calculate canopy volume, as it is exact for spherical canopies and likely to be reasonable for many ellipsoidal-shaped canopies. This method is a better estimate than measurements based on height and width, which do not take into account the irregular boundaries of the canopies (Sydnor et al. 1975). However, confirming the accuracy of the canopy volume determined in this present study would be difficult due to the large size of the trees.

Other limitations of the technique include the sampling method for trees and destructive samples, and the relatively subjective procedures involved with the digital image analysis. Increasing the number of trees assessed would allow better evaluation or confirmation of the regression model, and may allow inclusion of other explanatory variables such as canopy size. It should also provide more precise estimates of the parameters α, β and γ and hence predicted flower density. There will always be variability between trees, although the model may be improved by allocating different sized trees into categories. Predicted flower density could also be improved by improving the estimated flower density and proportion yellow (PY) measurements. To improve flower density estimates without total destructive sampling, sample number or sample size could be increased. In the current study, 5% of the tree canopy was sub-sampled for flower density. This could be increased to 10%.

In determining the proportion of yellow, the use of digital image analysis requires consideration of how factors such as light conditions, interference with neighbouring trees, and image collection procedure affect accuracy. Changing the brightness and contrast of the image before scanning and changing the threshold for the yellow flower detection in the video programme can alter the area of yellow. These subjective procedures must be carefully monitored, and ideally, the same operator should do all the digital image analysis. In this study, care was taken to prevent errors. Dense tree canopies must also be chosen for this technique, as flowers on the far side of the tree may be inadvertently included in the calculation of PY in sparse canopies. The dense canopy trees may therefore, give an over estimation of the number of flowers in the population. More photographs may also give a better representation of PY for the tree. In addition, solitary trees are needed to obtain the four photographs, which prevents

the random choice of plants. To study trees in groups, the image would need digital manipulation to remove unwanted trees from the background.

The average pod set for *A. baileyana* was 0.12% in this study, and ranged from zero to 0.36%. This was similar to the pod set results of Chapter 4. It is common for pod set to be low in acacias (Milton and Hall 1981, Tybirk 1989, Moncur et al. 1991, Tybirk 1993, Grant et al. 1994). In this study, low pod set may have been due to limited movement of pollinators between trees, since the 10 trees chosen for the study were solitary (to permit photography for digital analysis). In addition, pollination and fertilisation failure could occur if ineffective self pollination is common, as *A. baileyana* is highly self incompatible (Chapter 4). Pod and seed production may also be limited by adverse environmental conditions.

The mature *A. baileyana* trees studied in the Adelaide Hills produced an average of 5.5 million flowers that resulted in an average of 19,559 seeds per tree. In comparison, 2-year-old trees produced an average of 833 seeds for the purple leaf form and 1,798 seeds for the green leaf form (Table 4.7, Chapter 4). Maximum seed production for one mature trees was over 36,000. This is a very large reproductive output, confirming the weed potential of this species. Both young and mature trees are reproductively efficient.

Other species of *Acacia* that have become weeds show a similar reproductive output. For example, *A. saligna* and *A. cyclops*, Australian acacias that are weeds in south-western Cape Province of South Africa, can produce over 48,000 seeds per tree (Milton and Hall 1981). In comparison, *A. nilotica*, a species from South African and the Indian continent, is a major weed in the Mitchell grasslands of Queensland (Mackey 1997). It can produce three million flowers per tree, with seed production per tree reaching over 30,000 in South Africa (Tybirk 1989) and as much as 175,000 in Queensland (Carter et al. 1989). *Acacia baileyana* and *A. nilotica* have similar pod set, although *A. baileyana* had an average of 4.0 seeds per pod, compared to 10.8 for *A. nilotica* (Tybirk 1989). This accounts for the lower reproductive output of *A. baileyana*. This average seeds per pod of 4.0 (maximum 6.0) was lower than that previously recorded for *A. baileyana* (7.6 with a maximum of 12; Kenrick and Knox 1982).

Digital image analysis was useful in determining total numbers of flowers of *A. baileyana*. Despite the low pod set the reproductive output is still high, due to the high flower numbers. The extent to which *A. baileyana* is a major weed problem will depend on the number of seeds that germinate and grow. However with such a large seed set, even a low rate of germination and survival could allow this species to spread rapidly.

Development of HPLC methods for analysis of leaf anthocyanins

6.1 Introduction

No major differences were found between the green and purple leaf forms of *A. baileyana* in terms of their flowering, growth, breeding systems and reproductive efficiency (Chapters 3, 4 and 5). The only obvious difference between the two forms is the intensity of the purple leaf colour. This purple colour is due to the production of anthocyanins (Nozzolillo 1973). Anthocyanins are water-soluble, coloured flavonoid compounds, responsible for much of the orange and red colour, and most of the blue and purple tones in petals, fruits, leaves and roots of higher plants (Harborne 1967).

Accumulation of anthocyanins in *A. baileyana* occurs in juvenile leaves and their stems, in the pedicels of the inflorescences and in seedling plants (Plate 6.1). As young leaves and stems mature, the colour disappears from the tissue. The purple leaf colour can vary in intensity within a plant and between plants and throughout the seasons. The leaf colour of *A. baileyana* variety *purpurea* is intensely purple, while the green leaf form can also display a small amount of purple colour on young leaves. Very little is known about the anthocyanin chemistry of native plants, therefore it is not surprising that the compounds that contribute to leaf colour in *A. baileyana* have not been previously described. To enable manipulation of leaf colour in this horticulturally significant plant, an understanding of the chemistry of this trait is required.

The aim of this study was to develop a sensitive method to analyse anthocyanin pigments in *A. baileyana* leaves. High performance liquid chromatography (HPLC) was chosen as the method for analysis as it is a fast and powerful tool for separating individual components from complex mixtures. Reverse-phase HPLC was used, in which the liquid (mobile) phase is more polar than the stationary phase. Compounds bound to the column matrix by non-polar interactions are specifically eluted by the application of an increasing concentration of non-polar solvent, commonly acetonitrile. Reverse-phase HPLC is frequently used for non-polar or weakly polar compounds, and is therefore appropriate for the separation of anthocyanins.

HPLC conditions were modified to optimise the separation and resolution of individual anthocyanin compounds present in leaf extracts, from the two forms of *A. baileyana*. The modified conditions included examining extract concentration and dilution before loading onto column, solvent gradient, and column temperature. The efficiency of the anthocyanin extraction method and the effect of freezing leaf

Plate 6.1 Occurrence of the purple and green colouration in *A. baileyana*. A: Green petioles, leaves and leaf stems. Scale bar = 1.2 cm. B: Purple petioles, leaves and leaf stems. Scale bar = 1.2 cm. C: Purple and green pedicels of flowers. Scale bar = 0.75 cm. D: Purple and green seedlings. Scale bar = 2 cm.
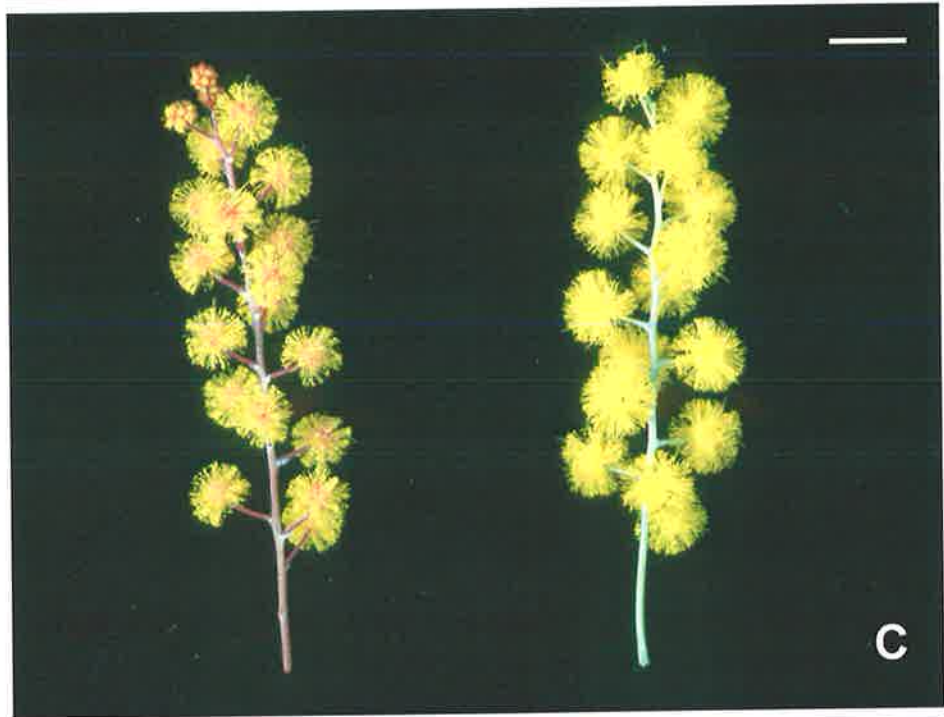
samples were also investigated. The optimised HPLC method was then used to identify the main anthocyanins present in leaves of variety *purpurea* (Chapter 7), and to compare the anthocyanin content of leaves with an allocated visual colour category (Chapter 8).

6.2 Development of HPLC methods

6.2.1 Leaf tissue extraction method

6.2.1.1 Plant material

Anthocyanins were isolated from the youngest, fully-formed leaves of *A. baileyana* and *A. baileyana* variety *purpurea,* from:

- Cuttings of three genotypes. The cutting material was grown in three temperature controlled growth cabinets, outside or shadehouse (refer to section 3.2.2 and 3.2.3 for cutting propagation and growth cabinet details).

- Samples taken in summer and winter from trees in the Waite Campus plantation (refer to section 4.2.1 for plantation details),

- Seedling plants grown outside, produced from 1995 crosses (refer to section 9.2.3.1 for cross pollination details).

Between three and five leaves (*c.* 0.5 g) were sampled and assessed for colour and allocated to one of four colour categories (1, 2, 3, 4), as described in Chapter 3. The more intense the purple colour the higher the colour category number.

6.2.1.2 Extraction of pigments

Fresh or frozen samples were frozen and ground to a fine powder in a mortar under liquid nitrogen. A sample (typically 0.1 g) of the powder was mixed with 10 mL of acidulated ethanol extraction solution [containing 95% ethanol: potassium chloride / hydrochloric acid at pH 1 (9:1)], heated to 70°C for 5 min, and then kept overnight at 4°C (method adapted from Price et al. 1995). The samples were centrifuged for 10 min and the supernatant stored at -80°C until required.

6.2.2 HPLC method

The purified anthocyanin samples were separated on a Beckman (Fullarton, CA, USA) System Gold HPLC equipped with a Vydac (W.R. Grace, Columbia, MD, USA) 201TP54 C_{18} reverse-phase column (250 x 4.5 mm i.d., 5 µm pore size). Elution with a solvent gradient made up from Buffer A (0.1% trifluoroacetic acid by volume in water) and Buffer B (0.085% trifluoroacetic, 80% acetonitrile by volume in water), prepared using a Gilson (Middleton, WI, USA) model 811C dynamic mixer, was performed at a flow rate of 0.6 mL/min using a Beckman model 126NM syringe pump. Buffer

components were filtered through a 0.45 μm PVDF (polyvinylidene fluoride) membrane (Millipore type HF) at the time of preparation and de-gassed under reduced pressure daily. All solvents were of HPLC grade. The column temperature was initially maintained at 30°C by an Eppendorf (Hamburg, Germany) model TC-50 column heater. The initial solvent gradient conditions (solvent gradient 1) are shown in Table 6.1.

Table 6.1 Solvent gradient 1 conditions.

Time (min)	Buffer B (%)	Duration (min)
0.00	0.00	5.00
5.00	35.16	25.00
30.00	100.00	10.00
45.00	0.00	5.00
55.00	—	—

For method development, leaf extracts were taken from the -80°C freezer, and mixed with varying volumes of Buffer A for loading onto the column. The samples were mixed by centrifugation and loaded automatically using a Beckman model 507e autosampler fitted with a 500 μL loading loop, and the absorbance of the eluent was monitored at 280 nm and 520 nm with a Beckman System Gold 168 photo-diode array detector.

The wavelength of 520 nm was used to measure the total content of anthocyanin compounds and the content of individual anthocyanin compounds. Data collection and analysis were performed with Beckman System Gold Nouveau software (version 1.6). Chromatograms drawn at both wavelengths were integrated after acquisition and peak areas were determined using the manufacturer's software. For clarity, the measured absorbance values in mAU (milli absorbance units) were divided by 100,000 in each case, to give scaled absorbance data.

6.2.3 Optimisation of methods

6.2.3.1 Trial 1 – Concentration of leaf extracts

Three different leaf extraction treatments were compared:
1) 0.1 g leaf material extracted in 10 mL extraction buffer.
2) 0.4 g leaf material extracted in 10 mL extraction buffer.
3) 0.1 g leaf material extracted in 10 mL extraction buffer and then concentrated four-fold.

Cuttings from six mother plants (genotype 2, details in Table 3.2) grown either outside or in a shadehouse, were used for this trial. Leaves of these cuttings can have an intense purple colour in cool

conditions (see Chapter 8). The leaves were assessed as colour category 3 for this trial, as these leaf samples were collected in April when the ambient temperature was just starting to drop.

The anthocyanins from either 0.1 g or 0.4 g of fresh leaf material were extracted in 10 mL of extraction buffer, as in section 6.2.1.2. For treatment 3, 2 mL of extract was concentrated to 0.5 mL on a centrivap concentrator (Labconco) at 35°C, connected to a cold trap (Labconco). A 350 μL sample (120 μL of extract plus 230 μL of Buffer A) was loaded on the HPLC column. The HPLC solvent gradient conditions used were as described in Table 6.1.

The HPLC chromatograms from two plant samples are presented (Fig. 6.1, Fig. 6.2); all the other samples showed similar results. Treatments 2 and 3 had about four times the anthocyanin content as treatment 1. Treatment 3 produced the best HPLC traces for all samples (Fig. 6.1, Fig. 6.2). The presence of excess ethanol in the samples may have contributed to the poor peak resolution for extracts obtained using treatments 1 and 2, causing the appearance of double peaks. The anthocyanin peaks obtained using treatment 3 required further resolution, as the first peak had a thick base at the front of the peak (fronting). This fronting could be due to a small band eluting just before the main peak, and/or an excessive volume of strong solvent injected with the sample. Therefore ethanol may still be affecting the peak resolution.

6.2.3.2 Trial 2 – Storage of leaves at -80°C prior to extraction

To examine the effect of storage of leaves at -80°C on the subsequent extraction and HPLC of anthocyanins, samples of both fresh and frozen leaves from cuttings (genotype 2) were compared. The frozen samples had been stored for one week at -80°C. Anthocyanins were extracted from both fresh and frozen leaves harvested from seven plants grown in one of three temperature controlled growth cabinets (Table 6.2). Cuttings grown in the coldest temperature cabinet had a colour category of 3 or 4, while cuttings grown in the warmer temperatures had a colour category of 2.

The anthocyanin extraction protocol described in section 6.2.1 was used with 0.1 g of leaf samples. 2 mL of extract sample was concentrated to 0.5 mL, on a centrivap concentrator, as previously described. A 350 μL sample (120 μL of extract plus 230 μL of Buffer A) was loaded onto the HPLC column. The HPLC solvent gradient conditions used was as described in Table 6.1. Areas under all the HPLC peaks, plus areas under the first peak (peak a) and second peak (peak b) were determined (Table 6.2). The amount of anthocyanins obtained at A_{520nm} in the sample was determined by integrating the area under the specific peaks. For clarity, the measured absorbance values in mAU (milli absorbance units) were divided by 100,000 to give scaled absorbance data.

The HPLC chromatograms from fresh and frozen samples from two plants are presented (Fig. 6.3, Fig. 6.4); all the other samples showed similar results. There was no difference between fresh and frozen

Fig. 6.1 HPLC chromatograms showing absorption profiles at $A_{520\,nm}$ for a genotype 2 cutting of colour category 3, grown outside. A: 0.1 g leaf material extracted in 10 mL extraction buffer. B: 0.4 g leaf material extracted in 10 mL extraction buffer. C: 0.1 g leaf material extracted in 10 mL extraction buffer and then concentrated four-fold. Please note that the scales differ for each absorption profile.
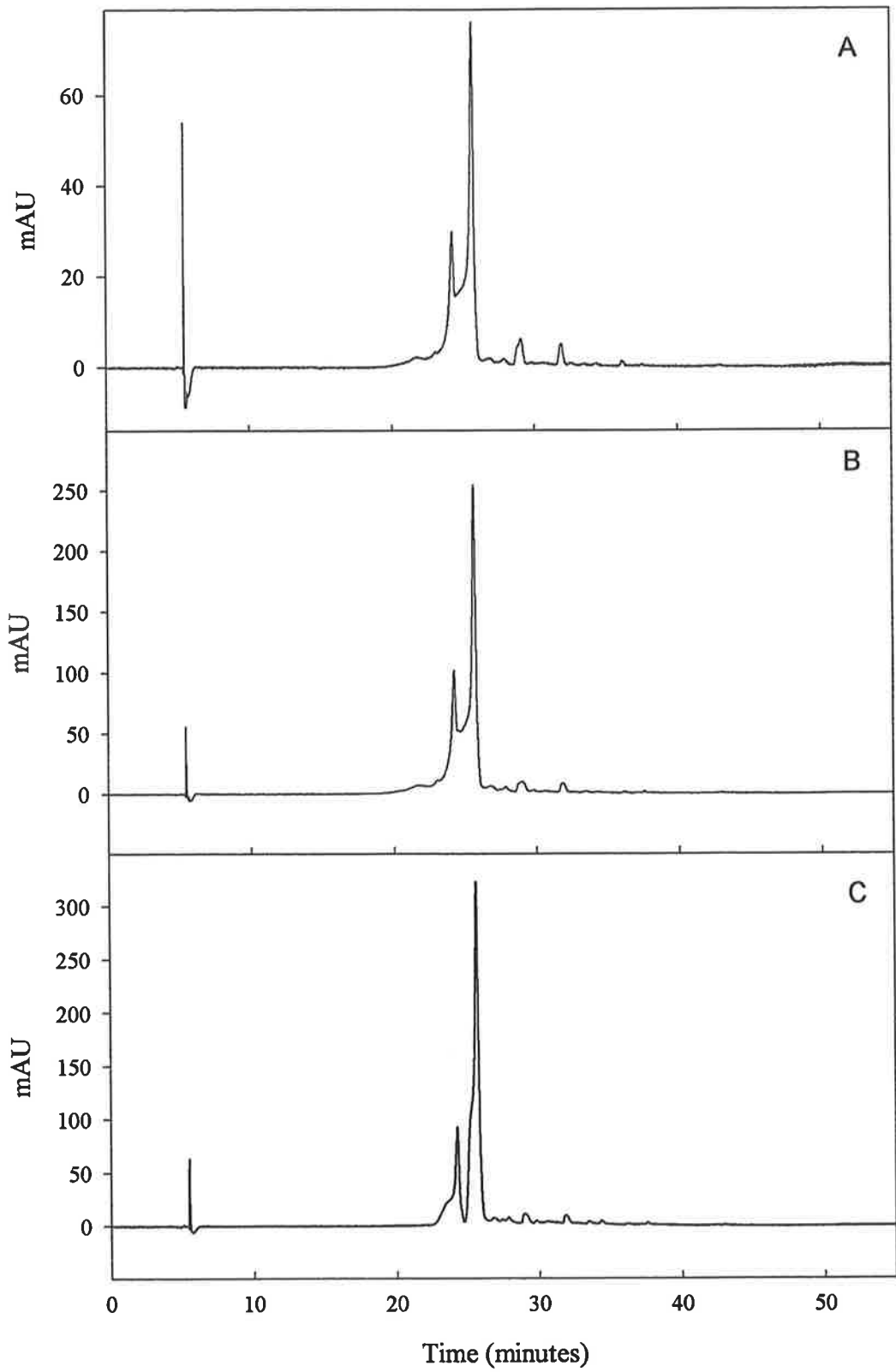
Fig. 6.2 HPLC chromatograms showing absorption profiles at A_{520nm} for a genotype 2 cutting of colour category 3, grown in a shadehouse. A: 0.1 g leaf material extracted in 10 mL extraction buffer. B: 0.4 g leaf material extracted in 10 mL extraction buffer. C: 0.1 g leaf material extracted in 10 mL extraction buffer and then concentrated four-fold. Please note that the scales differ for each absorption profile.
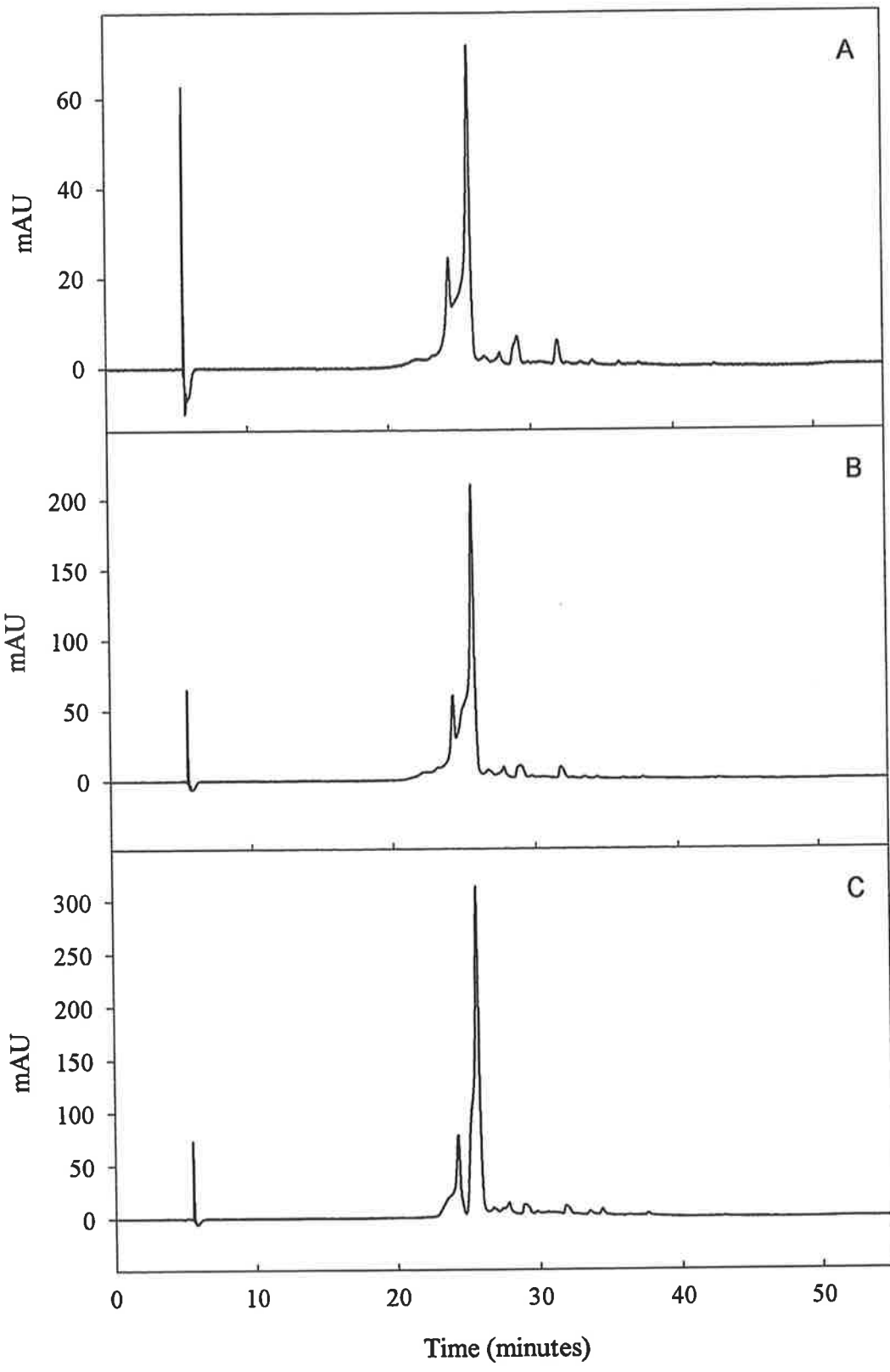
leaves in the resolution of peaks upon HPLC (Fig. 6.3, Fig. 6.4). There were differences between fresh and frozen samples in the amount of total anthocyanins, but this may be explained by the variation in total anthocyanin amount that can occur within one colour category (Table 6.2). Area under peak b was greater than for peak a, for all samples (Table 6.2). Resolution of the HPLC peaks required further improvement; the formation of fronting for the first peak (peak a) was again apparent.

Table 6.2 Anthocyanin content of fresh and frozen leaf samples of cuttings of genotype 2, determined by HPLC. Peak a is the first and Peak b the second peak eluted.

Plant no.	Sample	Colour category	Temperature of growth cabinets (max./min.°C)	Anthocyanin content (scaled mAU)		
				Sum of all peaks	Peak a	Peak b
1	frozen	3	13/9	68.6	7.8	46.9
	fresh	3		51.1	4.5	35.8
2	frozen	4	13/9	86.2	12.4	57.3
	fresh	4		65.1	7.9	49.9
3	frozen	2	23/19	12.5	1.2	8.5
	fresh	2		18.0	1.9	13.3
4	frozen	2	23/19	22.7	2.2	16.6
	fresh	2		24.8	1.8	19.4
5	frozen	2	18/13	25.8	2.7	18.4
	fresh	2		42.2	5.0	23.3
6	frozen	2	18/13	14.6	0.1	12.0
	fresh	2		33.0	2.0	29.7
7	frozen	2	18/13	6.3	0.0	3.0
	fresh	2		12.4	0.0	9.7

6.2.3.3 Trial 3 – Concentration of extract and subsequent dilution with Buffer A prior to HPLC

To improve resolution of HPLC peaks, extracts of leaf samples (Table 6.3) were evaporated to concentrate the anthocyanins, and diluted for column loading (Table 6.4). This was done to reduce the amount of ethanol in the loading solution, as it was assumed that ethanol was differentially lost by evaporation.

Table 6.3 Leaf sample details.

Plant no.	Plant type	Growing condition	Colour category	Comments
1	cutting (genotype A)	shadehouse	1	No anthocyanin
2	cutting (genotype B)	shadehouse	3	Fig. 6.5
3	seedling	outside	2	Low amounts of anthocyanins
4	seedling	outside	3	Fig. 6.6

Fig. 6.3 HPLC chromatograms showing absorption profiles at $A_{520\,nm}$ for a genotype 2 cutting (plant number 1 in Table 6.2), of colour category 3, grown at 13°C day and 9°C night in a growth cabinet. The first peak (peak a) and the second peak (peak b) are identified. A: Frozen leaf sample. B: Fresh leaf sample. Please note that the scales differ for each absorption profile.
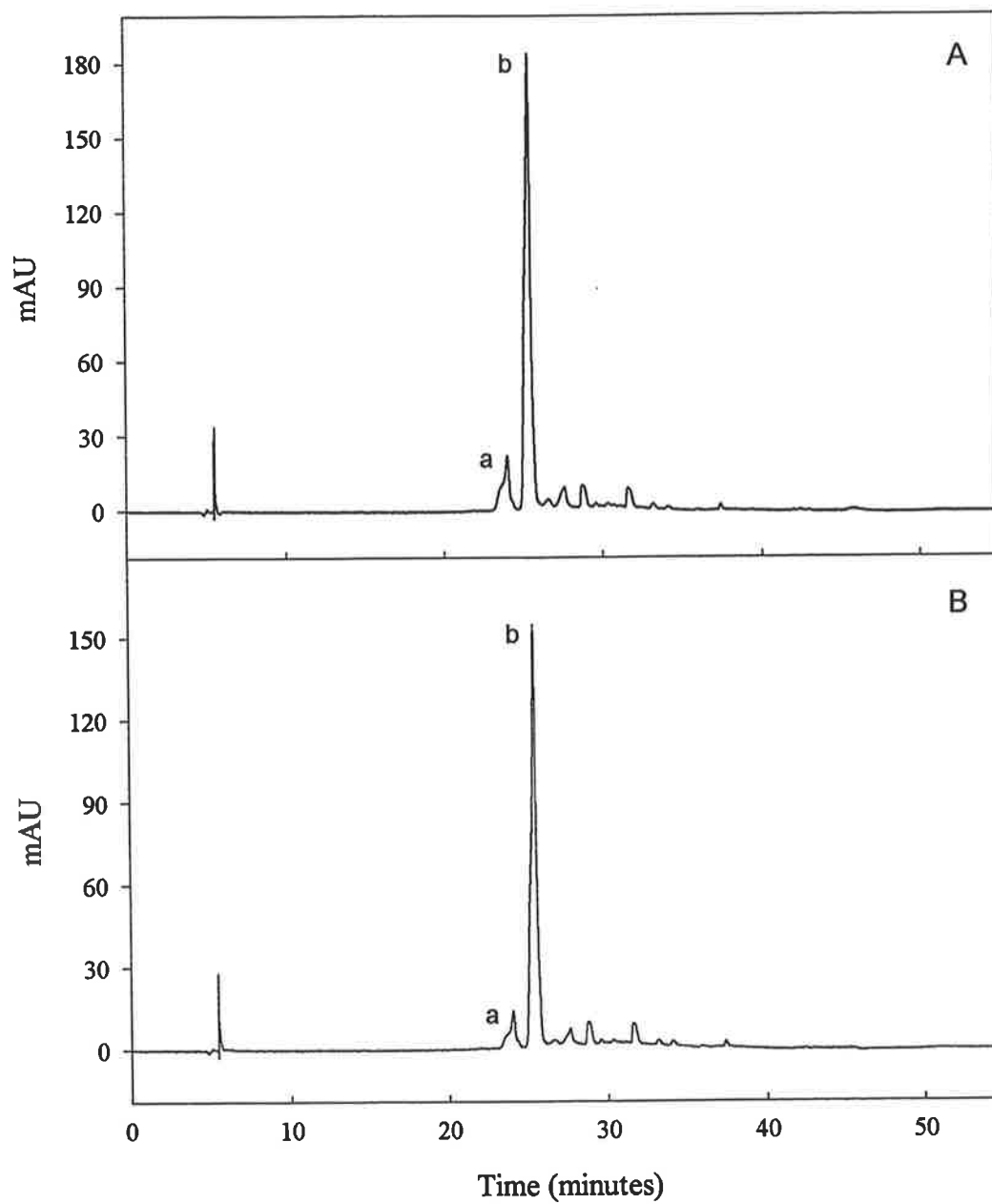
Fig. 6.4 HPLC chromatograms showing absorption profiles at $A_{520\ nm}$ for a genotype 2 cutting (plant number 6 in Table 6.2), of colour category 2, grown at 18°C day and 13°C night in a growth cabinet. The first peak (peak a) and the second peak (peak b) are identified. A: Frozen leaf sample. B: Fresh leaf sample. Please note that the scales differ for each absorption profile.
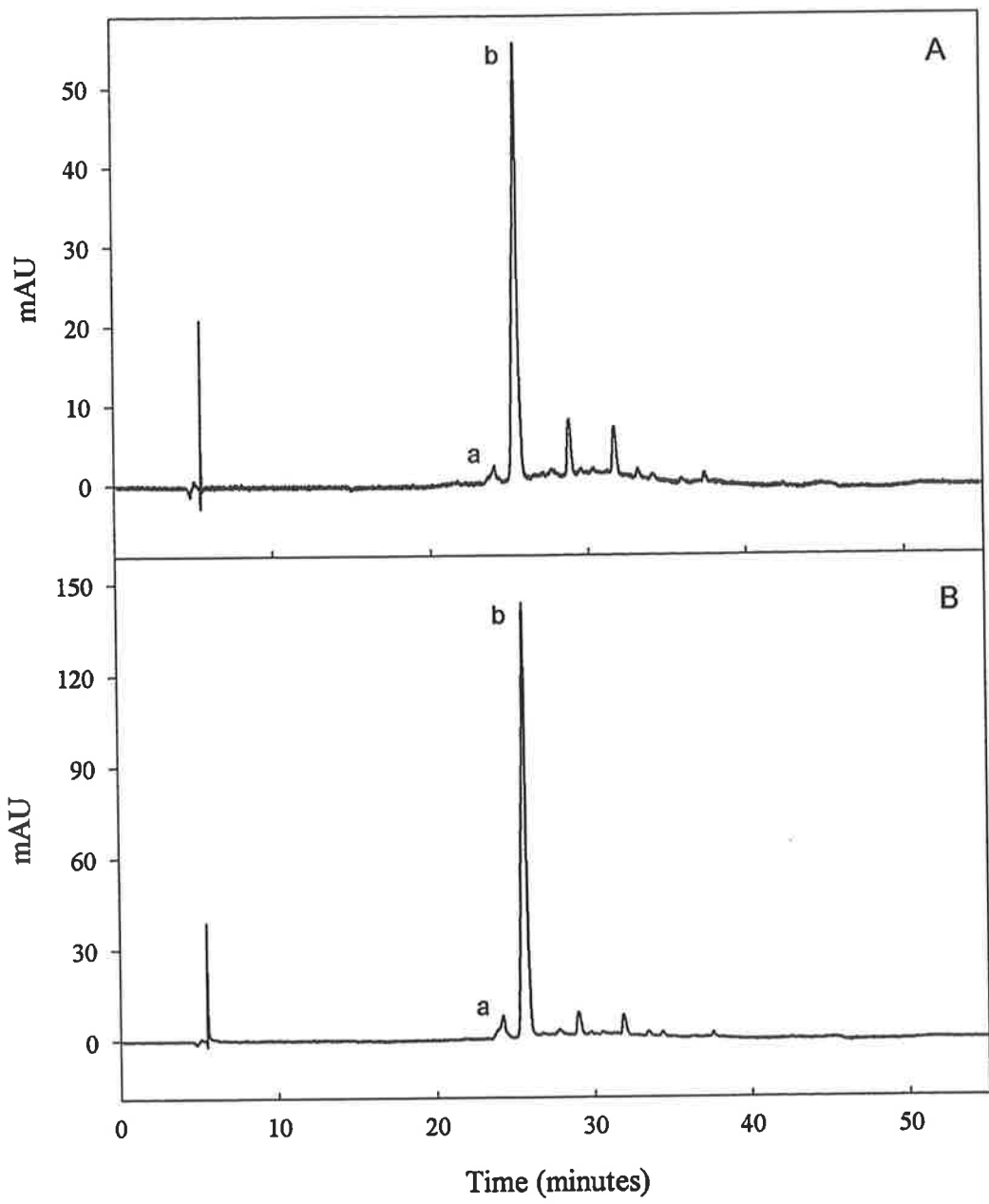
Table 6.4 Four concentration and dilution treatments.

Treatments	Concentration of anthocyanin extracts	Dilutions of extracts for column loading, with 500 μL loaded onto column
1	not concentrated	200 μL extract + 1.8 mL buffer A
2	concentrated from 2 mL to 0.5 mL	50 μL extract + 1.95 mL buffer A
3	concentrated from 2 mL to 0.3 mL	200 μL extract + 1.8 mL buffer A
4	concentrated from 2 mL to 0.5 mL	200 μL extract + 1.8 mL buffer A

Fresh leaf material from two cuttings (genotype A and B) grown in the shadehouse, and two seedling plants (1995 hybrids, see Chapter 9) grown outside (Table 6.3), were each subjected to the four treatments (Table 6.4). The extraction protocol and the HPLC solvent gradient conditions (Table 6.1) were as described earlier in sections 6.2.1 and 6.2.2.

The results from only two plants are shown (Fig. 6.5, Fig. 6.6), as the other two plants had low or no detectable anthocyanins (Table 6.3). Concentrating the extract and reducing the ratio of extract to loading buffer considerably improved the resolution of the eluting peaks (Fig. 6.5, Fig. 6.6), as compared to the previous trials. This is probably due to a reduction in the amount of ethanol. By concentrating the extract, the ethanol component of extraction buffer would be more readily lost on evaporation than the aqueous solution. Ethanol may have interfered with the binding and release of anthocyanins by the column. When the sample was not concentrated, a large solvent loading peak was apparent (Fig. 6.5A, Fig. 6.6A), due to the higher ethanol content of the extraction medium interfering with the detection system. Diluting the extract with Buffer A also increased the proportion of buffer, which allowed the compounds to firmly bind onto the column early in the chromatographic process, and to elute more distinctly as the gradient progressed.

Concentration of extract from 2 mL to either 0.3 mL or 0.5 mL, with 200 μL extract plus 1.8 mL Buffer A solution mixed together, and 500 μL loaded onto the column, gave the best results. In all subsequent trials, concentrating from 2 mL to 0.5 mL was used as this had the benefit of being quicker than concentrating to 0.3 mL. All peaks still did not resolve clearly, with peaks having a thick base at the tail end (tailing) (Fig. 6.5, Fig. 6.6). This tailing was most likely due to small peaks eluting just after each of the main peaks.

6.2.3.4 Trial 4 – Estimation of efficiency of anthocyanin extraction from leaf tissue

A high level of extraction was important if all anthocyanins present in leaf extracts were to be isolated. Trial 4 was conducted to confirm that the anthocyanin extraction method used was achieving extraction of greater than 90% of anthocyanins. Leaf samples were taken from ten plantation trees (details in

Fig. 6.5 HPLC chromatograms showing absorption profiles at $A_{520\ nm}$ for a genotype B cutting of colour category 3, grown in a shadehouse. A: Treatment 1 (extract not concentrated, 200 µL extract + 1.8 mL Buffer A). B: Treatment 2 (extract concentrated from 2 mL to 0.5 mL, 50 µL extract + 1.95 mL Buffer A). C: Treatment 3 (extract concentrated from 2 mL to 0.3 mL, 200 µL extract + 1.8 mL Buffer A). D: Treatment 4 (extract concentrated from 2 mL to 0.5 mL, 200 µL extract + 1.8 mL Buffer A). Note that 500 µL of diluted extract was loaded onto the column. Please note that the scales differ for each absorption profile.
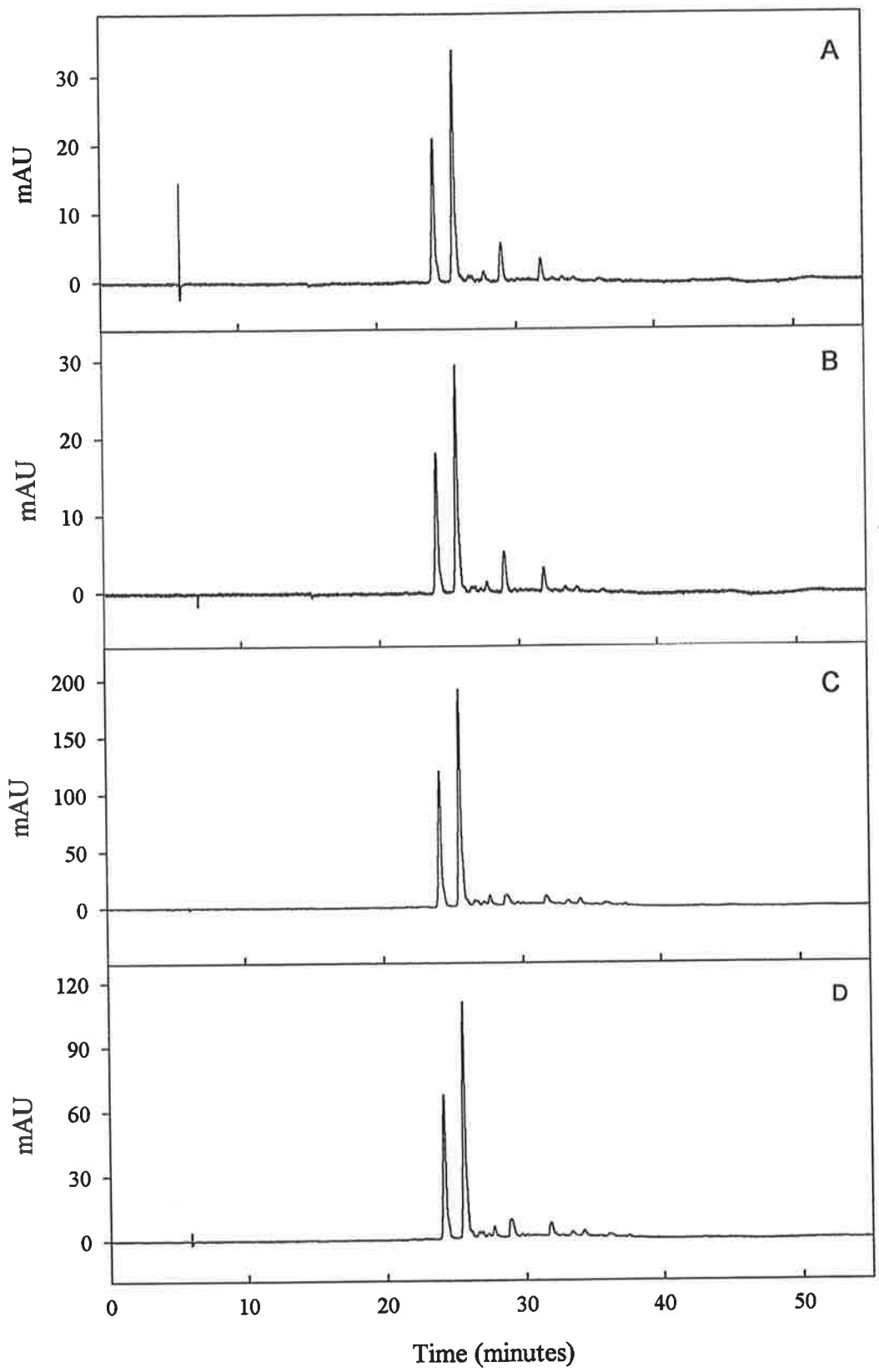
Fig. 6.6 HPLC chromatograms showing absorption profiles at $A_{520\,nm}$ for a seedling of colour category 3, grown outside. A: Treatment 1 (extract not concentrated, 200 µL extract + 1.8 mL Buffer A). B: Treatment 2 (extract concentrated from 2 mL to 0.5 mL, 50 µL extract + 1.95 mL Buffer A). C: Treatment 3 (extract concentrated from 2 mL to 0.3 mL, 200 µL extract + 1.8 mL Buffer A). D: Treatment 4 (extract concentrated from 2 mL to 0.5 mL, 200 µL extract + 1.8 mL Buffer A). Note that 500 µL of diluted extract was loaded onto the column. Please note that the scales differ for each absorption profile.
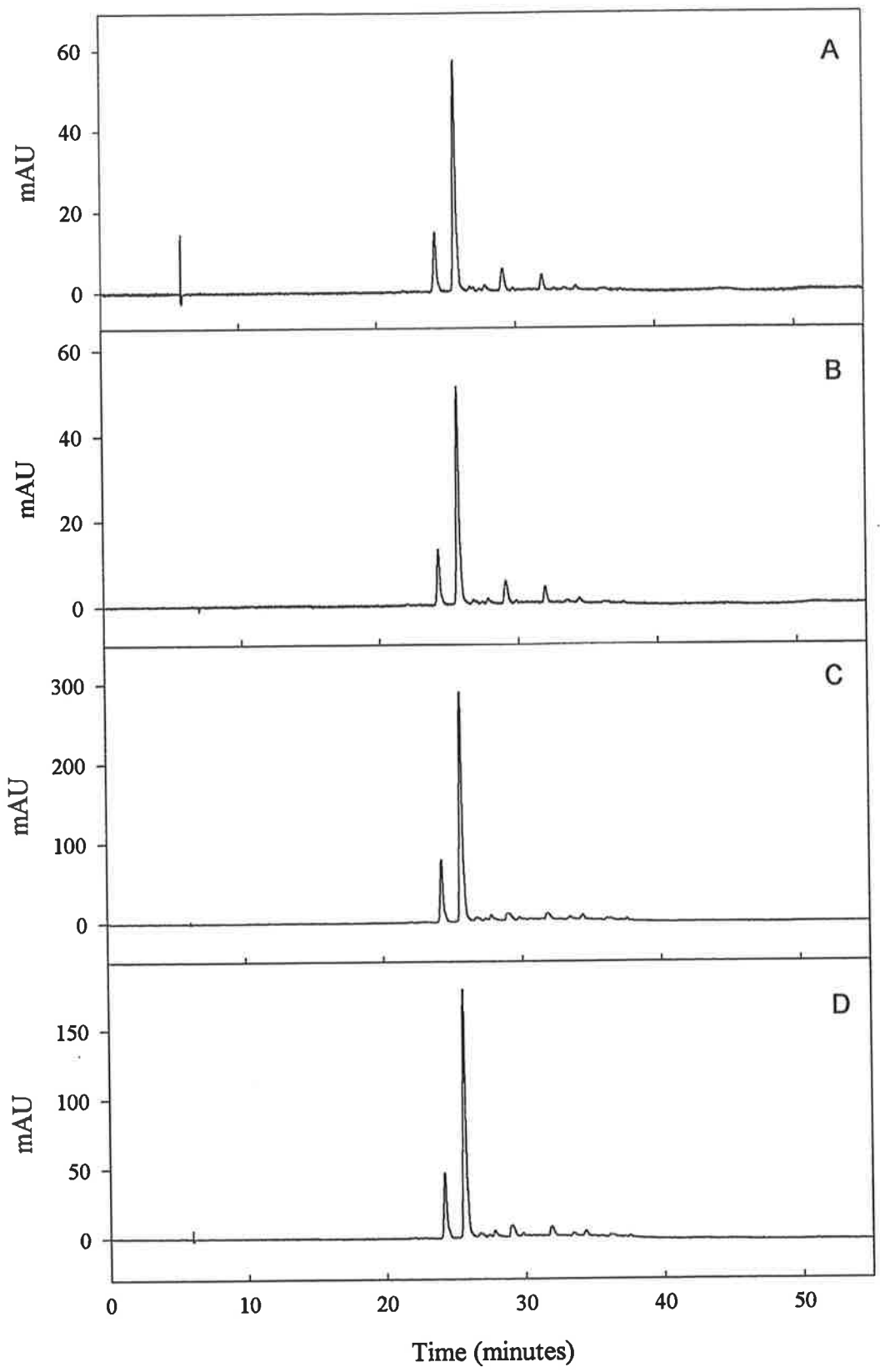
section 8.2.3.1) in summer and winter, and stored frozen. Leaves that had a colour category of 3 in summer, had a colour category of 4 in winter, due to the cooler temperatures (Table 6.5, Table 6.6). Leaves of colour category 2 in summer had the same colour category in winter.

Table 6.5 **Efficiency of anthocyanin extraction from leaves of *A. baileyana* sampled in summer.**
Total anthocyanin content of extractions 1 and 2 was measured at $A_{520\,nm}$.

Plant no.	Colour category	Total anthocyanin content (scaled mAU)		Total anthocyanins extracted (%)
		From extraction 1	From extraction 2	
1	3	33.3	1.4	96
2	3	90.9	2.8	97
3	3	120.8	3.7	97
4	3	119.7	3.7	97
5	3	47.1	1.5	97
6	2	19.1	0.0	100
7	2	7.7	0.0	100
8	2	9.3	0.0	100
9	2	9.4	0.0	100
10	2	7.7	0.0	100

Table 6.6 **Efficiency of anthocyanin extraction from leaves of *A. baileyana* sampled in winter.**
Total anthocyanin content of extractions 1 and 2 was measured at $A_{520\,nm}$.

Plant no.	Colour category	Total anthocyanin content (scaled mAU)		Total anthocyanins extracted (%)
		From extraction 1	From extraction 2	
18	4	76.4	2.2	97
19	4	114.9	4.0	97
20	4	119.8	4.4	96
21	4	117.1	4.8	96
22	4	103.8	4.6	96
23	2	12.1	0.0	100
24	2	5.8	0.0	100
25	2	5.5	0.0	100
26	2	7.3	0.0	100
27	2	6.7	0.0	100

These 20 samples were subjected to two sequential extractions. The first extraction followed the method described earlier (section 6.2.1). For the second extraction, the pellet formed following centrifugation of the first extract was re-extracted in 10 mL of buffer, heated at 70°C for 5 min and kept at 4°C overnight, and then centrifuged. 2 mL of the first and second extraction supernatants were concentrated to 0.5 mL. 200 µL concentrated extract plus 1.8 mL Buffer A solution were mixed together, and a 500 µL aliquot of this mixture loaded onto the column and subjected to HPLC using the

solvent gradient conditions described earlier (Table 6.1). The percentage of total anthocyanins extracted (measured at 520 nm) was calculated from the total anthocyanin content in the sample from the first extraction divided by the sum of the anthocyanin content from both extractions (Table 6.5, Table 6.6).

Greater than 96% of the anthocyanins from the intensely purple leaves (colour categories 3 and 4) were extracted with a single extraction. For the less intensely coloured leaves (colour category 2), 100% extraction was achieved. This confirms that a very high proportion of anthocyanins present in the 0.1 g of leaf material in 10 mL of extraction buffer, is extracted. No other extraction methods were tried due to the success of this method.

6.2.3.5 Trial 5 – Manipulation of HPLC solvent gradient conditions

The main anthocyanin peaks were eluted at about 25 min of chromatography (Fig. 6.5, Fig. 6.6). To improve resolution of these peaks, the gradient conditions were changed to extend the time taken to achieve 100% Buffer B. This should allow for a slower elution of the anthocyanin compounds over the different concentrations of Buffer B, and therefore increase their separation. Three new gradient conditions (Tables 6.7) were compared.

Table 6.7 Solvent gradient conditions for Trial 5.

Solvent gradient condition	Time (min)	Buffer B (%)	Duration (min)
2	0.00	0.00	5.00
	5.00	35.00	35.00
	40.00	100.00	5.00
	45.00	0.00	5.00
	50.00	0.00	5.00
	55.00	—	—
3	0.00	0.00	5.00
	5.00	13.00	13.00
	18.00	33.00	40.00
	58.00	100.00	3.00
	64.00	—	—
4	0.00	0.00	5.00
	5.00	15.00	5.00
	30.00	50.00	20.00
	50.00	100.00	5.00
	55.00	0.00	10.00
	65.00	—	—

A frozen leaf sample from a plantation tree (colour category 4) was used to test the different HPLC gradient conditions. The extraction followed the basic method (section 6.2.1), with a 2 mL extract

concentrated to 0.5 mL. 200 μL concentrated extract plus 1.8 mL Buffer A solution were mixed together, and three 500 μL aliquots of the mixture loaded onto the column, and subjected to HPLC using one of the three gradient conditions to be assessed.

The three new gradient conditions improved the resolution of the chromatograms (Fig 6.7), but the two main peaks (peaks a and b) still did not separate clearly. These chromatograms confirmed that the tailing was due to smaller peaks eluting with the main peaks. Therefore, peaks a and b each consisted of two peaks.

6.2.3.6 Trial 6 – Manipulation of HPLC gradient conditions and temperature of chromatography column

The HPLC gradient conditions and temperature of the chromatography column were changed in order to improve the resolution of peak separation. The gradient length was increased further from that of trial 5 (Table 6.9).

Combinations of gradient conditions and column temperatures were tested (Table 6.8). For test runs 1 to 5, the same extract supernatant was used, from one cutting (genotype 2), colour category 4, grown outside. For test run 6, a winter leaf sample from a plantation tree was used, as it had a colour category of 4. All samples followed the basic extraction method (section 6.2.1), with 2 mL of extract concentrated to 0.5 mL. 400 μL concentrated extract plus 3.6 mL Buffer A solution were mixed together, and five 500 μL aliquots of this mixture were loaded onto the column and subjected to HPLC using test runs 1 to 5 conditions for the cutting sample (Table 6.8). For the plantation sample, 200 μL concentrated extract plus 1.8 mL Buffer A solution were mixed together, and a 500 μL aliquot was loaded onto the column and subjected to HPLC using test run 6 conditions (Table 6.8).

Table 6.8 The gradient and column temperature conditions used for test runs 1 to 6.

Test run	Plant type	Colour category	Gradient no.	Temperature of column (°C)
1	cutting	4	1 (Table 6.1)	45
2	cutting	4	5 (Table 6.9)	45
3	cutting	4	6 (Table 6.9)	30
4	cutting	4	6 (Table 6.9)	40
5	cutting	4	6 (Table 6.9)	45
6	plantation tree	4	6 (Table 6.9)	45

Resolution of anthocyanin peaks improved under all conditions (Fig 6.8, Fig. 6.9). The best resolution occurred with gradient 6 and at a column temperature of 45°C (Fig. 6.9), with peaks a and b separating

Fig. 6.7 HPLC chromatograms showing absorption profiles at $A_{520\,nm}$ for a plantation tree of colour category 4. The first peak (peak a) and the second peak (peak b) are identified. A: Solvent gradient conditions 2 (Table 6.7). B: Solvent gradient conditions 3 (Table 6.7). C: Solvent gradient conditions 4 (Table 6.7). Please note that the scales differ for each absorption profile.
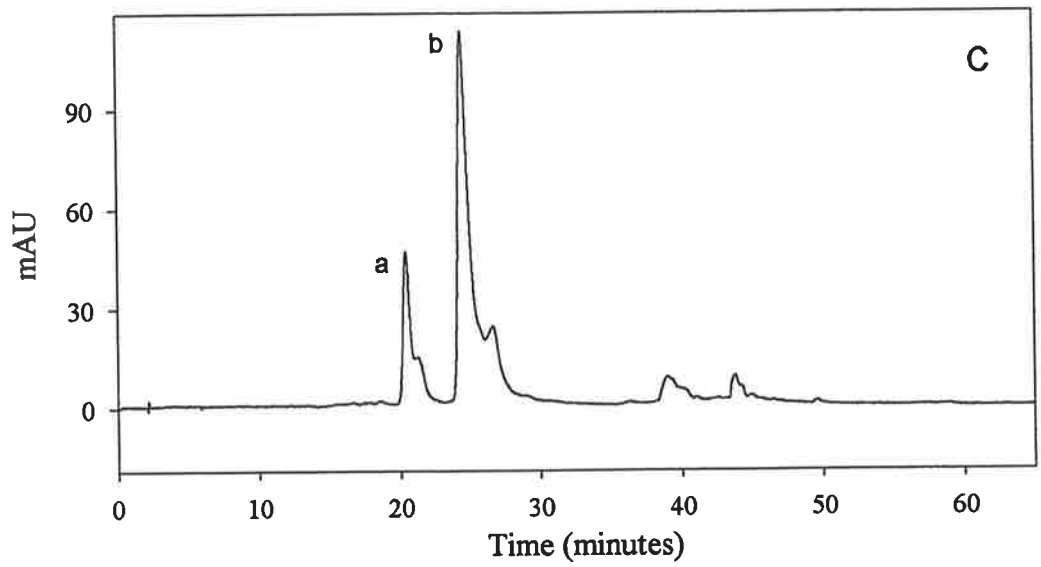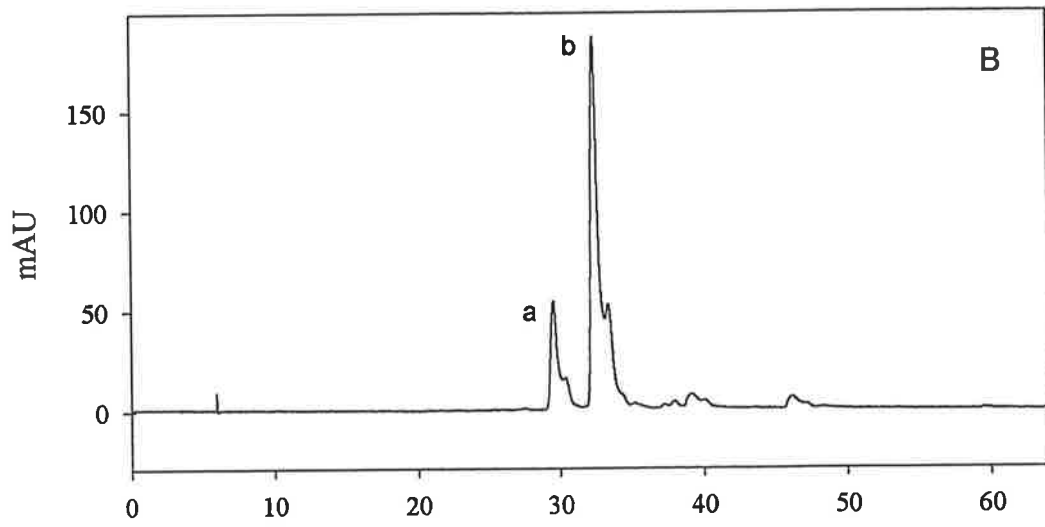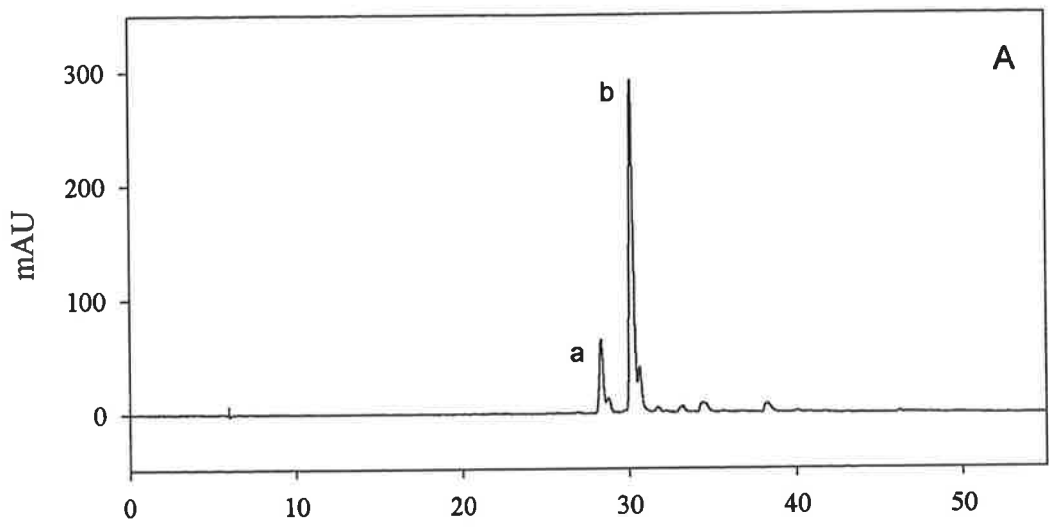
clearly into two peaks each. Increased temperature may have brought about the improvement in resolution of individual anthocyanin peaks, by increasing the rate at which the compounds bind and release from the chromatographic material.

Table 6.9 Solvent gradient conditions for Trial 6.

Solvent gradient condition	Time (min)	Buffer B (%)	Duration (min)
5	0.00	0.00	5.00
	5.00	50.00	40.00
	45.00	100.00	10.00
	55.00	0.00	5.00
	70.00	—	—
6	0.00	0.00	5.00
	5.00	50.00	50.00
	55.00	100.00	10.00
	65.00	0.00	5.00
	80.00	—	—

Figure 6.10 illustrates the optimised HPLC chromatogram of anthocyanins extracted from leaves of a plantation tree of *A. baileyana*, obtained using gradient 6 conditions and a column temperature of 45°C. There were 14 different peaks detected. Peaks a and b were successfully separated into peak 2/3 and peak 4/5, respectively.

6.3 Discussion

A protocol was developed whereby the highly efficient extraction of total anthocyanins from leaves of *A. baileyana* was achieved. Similarly, reverse-phase HPLC techniques were modified to optimise resolution of individual anthocyanin compounds. This was achieved by concentrating the leaf tissue extract and reducing the amount of sample loaded onto the HPLC column, thereby reducing the amount of ethanol solvent required. Ethanol may interfere with the binding and release of anthocyanins by the column. Optimal conditions were also achieved by increasing the column temperature, and by increasing the time of elution with Buffer B. The tailing peaks were resolved by these conditions, with the separation of the two main peaks into two separate peaks each.

These optimised conditions were used to identify the four main anthocyanins present in leaves of *A. baileyana* variety *purpurea* (Chapter 7), and to compare the anthocyanin content with four visual colour categories (Chapter 8).

Fig. 6.8 HPLC chromatograms showing absorption profiles at $A_{520\,nm}$ for a genotype 2 cutting of colour category 4, grown outside, at different solvent gradient conditions and column temperatures. A: Test run 1 (solvent gradient conditions 1 (Table 6.1), column temperature 45°C). B: Test run 2 (solvent gradient conditions 5 (Table 6.9), column temperature 45°C). C: Test run 3 (solvent gradient conditions 6 (Table 6.9), column temperature 30°C). D: Test run 4 (solvent gradient conditions 6 (Table 6.9), column temperature 40°C). The insert in each graph is a closeup of the main peaks to show the separation of the peaks more clearly.
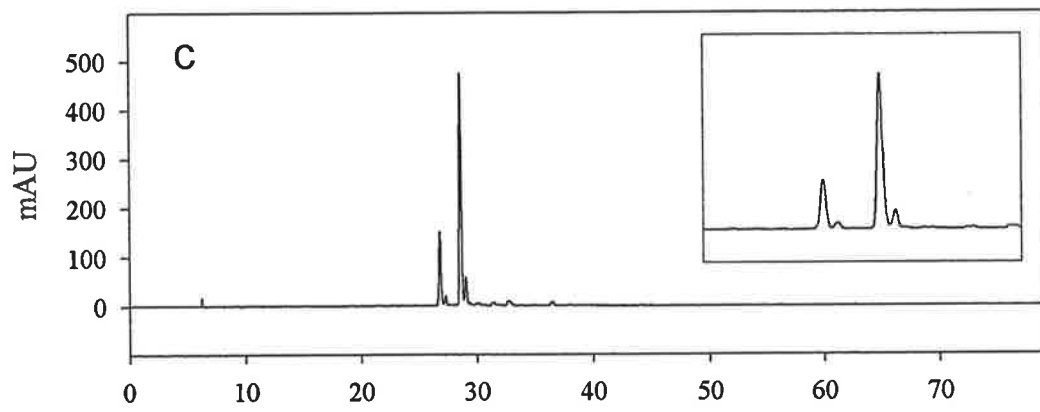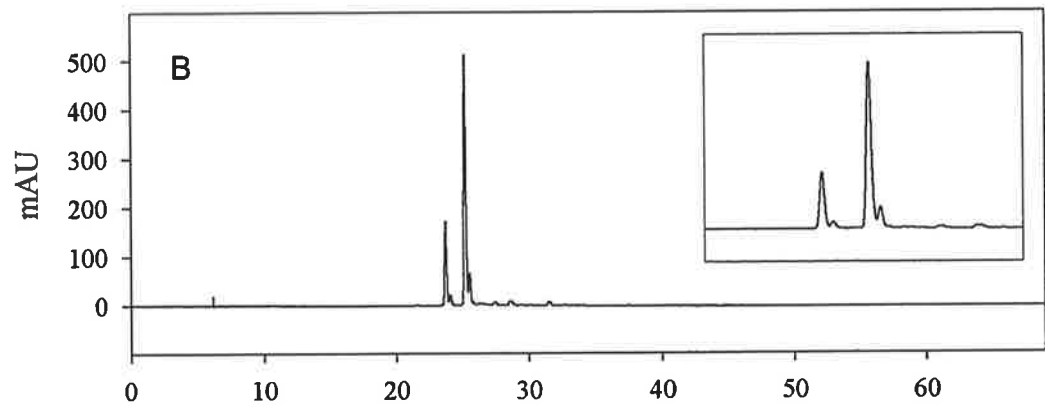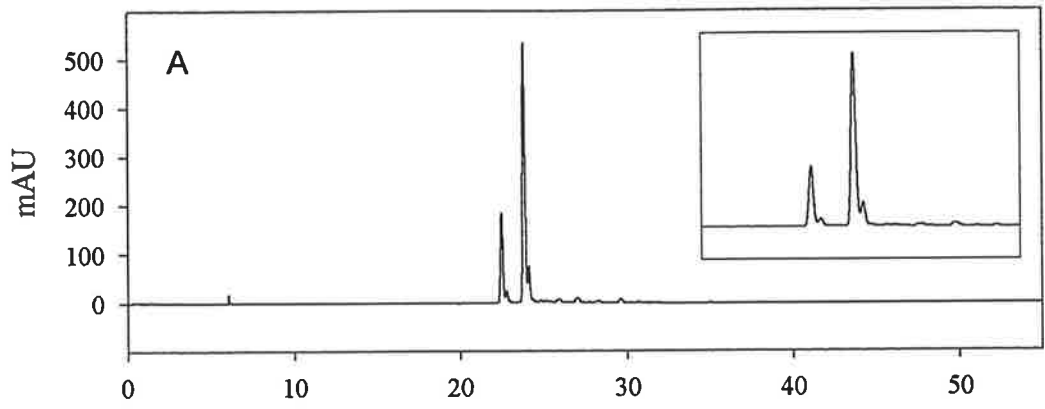
Fig. 6.9 HPLC chromatograms showing absorption profiles at $A_{520\,nm}$ from different plant material. A: Cutting (genotype 2) of colour category 4, test run 5 (solvent gradient conditions 6 (Table 6.9), column temperature 45°C). B: Plantation tree of colour rating 4, test run 6 (solvent gradient conditions 6 (Table 6.9), column temperature 45°C). The insert in each graph is another view of the main peaks to show the separation of the peaks more clearly.
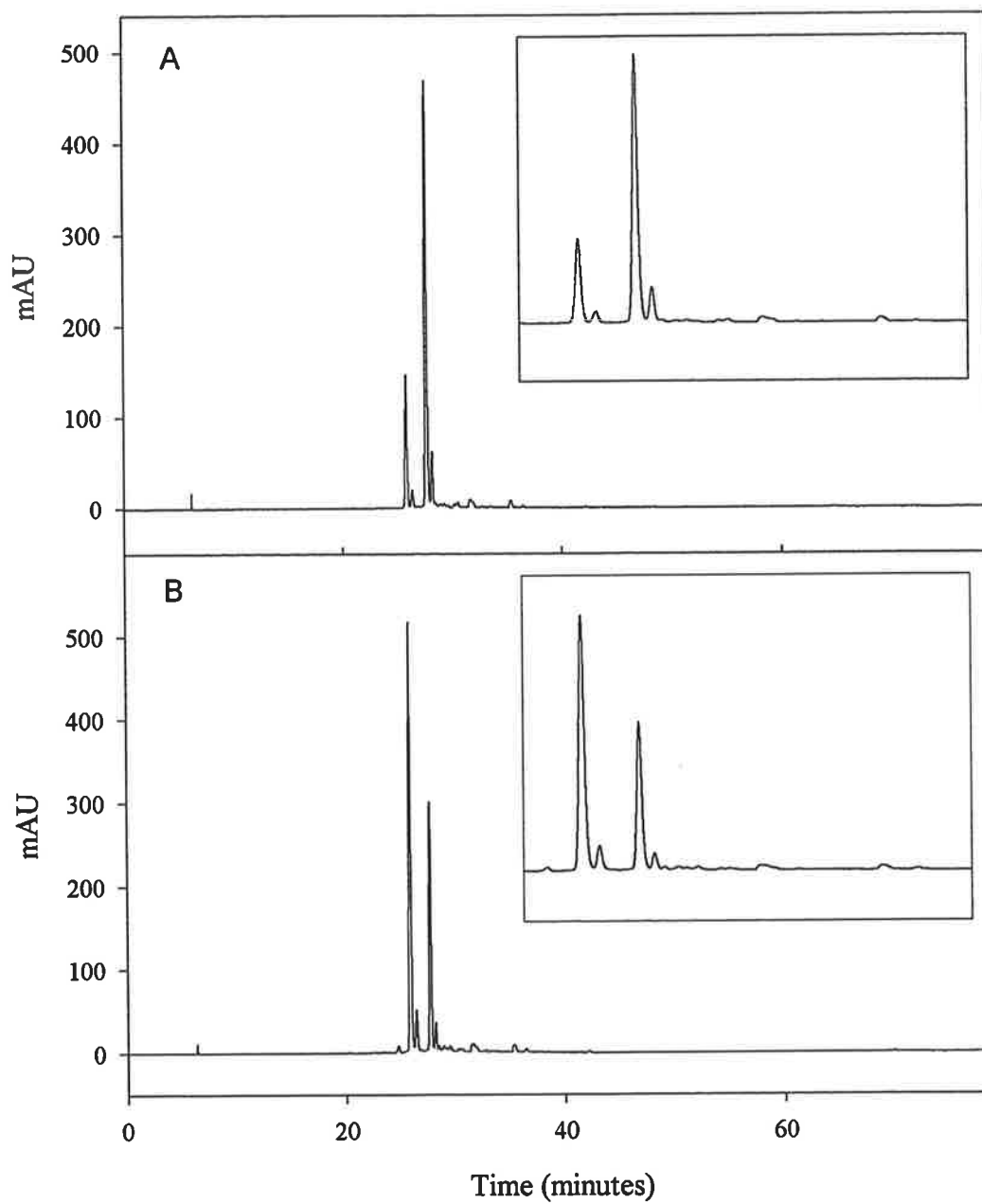
Fig. 6.10 HPLC chromatograms showing absorption profiles at $A_{520\ nm}$ for a plantation tree, of colour category 4, obtained using solvent gradient 6 conditions (Table 6.9) and a column temperature of 45°C. A: Chromatogram showing the total gradient time scale. B: Chromatogram showing a close up view of the main peaks from time 20 to 40 min, with the 14 peaks numbered.
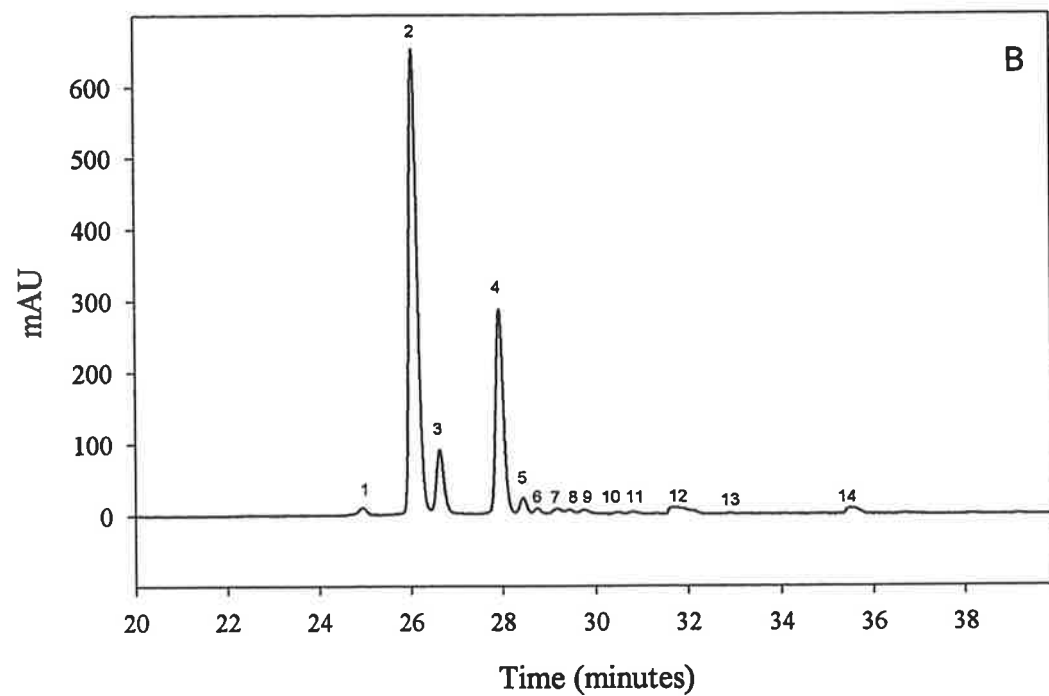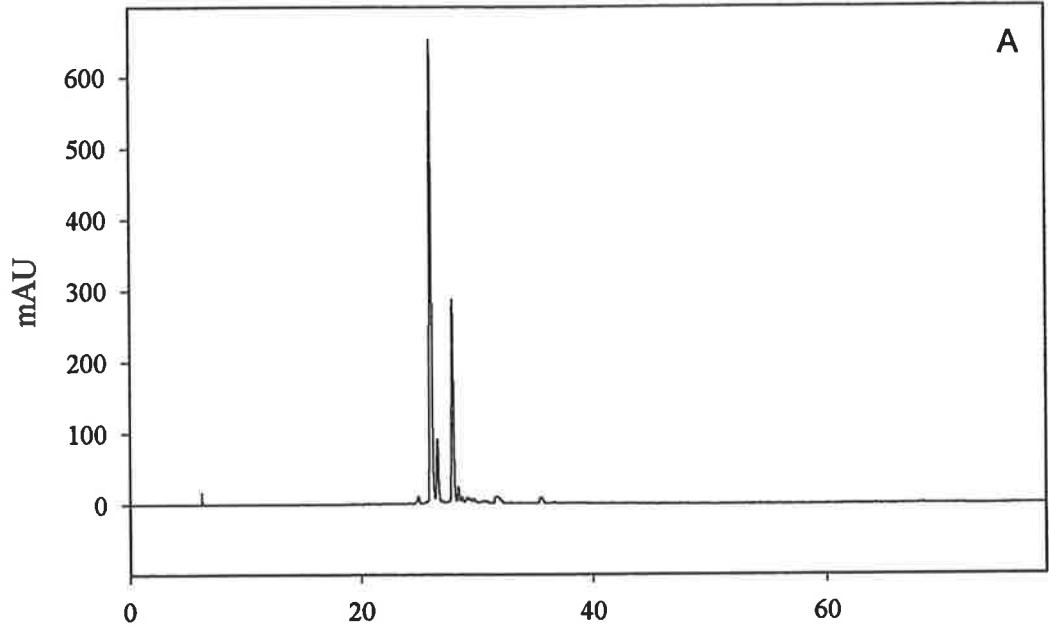
Purification and identification of anthocyanins from *Acacia* and *Banksia* species using high voltage paper electrophoresis[1]

7.1 Introduction

Variety in flower and foliage colour is essential to satisfy the demands of the commercial floriculture and horticulture industries. Identification of the anthocyanins responsible for the attractive colours of many plants may assist in the development of a breeding strategy for improved cultivars. Efficient methods for extraction of total anthocyanins and for the resolution of individual pigments from *A. baileyana* leaves were developed in Chapter 6. However, to easily identify individual anthocyanin compounds, interfering phenolic compounds need to be removed from the crude extract.

In particular, it is necessary to remove these phenolic compounds in order to identify the anthocyanin pigments using HPLC mass spectrometry. This may be done using high voltage paper electrophoresis (HVPE). Although HVPE has not been widely used for the separation of anthocyanins, Markakis (1960) isolated anthocyanins from cherries using this technique with a low pH buffer in which the anthocyanins were separated as cations. HVPE is, however, the method of choice for separating negatively-charged sulphonated phenolic compounds (Harborne 1989).

Theander (1957) successfully used paper electrophoresis, to separate aldehydes and ketones with good resolution, as their negatively charged hydroxy sulphonic acids (Fig. 7.1) in a bisulphite buffer at pH 4.7. Since sulphonic acids are strong acids, they are anionic in aqueous solution, except at very low pH. Anthocyanins react with bisulphite ions, as do aldehydes and ketones, through nucleophilic addition to form sulphonic acids. However, instead of forming hydroxy sulphonic acids, anthocyanins react to create stable, colourless chromen-4-sulphonic acids (Fig. 7.2). Thus by adapting the method

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[1] Asenstorfer RE, Morgan AL, Hayasaka Y, Sedgley M, Jones GP (2003) Purification of anthocyanins from species of *Banksia* and *Acacia* using high-voltage paper electrophoresis. *Phytochemical Analysis* **14**, 150–154 (Appendix 3). Robert Asenstorfer performed the high voltage electrophoresis, Yoji Hayasaka performed the liquid chromatography-ionspray mass spectrometry and Graham Jones gave advice on the methodology.

Fig. 7.1 Formation of an hydroxy-sulphonic acid from an aldehyde and bisulphite ion (Theander 1957).
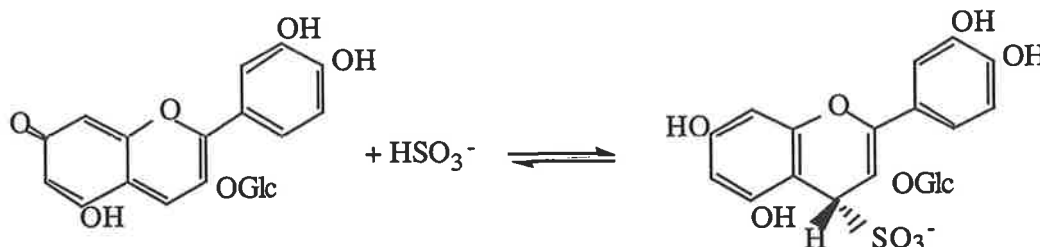
Fig. 7.2 Formation of the cyanidin bisulphite-addition product from cyanidin and bisulphite ion to give the chromen-4-sulphonic acid. Only one stereo-isomer is shown.

of Theander (1957), HVPE can be used for the separation and purification of anthocyanins as their anionic bisulphite adducts.

The aim of this study was to produce a mixed anthocyanin extract of sufficient purity from the purple juvenile leaves of *A. baileyana*. The main pigments from the purified extract can then be easily identified using HPLC mass spectrometry. Other plant species were also included in the development of the purification method. These were the red juvenile leaves of *Acacia glaucoptera*, the pink flowers of *Banksia menziesii* and the scarlet flowers of *B. coccinea*.

7.2 Materials and methods

7.2.1 Plant material

Anthocyanins were isolated from the three to five youngest (*c.* 0.5 g), fully-formed leaves from a single plant of *A. baileyana* variety *purpurea* (purple leaves, colour category 4, refer to Chapter 3 for details on the visual colour category technique) and *A. glaucoptera* (red-bronze leaves) (Plate 7.1A, B). Anthocyanins were also isolated from 50 flowers from a single inflorescence of *Banksia coccinea* (scarlet flowers) and *B. menziesii* (pink flowers) (Plate 7.1C, D).

Plate 7.1 Plant material used in the purification and identification of anthocyanins. A: Purple juvenile foliage of *A. baileyana* variety *purpurea*. Scale bar = 1 cm. B: Red-bronze juvenile foliage of *A. glaucoptera*. Scale bar = 1.3 cm. C: Scarlet coloured flower of *B. coccinea*. Scale bar = 2.4 cm. D: Pink coloured flower of *B. menziesii*. The closed flower buds are pink in colour, while the open flowers are yellow due to the presence of the pollen on the anthers. Scale bar = 2 cm.

7.2.2 Extraction of pigments

Fresh leaf or flower samples were frozen and ground to a fine powder in a mortar under liquid nitrogen. A sample (2 g) of the powder was mixed with acidulated ethanol extraction solution as described in section 6.2.1, to extract the anthocyanin pigments.

7.2.3 High voltage paper electrophoresis (HVPE)

Chromatography paper (Chr 3; Whatman, Clifton, NJ, USA), wetted with metabisulphite buffer (0.1 M, pH 4.2) was used to separate samples according to the procedure outlined by Tate (1981). A run time of 60 min with an electric field potential of 1400 V and a current of 150 mA was employed. The relative mobilities of the anthocyanins were compared with those of Orange G (1-phenylazo-2-naphthol-3,5-disulphonate: BDH; Poole, UK) as the anionic standard and fructose as the neutral standard. The position of fructose was identified using a silver nitrate stain (Trevelyan et al. 1950). After running, the papers were air dried and then fumed with concentrated hydrochloric acid to show the location of the anthocyanins. A major, red-coloured band with a relative mobility of approximately 0.35–0.40 was clearly observed for each of the samples, and this was eluted from the paper with 10% aqueous methanol. The eluent was concentrated by rotary evaporation, and further concentrated using a Sep-Pak classic (Waters, Milford, MA, USA) C_{18}-reverse-phase cartridge. The anthocyanin-rich material was eluted from the C_{18} cartridge with methanol, and samples were analysed for their anthocyanin composition using both HPLC and liquid chromatography-ionspray mass spectrometry.

7.2.4 HPLC analysis

Refer to Chapter 6 (section 6.2.2) for the basic HPLC method, and section 6.2.3.6 for the optimised conditions. The column temperature was maintained at 45°C. The gradient profile was: 0 min, 100% A, 0% B, increasing to 50% B at 5 min and 100% B at 55 min (solvent gradient 6 in Table 6.9). 2 mL of the extract was concentrated to 0.5 mL using a centrivap concentrator. 100 μL of this concentrated sample was mixed with 1.9 mL Buffer A and a 500 μL aliquot was then loaded onto the column.

7.2.5 Liquid chromatography-ionspray mass spectrometry (LC-MS)

The sample was loaded onto a Spherisorb (Waters) S5 OD52 HPLC column (250 x 1 mm i.d.) connected to a Rheodyne (Rohnert Park, CA, USA) model 8125 sample injector fitted with a 5 μL loop. The separations were carried out using Buffer C (0.05% trifluoroacetic acid, 5% acetonitrile by volume in water) and Buffer D (0.05% trifluoroacetic acid, 90% acetonitrile by volume in water) in a two-stage linear gradient system initially consisting of 0% C, 100% D, changing to 60% C in 60 min and then to 80% C over a further 30 min. A flow rate of 25 μL/min was achieved using an Applied

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Biosystems (Foster City, CA, USA) model 140B syringe pump. The HPLC column was connected to a Hewlett Packard (Palo Alto, CA, USA) model HP1100 UV-vis detector set to monitor 280 nm and 520 nm, followed by PE Sciex (Thornhill, Canada) mass spectrometer model API-300 equipped with a electrospray ion source. The mass spectrometer was operated in the positive ion mode and spectra were scanned from m/z 250 to m/z 1000 in 1.88 s. The ion spray and orifice potentials were set at 5.5 kV and 30 V, respectively. The curtain and nebuliser gases were nitrogen and air, respectively. All mass spectral data were processed using Bio-Multiview (PE Sciex) software (version 1.2ß3).

7.2.6 Sugar analysis

Sugars were analysed using gas-liquid chromatography according to the method of Chaplin (1982). The method involves methanolysis in the presence of methyl acetate followed by removal of hydrogen chloride by coevaporation with t-butyl alcohol and trimethylsilylation.

7.3 Results

Figures 7.3 and 7.4 are HPLC chromatograms of the crude leaf extract of *A. baileyana* before and after clean-up, respectively. Peaks 12 and 14 were removed by the HVPE bisulphite clean-up process (Fig 7.4). A number of compounds absorbing at 280 nm, but not at 520 nm can be seen in the crude extract (Fig. 7.3). The majority of these compounds were removed in the clean-up process, although a few compounds absorbing at 280 nm still remained in the purified sample (Fig. 7.4).

The mass spectrometry of the main peak (peak 4) of *A. baileyana* is shown in Figure 7.5. Figure 7.5A represents the HPLC mass spectrometry of the anthocyanin eluting at 27.2 min (peak 4) present in leaf extract prior to HVPE purification, whereas Fig 7.5B represents the HPLC mass spectrometry of the same component after purification. The compound was identified as cyanidin-3-glucoside on the basis of mass spectrometry and sugar analysis.

Cyanidin and delphinidin glucosides constituted the principal anthocyanins located in the leaves of *Acacia* species investigated, while the two *Banksia* species contained cyanidin and paeonidin-based anthocyanins (Table 7.1). The six carbon glycosidic entities of these pigments were either glucose or galactose. The five carbon glycoside (pentose moiety) was identified as an approximately equal mixture of xylose and arabinose.

Fig. 7.3 HPLC chromatograms showing absorption profiles of the crude leaf extract of *A. baileyana* before purification. A: Monitored at 280 nm. B: Monitored at 520 nm. Key to peak identity: peak 2, delphinidin-3-glucoside; peak 3, delphinidin-3-pentose-glucoside; peak 4, cyanidin-3-glucoside; peak 5, cyanidin-3-pentose-glucoside. Peaks 12 and 14 could not be identified as they were removed by the HVPE bisulphite clean-up process and were therefore not subject to mass spectrometry.
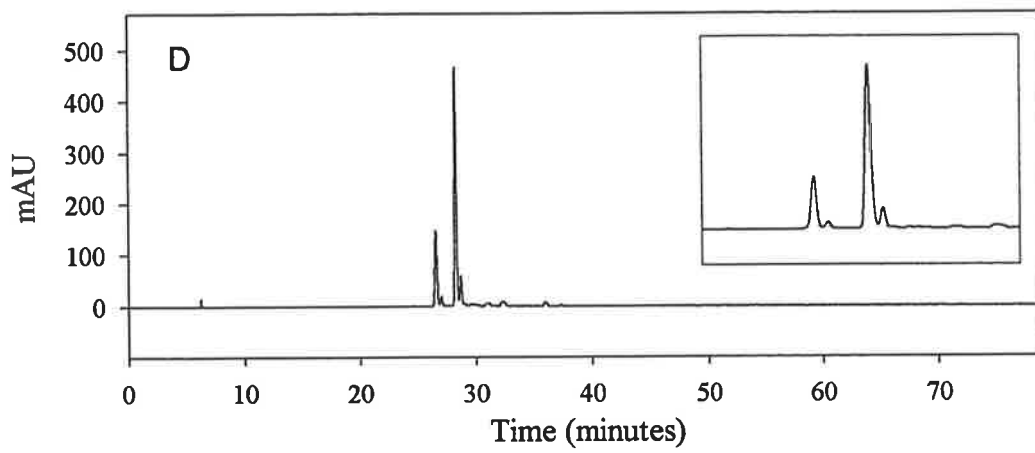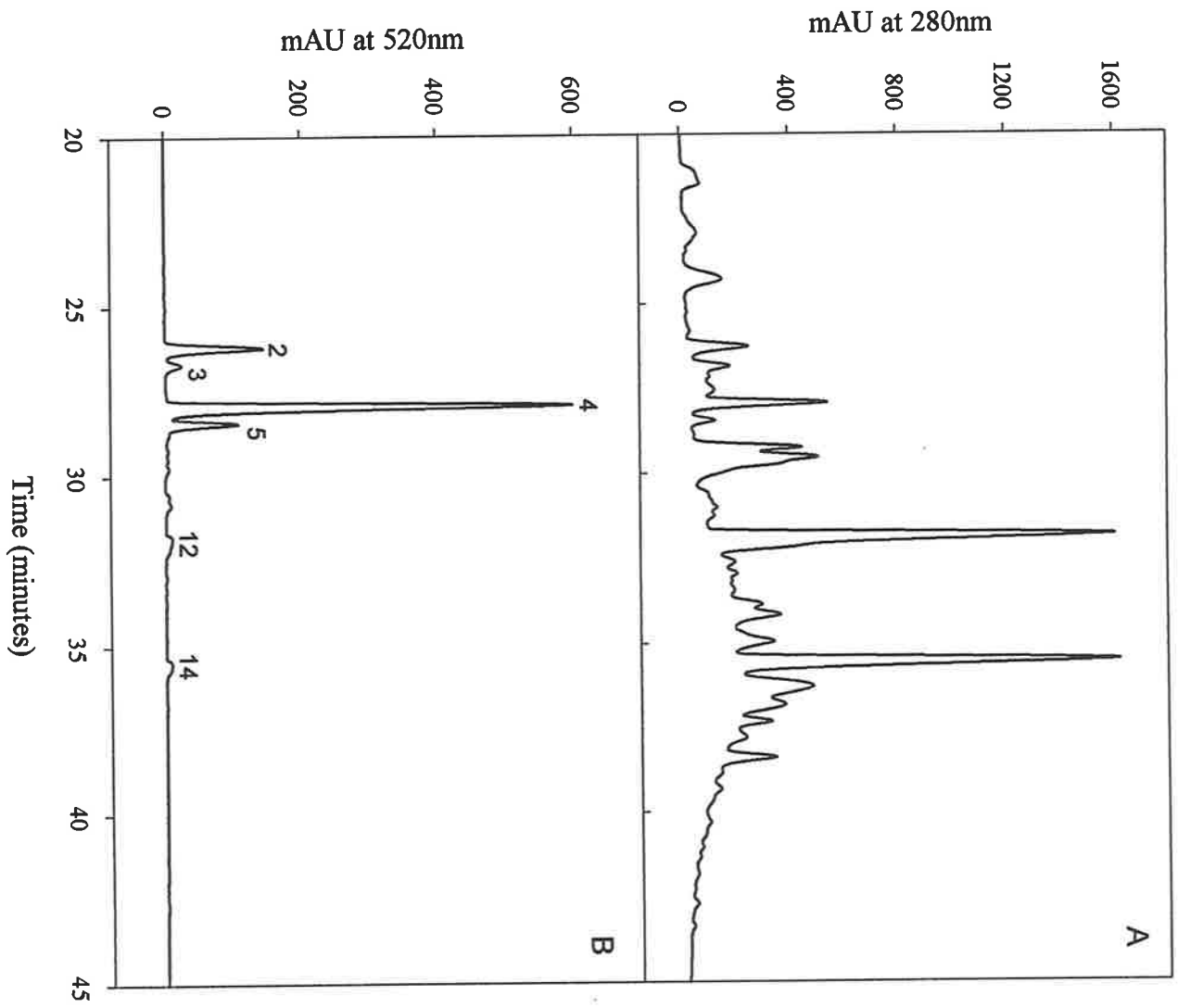
Fig. 7.4 HPLC chromatograms showing absorption profiles of the crude leaf extract of *A. baileyana* following purification using the HVPE bisulphite process. A: Monitored at 280 nm. B: Monitored at 520 nm. Key to peak identity: peak 2, delphinidin-3-glucoside; peak 3, delphinidin-3-pentose-glucoside; peak 4, cyanidin-3-glucoside; peak 5, cyanidin-3-pentose-glucoside.
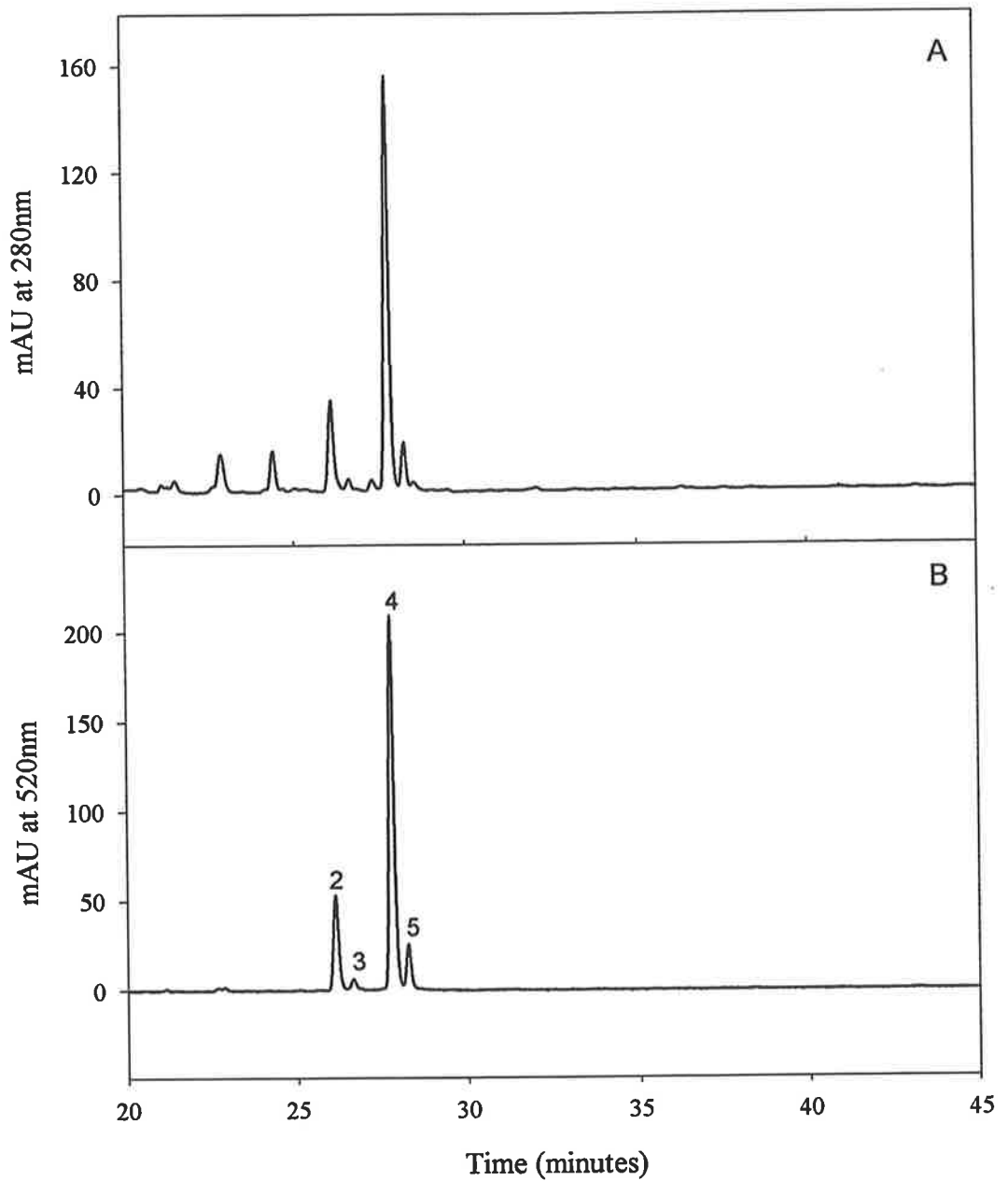
Fig. 7.5 HPLC mass spectrometry of the major anthocyanin pigment (peak 4 in Figs. 7.3 and 7.4), retention time 27.2 min, present in *A. baileyana*. A: Measured before HVPE bisulphite purification. B: Measured after HVPE bisulphite purification.

A

Spectrum from 20 scans

B

Spectrum from 29 scans
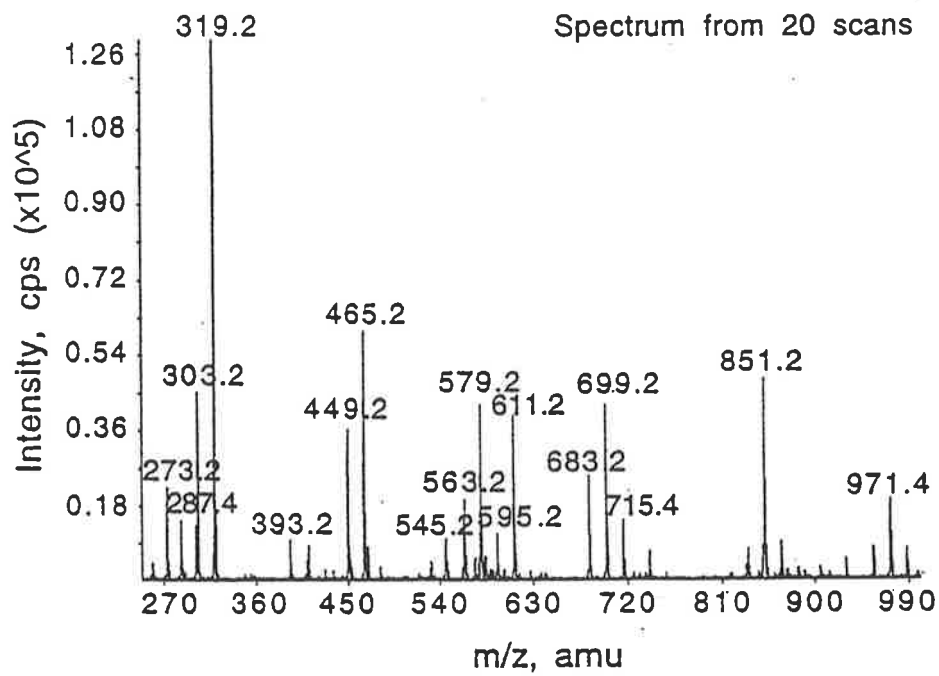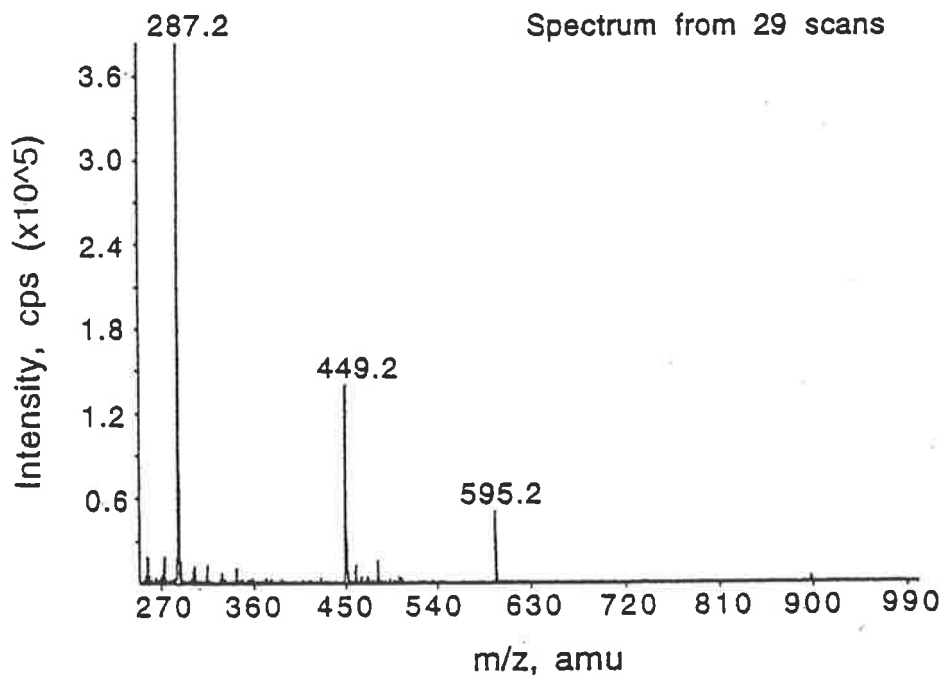
Table 7.1 Major anthocyanin pigments identified in the flowers of two _Banksia_ species and in the leaves of two _Acacia_ species. The absorbance maxima and the _m/z_ values of the glycoside and the aglycone of the anthocyanins are presented.

Anthocyanin (in order of elution in the HPLC)	Absorbance λ_{max} (nm)	Mass (_m/z_) Glycoside (M+H)	Fragment	Aglycone
Banksia coccinea				
cyanidin-3-galactoside	513	449.2		287.2
cyanidin-3-glucoside	513	449.2		287.2
paeonidin-3-galactoside	513	463.2		301.4
cyanidin-3-pentoside[x]	514	433.2		287.2
Banksia menziesii				
cyanidin-3-galactoside	513	449.0		287.2
cyanidin-3-glucoside	513	449.0		287.2
paeonidin-3-glucoside	513	463.2		301.4
Acacia baileyana				
delphinidin-3-glucoside	521	465.2		303.2
delphinidin-3-pentose-glucoside[x]	521	611.4	465.2	303.2
cyanidin-3-glucoside	513	449.2		287.2
cyanidin-3-pentose-glucoside[x]	513	595.2	449.2	287.2
Acacia glaucoptera				
delphinidin-3-glucoside	521	465.2		303.2
cyanidin-3-glucoside	513	449.0		287.2

[x] Pentose moiety was an approximately equal mixture of xylose and arabinose

7.4 Discussion

Both *Acacia* and *Banksia* (Proteaceae) are important genera in the floriculture industry. *Acacia* species are presently grown overseas for cut flowers and in Australia mainly for cut foliage, while *Banksia* species are cultivated in Australia and overseas for cut flower production. The importance of these genera is due to their attractive and colourful flowers and foliage. The identification of colour pigments is important to the success of breeding programs, which aim to produce novel coloured cultivars, but anthocyanins have previously been poorly characterised in these genera (Gascoigne et al. 1948).

A new method has been developed for the isolation and rapid identification of anthocyanins from the leaves of *Acacia* species and the flowers of *Banksia* species based on the use of high voltage paper electrophoresis with bisulphite buffer. Using this method, anthocyanin pigments were successfully purified as their negatively charged bisulphite-addition compounds from crude extracts of plant tissue. In conjunction with liquid chromatography-ionspray mass spectrometry, the method enabled the anthocyanins from these species to be identified.

High voltage paper electrophoresis provided an effective method for isolating anthocyanins from complex plant extracts by removing interfering phenolic material, which did not react with bisulphite and significantly improved identification of anthocyanins by mass spectrometry. The determination of the anthocyanins using mass spectrometry prior to the HVPE preparative step was difficult, but after purification the anthocyanins were clearly identifiable. The clean-up process removed the majority of compounds absorbing at 280 nm. A few non-anthocyanin compounds were, however, not removed. These compounds could not be unambiguously identified. It is possible that these peaks are due to anionic sulphonated flavonoids previously identified in herbaceous plants (Harborne 1976). Only the four main anthocyanin peaks absorbing at 520 nm remained after purification, as the trace peaks, including peaks 12 and 14, were removed.

Cyanidin and delphinidin glucosides constituted the principal anthocyanins located in the leaves of the *Acacia* species investigated. Nozzolillo (1973) noted that *A. baileyana* contained anthocyanins in the epidermis of the hypocotyl stem and the cotyledon leaves but the pigments were not identified. Cyanidin mono-glycoside has been identified previously in the fruit of *A. baileyana* (Gascoigne et al. 1948). Legume seedlings have a diverse range of anthocyanins, including cyanidin, delphinidin, malvidin, paeonidin and petunidin (Harborne 1971). The range of anthocyanins is not surprising as the legume family contains more than 12,000 species (Harborne 1971), although the most common anthocyanin aglycone identified from a small survey of 40 legume seedling species was cyanidin (Nozzolillo and McNeil 1985).

The flowers of the two *Banksia* species contain mainly cyanidin and paeonidin based anthocyanins. Previous studies have reported that anthocyanins are present in flowers of *B. menziesii* (Bickford and Sedgley 1994), and delphinidin has been identified as the main anthocyanidin of the red flowers of *B. ericifolia* (Gascoigne et al. 1948). Delphinidin-, cyanidin-, petunidin- and malvidin-containing anthocyanins have been identified in some species of the Proteaceae family, namely *Conospermum, Grevillea, Telopea* and *Lambertia* (Gascoigne et al. 1948), but the current HPLC mass spectrometry data provide solid evidence for the presence of paeonidin in *B. coccinea* and *B. menziesii*.

Glucose and galactose were identified as the main sugar moities. These sugars are commonly found bonded to anthocyanidins (Mazza and Miniati 1993). The pentose moiety was identified as an approximately equal mixture of xylose and arabinose. However, the low concentrations of pigments containing these pentoses did not allow for their accurate quantitative determination.

The utilisation of the bisulphite-addition product for the separation and purification of anthocyanins is novel and may provide a useful analytical and preparative tool when combined with ion-exchange techniques. The relative simplicity of this purification method suggests that it has wide application for the identification of anthocyanins in nature and may assist in the commercial development of floriculture crops. In addition, this technique may also be used to describe these new cultivars under Plant Breeders Rights by providing a rapid and reliable method of characterising the anthocyanins present.

Development and validation of four colour categories to assess leaf colour

8.1 Introduction

The flavonoid pathway leading to anthocyanin biosynthesis is controlled by multiple regulatory genes and induced by various developmental and environmental factors (Harborne 1965, Taylor and Briggs 1990). The main endogenous factors known to affect the colour of anthocyanin-containing tissue are the chemical nature of the anthocyanin, the anthocyanin concentration, the pH, the co-occurrence of several anthocyanins, and the presence of copigments (Brouillard 1983). Anthocyanins are synthesised in specific tissues at defined times in response to various environmental conditions (Christie et al. 1994, Dixon and Paiva 1995). Two of the environmental factors that have a major influence on the colour of anthocyanin-containing tissue are light and temperature (Grisebach 1982, Christie et al. 1994). Continuous UV, blue, red and far-red light may lead to an increase in enzymatic activity of the flavonoid biosynthetic pathway, with specific light responses for individual plant systems (Mancinelli 1983). Anthocyanins can also increase if plants are stressed as a result of cold temperatures (Christie et al. 1994), with foliar anthocyanins generally accumulating with the onset of cold weather (McClure 1975).

Experiments to determine the environmental conditions affecting anthocyanin expression and inheritance of the purple leaf colour of *A. baileyana* (Chapter 9) require the assessment of large numbers of plants for colour. Therefore, a quick and reliable method of assessing the leaf colour of *A. baileyana* was needed. This involved the definition by eye of four colour categories that could be used as a grading technique to classify the leaf colour of both the green and purple forms of *A. baileyana*. To validate this technique, the four colour categories were compared to data obtained using a colour meter (Voss 1992) and to the anthocyanin content of leaves determined by HPLC. It was feasible to compare the four colour categories with both these other methods, as the number of leaf samples used in the validation was relatively small.

The specific colour meter measurements were hue angle, chroma and lightness, and these values were compared to the allocated colour category for each leaf sample. Hue angle represents the perception of colour as red, orange, yellow, green, blue or purple, or the intermediates of these colours (Voss and

Hale 1998). Chroma is the degree of departure from grey of the same lightness (Voss and Hale 1998), and lightness distinguishes light and dark colours (Cleland 1931).

For anthocyanin content determined by HPLC, the sum of all peak areas and specific anthocyanin peak areas were compared separately with the four colour categories. This was to determine if any of the individual anthocyanin peaks were more important in predicting the colour categories. Seasonal and temperature interactions were also investigated to determine if colour and anthocyanin peak areas varied from summer to winter for plants grown outside, and to determine temperature interactions for plants grown in controlled environment growth cabinets.

8.2 Materials and methods

8.2.1 Definition of the colour categories

Four defined colour categories were used to classify the colour of each leaf sample, as has been previously stated in Chapter 3 (section 3.2.1). The colour categories were established by examining (by eye) the youngest, fully-expanded leaves of *A. baileyana*, and, based on the range of visible colour observed, the following were defined: category 1 (pure green), category 2 (between a trace and approximately half maximal purple colouration), category 3 (more purple colour than those in category 2, but not totally purple) and category 4 (intense purple) (see Plate 3.1A). Between three and five leaves were assessed per plant. The allocated colour category of a leaf sample was compared to values from a colour meter and HPLC-determined anthocyanin content.

8.2.2 Colour meter

8.2.2.1 Plant material

Plants were germinated from seeds on 23 April 1996. The purple leaf form was grown from three seed lots obtained from two sources: Nindethana Seed Service (two seed lots; Albany, Western Australia) and Blackwood Seeds (one seed lot; Mylor, South Australia). Two seed lots of the green form were obtained from the same sources (Nindethana Seed Service and Blackwood Seeds). Seeds were germinated as described in Chapter 4 (section 4.2.1) but placed into trays containing 1:1:1 sand: perlite: peat. Seedlings were potted up into 10 cm diameter pots containing native plant soil mix (refer to section 3.2.2, Chapter 3, for details). Approximately 140 seedlings were each placed randomly into a shadehouse, outside, or in a controlled environment growth cabinet on 5 August 1996. The conditions in the growth cabinet were 13.0°C day and 9.0°C night (13/9°C, L), with 12 hours daylight and 12 hours without light (refer to section 3.2.3 for details). Plants in the three treatments were

assessed for colour using the colour category technique on 14 July 1997. For each plant, the colour category was recorded and one representative leaf was collected. The leaf was stored in a sealed plastic bag at 4°C until it was assessed with the colour meter the same day.

8.2.2.2 Colour meter

A Minolta Chroma Meter (Tristimulus Colour Analyzer, CR-300, Minolta, New Jersey, USA), with a measuring diameter of 8 mm, was used to measure the colour of the leaves. The leaf sample was placed on the manufacturer's standard white calibration tile and the colour was recorded once. The meter was calibrated after every ten leaves using the white calibration tile.

8.2.2.3 Comparison of the colour categories with the colour meter

Representative leaf samples, taken from the 420 plants growing under the three different conditions, were assigned to one of the four colour categories and this allocation was compared to the leaf colour meter results. The data from the three environmental conditions were pooled to increase the sample size. A number of colour meter parameters were compared, namely hue angle (H°), chroma (C), lightness (L) from the L*C*H° colour system.

8.2.3 HPLC-determined anthocyanin content

8.2.3.1 Plant material

Plants grown from cuttings

Plants grown from cuttings were placed into three controlled environment growth cabinets in August 1997, to provide leaves of the same genotype grown under three temperature conditions. Refer to Chapter 3 for the plant material (section 3.2.1), propagation of cuttings (section 3.2.2) and growth cabinet conditions (section 3.2.3). Two genotypes were used in the experiment. Genotype 1 (purple leaf form) originated from a colour category 4 mother plant (refer to Table 3.2 in Chapter 3), produced leaves allocated to colour categories 2, 3 and 4 when placed into the growth cabinets. Genotype 6 (green leaf form) originated from a colour category 2 mother plant (refer to Table 3.2 in Chapter 3), produced leaves allocated to category 1 when placed into growth cabinets. Some of these cuttings had also been used to investigate the temperature requirements for flowering (Chapter 3) and for the development of the HPLC method (Chapter 6). The temperatures in the three cabinets were 13.0°C day and 9.0°C night (13/9°C, L), 18.3°C day and 13.3°C night (18/13°C, M) or 23.3°C day and 19.3°C night (23/19°C, H).

Leaf colour from cutting material was assessed using the four colour categories and these data compared to the anthocyanin content determined by HPLC. Between three and five representative leaves were collected from a total of 41 plants in March 1998. Leaves were first assessed for colour and then stored at -80°C until analysis by HPLC (Appendix 1, Table 7). For Genotype 6 (green leaf form), three plants came from each of the three growth cabinets. For Genotype 1 (purple leaf form), 16 plants came from the 13/9°C growth cabinet, 5 plants from the 18/13°C growth cabinet and 11 plants from the 23/19°C growth cabinet.

Plantation trees

One-year-old *A. baileyana* seedlings were established in the Waite Campus plantation in June 1996. The plantation had previously been used to study growth rates, flowering and seed production (Chapter 4). Chapter 4 (section 4.2.1) gives details on seed germination and plantation information. Ten trees from the plantation were assessed for leaf colour using the four colour categories in winter (August 1997) and again in summer (January 1998). At the same time, between three and five representative leaves were collected and stored at -80°C for analysis of anthocyanin content by HPLC. Leaves from the trees varied in colour between category 2 and 4 (Appendix 1, Table 8). There were no trees in the plantation with leaves allocated to colour category 1 (pure green). The temperature conditions for the summer and winter samples are shown in Table 8.1. The complete temperature data for 1997 and 1998 is shown in Appendix 2, Table 1.

Table 8.1 Temperature conditions during the months of winter 1997 and summer 1998, recorded at the Waite Campus, The University of Adelaide.

Date	Temperature (°C)			
	Maximum	Mean maximum	Minimum	Mean minimum
June 1997	22.1	15.1	4.5	8.8
July 1997	17.6	13.7	4.4	6.8
August 1997	19.9	14.5	4.0	7.9
Average	19.9	14.4	4.3	7.8
November 1997	40.1	25.3	7.8	14.9
December 1997	33.2	25.5	9.8	14.4
January 1998	38.6	28.0	9.7	16.0
Average	37.3	26.3	9.1	15.1

8.2.3.2 Extraction of colour pigments and HPLC-determined anthocyanin content

Extracts of the colour pigments were prepared from a frozen sample (c. 0.1g) of between three and five of the youngest, fully expanded leaves per plant. The method of extraction is described in Chapter 6 (section 6.2.1). The extracts were analysed by HPLC at 520 nm to determine the anthocyanin content. It was assumed that any peaks absorbing at 520 nm were anthocyanins. Refer to Chapter 6 for the basic details of the HPLC analytical method (section 6.2.2). Changes to the method are the same as for Chapter 7 (section 7.2.4).

Measuring absorbance at 520 nm identified 16 peaks, although not all leaf sample extracts contained all the peaks. The 16 peaks were assigned in sequence of elution, with the first peak eluted named peak 1, and the remaining peaks subsequently named 2, 3, etc. The amount of anthocyanin obtained at $A_{520\,nm}$ in the sample was determined by integrating the area under all of the peaks or under specific peaks. For clarity, the measured absorbance values in mAU (milli absorbance units) were divided by 100,000 in each case, to give scaled absorbance data.

As given in Chapter 7, the major anthocyanins (peaks 2, 3, 4 and 5) from a plant allocated to colour category 4 were identified (refer to Table 7.1), as:

- delphinidin-3-glucoside (peak 2)
- delphinidin-3-xylose-glucoside and delphinidin-3-arabinose-glucoside (peak 3)
- cyanidin-3-glucoside (peak 4), and
- cyanidin-3-xylose-glucoside and cyanidin-3-arabinose-glucoside (peak 5).

With each set of multiple samples, chromatography of the first sample was repeated at the end to check for uniformity of HPLC analysis.

8.2.3.3 Comparison of the colour categories with HPLC-determined anthocyanin content

Representative leaf samples, taken from cuttings or from the plantation trees, were assigned to one of the four colour categories and compared to their HPLC-determined anthocyanin content. The anthocyanin content was determined as either the area under each of the two major peaks (peaks 2 and 4) or the sum of all peak areas. The area under peak 5 was also measured for the cutting-grown material, as it had a similar peak area to peak 2 (see Fig. 8.2; Appendix 1, Table 7). The anthocyanin content of leaves was compared for plants grown from cuttings at the three growth cabinet temperatures, and from samples collected from the plantation in both winter and summer.

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Discriminant analysis was used to allocate the leaf sample of each plant to one of the four colour categories on the basis of the HPLC-determined anthocyanin content, thereby separating the HPLC data into four groups. Discriminant analysis (DA) is a method that classifies data into one of a number of groups on the basis of the observations made (Mardia et al. 1979). How well these four HPLC data groups relate to the four colour categories was then expressed as a percentage of agreement between methods.

For leaf data from plants grown from cuttings and from the plantation, DA was applied to either the area under two major peaks (peaks 2 and 4) or the sum of all peak areas. Discriminant analysis was also applied to peak 5 for cutting-grown material.

The DA classification rule for allocating plants is based on one or more linear discriminant functions. Relevant examples of the method are shown in the Appendix 1. The number of discriminant functions is equal to the minimum of the number of colour categories minus 1, and to the number of explanatory peak areas. Only one discriminant function is necessary to discriminate between the colour categories, if the function explains over 80% of the total variation. Using the discriminant functions, discriminant scores can be obtained for each plant. The mean discriminant scores for each colour category are then calculated. The plant is then assigned to one of the categories on the basis of the 'distance' of its discriminant scores from the mean discriminant score of each colour category. The 'distance' is given by:

$$D_k^2 = \sum_{j=1}^{n} \left(\mathrm{LD}_j - \bar{y}_{jk} \right)^2$$

LD_j is the jth discriminant function score for a leaf sample. $\bar{y}_{jk}$ is the mean of the jth discriminant function scores (LD_j) for the kth colour category and thus a D_k^2 score is obtained for each of the visual colour categories. The leaf sample is allocated to the colour category that has the smallest 'distance' (D_k^2) value. In all cases it is assumed that each leaf sample has an equal chance, *a priori*, of being in each of the four colour categories.

Splus for Windows V3.3R1 (Mathsoft Inc.) was used for all statistical analysis. The discriminant analysis was performed using the linear discriminant analysis function (Venables and Ripley 1994).

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Fig. 8.1 Relationship between the assessment of *A. baileyana* leaf colour using the four colour categories and the Minolta Colour Meter. A: Hue angle. B: Chroma. C: Lightness.
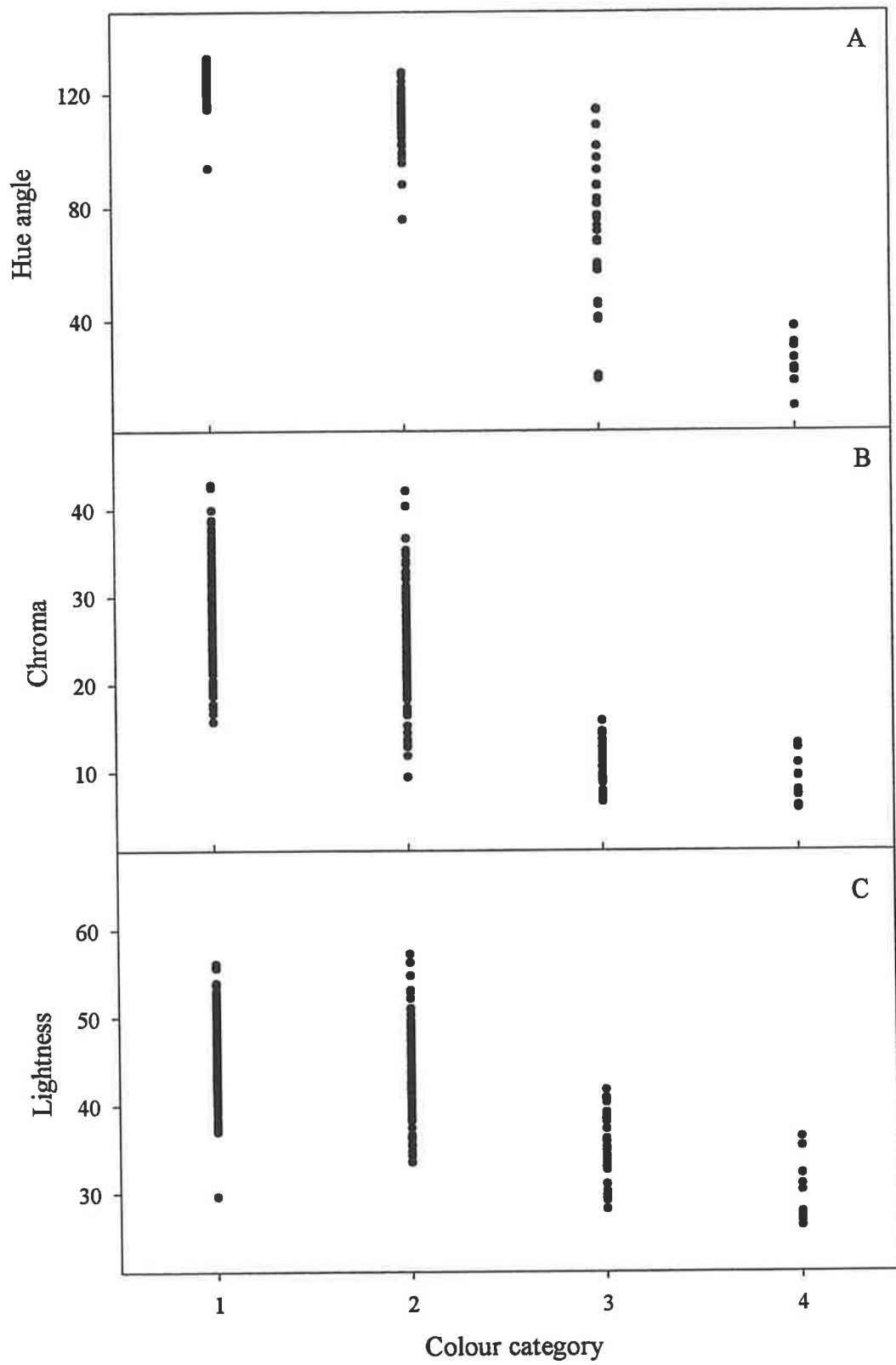
8.3 Results

8.3.1 Colour meter

In general, colour meter data discriminated poorly between the four colour categories (Fig. 8.1). The greener plants (colour categories 1 and 2) had a greater hue angle, towards the green colour range, while the pure purple leaf form (colour category 4) had a lower hue angle, in the red colour range. However, the hue angle for plants in category 3 varied greatly, between the green and red limits. This is illustrated in Plate 3.1A (Chapter 3), where leaves allocated to category 3 contain both purple and green areas. The hue angle did, however, separate category 1 from category 4, which were the extremes in colour. For chroma and lightness, there was a greater range in values for plants in categories 1 and 2, than for plants in categories 3 and 4. The purple leaf plants had lower chroma and lightness than the greener phenotypes.

8.3.2 HPLC-determined anthocyanin content

8.3.2.1 Plants grown from cuttings

Plants grown from cuttings in colour category 4 had the highest total content of anthocyanins, while those of category 1 had the lowest (Table 8.2; Fig. 8.2; Appendix 1, Table 7). Genotype 1 plants, had leaves with purple colouring (colour categories 2, 3 and 4), had up to 15 peaks detected by HPLC at 520 nm: the main ones were peaks 2, 3, 4, 5, 12 and 14 (Fig. 8.2; Appendix 1, Table 7). The ratio of the areas of peaks 2 and 4 to the total anthocyanin content was constant in these Genotype 1 plants (Table 8.2). For these leaf extracts, peak 4 (cyanidin-3-glucoside) was the largest, comprising approximately 70% of the total anthocyanin content. For Genotype 6, all plants were assessed as green (colour category 1), but peaks 12 and 14 were detected (Fig. 8.2; Appendix 1, Table 7).

Plants of colour category 4 were produced only at the lowest temperatures of 13/9°C, while plants of category 2 were produced only at the highest temperatures of 23/19°C (Table 8.3). However, leaf colour categories 1 and 3 were produced in plants that had been growing at any of the three growth cabinet temperatures. For the green phenotype (Genotype 6) no purple colouring was observed at any of the three temperatures.

Discriminant analysis was used to distinguish between the four colour categories on the basis of the HPLC-determined anthocyanin content. For the sum of all the peak areas, there was 85% agreement between the four colour categories and HPLC-determined anthocyanin content, with six plants

misclassified (Table 8.4). Four of these misclassifications were between colour categories 2 and 3, and two misclassifications were between categories 3 and 4 (Appendix 1, Table 2). When only the area of peak 4 was used as the predictor of external colour, discrimination between the four categories was possible (Fig. 8.2), with 88% agreement between the two methods (Table 8.4; Appendix 1, Table 2). Five plants were misclassified in this analysis and these outliers included the same outliers as for the sum of all peaks (Appendix 1, Table 2). When only the area of peak 2 was used as the predictor of

Table 8.2 A comparison of the HPLC-determined anthocyanin content (mean ± standard error) of leaf extracts from A. baileyana, and the allocated colour category for the sample, independent of temperature. Plants were grown from cuttings. Figures in parentheses are the percentages of the anthocyanin content of the individual peaks relative to the sum of all peaks.

Colour category	No. of leaf samples	Genotype	Average anthocyanin content (scaled mAU) ± s.e.		
			Sum of all peaks	Peak 2	Peak 4
4	8	1	54.2 ± 4.55	3.9 ± 0.56 (7)	39.2 ± 3.32 (72)
3	16	1	30.0 ± 2.34	2.0 ± 0.17 (7)	21.3 ± 1.68 (71)
2	8	1	12.6 ± 1.33	1.1 ± 0.25 (9)	8.6 ± 0.86 (68)
1	9	6	1.9 ± 0.09	0.0 ± 0.00 (0)	0.0 ± 0.00 (0)

Table 8.3 A comparison of the HPLC-determined anthocyanin content (mean ± standard error) of leaf extracts from A. baileyana grown under three temperature regimes, and the allocated colour category for the sample. Plants were grown from cuttings. Figures in parentheses are the percentages of the anthocyanin content of the individual peaks relative to the sum of all peaks.

Colour category	No. of leaf samples	Temperature °C (max./min.)	Average anthocyanin content (scaled mAU) ± s.e.		
			Sum of all peaks	Peak 2	Peak 4
4	8[x]	13/9	54.2 ± 4.55	3.9 ± 0.56 (7)	39.2 ± 3.32 (72)
3	8[x]	13/9	37.0 ± 2.15	2.5 ± 0.21 (7)	26.0 ± 1.67 (70)
3	5[x]	18/13	24.6 ± 3.28	1.5 ± 0.16 (6)	17.5 ± 2.71 (71)
3	3[x]	23/19	20.2 ± 1.57	1.6 ± 0.20 (8)	15.0 ± 1.10 (75)
2	8[x]	23/19	12.6 ± 1.33	1.1 ± 0.25 (9)	8.6 ± 0.86 (68)
1	3[y]	13/9	2.2 ± 0.15	0.0 ± 0.00 (0)	0.0 ± 0.00 (0)
1	3[y]	18/13	1.7 ± 0.12	0.0 ± 0.00 (0)	0.0 ± 0.00 (0)
1	3[y]	23/19	1.9 ± 0.03	0.0 ± 0.00 (0)	0.0 ± 0.00 (0)

[x] Genotype 1
[y] Genotype 6

Fig. 8.2 HPLC chromatograms showing absorption profiles at A$_{520nm}$ for leaf extracts of *A.* *baileyana*, representing each of the four colour categories. Samples were from plants grown from cuttings. A: Colour category 4 (Genotype 1). B: Colour category 3 (Genotype 1). C: Colour category 2 (Genotype 1). D: Colour category 1 (Genotype 6). The main peaks are numbered according to their elution time.
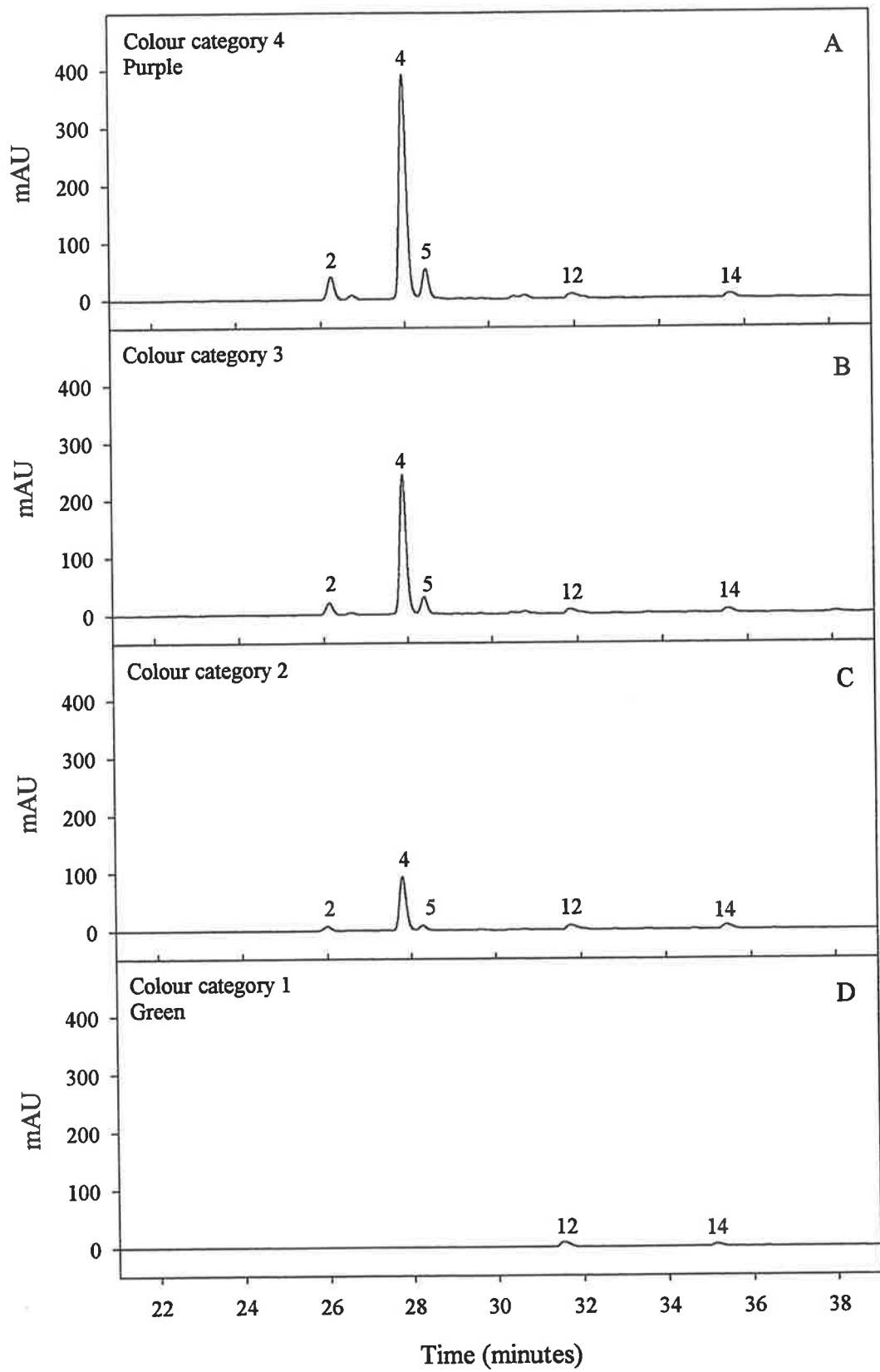
external colour, there was 71% agreement between the two methods, with 12 plants misclassified, while for peak 5, seven plants were misclassified (Table 8.4).

Table 8.4 Levels of 'agreement', calculated by discriminant analysis, between HPLC-determined anthocyanin content of *A. baileyana* leaf extracts and the allocated colour category for the sample. Plants were grown from cuttings.

Anthocyanin content	Agreement between methods (%)	No. of plants misclassified (n=41)
Sum of all peaks	85	6
Peak 4	88	5
Peak 2	71	12
Peak 5	83	7

8.3.2.2 Plantation trees

The dominant anthocyanin peaks for plantation trees were 2, 3, 4, 5, 12 and 14, with up to 15 peaks present in total (Fig. 8.3; Fig. 8.4; Appendix 1, Table 8). For each of the ten plants assessed, the sum of all peak areas was similar in winter and summer (Table 8.5; Appendix 1, Table 8). The colour of plants 1 to 5, varied between winter and summer; with plants classified as colour category 4 in winter and category 3 in summer. In leaf extracts from these plants, peak 4 (cyanidin-3-glucoside) was larger in winter, while peak 2 (delphinidin-3-glucoside) was larger in summer (Fig. 8.3, Table 8.5). For plants 6 to 10, peak 4 was larger than peak 2 in both winter and summer, although the assessed colour (category 2) did not differ between the seasons (Table 8.5, Fig. 8.4). No plants in the plantation were scored as colour category 1.

Discriminant analysis was used to distinguish between the three colour categories present on the basis of the HPLC-determined anthocyanin content. For the sum of all peak areas, there was 75% agreement between the four colour categories and HPLC-determined anthocyanin content, with five plants misclassified (Table 8.6). Misclassification occurred between colour categories 3 and 4 only (Appendix 1, Table 4), with 50% of the plants numbered 1 to 5 misclassified. When the area of peak 4 only was used as the predictor of external colour, there was total agreement between methods, with no plant misclassified (Table 8.6; Appendix 1, Table 4). Only one plant was misclassified when the area of peak 2 was used as the predictor of external colour, giving 95% agreement between methods (Table 8.6).

Table 8.5 A comparison of the HPLC-determined anthocyanin content (mean ± standard error) of leaf extracts from *A. baileyana*, grown in the Waite Campus plantation in winter and summer, and the allocated colour category for the sample. Figures in parentheses are the percentages of the anthocyanin content of the individual peaks relative to the sum of all peaks.

Colour category	Plant no.	No. of leaf samples	Season	Average anthocyanin content (scaled mAU) ± s.e.		
				Sum of all peaks	Peak 2	Peak 4
4	1 to 5	5	winter	108.3 ± 8.69	14.8 ± 1.83 (14)	72.2 ± 7.12 (67)
3	1 to 5	5	summer	98.8 ± 13.69	52.6 ± 10.31 (53)	28.6 ± 2.24 (29)
2	6 to 10	5	winter	8.2 ± 1.59	0.5 ± 0.21 (6)	4.1 ± 1.08 (50)
2	6 to 10	5	summer	11.7 ± 0.80	3.1 ± 0.64 (26)	4.6 ± 0.41 (39)

Table 8.6 Levels of 'agreement', calculated by discriminant analysis, between HPLC-determined anthocyanin content of *A. baileyana* leaf extracts and the allocated colour category for the sample. Samples were from plants grown in the Waite Campus plantation.

Anthocyanin content	Agreement between methods (%)	No. of plants misclassified (*n*=20)
Sum of all peaks	75	5
Peak 4	100	0
Peak 2	95	1

8.4 Discussion

The four colour categories provided a quick yet reliable method of assessing the anthocyanin content of the young leaves of *A. baileyana*. Discrimination between the four colour categories was possible when compared to the anthocyanin content determined by HPLC analysis. Routine analysis of leaf colour from hundreds of plant samples by HPLC would be very time consuming in comparison to assessment using the colour categories, due to the time involved with extraction and separation of anthocyanins. The use of the colour categories therefore, appears to be an objective and facile means

Fig. 8.3 HPLC chromatograms showing absorption profiles at $A_{520\,nm}$ of leaf extracts from an *A. baileyana* tree (plant 2; Appendix 1, Table 8) in the Waite Campus plantation. A: Summer leaf sample, colour category 3. B: Winter leaf sample, colour category 4. The main peaks are numbered according to their elution time.
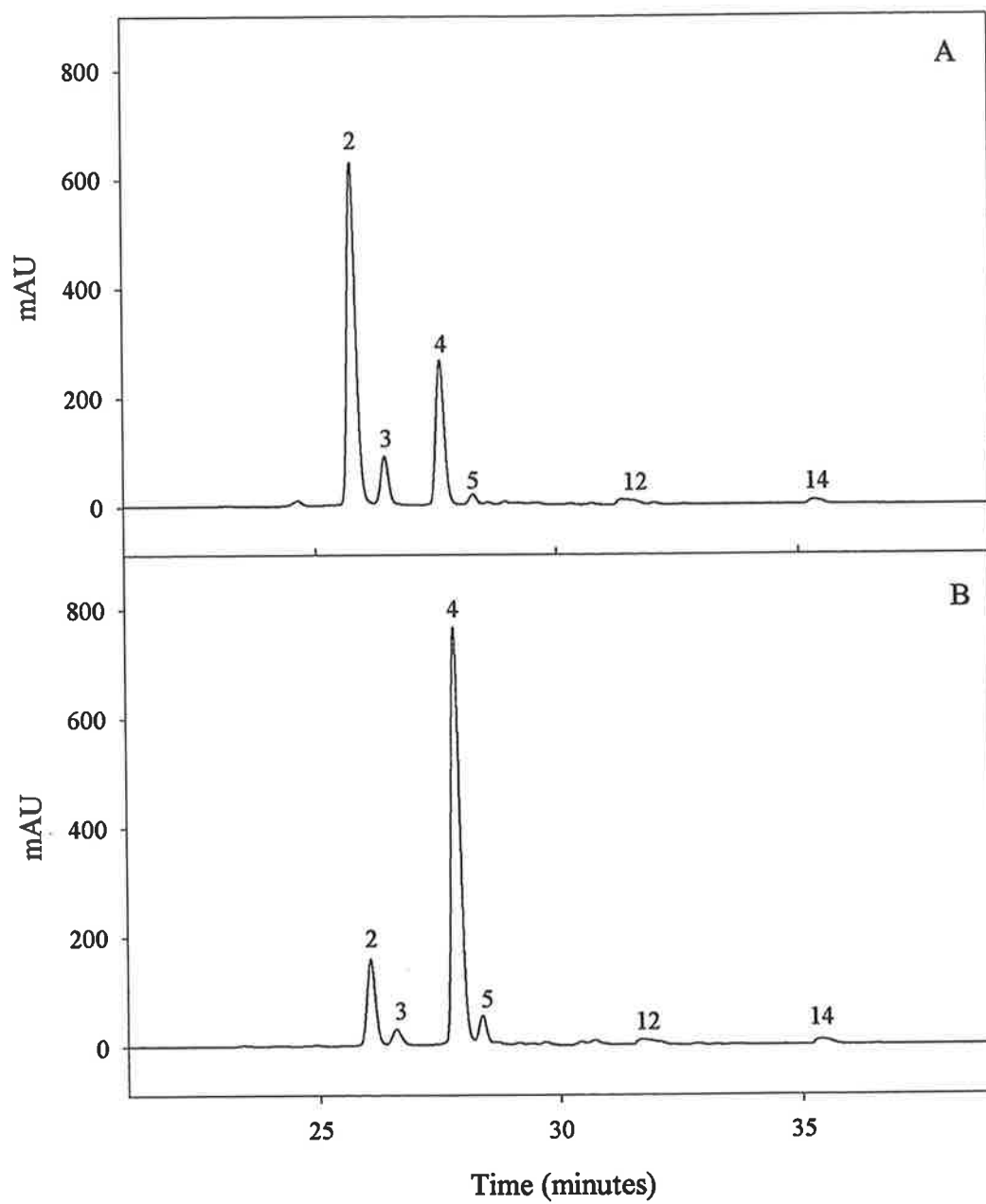
Fig. 8.4 HPLC chromatograms showing absorption profiles at A_{520nm} of leaf extracts from an *A. baileyana* tree (plant 7; Appendix 1, Table 8) in the Waite Campus plantation. A: Summer leaf sample, colour category 2. B: Winter leaf sample, colour category 2. The main peaks are numbered according to their elution time.
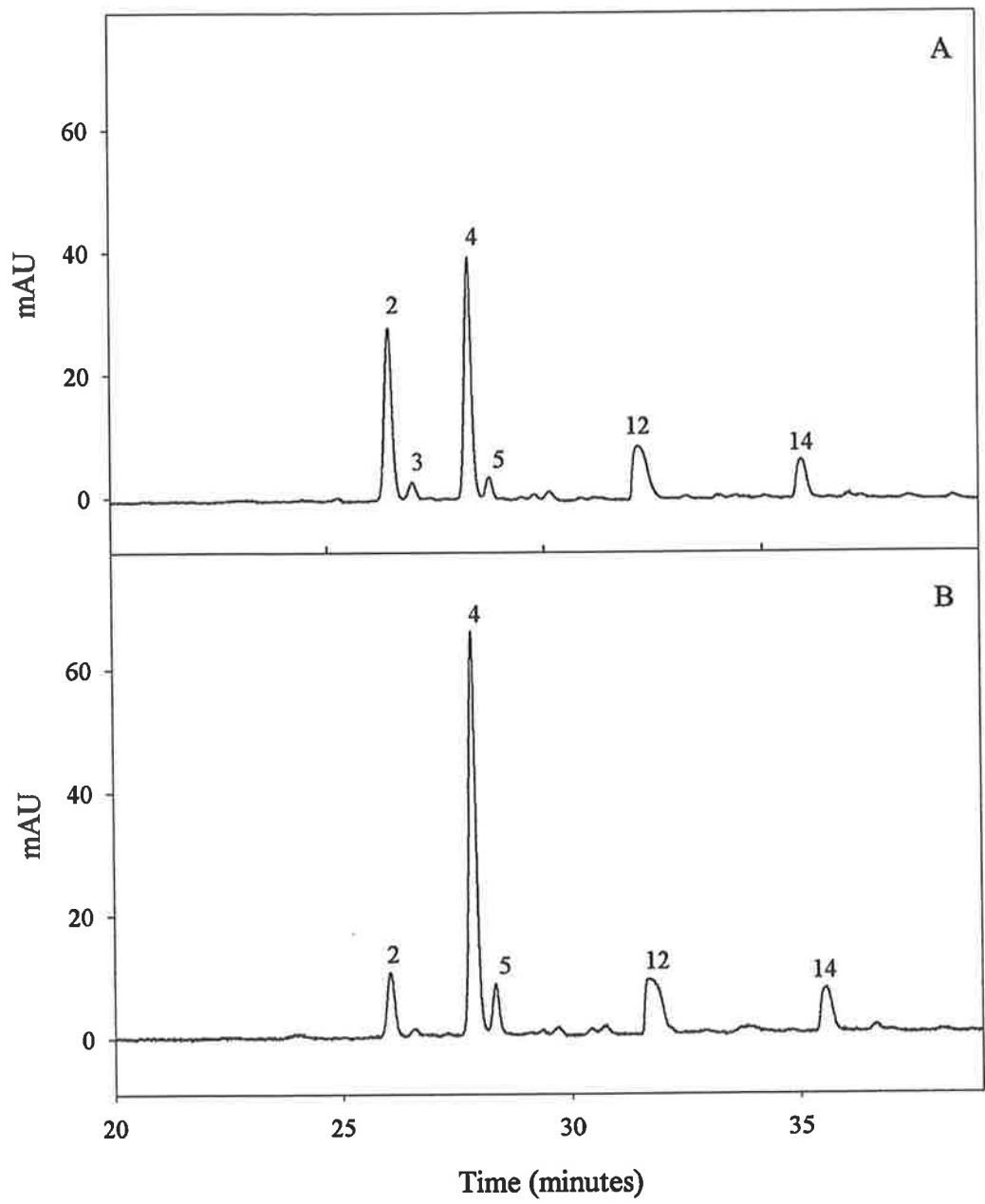
of recording leaf colour and should prove useful in future studies on inheritance of leaf colour and expression of anthocyanin accumulation.

The colour meter data did not clearly separate plants into the four colour categories, as there was a wide variation in hue, chroma and lightness within and between each of the colour categories. This may be due to variation in colour across each leaf of *A. baileyana*. For the intensely purple leaves (colour category 4) all of the leaf is pigmented, while for colour categories 2 and 3, only some cells are pigmented (Plate 3.1A). The allocation of a leaf sample to a colour category is determined by obtaining a general overall appearance of the leaf, however the colour meter measures only a small area. Therefore, small shifts in colour meter location could give different readings if the leaf sample is not uniform in colour. Also, since *A. baileyana* leaves are bipinnate, the colour meter does not necessarily sit over leaf tissue alone, but may partially sit over the supporting white tile. A number of meter readings taken at different places within the leaf sample may provide a more accurate representation of leaf colour, and would be more time consuming.

Measurement of the anthocyanin area under peak 4 (cyanidin-3-glucoside) by HPLC alone provided better discrimination between the colour categories than for peak 2 or the total sum of all peaks. This suggests that the level of cyanidin-3-glucoside, in particular, is important in determining leaf colour. This could be because peak 4 is the major peak and thus the integration of this peak will be more accurate than that of the others. Another possibility is that in the vacuolar environment within these plants, cyanidin-3-glucoside may be copigmented with itself or with other copigments, which would lead to a greater stability for its flavylium form and thus have enhanced colour (Mazza and Miniati 1993). Other anthocyanins, if not stabilised in this way, would be hydrated to a greater extent and their colour would not be truly representative of their concentration (Ken Markham, personal communication).

No purple colouring was visually observed for category 1 plants (Genotype 6) grown from cuttings, but two peaks (12 and 14) were detected by HPLC. It is difficult to confirm that peaks 12 and 14 are anthocyanins or other types of pigments, as the spectral data identified very low, poor resolution peaks at 520 nm (data not shown). As these pigments are present at 520 nm they do contribute red-type colour. Other metabolic pathways may produce red carotenoids, and a variety of structural modifications can shift absorbance towards longer wavelengths. For example, lycopene and rhodoxanthin can absorb at wavelengths above 500 nm (Lee 2002 and references within). Therefore, these pigments may be red carotenoids.

It is likely that the amount of peaks 12 and 14 detected by HPLC was insufficient to be detected by the eye. Chlorophyll may also be masking the visual detection of these colour pigments at such low concentrations, or the pH of the vacuoles may produce colourless pigments (Asen et al. 1972, Gould et al. 2002). The mature green to blue-grey leaves of *A. baileyana* may also contain colour pigments. It would be interesting to compare the young leaves of both the green and purple leaf forms with their mature leaves, to determine if colour pigments are present in the older leaves.

Genotype 1 plants grown from cuttings in the growth cabinets all produced purple colouration. Plants were assessed as colour category 4 only when they were grown at the lowest temperatures (13/9°C). In contrast, category 3 plants were recorded at all three growth cabinet temperatures, while colour category 2 plants occurred only at the highest temperatures (23/19°C). The interaction between genotype and environment clearly plays a role in determining the capacity for purple leaf colour to develop. However, plants grown from cuttings of Genotype 6 were only ever scored as 'pure green' (category 1), even when grown at the lowest temperatures of 13/9°C. Therefore, cool temperature did not affect the colour of these plants. This was similar to the plantation trees that were assessed as colour category 2 in both winter and summer.

For plants grown from cuttings, the highest total HPLC-determined anthocyanin was for plants assessed as colour category 4. However, this was not the case for the leaf samples taken from the plantation. The total anthocyanin content was similar for the intense purple leaf samples taken in winter and summer, however there was a shift in the intensity of purple colour from category 4 to category 3 from winter to summer. This change in the colour intensity between winter and summer could be due to a seasonal difference in vacuolar pH. A drop in pH would enhance the red colour (Mazza and Miniati 1993) and possibly the purple colour, if the purple were a mix of flavylium red and chlorophyll green. This may occur in winter and cause the enhanced colouration.

Within one tissue type the distribution of flavonoids may not be uniform (Grisebach 1982). Therefore, it is also possible that in winter more anthocyanin pigments are located near the outer surface of the leaves than in summer, which would account for seasonal differences in colour expression. Anthocyanins have been observed in the epidermis of cotyledon leaves of *A. baileyana* (Nozzolillo 1973) but the season of sampling was not mentioned. The number and size of vacuoles containing anthocyanins could also vary between seasons, affecting the colour. This has been found for apples, with variations in the proportion of red cells in the skin and size of the vacuoles containing anthocyanins affecting their colour (Lancaster et al. 1994). Anthocyanic vacuolar inclusions (Markham et al. 2000) may be present at different densities between the seasons, which may affect the

colour. Future work could involve locating the anthocyanins in the leaf, to determine if the location varies between seasons.

Anthocyanins were produced in both winter and summer in *A. baileyana* growing in the Waite Campus plantation. The accumulation of anthocyanins seems to only occur in the juvenile foliage, but new foliage is produced all year around (refer to Fig. 4.1, Chapter 4). Anthocyanin composition also varied between winter and summer for the intense purple leaf plants. The level of cyanidin-3-glucoside (peak 4) was greater in winter and delphinidin-3-glucoside (peak 2) was greater in summer for those trees classified as colour category 4 in winter and colour category 3 in summer. Copigmentation of anthocyanins with flavonoids, such as flavonols, or self association, is known to intensify colour. This effect, however, is reduced at high temperatures (Mazza and Miniati 1993). It is therefore possible that the change in the amount of cyanidin-3-glucoside and delphinidin-3-glucoside observed between winter and summer, and the difference in leaf colour between the seasons, is due to the variable effect of temperature on copigmentation. This should be investigated further.

For the purple leaf plants in the growth cabinets, there was no difference in leaf anthocyanin composition, with cyanidin-3-glucoside (peak 4) always being the dominant pigment. This is despite the highest (23/19°C) and lowest temperatures (13/9°C) in the growth cabinet being similar to the summer (26/15°C) and winter (14/8°C) mean temperatures in the plantation. Therefore, differences in light between winter and summer may also affect the anthocyanin composition of leaves from plantation trees. This is known to occur with other flavonoids. For example, the increasing levels of UV light in summer have been shown to increase both the total level of flavonols, and the quercetin: kaempferol ratios in plants (Ryan et al. 1998). This would result in a very different copigmentation environment for the anthocyanins in summer compared to winter, and could lead to colour modification. The resultant colour would be dependant upon which flavonols copigment with which anthocyanins and how this is influenced by other factors such as relative pigment and copigment concentrations, temperature and pH (Mazza and Miniati 1993).

The flavonoid biosynthetic pathway is most likely operating for the four colour categories, as HPLC peaks were present for all the categories. Light and/or temperature may regulate the expression of genes encoding enzymes of the flavonoid pathway in *A. baileyana* and, as a result, levels and types of anthocyanins (and other colour pigments) present in leaves would vary depending on the environmental conditions. The rate of flux down the cyanidin and delphinidin branches of the biosynthesis pathway may therefore vary under different environmental conditions. Some genes involved in anthocyanin biosynthesis have been found to be cold-temperature regulated in maize (Christie et al. 1994), while some are light regulated (Dooner et al. 1991). For example, in Japanese parsley, expression of the

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gene encoding dihydroflavonol-4-reductase (DFR) is strongly induced by low temperature (Hasegawa et al. 2001), while for juvenile English ivy, DFR activity responds to carbohydrate and light (Murray and Hackett 1991). This enzyme is one of at least three enzymes required for converting the colourless dihydroflavonols to coloured anthocyanins (Holton and Cornish 1995). The difference between the anthocyanin aglycones, delphinidin and cyanidin, is the presence of an extra hydroxyl group on the B-ring of delphinidin. Environmental conditions could favour the expression of either the flavonoid 3',5'-hydroxylase enzyme which catalyses hydroxylation to the delphinidin aglycone, or the flavonoid 3'-hydroxylase enzyme which catalyses the synthesis of cyanidin aglycone. Therefore, the varied levels of light and/or temperature during leaf development could affect the amount of cyanidin and delphinidin derivatives produced. The influence of light could be investigated further, by growing plants in controlled environment growth cabinets under different light conditions and determining if there is a difference in hydroxylase enzyme activities as has been observed for other flavonoids (Ryan et al. 1998).

This research has developed and validated a quick and objective method of assessing the leaf colour of *A. baileyana*. The four defined colour categories closely reflected the amount of cyanidin-3-glucoside in young leaves. This method of assessing leaf colour can therefore confidently be used in the following studies where the inheritance and expression of the purple leaf colour is investigated (Chapter 9).

Anthocyanin expression and inheritance of *Acacia baileyana* purple leaf colour

9.1 Introduction

The obvious phenotypic difference between the green and purple leaf forms is the colour of the young leaves. Reproductively, both forms are similar in that they are outcrossing, have similar pod set, the ability to reproduce from seed six months after flowering (refer to Chapter 4), and have similar temperature requirements for flowering (refer to Chapter 3). The purple form is as reproductively efficient as the green form, but is not currently present in weed populations. This may be because the purple form has been cultivated only since the 1970s (Bill Molyneux, personal communication) and thus the frequency of the gene(s) for this colour trait may be relatively low. As a consequence, the purple leaf form may not have had the time to spread from garden plantings to native bush. In addition, the purple colour may be due to a recessive gene(s), thus the offspring would have a higher likelihood of being the prevalent green leaf form in a weed population.

It was shown in Chapter 8 that plants of all the four colour categories produce colour pigments, including the pure green form (colour category 1), albeit at very low concentrations. Therefore, the flavonoid pathway (Fig. 2.1, Chapter 2) leading to the biosynthesis of anthocyanins is most likely operating in all colour categories. The main genes involved in anthocyanin production are either structural or regulatory. Many genes control the anthocyanin biosynthesis pathway, and different allelic combinations may lead to both qualitative and quantitative differences in the distribution of pigments (Reddy et al. 1995). In general, changes in structural genes should change the activity of only one flavonoid enzyme and not influence the enzymes of other steps. Regulatory genes, however, affect the patterns and intensity of flavonoid biosynthesis by influencing the activity of more than one flavonoid enzyme (Forkmann 1994). These genes can influence flavonoid concentration by enhancing or reducing pigment synthesis, by enzymatic or chemical degradation or by preventing pigment accumulation (Forkmann 1994).

The anthocyanin regulatory genes can also control the expression of structural genes in response to various developmental and environmental factors (Taylor and Briggs 1990, Dooner et al. 1991). Although the genetic constitution of a plant determines its ability to synthesise anthocyanin pigments, environmental factors can influence the level of synthesis (Taylor and Briggs 1990). High light

intensities, as well as low temperature, are important triggers for synthesis of anthocyanins (Grisebach 1982, Christie et al. 1994, Mol et al. 1996). It has been suggested that anthocyanin production in foliage may allow the plant to develop resistance to a number of environmental stresses (Chalker-Scott 1999). The function of anthocyanins is poorly understood, although a number of hypotheses have been presented in recent papers, including photoprotection, antioxidant activity and osmoregulation, as discussed in Chapter 2 (Chalker-Scott 1999, Field et al. 2001, Hoch et al. 2001, Chalker-Scott 2002, Gould et al. 2002, Lee and Gould 2002, Steyn et al. 2002). Anthocyanins may in fact have developed a number of functions over time, as shown by the variability in anthocyanin pigmentation in different plant material, the accumulation of anthocyanins as a result of different environmental and developmental triggers, and the different localisations of the pigments within the plant cell (Gould et al. 2002).

In order to further develop *A. baileyana* as a horticultural commodity, the factors that influence leaf colour must be determined. This chapter examined the environmental conditions that control leaf colour expression and the inheritance of the purple leaf colour. The information may help to manipulate leaf colour as well as to produce desirable colour forms through breeding programs. The first aim was to characterise the temperatures that enhance the expression of the purple leaf pigmentation. This work will expand on the preliminary studies described in Chapter 8. The second aim examined the inheritance of anthocyanins, by studying the offspring of crosses between the purple and the green forms. This may give an indication as to whether the purple form is the result of a recessive gene and help explain why this form is not observed in weed populations. For both the colour expression and inheritance studies, plants were assessed using the four colour categories described in Chapter 8.

9.2 Materials and methods

9.2.1 Assessment of leaf colour

The leaf colour of *A. baileyana* was assessed using the four colour categories mentioned in Chapter 3 and discussed further in Chapter 8. Between three and five of the youngest, fully expanded leaves per plant were assessed for colour, with each plant assigned to one of four colour categories. The categories varied from category 1 (pure green) to category 4 (intense purple).

9.2.2 Expression of leaf colour

9.2.2.1 Plants outside (plantation)

A plantation of one-year-old seedlings was established in the Waite Campus plantation in June 1996. The plantation had previously been used to study growth, flowering and seed production (Chapter 4), and to determine anthocyanin content of leaf samples (Chapter 8). Leaf colour of plants was assessed using the four colour categories in November 1997 (spring), February 1998 (summer), May 1998 (autumn) and August 1998 (winter). Refer to Appendix 2, Table 1 for environmental conditions.

9.2.2.2 Plants in modified temperature environments

Plant material grown from seeds or from cuttings was randomly placed into three growth cabinets, in a shadehouse or outside. The treatments were as follows:

- L: low temperature growth cabinet (13.0°C day / 9.0°C night; 13/9°C),
- M: medium temperature growth cabinet (18.3°C day / 13.3°C night; 18/13°C),
- H: high temperature growth cabinet (23.3°C day / 19.3°C night; 23/19°C),
- outside at the Waite Campus, The University of Adelaide.
- shadehouse at the Waite Campus; 75% shade and temperature similar to outside conditions.

Refer to Chapter 3 (section 3.2.3) for details on the experimental conditions, including the light and growing conditions.

Seedlings

Seeds were germinated as described in Chapter 4 (section 4.2.1), on 23 April 1996. They were germinated in trays containing 1:1:1 sand: perlite: peat. Seedlings were potted up into 10 cm diameter pots containing native plant soil mix in June 1996. Refer to Chapter 3 (section 3.2.2) for specifications of the native plant soil mix. Approximately 140 plants were placed randomly into each of the five environmental treatments on 5 August 1996. Plants in the five treatments were allocated to one of the four colour categories on 14 July 1997. Plants were then moved outside on 30 July 1997 and again assessed for colour on 4 October 1997. Seedlings from the 13/9°C treatment, shadehouse and outside, had previously been used for the comparison of the colour categories with the colour meter (section 8.2.2, Chapter 8).

Cuttings

Refer to Chapter 3 for details on plant material (section 3.2.1), propagation (section 3.2.2) and growth cabinet conditions (section 3.2.3). The cutting material consisted of three green leaf phenotypes and three purple leaf phenotypes (Genotypes 1, 2, 3, 5, 6, 7 in Table 3.2, Chapter 3). For the green leaf

forms, cuttings originated from colour category 2 mother plants, while for the purple leaf forms, cuttings originated from category 4 mother plants. About 48 purple leaf cuttings and about 10 green leaf cuttings were randomly placed into four treatments on 1 August 1997. The four treatments included the low (13/9°C) and high temperature (23/19°C) growth cabinets, outside and shadehouse, as described previously. No cutting material was put into the medium temperature (18/13°C) cabinet. Plants were transferred between high and low temperature treatments on 6 January 1998. Leaf colour was assessed using the colour categories on 6 January 1998, before the plant transfer and, after transfer, assessed for colour approximately weekly until 5 April 1998. The mother plants from which the cutting material was taken were assessed for colour in October 1997. The cuttings had previously been used to investigate the temperature and light conditions for flowering (Chapter 3) and to determine anthocyanin content (Chapter 8).

9.2.3 Inheritance of the purple leaf colour

9.2.3.1 1995 crosses

To investigate the inheritance of the purple leaf colour, the green leaf form was crossed with the purple leaf form in July 1995 (Table 9.1). Five green leaf plants were also crossed with five purple leaf plants, all from the Adelaide Hills, South Australia, while five purple leaf plants from the Adelaide Hills were crossed with five green leaf plants from the Waite Campus, The University of Adelaide (Table 9.1). Refer to Chapter 4 (section 4.2.3) for details on the technique used for the cross pollinations.

Table 9.1 Details of the inter-variety crosses conducted between the green (G) and purple (P) leaf forms of *A. baileyana* in 1995.

Female (pollen recipient)			Male (pollen source)		
Plant code	Location in South Australia	Colour category	Plant code	Location in South Australia	Colour category
G3	Woodside	2	P8	Mylor	4
G5	Mylor	2	P8	Mylor	4
G4	Lenswood	2	P8	Mylor	4
G2	Lenswood	2	P7	Mylor	3
G6	Balhannah	2	P7	Mylor	3
P2	Woodside	4	G7	Waite Campus	2
P6	Mylor	4	G7	Waite Campus	2
P1	Woodside	4	G7	Waite Campus	2
P5	Woodside	4	G8	Waite Campus	2
P3	Mylor	4	G8	Waite Campus	2

Seeds from the cross pollinations were collected in January 1996 and germinated on 19 June 1996, using the hot water treatment. Refer to Chapter 4 (section 4.2.1) for details of the hot water germination treatment. The seeds were sown into trays containing vermiculite and placed in a 25°C glasshouse. Refer to Appendix 2, Table 2 for details on seed germination and pollination results. Seedlings were potted into 10 cm diameter pots containing native plant soil mix and placed in a 25°C glasshouse on 24 September 1996. Refer to Chapter 3 (section 3.2.2) for specifications of the native plant soil mix. The seedlings were re-potted into 15 cm pots on 18 February 1997. Plants were moved outside on 9 July 1997, and assessed for leaf colour on 2 October 1997. Leaf colour of the parent plants was assessed in October 1997.

9.2.3.2 1996 crosses

In July 1996, a number of different crosses (intra-variety, inter-variety and self treatments) were performed on three pollen recipient (i.e. female) plants all located in the Adelaide Hills (Table 9.2). Refer to Chapter 4 (section 4.2.3) for details on the pollination technique. The plants used for the controlled pollinations in Chapter 4 were also used to study the inheritance of the purple leaf colour. The seeds from the controlled pollinations were collected in January 1997, and germinated on 12 March 1997, using the hot water treatment. Refer to Chapter 4 (section 4.2.1) for details of the hot water germination treatment. The seeds were sown into trays containing vermiculite and placed in a 25°C glasshouse. Seedlings were potted into 10 cm diameter pots containing native plant soil mix and placed in a 25°C glasshouse on 23 May 1997. Refer to Chapter 3 (section 3.2.2) for specifications of the native plant soil mix. The seedlings were re-potted into 15 cm pots on 1 March 1998. Plants were moved outside on 16 June 1998, and assessed for leaf colour on 2 October 1998. Leaf colour of the parent plants was assessed in October 1997.

9.2.3.3 Statistical analysis

Chi-square analysis was used to compare observed data with expected ratios in order to interpret the inheritance of anthocyanins. Colour categories 1 and 2 were combined to represent the green leaf form and colour categories 3 and 4 were combined to represent the purple form for Chi-square analysis. To test for homozygous dominant green and recessive purple parents, the leaf colour data were analysed for the expected ratio of 1:0 (green: purple). For completion, all data were analysed by Chi-square, even though normally it is considered inappropriate to apply a Chi-square analysis if the frequency of any class is less than five (Ayala and Kiger 1984).

Table 9.2 Details of pollinations conducted between the green (G) and purple (P) leaf forms of *A. baileyana* in 1996.

Female (pollen recipient)			Male (pollen source)			Pollination treatment
Plant code	Location in South Australia	Colour category	Plant code	Location in South Australia	Colour category	
P5	Woodside	4	P5	Woodside	4	self
			P3	Mylor	4	intra-specific
			G5	Mylor	2	inter-specific
			G3	Woodside	2	inter-specific
G2	Lenswood	2	G2	Lenswood	2	self
			G5	Mylor	2	intra-specific
			P5	Woodside	4	inter-specific
			P3	Mylor	4	inter-specific
G5	Mylor	2	G5	Mylor	2	self
			G3	Woodside	2	intra-specific
			P5	Woodside	4	inter-specific
			P3	Mylor	4	inter-specific

9.3 Results

9.3.1 Expression of leaf colour

9.3.1.1 Plants outside (plantation)

Purple foliage (colour category 4) was produced only in autumn and winter for plants grown outside (Table 9.3). This coincided with low temperatures. Only a few plants had all green foliage (colour category 1). Ninety-five of the 120 trees in the plantation were assessed as the green leaf form (colour category 1 and 2). The remaining 25 trees expressed purple leaf colour (categories 3 and 4) in spring, autumn and winter, however in summer some of the purple phenotypes were assessed as category 2. The majority of the green leaf forms were colour category 2 for all seasons, while the majority of the purple forms were category 3 in spring and summer and category 4 in autumn and winter.

9.3.1.2 Plants in modified temperature environments

Seedlings

For seedling plants in the growth cabinets, colour category 4 was only recorded for plants at the low temperature of 13/9°C (Table 9.4). Only two of the seedling plants grown outside and one in the shadehouse were recorded as colour category 4. The temperature outside and in the shadehouse (14/7°C) was similar to the low temperature growth cabinet of 13/9°C, but more category 4 plants

were present in the growth cabinet. The majority of plants in the growth cabinets and shadehouse were assessed as category 1, while the majority of outside grown plants were category 2. The high and medium temperatures in the growth cabinets resulted in a similar number of plants at each colour category.

Table 9.3 Leaf colour of the 120 *A. baileyana* plantation trees assessed during each season, and the mean temperature of the season.

Colour category	Number of plants			
	Spring (Nov. 1997)	Summer (Feb. 1998)	Autumn (May 1998)	Winter (Aug. 1998)
1	0	2	4	3
2	95	100	91	92
3	25	18	6	0
4	0	0	19	25
Temperature (°C) (mean max. / mean min.)	25/15	27/15	19/11	16/9

Table 9.4 Leaf colour of *A. baileyana* seedlings grown under five environmental conditions. Leaf colour was assessed in July 1997. The mean maximum and minimum outside temperatures for the month of July were 14°C and 7°C, respectively.

Colour category	Number of plants			Shadehouse	Outside
	Growth cabinets (temp. °C, mean max./mean min.)				
	Low (13/9°C)	Medium (18/13°C)	High (23/19°C)		
1	91	129	126	121	35
2	28	9	9	13	93
3	14	2	5	4	8
4	7	0	0	1	2

More plants expressed purple foliage (colour category 4) following their transfer from the growth cabinet and shadehouse to outside (Table 9.5). This was particularly the case for the plants that had previously been in the low (13/9°C) and medium (18/13°C) temperature growth cabinets. The majority of plants were recorded as colour category 2, following their transfer outside. For plants grown outside throughout, the number of plants allocated to each colour category varied between the two assessment dates, with fewer plants assessed as category 1 in October (Table 9.4, Table 9.5).

Table 9.5 Leaf colour of *A. baileyana* seedlings outside, after previously been grown in either a growth cabinet or shadehouse. The colour of seedlings that had always been grown outside are also presented for comparison. Leaf colour was assessed in October 1997, two months after transfer. The mean maximum and minimum outside temperatures for the month of October were 21°C and 12°C, respectively.

Colour category	Number of plants			Shadehouse	Outside
	Growth cabinets (temp. °C, mean max./mean min.)				
	Low (13/9°C)	Medium (18/13°C)	High (23/19°C)		
1	9	14	18	12	18
2	102	104	94	109	105
3	15	12	11	16	14
4	13	7	4	3	3

Cuttings

All cutting material originating from purple (colour category 4) mother plants had intense purple foliage when grown in the 13/9°C growth cabinet (Table 9.6). However, when these plants were transferred to the warmer 23/19°C cabinet, leaves lost some of their purple pigmentation (Table 9.6, Fig. 9.1). In these cuttings, colour began to change after approximately one week, and was stable after approximately one month (Fig. 9.1). In contrast, the foliage of plants originating from category 4 mother plants, which had a tinge of purple colour (category 2) while in the warm growth cabinet, became more purple when the plants were moved to the low temperature cabinet (Table 9.6, Fig. 9.2). It took about three weeks for the colour change to be detected and about two months for the colour to stabilise (Fig. 9.2). All of the cuttings originating from a green (category 2) mother plant had green (category 1) foliage (Table 9.6). Transferring this cutting material between the low and high temperature cabinets did not alter the leaf colour.

For the cutting material originating from purple leaf (category 4) mother plants, a higher number had intense purple foliage when grown outside compared to those growing in the shadehouse environment (Table 9.7). The majority of these plants had strong purple colouration, with no category 1 plants recorded. Foliage of cutting material originating from green (colour category 2) mother plants, were assessed as colour categories 1 and 2 for the outside plants and colour category 1 for the shadehouse plants.

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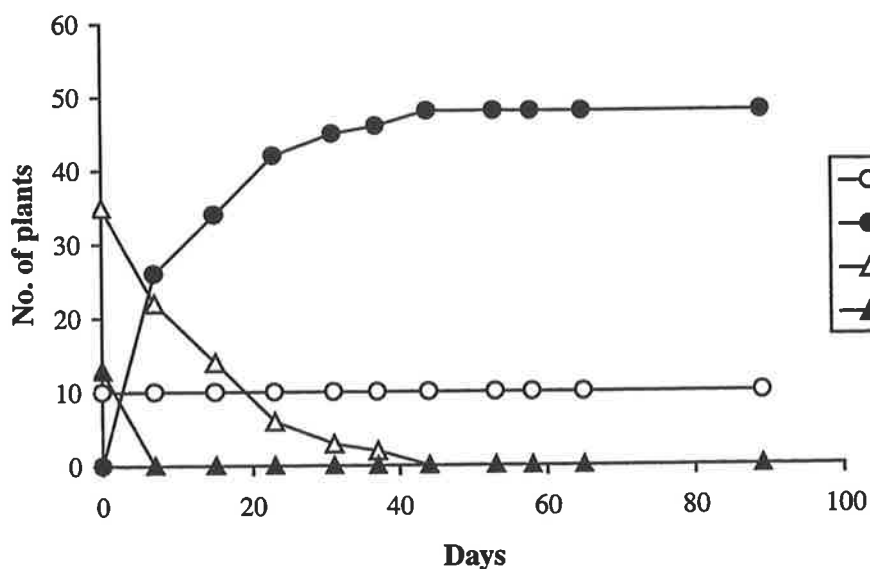
Fig. 9.1 Change in leaf colour of *A. baileyana* plants grown from cuttings, after transfer from the 13/9°C to the 23/19°C growth cabinet. Each line represents those plants allocated to a particular colour category, as described in the legend. All category 1 plants were derived from category 2 mother plants, whereas category 4 mother plants were the source for all cutting material allocated to colour categories 2, 3 and 4.

Table 9.6 Leaf colour of *A. baileyana* plants, grown from cuttings, before and after transfer between the high (23/19°C) and low (13/9°C) temperature growth cabinets. Leaf colour was initially assessed on 6 January 1998, immediately before transfer, and re-assessed on 5 April 1998.

Colour category of mother plant	Colour category	Number of plants			
		Low to high temperature transfer		High to low temperature transfer	
		Low (13/9°C)	High (23/19°C)	High (23/19°C)	Low (13/9°C)
4	1	0	0	0	0
	2	0	48	48	0
	3	35	0	0	32
	4	13	0	0	16
2	1	10	10	11	11
	2	0	0	0	0
	3	0	0	0	0
	4	0	0	0	0
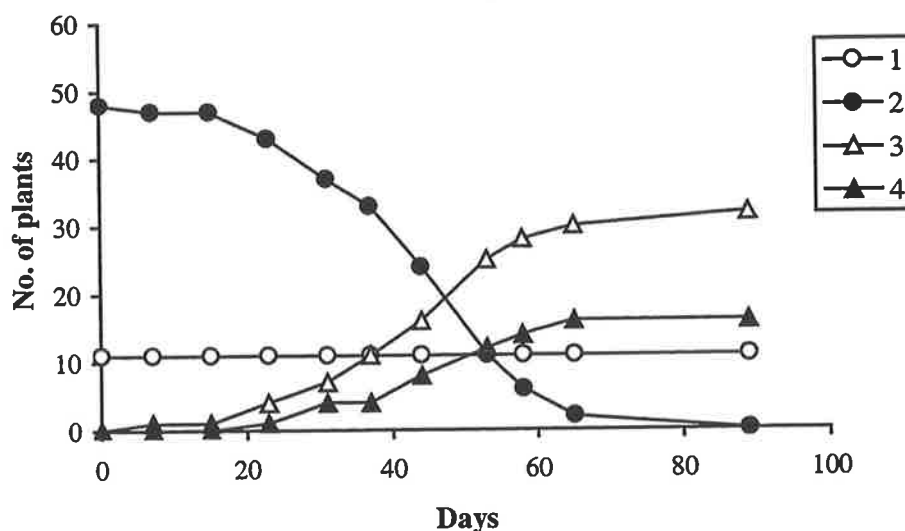
Fig. 9.2 Change in leaf colour of *A. baileyana* plants grown from cuttings, after transfer from the 23/19°C to the 13/9°C growth cabinet. Each line represents those plants allocated to a particular colour category, as described in the legend. All category 1 plants were derived from category 2 mother plants, whereas category 4 mother plants were the source for all cutting material allocated to colour categories 2, 3 and 4.

Table 9.7 Leaf colour of *A. baileyana* plants grown from cuttings, located in the shadehouse and outside. Leaf colour was assessed on 21 April 1998. The mean maximum and minimum outside temperatures for the month of April 1998 were 20°C and 12°C, respectively.

Colour category of mother plant	Colour category	Number of plants	
		Shadehouse	Outside
4	1	0	0
	2	5	3
	3	39	20
	4	3	28
2	1	9	2
	2	0	9
	3	0	0
	4	0	0

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9.3.2 Inheritance of the purple leaf colour

The results of the inheritance studies performed on *A. baileyana* in 1995 and 1996 are recorded in Tables 9.8 and 9.9, respectively. Crosses between the green leaf forms, either from selfing or intra-variety crossing, resulted in green leaf colour offspring (colour categories 1 and 2) (Table 9.9). In contrast, the crosses between the purple forms (selfing and intra-variety crossing) produced mainly purple leaf offspring (colour categories 3 and 4) (Table 9.9). Crosses between the purple and green leaf forms produced offspring mainly green in leaf colour (colour categories 1 and 2) (Table 9.8, Table 9.9). Nearly all the Chi-square analyses supported the hypothesis that the parents are homozygous. The exceptions were for two purple and green crosses (P5 x G8 and P3 x G8), which produced green and purple offspring (Table 9.8).

9.4 Discussion

9.4.1 Expression of leaf colour

Both developmental and environmental factors affect anthocyanin accumulation in *A. baileyana*. For outside grown plants, purple colour in juvenile foliage is present all year around, as there is continuous growth (refer to Fig. 4.1, Chapter 4), but it is also induced by particular environmental conditions. Anthocyanin expression in the juvenile foliage of *A. baileyana* is strongly induced by low temperatures, as was found in Chapter 8, and high light intensity. Intense purple pigmentation was produced during the cool months for outside grown plants, at temperatures below 21°C maximum and 12°C minimum, and in plants grown in the coolest growth cabinet of 13°C maximum and 9°C minimum. Therefore, in order to enhance the purple colour, *A. baileyana* plants should be grown in cool environments.

As discussed in Chapter 8, the genotype of the plant also contributes to colour of the leaves. Some plants never expressed any purple colour in the leaves, even when exposed to low temperatures. Cutting material was used to study the genotype effects, to overcome seedling variability, as the potential leaf colour is obvious from the mother plant. The use of cuttings also overcomes problems with genotypic variability in seedlings. Cuttings taken from plants with intense purple leaves (colour category 4) were recorded as categories 3 or 4 when grown at low temperatures (13/9°C), and were recorded as category 2 at high temperatures (23/19°C), but were never pure green. In contrast, cuttings taken from mother plants with green (category 2) foliage were all green (category 1) at the low and high temperatures.

Table 9.8 The leaf colour of the female and male A. *baileyana* parents, and their F_1 offspring, and the Chi-square analysis of leaf colour, in 1995. The Chi-square analysis (χ^2) of the combined colour category data was used to test for homozygous dominant green leaf, and recessive purple leaf parents. Leaf colour was assessed on 2 October 1997.

Female (pollen recipient)		Male (pollen source)		Number of F_1 offspring in the colour category						Expected ratio for 1+2 (green) & 3+4 (purple) colour categories	χ^2	Probability	H_o
				Individual				Combined					
Plant code	Colour category	Plant code	Colour category	1	2	3	4	1+2 (green)	3+4 (purple)				
G3	2	P8	4	10	92	0	0	102	0	1:0	0.00	1.000	accept
G5	2	P8	4	5	65	0	0	70	0	1:0	0.00	1.000	accept
G4	2	P8	4	2	18	0	0	20	0	1:0	0.00	1.000	accept
G2	2	P7	3	19	231	0	0	250	0	1:0	0.00	1.000	accept
G6	2	P7	3	0	29	0	0	29	0	1:0	0.00	1.000	accept
P2	4	G7	2	0	14	0	0	14	0	1:0	0.00	1.000	accept
P6	4	G7	2	15	149	0	0	164	0	1:0	0.00	1.000	accept
P1	4	G7	2	13	138	0	2	151	2	1:0	0.03	0.872	accept
P5	4	G8	2	0	43	28	52	43	80	1:0	52.03	0.000	reject
P3	4	G8	2	7	53	32	6	60	38	1:0	14.73	0.0001	reject

Table 9.9 The leaf colour of the female and male A. *baileyana* parents, and their F_1 offspring, and the Chi-square analysis of leaf colour, in 1996. The Chi-square analysis (χ^2) of the combined colour category data was used to test for homozygous dominant green leaf, and recessive purple leaf parents. Leaf colour was assessed on 2 October 1998.

Female (pollen recipient)		Male (pollen source)		Number of F_1 offspring in the colour category						Expected ratio for 1+2 (green) & 3+4 (purple) colour categories	χ^2	Probability	H_o
Plant code	Colour category	Plant code	Colour category	Individual				Combined					
				1	2	3	4	1+2 (green)	3+4 (purple)				
P5	4	P5	4	0	0	0	3	0	3	0:1	0	1.00	accept
		P3	4	0	4	31	77	4	108	0:1	0.14	0.71	accept
		G5	2	16	97	0	0	113	0	1:0	0	1.00	accept
		G3	2	26	76	0	0	102	0	1:0	0	1.00	accept
G2	2	G2	2	5	17	0	0	22	0	1:0	0	1.00	accept
		G5	2	60	55	0	0	115	0	1:0	0	1.00	accept
		P5	4	30	86	0	0	116	0	1:0	0	1.00	accept
		P3	4	25	86	0	0	111	0	1:0	0	1.00	accept
G5	2	G5	2	2	2	0	0	4	0	1:0	0	1.00	accept
		G3	2	21	81	0	0	102	0	1:0	0	1.00	accept
		P5	4	20	82	0	0	102	0	1:0	0	1.00	accept
		P3	4	20	74	0	0	94	0	1:0	0	1.00	accept

The transition in leaf colour in response to alternating cool / warm temperatures in plants of *A. baileyana* was similar to the response in young leaves of *Cotinus coggygria* (Oren-Shamir and Levi-Nissim 1997). Anthocyanin pigmentation was lost within a week, when *A. baileyana* plants were transferred from the cool to the warm environment. In contrast, plants that were moved from the warm (23/19°C) to cool (13/9°C) temperatures took over three weeks to develop intense pigmentation on the newest growth. This delay in expression of purple pigmentation is likely due to the slower leaf growth that occurs at the lower temperatures.

High light intensity may also be important in inducing anthocyanin pigmentation in *A. baileyana*. When cuttings taken from category 4 mother plants were grown outside, the majority were also assigned to this colour category. In contrast, the majority of plants grown in the shadehouse, which had 75% shade but similar temperatures to outside, were assessed as category 3. Similarly, cuttings taken from category 2 mother plants had pure green leaves (colour category 1) in the shadehouse, while the majority grown outside produced leaves with a small amount of purple colouring (category 2). Thus the low light in the shadehouse may have reduced anthocyanin accumulation.

Differences also occurred between the leaf colour of seedlings grown in the growth cabinets and those grown outside. The majority of seedlings grown in the shadehouse and at the various temperatures in the growth cabinets had leaves of category 1, while those grown outside were mainly category 2. Therefore, the conditions within the growth cabinets and shadehouse were more suitable for producing plants of green leaf colour than the outside conditions. There was an increase in the purple pigmentation after the seedling plants in the growth cabinets and shadehouse were moved outside, with the majority of plants re-assessed as category 2. There was also an increase in the number of category 4 plants in all environmental treatments. This increase in purple pigmentation may be due to high light intensity, as the ambient (outside) temperature during assessment was warm (21°C maximum / 12°C minimum). An increase in pigmentation was also seen in plants grown outside continuously when they were assessed in October, compared with July, probably due to higher light intensity and increased daylength later in the year. Therefore, it seems that pure purple plants are only produced in the growth cabinets at the low temperature but can occur at warmer temperatures if grown outside under suitable light conditions. The light emitted by the halide lamps used in the growth cabinets had a very low component in the UVB range, which may limit anthocyanin accumulation at the warmer temperatures. Light may affect copigmentation and result in colour modification, as explained in Chapter 8.

In contrast to plants in the growth cabinets, the majority of plants grown outside in the plantation displayed some level of purple pigmentation (colour categories 2, 3 and 4), with few plants assessed as category 1. Ninety-five of the 120 trees in the plantation were the green leaf form (categories 1 and 2) and 25 were the purple form (categories 3 and 4) during autumn, winter and spring. Only 18 plants

were assessed as the purple form in summer. The intense bright light during summer may make it difficult to distinguish between colour categories 2 and 3.

As discussed in Chapter 8, certain levels of light or temperature may favour the synthesis of delphinidin aglycone or cyanidin aglycone. The amount of cyanidin-3-glucoside (peak 4) was found to discriminate between the four colour categories better than the total anthocyanin content of the leaves. Its amount was greater in winter. Therefore, cool temperatures may induce the anthocyanin pathway which results in greater cyanidin-3-glucoside (peak 4) production. This is similar to what is known to occur in Japanese parsley (*Oenanthe stolonifera*) where cyanidin is induced at low temperatures (Hasegawa et al. 2001). Therefore, in winter, the driving force of the anthocyanin and flavonoid pathway seems to be towards cyanidin. In contrast, high light intensities during summer may result in greater delphinidin-3-glucoside (peak 2) production.

9.4.2 Inheritance of purple leaf colour

The inheritance data indicated that the green leaf colour of *A. baileyana* is a dominant trait and the purple colour is recessive, as most of the progeny were green in colour. This is in contrast to purple basil (*Ocimum basilicum*), where anthocyanin expression is dominant and the green trait is recessive (Phippen and Simon 2000). The inheritance data generally supported the concept of mainly homozgous green leaf parents, as the offspring were mainly green in colour. The exception was for the two 1995 crosses (P5 x G8 and P3 x G8) which produced green and purple offspring. The G8 pollen source (green leaf male parent) may in fact be heterozygous. For a one gene system, the number of offspring for these crosses involving heterozygous dominant green parent (G8) and homozygous recessive purple parent (P5 or P3) would be 50% purple and 50% green. As this was not the outcome and the number of green and purple offspring between the P5 x G8 and P3 x G8 crosses were different, a number of alleles and/or two or more genes may be involved. Thus, the genetics of leaf colour of *A. baileyana* may be complex.

While dominance appears to be complete, the continuum in leaf colour suggests that colour intensity of *A. baileyana* is most likely a quantitative trait. A problem with the interpretation of the inheritance data was the grouping of colour categories 1 and 2 together to represent the green leaf form, and the grouping of colour categories 3 and 4 together to represent the purple leaf form. However, previous data from the plantation trees clearly separated the forms into these colour categories during assessment in winter. Precise measurements of the leaf colour such as determination of the anthocyanin content may be more appropriate than the grouping of colour into four categories.

Quantitative traits may be controlled by a large number of unlinked genes each with a small effect on

the phenotype (polygenic model; Allard 1960, Lamb 2000). Alternatively, these traits could be controlled by a few loci with relatively large effects (oligogenic model, Allard 1960), with environmental variation masking any discrete phenotypes. Polygenes and oligogenes can mutate and recombine and may show linkage, gene interactions and dominance or additive action (Lamb 2000). Additive action is probably not occurring for *A. baileyana* as the F_1 offspring are not intermediate in colour between the two parental types. Elucidation of the number of genes requires further investigations, by initially creating F_2 and back cross populations. Owing to environmental variation, quantitative traits do not usually fall into classes that precisely reflect the genotype (Ayala and Kiger 1984). This often makes it difficult to determine the number of genes affecting quantitative traits (Ayala and Kiger 1984, Lamb 2000). Moffett and Nixon (1958) looked at inheritance of four leaf characters, not including anthocyanins, of *A. decurrens* and *A. mearnsii*. They suggested that these leaf characters were polygenic in inheritance.

The purple leaf form probably developed from a mutation which occurred during cultivation, as it does not seem to occur in the endemic or weed populations and has been observed only since the 1970s. This mutation appears to affect only the leaf, stem and flower pedicle colour and not influence growth and time to flowering, breeding systems and pod set (refer to Chapter 4). The absence of purple leaf plants in weed populations may partly be due to the relatively short period of cultivation of the variety (30 years). In addition, the recessive nature of the purple leaf form may also explain why these plants are not present in weed populations. If purple plants were encroaching into native bush, crosses between them and the existing green leaf *A. baileyana* weeds would most likely result in green leaf offspring.

The purple leaf form is likely to be due to a 'loss of function' mutation. There may have been more than one mutation resulting in different purple forms, occurring in the structural or regulatory genes or in the promotor. For example, flavonoid 3'-hydroxylase is regulated only in the red form of *Perilla frutescens* (Kitada et al. 2001). Phippen and Simon (2000) hypothesised that a blockage occurs at flavonoid 3'-hydroxylase for green leaf basil. They suggested that if all the structural genes were intact, without the functioning regulatory genes, anthocyanin production will not occur. Another controlling enzyme was found to be dihydroflavonol reductase for mature green leaf ivy (*Hedera helix*) (Murray and Hackett 1991). In white grapes, the UFGT (UDP glucose-flavonoid 3-*o*-glucosyl transferase) gene was present but not expressed (Boss et al. 1996b). This lack of expression could result from mutation of this structural gene or mutation of a separate regulatory gene controlling its expression (Boss et al. 1996b). Promotor mutations can affect the way gene transcription is regulated (Winter et al. 1998), and perhaps result in a more efficient promotor and thus more transcription of the structural genes. Another possibility is that the wild type gene(s), in fact, determine the synthesis of a

repressor regulatory protein, which is missing or inactive in the mutant purple form. The binding of the repressor to the operator gene could prevent or limit the transcription of the structural genes. Therefore, a purple mutant could induce anthocyanin production by unblocking the pathway. Peach (*Prunus persica*) (Chaparro et al. 1995) and rice (*Oryza sativa*) (Reddy et al. 1995) putatively have this system of a dominant inhibitor of pigmentation.

Anthocyanin production is possibly totally blocked in the green leaf form, while anthocyanins are produced in the purple form. The green leaf plants do have the ability to produce very low amounts of purple pigmentation under certain environmental conditions. Thus, the regulatory genes for the anthocyanin pathway in the purple leaf plants may be constitutive, while in green leaf plants they are silenced (i.e. colour category 1) yet can be induced under stress (i.e. colour category 2). Thus the regulatory genes of *A. baileyana* could control the intensity of the purple leaf colour, resulting in plants grown under different conditions being allocated to different colour categories. There may be a partial block in the flavonoid and anthocyanin pathway but a stress-induced regulatory gene in the green form could up-regulate the pathway under cool temperatures and appropriate light conditions. This suggests that the structural genes are still present and functional. Mutations are therefore more likely to be the result of a change in a regulatory gene(s) as opposed to a structural gene. Regulatory genes can also alter the pattern of pigmentation across a leaf (Martin and Gerats 1993), as leaves of colour categories 2 and 3 have some cells pigmented while others are green.

As stated in Chapter 8, the enzymes that control the formation of delphinidin or cyanidin aglycones are flavonoid 3',5'-hydroxylase and flavonoid 3'-hydroxylase (Fig. 2.1, Chapter 2). The low production of anthocyanins in the green leaves may be the result of silencing of the flavonoid hydroxylase group of enzymes. In contrast, there may be an induction of the hydroxylase enzymes and an accumulation of anthocyanins in response to temperature and light stress by the purple leaves. The control of the anthocyanin pathway by a regulatory gene need not be at one specific point in the pathway but could affect all of these hydroxylase enzymes.

To study the transcriptional control of gene expression in the flavonoid and anthocyanin pathways, the structural genes of the green and purple leaf forms could be compared using real time PCR, reverse transcription PCR and Northern blot analysis. Fragments of cDNA fragments of genes from the flavonoid pathway could be isolated using the polymerase chain reaction technique and then used as probes for determining the expression of the flavonoid pathways genes of *A. baileyana*. An increase in gene expression of the phenylpropanoid pathway prior to anthocyanin production in green leaves when compared to purple leaves would be a strong indicator as to the potential control site(s). It would also be interesting to compare activity of the structural genes between colour categories 1, 2, 3 and 4 to see if the green form is down regulated. Alternatively, enzyme activity could be compared between the

forms using Western blot analysis.

The function of anthocyanins in the leaves of *A. baileyana* is unknown (see Chapter 2). It may confer biotic or abiotic stress tolerance, especially in young leaves that are susceptible to photoinhibition of the photosynthetic pigments ('photoprotection'), and cold tolerance during the winter months ('osmoregulation') (Chalker-Scott 2002, reviewed in Lee and Gould 2002, Steyn et al. 2002). It is, of course, possible that the accumulation of anthocyanins does not have any function, and that the purple form does not have any advantage or disadvantage over the typical green form. This will be discussed further in the general discussion (Chapter 10).

The results of this study will enable horticulturists to take advantage of the diversity in *A. baileyana* foliage colour. Appropriate leaf colour forms can be crossed to produce progeny of a desired colour, and these can be simply segregated into four colour categories using the developed method. Cuttings are the preferred method of propagation as the colour of the progeny is known. Plants grown from seed will not reliably produce purple colour forms due to the dominance of the green colour. The likelihood of purple seedlings can be increased, however, by collecting the seed from colour category 4 trees located in an exclusively *A. baileyana* variety *purpurea* plantation. To produce foliage of an intense and uniform purple colour, plants should be grown at cool temperatures.

Chapter Ten

General Discussion

Acacia baileyana has been cultivated since the late 1800s. Its prolific flowering, precocity, fast growth, attractive form and flowers, and variations in foliage colours are attributes that can be exploited for cut flowers, cut foliage and pot plants. The typical green leaf form is popular as a garden plant, but unfortunately it has escaped from cultivation and invaded natural ecosystems to become an environmental weed. In contrast, the variety *purpurea*, which has only been cultivated since the 1970s, has not yet been recorded as a weed. The aim of this research was to develop an understanding of the fertility and leaf colour in *A. baileyana*, for both its future commercial development and management of invasiveness.

A number of environmental factors influence flowering. Many acacias flower in response to rainfall (Kenrick 2003, Stone et al. 2003 and references within) but the strict flowering season of most species suggests that flowering is related to other environmental variables such as temperature (Davies 1976, Sedgley 1989). In fact, it was found in this study that *A. baileyana* has obligatory temperature requirements for both floral initiation and flowering. Warm temperatures (above 18°C maximum / 13°C minimum) were required for floral bud initiation and cool temperatures (below 16°C maximum / 9°C minimum) were required for flowering. High temperatures may be an important controlling influence, by blocking flower formation and development, and low temperature may be the passive condition, permitting flowering (King and Gocal 1999). Therefore, *A. baileyana* must be grown in regions with both warm and cool seasons for flowering to occur. This is similar to *A. pycnantha*, in which plants require cool temperatures (less than 19°C) for flowering (Sedgley 1985). A few floral buds can be produced all year around for *A. pycnantha* but anthesis occurs only once a year in winter (Buttrose et al. 1981). Consequently, resources used for floral bud formation at other times are wasted. Because flower buds are produced only annually in *A. baileyana*, it is relatively efficient in managing its resources.

Light was also found to affect flower production of *A. baileyana* in this study. Low light reduced the number of plants with flowers, with only half of the plants grown in the shadehouse (75% of light excluded) flowering when compared with plants grown outside. Similarly, no plants of *A. pycnantha* flowered under similar shadehouse conditions, as floral development was inhibited at an early stage (Sedgley 1985). Therefore, to achieve good flowering, *A. baileyana* plants should not be grown in shady locations. Sedgley (1985) suggested that the sensitivity of the flowering process to changes in environmental conditions might have contributed to the diversity and large number of *Acacia* species found in Australia.

An understanding of the influence of environmental conditions on flowering is crucial for nursery production of indoor flowering pot plants (King and Gocal 1999). Advancing or delaying the flowering period are valuable tools for targeting key markets. Many plants used as pot plants flower in response to photoperiodic conditions (Larson 1980), and flowering time can be manipulated by maintaining long-day or short-day conditions in the production environment (Larson 1980). Such a strategy has been used with chrysanthemums to extend flowering time (Crater 1992). Photoperiod control of flowering most likely cannot be used to manipulate the flowering period of *Acacia*, as photoperiod does not appear to affect flowering (Sedgley 1985). However, in this study, peak flowering of *A. baileyana* was advanced by four months by exposing plants to warm temperatures to initiate buds and then to cool temperatures to initiate flower opening. This treatment also doubled the number of plants that flowered. Therefore, this is an alternative approach to manipulate flowering.

This new knowledge of the temperatures required to induce flowering in *A. baileyana* has the potential to be applied in commercial flower operations that have access to controlled environment rooms. For example, for indoor flowering pot plants for Mother's Day in May, plants could be kept outside over summer if the average temperature is above 18°C maximum and 13°C minimum. Once the plants reach the medium bud stage in January or February, they should be placed in an environment at 16°C maximum and 9°C minimum or lower to advance flowering. Even though the potential to manipulate flowering time of *A. baileyana* exists, the reality is that it would be costly. Shading of a glasshouse may be sufficient to reduce temperature, thereby avoiding costly cooling, but then lower photosynthetic input may result in poorer flower production, as shown in this study. The production of flowering pot plants derived from cuttings would take over a year under natural conditions, while the manipulation of flowering time would require heating and cooling. In comparison, a chrysanthemum pot plant takes about 15 weeks to produce, and can be easily manipulated for out-of-season flowering as plants flower under short days (Crater 1992). Supplementary lighting is used to keep chrysanthemum plants vegetative when natural day lengths are short, while shading with black cloth is used to induce flowering when day lengths are too long (Crater 1992). Therefore, photoperiodic responsive plants would be easier and cheaper to manipulate then plants that flower in response to temperature.

Compact plants are produced for the pot plant market by pruning and by the application of growth regulators, which can also enhance flowering (Sedgley and Parletta 1993). The growth retardant paclobutrazol has been shown to reduce the height of *Acacia* species (Parletta and Sedgley 1998). The prostrate form of *A. baileyana* (Elliot and Jones 1984, Whibley and Symon 1992) may be suitable as a indoor flowering plant and would reduce the need for chemical retardant applications. The flowering pot plant must also have a shelf life in excess of 14 days under indoor home or office conditions (Lamont 1987), which is the typical flowering duration of *A. baileyana* (Boden 1969). *Chamelaucium uncinatum* (Geraldton wax), an Australian native plant that is a very popular cut flower, can remain in flower in a pot for up to six weeks in simulated home conditions (Lamont 1987). Future research could

aim to produce good quality flowering pot plants of *A. baileyana* at different times of the year, by controlling the time of transfer from warm to cool conditions, and by mother-plant selection and plant-shape treatments, if production is cost effective.

Acacia baileyana was nominated as a promising cut flower species (Horlock et al. 2000), but its development as a cut flower, particularly for the export market, is limited because of its relatively short vase life. Post-harvest solutions are essential for acacia stems, as they have a short vase-life due to presence of pectins and gums in the vessels (Williamson and Milburn 1989, cited in Sedgley and Parletta 1993). In France and Italy, to achieve a vase life of seven days, *Acacia* cut flowers are harvested just prior to flower opening and placed into high temperature and high humidity rooms to force the flowers into full bloom, whilst being maintained in a post-harvest solution, as cool temperatures are not required for flower opening (Sedgley 1989). Given their location, growers of *A. baileyana* in France and Italy are well placed to supply the European market, and a seven day vase life may be sufficient. Work by Horlock et al. (2000) in Australia has extended the post-harvest life of *A. baileyana* from two to three days to between seven and ten days, using a similar post-harvest protocol to the European method. Even though this is a substantial improvement in vase-life, it would need to be extended in order to maximise the export market potential. It may be possible to select for plants with long vase-life and then propagate these selections vegetatively. This has been done with *Chamelacium uncinatum,* as vase-life ranges from about 5 to 20 days (Manning 1995, cited in Manning et al. 1996). Adequate flowering is also required for good quality cut flowers. As shown in this study, temperature and light during bud formation and anthesis will influence the production of flowers.

At present, *A. baileyana* is most suited as a garden plant and for cut foliage. Its appeal as cut foliage is due to its attractive bipinnate leaves and range of leaf colours from green through to purple. The uniqueness of variety *purpurea* provides diversity in colour within a foliage market that is predominantly green. Variety in plant colour is essential to satisfy the demands of the commercial horticulture industry, and manipulation of the colour relies on understanding the chemistry, inheritance and environmental control of the trait.

To characterise the type of anthocyanins in leaf tissue of *A. baileyana*, efficient extraction, separation, purification and identification techniques were developed in this study. Rapid purification and identification of anthocyanins was achieved using high voltage paper electrophoresis with bisulphite buffers, in conjunction with mass spectrometry. High voltage paper electrophoresis removed phenolic compounds that interfered with mass spectrometry. These methods were used successfully to identify the main anthocyanins in the leaves of *A. baileyana* and *A. glaucoptera,* and in the flowers of *Banksia coccinea* and *B. menziesii. Acacia* leaves contained cyanidin and delphinidin derivatives, while the *Banksia* flowers contained cyanidin and paeonidin based pigments. Cyanidin-3-glucoside and

delphinidin-3-glucoside were the two main pigments of *A. baileyana* leaves. The concentration of cyanidin-3-glucoside was the prime determinant of leaf colour.

The methods of extraction, separation, purification and identification of anthocyanins developed in this study may be suitable for other plant material (leaves and flowers) and may assist in the commercial development of floricultural crops. The methods could also be used to characterise the anthocyanin pigments of new colour cultivars developed under Plant Breeders Rights. Currently, Royal Horticultural Society Colour Charts are used for variety descriptions in the Plant Varieties Journal. The developed methods would provide a more definitive identification of a coloured cultivar and avoid the problems associated with subjective assessment using colour charts.

The variety *purpurea* has been cultivated for only about 30 years and does not occur in the endemic populations in New South Wales, Australia. The purple form is therefore likely to be a new mutation, in which the flavonoid pathway may be up-regulated, producing a greater concentration of anthocyanins. The green form appears to produce trace amounts of anthocyanins, although these are visible only under certain environmental conditions. The presence of anthocyanins in the green form needs to be confirmed. The regulation of anthocyanin production of *A. baileyana* must be complex as the main production of anthocyanins occurs in the cool months and only in juvenile foliage.

The production of concentrated anthocyanins in the purple leaf form may not confer any benefit in performance over the typical green leaf form, but may be a by-product from the production of other flavonoids. Holton and Cornish (1995) stated that mutations in genes of anthocyanin biosynthesis do not usually affect growth and development. Therefore, increased pigmentation may not necessarily be an advantage or disadvantage. However, anthocyanins could have some photoprotective, antioxidant or osmoregulatory properties to reduce plant stress from changes in environmental conditions (Chalker-Scott 2002, Gould et al 2002, Lee and Gould 2002). Anthocyanins in juvenile, expanding leaves may occur for photoprotection, as the leaves are more susceptible to photoinhibition and photobleaching of photosynthetic pigments than mature leaves (Steyn et al. 2002), or for cold tolerance (Chalker-Scott 2002). Nozzolillo (1973) noted that *A. baileyana* contained anthocyanins in the epidermis of the hypocotyl stem and the cotyledon leaves. A photoprotection role of anthocyanins is feasible if accumulation also occurs in the epidermis of juvenile leaves. Leaves containing anthocyanins could also have better photosynthetic performance under strong light, as has been reported for red versus green leaves in several plant species (reviewed in Lee and Gould 2002). However, if photosynthetic performance was enhanced in variety *purpurea* one might expect to see increased growth or reproductive output, but this was not the case. In fact, it is the typical green form which is a weed. The lack of response may be due to plants growing under ideal conditions. Perhaps under more stressful growing conditions than those experienced in this study, a difference between the two forms would be noticed. More informed speculation on anthocyanin function in *A. baileyana*, will require comparisons

between the green and purple leaf forms in the areas of anthocyanin location in the leaf tissue, photosynthetic performance under high light and cool temperatures, and osmoregulation, by measuring plant water potential and stomatal conductance under high / low water stress conditions.

Another function of anthocyanins of *A. baileyana* may be to attract pollinators, such as birds (Faegri and van der Pijl 1979, or deter predators. If birds were attracted to the purple leaf form, due to the purple pedicels of the inflorescence, purple foliage and the extra-floral nectaries, one might expect a higher pod set and/or greater proportion of the seeds to be outcrossed. However, the purple form of *A. baileyana* in this study did not produce higher pod set compared to the green form. This may be because red-attractant pollinators, such as birds, are not important pollinators of *A. baileyana*, or were not abundant in the study area.

The green foliage trait was found to be dominant over purple leaf colour, but the intensity of leaf colour of *A. baileyana* seems to be a quantitative trait that may be controlled by many genes. Determining the number of genes involved in anthocyanin synthesis needs further research. Due to the recessive nature of the purple form, seeds from *A. baileyana* variety *purpurea* may not necessarily be true to type. Therefore, to obtain purple leaf offspring, plants should be derived from cuttings, or seeds collected from a variety *purpurea* plantation.

Anthocyanin expression in the young leaves of *A. baileyana* was strongly induced at low temperatures and at high light intensity. In addition, the anthocyanin composition was different between the seasons, with the content of cyanidin-3-glucoside higher in summer leaf samples, while delphinidin-3-glucoside was higher in winter samples. Therefore, the different anthocyanin pigments may have a different response to change in light and/or temperature. Intense purple leaves from variety *purpurea* were produced only at temperatures below a mean of 21°C maximum and 12°C minimum when plants were grown outside, or at a constant 13°C day and 9°C night temperatures when plants were housed in growth cabinets. These findings indicate that to obtain intense purple foliage from variety *purpurea*, plants should be grown at cool temperatures.

The development of novel forms of *A. baileyana* by plant breeding is reliant on selection of superior plants, the development of hybridisation techniques and reliable propagation methods. For example, the plant selection of 'Scarlet Blaze', a form of *A. leprosa*, has resulted in a novel form with red flowers (Royal Botanic Gardens, Melbourne, 1998). Hybridisation between selected plants of *A. baileyana* or with other acacia species could result in superior plants, which are suitable as cut flowers, cut foliage or pot plants. Knowledge of the reproductive biology and the flowering behaviour of a species is essential before successful crossing methods can be developed. It was found in this study that both the green and purple leaf forms have a similar temperature requirement for flowering, and a similar reproductive system: both are protogynous, outcrossing, highly self-incompatible, and flower by two years of age. A reliable cross pollination technique was developed in this study for *A. baileyana* by exploiting the

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outcrossing mechanism of the flowers. Emasculation was not necessary, as the flowers are protogynous and self-incompatible. Controlled cross pollinations producing an average pod set of 23%, which was a significant increase in pod set from self pollinations (0.36%) and open pollinations (less than 0.41%). In addition, controlled cross pollinations increased the number of seeds per pod.

Interspecific hybridisation is the most likely method to result in desirable new forms. Natural hybrids are known to occur between A. *baileyana* and other acacias (Cheel 1935, Moffett 1965b, Pedley 1987, Whibley and Symon 1992, Blood 2001), therefore controlled hybridisation must be possible. Hybrids may be superior in foliage and flower quality and ornamental value. For example, hybrid forms of A. *retinodes* and A. *dealbata* are popular sources of cut flowers in France and Italy (Horlock et al. 2000). It may be necessary to store pollen to enable cross pollinations to be performed on *Acacia* species that flower at different times of the year. It is already known that viable pollen of A. *auriculiformis*, A. *iteaphylla*, A. *karroo* and A. *mangium* can be stored successfully if it is vacuum dried and kept at −18°C (Sedgley and Harbard 1993). In addition, novel forms could be produced through mutation breeding (by x-ray or γ-irradiance) and induced autoploidy (by using colchicine) (Langton 1987).

Acacia baileyana can be propagated from seeds or by cuttings. The latter is the preferred method of propagating plants with desirable characteristics, for example purple foliage, as the clonal progeny is identical to the mother plant. In addition, the time to flowering is reduced with clonal material, as the non-flowering juvenile phase is avoided. The success of cutting propagation in this study was, however, poor: an average of only 11% of cuttings formed roots. Some mother plants provided better cuttings than others, as the strike rate varied between 1 to 29% depending on the plant source. It is known that root formation in cuttings is dependent on the physiological and environmental condition of the plant, and these are influenced by the time of year material is taken. Schwarz et al. (1999) found that cuttings of A. *baileyana* collected in autumn had a three-fold greater strike-rate than material collected in spring. The poor strike rate in the current study may be due to the cuttings being taken in summer. In order to improve the strike rate, cuttings should be taken in the cooler month of autumn from plants with juvenile foliage. Further work is required in the selection and optimum maintenance of mother plants for vegetative propagation.

Maximum flower number for two-year old A. *baileyana* plants in this study was over 300,000, which resulted in more than 8,000 seeds. Estimated flower numbers for ten mature trees in a weed population ranged from 1.25 to 13.2 million (average 5.5 million), with a maximum seed production of 36,387. Both young and mature trees are reproductively efficient. Pod set was low (average 0 to 0.41%) but comparable with other acacias (Milton and Hall 1981, Tybirk 1989, Moncur et al. 1991, Tybirk 1993, Grant et al. 1994, reviewed in Kenrick 2003). This demonstrates that the efficient reproductive system in A. *baileyana* is due mainly to the very high number of flowers produced, and not due to effective cross pollination strategies. This is exacerbated in the experimental site as temperatures are close to

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ideal for floral initiation and development, as determined in the study. This very high seed production is likely to contribute to its status as a weed in the Adelaide Hills. Future research should investigate the success of seed dispersal, soil seed storage, seed germination and plant survival, to determine the extent of the weed potential.

Total flower numbers were estimated using digitised photographic images. This is a new method that has potential to be used on other flowering and fruiting trees to estimate yield. Comparisons with total destructive sampling would be needed to validate the method on other plants. The ease of using this method may be improved through the use of digital cameras and advances in image software.

Both the green and purple leaf forms of *A. baileyana* have the potential to become weeds, due to their fast growth, early flowering age, and high seed production. These attributes are common in other weeds. For example, *Acacia nilotica* ssp. *indica*, an aggressive weed in Queensland, produces numerous long-lived seeds, which are dispersed over long distances, and is fast growing (Carter 1994). *Pittosporum undulatum*, another environmental weed, produces seeds at a young age and is also fast growing (Gleadow and Ashton 1981). The purple form of *A. baileyana* has not been observed as a weed. This may be due to its relatively short period in cultivation, and / or to the recessive nature of the purple trait.

As there was no difference in reproductive output between the purple and green leaf forms, all forms of *A. baileyana*, including hybrids, should be regarded as potential environmental weeds. If *A. baileyana* is to be promoted for horticulture, strategies must be in place to prevent further invasion of plants into native ecosystems. For example, such a strategy has been proposed for the Australian olive industry, where the olive plant is a major environmental weed but the fruit is an important commercial product (Spenneman and Allen 2000). Wide buffer areas between plantings and native habitat, and an undertaking by industry to assume the management of abandoned groves are important components of this strategy. Plantations of all forms of *A. baileyana* and their hybrids must be managed carefully to prevent their spread into native bush. Ideally, plantations should not be established near native bush. Regular harvesting of the cut stems will also limit seed escape. The cut flower, foliage and pot plant products are not considered to be vectors for the dispersal of seed, unless seed pods are attached.

Great potential exists for the commercial development of *A. baileyana* as a pot plant and for its flowers, but in particular for its foliage. This study has provided information critical for the production of improved forms of *A. baileyana*. Given the potential for *A. baileyana* to become a weed, any commercialisation should include a strategy to prevent the spread of plants into the surrounding environment.

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Discriminant analysis method and HPLC-determined anthocyanin content data from Chapter 8

Introduction

Details of the discriminant analysis method used in Chapter 8 are described below. Only the discriminant analysis details of the sum of all peak areas and the area of peak 4 are presented. In particular, more details are shown for the sum of all peaks for the plants grown from cuttings, for clarity of the method.

Plants grown from cuttings

Sum of all peak areas

For the sum of all the peak areas for leaf samples grown from cuttings, at three temperatures, only one discriminant function was found to be necessary. The discriminant function is:

$$LD_1 = -2.96 + (1.20 \times 10^{-6}) \text{ (peak 1 + peak 2 +........+ peak 16)}$$

The above equation was used to obtain the discriminant scores for each plant sample (Appendix 1, Table 2). Mean discriminant scores were then obtained for each visual colour category (Appendix 1, Table 1). To determine the allocation of a plant sample to a colour category, the distance of the individual leaf sample discriminant score from the mean discriminant scores for each of the colour categories was calculated. The leaf was allocated to the category with the smallest distance. For example, the distance of plant one from the mean colour category 1 is: $D_k^2 = (4.61 + 2.73)^2 = 53.83$. Similarly, the distance from colour category 2 is 36.70, colour category 3 is 15.81 and colour category 4 is 1.14. Therefore, the first plant sample is allocated to colour category 4, as this is the smallest distance. From Appendix 1, Table 2 it can be seen that a total of 6 plants have been misclassified, giving an 85% agreement between methods. All the misclassifications were between categories 2 and 3, and between 3 and 4.

Table 1 Mean discriminant scores for the four colour categories, for the sum of all peak areas.

	Colour category			
	1	2	3	4
Mean LD$_1$ score	-2.73	-1.45	0.63	3.54

Table 2 Discriminant scores for the sum of all peak areas and peak 4 area, to predict the leaf colour categories, for plants grown from cuttings, at three different temperatures. Misclassified categories are boxed.

Plant sample	Allocated colour category	Temperature °C	Sum of all peak areas		Peak 4 area	
			Discriminant score (LD$_1$)	Predicted colour category	Discriminant score (LD$_1$)	Predicted colour category
1	4	13/9	4.61	4	4.76	4
2	4	13/9	2.81	4	2.65	4
3	4	13/9	3.38	4	3.39	4
4	4	13/9	2.91	4	3.26	4
5	4	13/9	2.35	4	2.45	4
6	4	13/9	1.38	[3]	1.49	[3]
7	4	13/9	4.68	4	4.91	4
8	4	13/9	6.22	4	6.29	4
9	3	13/9	0.69	3	0.49	3
10	3	13/9	1.75	3	1.68	3
11	3	13/9	1.44	3	1.46	3
12	3	13/9	1.77	3	1.80	3
13	3	13/9	0.42	3	0.39	3
14	3	13/9	1.61	3	1.60	3
15	3	13/9	1.32	3	1.29	3
16	3	13/9	2.81	[4]	2.88	[4]
17	1	13/9	-2.73	1	-2.87	1
18	1	13/9	-2.68	1	-2.87	1
19	1	13/9	-2.67	1	-2.87	1
20	3	18/13	0.23	3	0.31	3
21	3	18/13	0.85	3	1.03	3
22	3	18/13	-0.75	[2]	-0.84	[2]
23	3	18/13	-1.11	[2]	-1.18	[2]
24	3	18/13	0.71	3	0.90	3
25	1	18/13	-2.78	1	-2.87	1
26	1	18/13	-2.73	1	-2.87	1
27	1	18/13	-2.75	1	-2.87	1
28	2	23/19	-1.17	2	-1.14	2
29	2	23/19	-0.68	2	-0.81	2
30	2	23/19	-1.85	2	-1.81	2
31	2	23/19	-1.44	2	-1.46	2
32	2	23/19	-1.53	2	-1.58	2
33	2	23/19	-1.07	2	-1.05	2
34	2	23/19	-1.99	2	-1.93	2
35	2	23/19	-1.83	2	-1.81	2
36	3	23/19	-0.62	[2]	-0.38	3
37	3	23/19	-0.17	3	-0.06	3
38	3	23/19	-0.81	[2]	-0.69	[2]
39	1	23/19	-2.73	1	-2.87	1
40	1	23/19	-2.74	1	-2.87	1
41	1	23/19	-2.73	1	-2.87	1

Peak 4 area

Only one discriminant function was necessary for the anthocyanin content of peak 4. The discriminant function for peak 4 is:

$LD_1 = -2.87 + (1.66 \times 10^{-6})peak4.$

This equation was used to obtain the peak 4 discriminant scores for each plant sample (Appendix 1, Table 2). Mean discriminant scores were then obtained for each colour category (Appendix 1, Table 3). Leaves were classified into the four colour categories, on the basis of the minimum distance of their discriminant score to the mean discriminant score for each colour category. An 88% agreement between methods was obtained, with five plants being misclassified (Appendix 1, Table 2).

Table 3 Mean discriminant scores for the four colour categories, for peak 4 area.

	Colour category			
	1	2	3	4
Mean LD_1 score	-2.87	-1.45	0.67	3.65

Plantation

Sum of all peak areas

For the total sum of all peak areas, only the first discriminant function was necessary to discriminate between the colour categories. The discriminant function is given by the equation below and was used to obtain discriminant scores (Appendix 1, Table 4).

$$LD_1 = -4.08 + (5.63 \times 10^{-7})(peak\ 1 + peak\ 2 + \ldots\ldots + peak\ 16)$$

Plants were classified into colour categories on the basis of the minimum distance of their discriminant score to the mean discriminant score for each colour category (Appendix 1, Table 5). A 75% agreement occurred between the two methods, with five plants misclassified (Appendix 1, Table 4).

Peak 4 area

The discriminant analysis function for peak 4 area is:

$LD_1 = -4.28 + (1.22 \times 10^{-6})peak4.$

Plants were classified into colour categories on the basis of the minimum distance of their discriminant score (Appendix 1, Table 4) to the mean discriminant score (Appendix 1, Table 6) for each colour

category. Discriminant analysis of peak 4 data classified all leaves into the correct categories, giving 100% agreement between methods (Appendix 1, Table 4).

Table 4 First discriminant score for the sum of all peak areas and peak 4 area, to predict the leaf colour categories, for leaf samples taken in summer and winter from the plantation. Misclassified categories are boxed.

Plant	Season	Allocated colour category	Sum of all peak areas		Peak 4 area	
			Discriminant score (LD_1)	Predicted colour category	Discriminant score (LD_1)	Predicted colour category
1	summer	3	3.88	[4]	-0.14	3
2	summer	3	-0.49	3	-0.71	3
3	summer	3	0.21	3	-1.14	3
4	summer	3	1.58	3	-1.64	3
5	summer	3	2.27	[4]	-0.32	3
1	winter	4	3.65	4	7.71	4
2	winter	4	1.01	[3]	2.91	4
3	winter	4	1.35	[3]	3.50	4
4	winter	4	1.47	[3]	3.50	4
5	winter	4	2.65	4	5.04	4
6	summer	2	-3.57	2	-3.81	2
7	summer	2	-3.32	2	-3.79	2
8	summer	2	-3.42	2	-3.73	2
9	summer	2	-3.34	2	-3.53	2
10	summer	2	-3.43	2	-3.69	2
6	winter	2	-3.74	2	-3.94	2
7	winter	2	-3.77	2	-4.00	2
8	winter	2	-3.47	2	-3.53	2
9	winter	2	-3.33	2	-3.39	2
10	winter	2	-3.76	2	-4.02	2

Table 5 Mean discriminant scores for the three colour categories, for the sum of all peak areas.

	Colour category		
	2	3	4
Mean LD_1 score	-3.52	1.49	2.03

Table 6 Mean discriminant scores for the three colour categories, for peak 4 area.

	Colour category		
	2	3	4
Mean LD_1 score	-3.74	-0.79	4.53

Table 7 HPLC-determined anthocyanin content for the 16 peaks and the sum of all peak areas, for 41 leaf samples taken from plants produced from cuttings. The plants were grown at three different temperatures in growth cabinets. The colour categories of the plant samples are also presented.

Plant no.	Genotype	Colour category	Temp. (°C) (max./min.)	Sum of all peak areas	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
									Anthocyanin content under peaks (scaled mAU)										
1	1	4	13/9	63.3	0.0	4.6	1.0	45.9	6.5	0.0	0.3	0.3	0.3	0.5	0.9	1.7	0.0	1.3	0.0	0.0
2	1	4	13/9	48.0	0.0	4.5	0.9	33.2	4.6	0.2	0.2	0.2	0.2	0.4	0.8	1.5	0.0	1.1	0.0	0.2
3	1	4	13/9	52.9	0.0	4.4	0.8	37.6	5.2	0.0	0.3	0.2	0.2	0.5	0.9	1.6	0.0	1.2	0.0	0.0
4	1	4	13/9	49.0	0.0	2.8	0.6	36.9	4.9	0.0	0.0	0.0	0.2	0.3	0.8	1.4	0.0	1.1	0.0	0.0
5	1	4	13/9	44.3	0.0	2.3	0.6	32.0	5.1	0.0	0.0	0.2	0.2	0.4	0.8	1.6	0.0	1.1	0.0	0.0
6	1	4	13/9	36.2	0.0	1.8	0.4	26.3	4.0	0.0	0.0	0.0	0.0	0.3	0.7	1.5	0.0	1.2	0.0	0.0
7	1	4	13/9	63.9	0.0	4.4	0.9	46.8	6.5	0.0	0.2	0.3	0.3	0.4	0.8	1.7	0.0	1.2	0.2	0.2
8	1	4	13/9	76.5	0.0	6.7	1.1	55.1	6.9	0.4	0.3	0.3	0.3	0.6	1.3	1.7	0.0	1.2	0.3	0.3
9	1	3	13/9	30.3	0.0	2.9	0.5	20.2	2.1	0.0	0.0	0.0	0.0	0.3	0.6	1.4	0.2	1.2	0.4	0.5
10	1	3	13/9	39.3	0.0	2.9	0.4	27.4	3.2	0.0	0.0	0.0	0.2	0.4	0.7	1.5	0.2	1.1	0.5	0.8
11	1	3	13/9	36.6	0.0	2.3	0.4	26.0	3.2	0.0	0.0	0.0	0.2	0.3	0.6	1.4	0.0	1.1	0.4	0.7
12	1	3	13/9	39.5	0.0	2.8	0.6	28.1	3.4	0.0	0.0	0.0	0.2	0.4	0.7	1.6	0.0	1.1	0.3	0.3
13	1	3	13/9	28.1	0.0	1.4	0.3	19.6	2.9	0.0	0.0	0.0	0.2	0.2	0.4	1.3	0.0	0.9	0.3	0.6
14	1	3	13/9	38.2	0.0	3.3	0.5	26.9	3.1	0.0	0.0	0.0	0.0	0.3	0.6	1.4	0.0	1.0	0.5	0.6
15	1	3	13/9	35.7	0.0	2.4	0.5	25.0	3.0	0.0	0.0	0.0	0.0	0.4	0.7	1.5	0.0	1.2	0.3	0.7
16	1	3	13/9	48.2	0.0	2.2	0.5	34.6	4.7	0.0	0.0	0.0	0.2	0.4	0.8	1.5	0.3	1.2	0.8	1.0
17	6	1	13/9	1.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.3	0.0	0.6	0.0	0.0
18	6	1	13/9	2.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.6	0.0	0.7	0.0	0.0
19	6	1	13/9	2.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.7	0.0	0.8	0.0	0.0

Table 7 cont. HPLC-determined anthocyanin content for the 16 peaks and the sum of all peak areas, for 41 leaf samples taken from plants produced from cuttings. The plants were grown at three different temperatures in growth cabinets. The colour categories of the plant samples are also presented.

Plant no.	Genotype	Colour category	Temp. (°C) (max./min.)	Sum of all peak areas	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
20	1	3	18/13	26.7	0.0	1.6	0.3	19.1	2.4	0.0	0.0	0.0	0.0	0.2	0.4	1.3	0.0	1.1	0.0	0.3
21	1	3	18/13	31.9	0.0	1.8	0.3	23.5	2.8	0.0	0.0	0.0	0.2	0.2	0.4	1.4	0.0	1.1	0.0	0.2
22	1	3	18/13	18.6	0.0	1.2	0.0	12.2	1.4	0.0	0.0	0.0	0.3	0.0	0.3	1.3	0.0	1.0	0.3	0.6
23	1	3	18/13	15.5	0.0	1.1	0.0	10.2	1.2	0.0	0.0	0.0	0.0	0.0	0.0	1.2	0.0	0.9	0.3	0.6
24	1	3	18/13	30.6	0.0	1.9	0.3	22.7	2.5	0.0	0.0	0.0	0.2	0.0	0.4	1.2	0.0	1.1	0.0	0.3
25	6	1	18/13	1.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.1	0.0	0.4	0.0	0.0
26	6	1	18/13	1.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.3	0.0	0.6	0.0	0.0
27	6	1	18/13	1.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.2	0.0	0.6	0.0	0.0
28	1	3	23/19	19.6	0.0	1.4	0.0	15.0	1.1	0.0	0.0	0.0	0.0	0.0	0.0	1.2	0.0	0.9	0.0	0.0
29	1	3	23/19	23.1	0.0	2.0	0.2	16.9	1.4	0.0	0.0	0.0	0.2	0.0	0.0	1.3	0.0	0.9	0.0	0.2
30	1	3	23/19	17.9	0.0	1.4	0.0	13.1	1.0	0.0	0.0	0.0	0.0	0.0	0.0	1.4	0.0	1.0	0.0	0.0
31	1	2	23/19	14.9	0.0	0.9	0.0	10.4	1.0	0.0	0.0	0.0	0.0	0.0	0.0	1.5	0.0	1.1	0.0	0.0
32	1	2	23/19	19.0	0.0	2.6	0.2	12.4	1.0	0.0	0.0	0.0	0.2	0.0	0.0	1.6	0.0	1.0	0.0	0.0
33	1	2	23/19	9.3	0.0	0.5	0.0	6.4	0.5	0.0	0.0	0.0	0.0	0.0	0.0	1.1	0.0	0.8	0.0	0.0
34	1	2	23/19	12.7	0.0	0.9	0.0	8.5	0.8	0.0	0.0	0.0	0.2	0.0	0.0	1.3	0.0	1.0	0.0	0.0
35	1	2	23/19	11.9	0.0	1.1	0.0	7.8	0.7	0.0	0.0	0.0	0.0	0.0	0.0	1.2	0.0	0.9	0.0	0.2
36	1	2	23/19	15.7	0.0	1.4	0.0	10.9	0.9	0.0	0.0	0.0	0.2	0.0	0.0	1.3	0.0	1.0	0.0	0.0
37	1	2	23/19	8.1	0.0	0.3	0.0	5.7	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.9	0.0	0.7	0.0	0.0
38	1	2	23/19	9.4	0.0	0.7	0.0	6.4	0.6	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0	0.7	0.0	0.0
39	6	1	23/19	1.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.3	0.0	0.6	0.0	0.0
40	6	1	23/19	1.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.3	0.0	0.5	0.0	0.0
41	6	1	23/19	1.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.3	0.0	0.5	0.0	0.0

Table 8 HPLC-determined anthocyanin content for the 16 peaks and the sum of all peak areas, for 20 leaf samples taken from 10 trees in winter and summer from the plantation. The colour categories of the plant samples are also presented.

Plant no.	Colour category	Season	Anthocyanin content under peaks (scaled mAU)																
			Sum of all peak areas	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	3	summer	141.3	1.5	81.7	11.1	33.9	2.6	1.0	1.3	0.9	1.2	0.4	0.7	3.0	0.2	1.6	0.2	0.0
2	3	summer	63.9	0.0	22.2	2.6	29.2	3.0	0.5	0.6	0.5	0.4	0.3	0.5	2.6	0.0	1.5	0.0	0.0
3	3	summer	76.0	0.0	37.6	3.0	25.7	2.5	0.5	0.6	0.7	0.5	0.2	0.4	2.8	0.0	1.5	0.0	0.0
4	3	summer	100.5	0.0	61.4	6.3	21.6	2.8	0.9	1.0	0.9	0.8	0.4	0.4	2.8	0.0	1.2	0.0	0.0
5	3	summer	112.7	1.0	59.9	6.0	32.4	3.7	0.8	1.1	0.7	1.0	0.4	0.7	3.1	0.2	1.7	0.0	0.0
1	4	winter	137.2	0.3	19.3	3.9	98.2	6.0	0.7	0.6	0.4	0.7	0.7	1.3	2.7	0.3	2.1	0.0	0.0
2	4	winter	90.3	0.4	8.7	2.3	58.9	9.0	0.9	0.8	0.7	0.3	1.7	2.6	2.4	0.0	1.6	0.0	0.0
3	4	winter	96.3	0.0	14.1	1.6	63.7	9.8	0.0	0.0	0.5	0.4	2.1	0.0	2.3	0.3	1.5	0.0	0.0
4	4	winter	98.5	0.0	14.2	2.2	63.7	11.7	0.0	0.0	0.4	0.7	0.5	1.2	2.5	0.0	1.4	0.0	0.0
5	4	winter	119.6	0.3	17.7	3.4	76.3	13.1	0.0	0.6	0.5	0.8	0.7	1.4	2.8	0.0	2.0	0.0	0.0
6	2	summer	8.8	0.0	1.2	0.2	3.8	0.3	0.0	0.0	0.0	0.2	0.0	0.0	2.1	0.0	1.0	0.0	0.0
7	2	summer	13.4	0.0	5.2	0.3	4.0	0.3	0.0	0.0	0.4	0.3	0.0	0.0	2.1	0.0	0.8	0.0	0.0
8	2	summer	11.7	0.0	3.4	0.4	4.5	0.4	0.0	0.0	0.0	0.0	0.0	0.0	2.0	0.0	1.0	0.0	0.0
9	2	summer	13.1	0.0	3.2	0.0	6.1	0.4	0.0	0.0	0.0	0.0	0.0	0.0	2.3	0.0	1.1	0.0	0.0
10	2	summer	11.5	0.0	2.7	0.3	4.8	0.3	0.0	0.0	0.0	0.0	0.0	0.0	2.3	0.0	1.1	0.0	0.0
6	2	winter	6.0	0.0	0.2	0.0	2.8	0.2	0.0	0.0	0.0	0.0	0.0	0.0	1.6	0.0	1.2	0.0	0.0
7	2	winter	5.4	0.0	0.2	0.0	2.2	0.3	0.0	0.0	0.0	0.0	0.0	0.0	1.8	0.0	0.9	0.0	0.0
8	2	winter	10.9	0.0	0.8	0.3	6.1	0.6	0.0	0.0	0.0	0.0	0.0	0.0	1.9	0.0	1.2	0.0	0.0
9	2	winter	13.3	0.0	1.2	0.0	7.3	0.8	0.0	0.0	0.0	0.0	0.0	0.2	2.3	0.3	1.2	0.0	0.0
10	2	Winter	5.6	0.0	0.2	0.0	2.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.1	0.0	1.2	0.0	0.0

Environmental data and 1995 cross pollination results referred to in Chapter 9

Table 1 The outside environmental conditions at the Waite Campus, The University of Adelaide, for 1997 and 1998.

Year	Month	Temperature °C				Daylength h	Mean solar radiation MJm^{-2}	Mean photosynthetic photon flux density µmolm^{-2}s^{-1} [x]
		Max.	Mean max.	Min.	Mean min.			
1997	January	37.9	28.4	9.2	17.8	14	26.2	1117
	February	39.3	31.6	12.9	19.8	13.5	23.8	1015
	March	28.0	22.3	9.5	13.3	12.5	18.3	840
	April	29.4	22.1	8.7	13.3	11	12.6	657
	May	24.4	16.8	5.3	10.5	10.5	7.3	397
	June	22.1	15.1	4.5	8.8	10	6.1	348
	July	17.6	13.7	4.4	6.8	10	7.8	448
	August	19.9	14.5	4.0	7.9	11	8.6	452
	September	22.9	17.2	8.0	10.4	11	12.5	654
	October	33.3	20.8	6.4	11.6	13	17.8	785
	November	36.9	25.3	7.8	14.9	14	22.3	917
	December	33.2	25.5	9.8	14.4	14.5	25.3	1004
1998	January	38.6	28.0	9.7	16.0	14	25.6	1053
	February	38.8	27.2	9.4	15.3	13.5	25.2	1072
	March	38.1	25.7	10.4	15.2	12.5	18.5	849
	April	30.9	19.7	8.8	11.9	11	12.0	629
	May	25.2	18.5	6.3	11.1	10.5	8.6	472
	June	23.7	15.0	3.8	8.3	10	6.2	354
	July	16.3	13.3	3.3	7.1	10	6.3	360
	August	22.1	16.1	5.8	9.2	11	10.1	527
	September	27.6	19.1	6.1	10.6	11	14.0	730
	October	28.8	19.6	5.9	10.6	13	17.5	775
	November	32.7	23.4	7.9	12.5	14	24.6	1009
	December	39.0	26.5	9.3	15.3	14.5	25.6	949

[x] Mean photosynthetic photon flux density = mean solar radiation x 0.45 x 10^6 x 4.6 / (daylength hours x 60 x 60). The solar radiation data were converted to photosynthetic photon flux density assuming a flat spectral distribution curve between 400–700 nm.

Table 2 Details of the crosses performed in 1995 and the number of pods and seeds produced.

Pollen recipient (female)	Location in SA	Colour category	Pollen source (male)	Location in SA	Colour category	Total no. flowers pollinated	No. pods	No. seeds	No. seeds germinated (% germinated)
P5	Woodside	4	G8	Waite Campus	2	866	35	134	125 (93)
P3	Mylor	3	G8	Waite Campus	2	655	29	115	104 (90)
P2	Woodside	4	G7	Waite Campus	2	872	8	19	17 (89)
P6	Mylor	4	G7	Waite Campus	2	773	35	192	169 (88)
P1	Woodside	4	G7	Waite Campus	2	1129	67	241	159 (66)
G3	Woodside	2	P8	Mylor	4	957	25	123	112 (91)
G5	Mylor	2	P8	Mylor	4	727	17	72	71 (99)
G4	Lenswood	2	P8	Mylor	4	604	14	44	43 (98)
G2	Lenswood	2	P7	Mylor	3	674	47	276	254 (92)
G6	Balhannah	2	P7	Mylor	3	651	8	36	34 (94)

Appendix 3

Published papers

- Morgan A, Sedgley M (2002) Environmental control of bud formation and flowering of clonal *Acacia baileyana* F. Muell. for ornamental horticulture. *Australian Journal of Experimental Agriculture* **42**, 211–216.

- Morgan A, Carthew SM, Sedgley M (2002) Breeding system, reproductive efficiency and weed potential of *Acacia baileyana. Australian Journal of Botany* **50**, 357–364.

- Morgan A, Adams P, Kolesik P, Sedgley M, Carthew S, Haskard K (2002) Using digital image analysis to estimate flower numbers of Cootamundra wattle (*Acacia baileyana* F. Muell.) and hence determine seed production and weed potential. *Plant Protection Quarterly* **17**, 162–167.

- Asenstorfer RE, Morgan AL, Hayasaka Y, Sedgley M, Jones GP (2003) Purification of anthocyanins from species of *Banksia* and *Acacia* using high-voltage paper electrophoresis. *Phytochemical Analysis* **14**, 150–154.

Morgan, A., Carthew, S. M. & Sedgley, M. (2002). Breeding system, reproductive efficiency and weed potential of A. baileyana. *Australian Journal of Botany, 50*(3), 357-364.

Morgan, A., Adams, P., Kolesik, P., Sedgley, M. Carthew, S. & Haskard, K. (2002). Using digital image analysis to estimate flower numbers of Cootamundra wattle (Acacia baileyanna F.Muell.) and hence determine seed production and week potential. *Plant Protection Quarterly, 17*(4), 162-167.

Asenstorfer, R. E., Morgan, A. L., Hayasaka, Y., Sedgley, M. & Jones, G. P. (2003). Purification of anthocyanins from species of Banksia and Acacia using high-voltage paper electrophoresis. *Phytochemical Analysis, 14*(3), 150–154.