PIGMENTS IN GERALDTON WAX (CHAMELAUCIUM UNCINATUM SCHAUER) VARIETIES

Klyne A.M., Plummer J.A.  Grows D.J.  Spadek Z., Best W.  Hall D.J.
Plant Sciences,  Agriculture Western  The Chemistry  Innovating
The University of  Australia  Centre (WA),  Horticulture
Western Australia,  South Perth  East Perth  Australia,
Stirling Highway  WA 6151  WA 6004  Bayswater
Crawley WA 6009  Australia  Australia  WA 6053
Australia
aklyne@cyllene.uwa.edu.au

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Abstract

Waxflowers (Chamelaucium) are the leading Australian native plants cultivated for the floriculture industry but the market is limited by the currently available varieties. Colours, especially white and purple, are only available for short periods during the season.

Early work on the flavonoids in Chamelaucium uncinatum indicated that the main pigments were delphinidin and malvidin glycosides. The current project aims to clarify the pigment composition in Geraldton Wax. Information on the genetic basis for colour will be useful for the breeding of new varieties with novel colours.

Thin layer chromatography and HPLC data have been used to distinguish the pigments in three cultivars representing the standard colours: ‘Purple Pride’ (purple), ‘CWA Pink’ (pink) and ‘Alba’ (white).

1. Introduction

Waxflowers are grown commercially for the floriculture industry where they are generally used as ‘filler’ species (Manning et al., 1996). On the European market, waxflowers are ranked in the top 20 (Considine and Growns, 1998) and they are the major export cutflower from Australia (Growns et al., 2000). Several species are used with the majority of varieties belonging to Chamelaucium uncinatum (Geraldton Wax). Flowers range in size from 9 to 25.6mm and have five waxy petals around a large central nectary disk. Petal colours range from white through pinks to mauve and purple, with the deep pinks and purples being most desirable commercially but rarest in nature. Other closely related genera such as Verticordia and Darwinia show a wider range of colours including reds, oranges and yellows but rarely have the plant form and vigour of waxflowers. A breeding program has commenced to introduce novel colours, such as reds and yellows, into waxflowers (Webb et al., 1997, Growns et al., 2000).

Anthocyanins are the major floral pigments in plants (Mol et al., 1998), contributing colours ranging from reds through purples to blues, and they are produced through the anthocyanin biosynthetic pathway (Mulder-Krieger and Verpoorte, 1994). The anthocyanidins are the primary product of the pathway (Fig. 1) with pigments being modified in vivo by glycosylation, and sometimes acylation. These modified pigments are referred to as anthocyanins and are found in the vacuoles of pigmented cells. The modifications serve several functions: to stabilise the anthocyanins, alter the colour and allow the association of anthocyanins with themselves or with co-pigments (Goto and Kondo, 1991). Anthocyanin colour is dependent on the primary structure of the pigment, modifications with sugar and acyl groups, the location of the pigmented tissue and the vacuolar environment (Mol et al., 1998).

Previous work indicated that the pigments of Chamelaucium uncinatum are delphinidin and cyanidin based (Fig. 1) (Gascoigne et al., 1948; Lowry, 1976) but there
was minimal information about the extent of modifications to the anthocyanidins. This study compares the pigment composition of several *Chamelaucium* cultivars representing the range of colours currently available: white ‘Alba’, pink ‘CWA Pink’ and purple ‘Purple Pride’.

### 2. Materials and Methods

#### 2.1. Plant material

Bunches of ‘Purple Pride’, ‘CWA Pink’ and ‘Alba’ flowers were picked from plants grown at the Agriculture Western Australia Field Station under natural lighting. Mature flowers at maximum pigment development were selected for analysis. Petals were excised, weighed then freeze-dried.

#### 2.2. Pigment analysis

Freeze dried material was extracted with 95% methanol containing 1% trifluoroacetic acid. Samples were filtered and concentrated under nitrogen then stored at −20°C. Replicate extractions of the material were performed.

Aliquots (100-200 µl) of extracts containing anthocyanins were fully hydrolysed by adding an equal volume of 4M hydrochloric acid and heating at 98°C for one hour. For partial hydrolysis, samples were removed from heat after 20 minutes. Samples were extracted with amyl alcohol before concentration and storage at −20°C.

Samples were spotted onto cellulose thin layer chromatography plates and allowed to dry. Plates were developed in HCL:formic acid:water (7.1:51.4:41.4; Andersen and Francis, 1985).

Anthocyanins were analysed on a Polymer Laboratories PLRP-S column (5 µm particle size) with a PLRP-S Guard Column (Hong and Wrolstad, 1990) on a Waters HPLC system with a Photodiode Array detector, measuring absorbance from 210 nm to 600 nm. Data were analysed using the Millenium32 software package from Waters and Microsoft Excel.

### 3. Results

Thin layer chromatography of hydrolysed pigments indicated that there were at least two anthocyanidins present in ‘Purple Pride’ petals. Comparison of hydrolysed ‘Purple Pride’ extract Rf values and spot colour, with blueberry and strawberry extracts (Curtright *et al.*, 1996) suggested that the anthocyanidins were delphinidin and petunidin or cyanidin (Table 1). Partial hydrolysis experiments indicated that the anthocyanins in ‘Purple Pride’ petals may be diglycosylated as extra spots with intermediate Rf values, compared to the unhydrolysed and completely hydrolysed samples, were evident on the thin layer plates (Table 1).

*Chamelaucium uncinatum* varieties produced anthocyanins in their petals. Peaks were detected corresponding to compounds that absorb in the 475 nm – 560 nm wavelength range with a secondary peak at approximately 275 nm, which is characteristic of anthocyanin pigments (Harborne, 1967). The total concentration of pigments, as estimated from peak height at 520 nm, in ‘Purple Pride’ was approximately eight times the level found in ‘CWA Pink’ while ‘Alba’ had no detectable pigments at 520 nm.

Six peaks with absorbance spectra characteristic of anthocyanins were found in ‘Purple Pride’ (Fig. 2A), when analysed at 520 nm. The difference in retention times indicated that these six peaks had distinct structures and therefore represented six anthocyanin pigments. ‘CWA Pink’ also produced six anthocyanin peaks (Fig. 2B). The ‘Purple Pride’ and ‘CWA Pink’ pigment peaks corresponded in retention times and absorbance spectra but differed in the proportion of each peak present (Fig. 3). ‘Purple Pride’ had three major peaks with Peak 1 accounting for 47% of the absorbance at 520
nm while Peak 4 and Peak 6 had 25% and 17% respectively. ‘CWA Pink’ had more of Peak 6 (47%) and approximately equal amounts of Peaks 1 and 4 (20% each).

‘Purple Pride’ and ‘CWA Pink’ had three major peaks that absorbed strongly in the regions 340 nm - 390 nm and 250 nm - 270 nm with a shoulder at 300 nm (Table 2), which is indicative of flavonols (Harborne, 1967). In contrast, ‘Alba’ contained only the second and third of these common flavonol peaks and several additional smaller peaks (less than 10% area) (Table 2).

4. Discussion

This study supports earlier reports of delphinidin and cyanidin derived pigments in *Chamelaucium uncinatum* (Gascoigne *et al.*, 1948; Lowry, 1976). Flavonoid 3’5’-hydroxylase catalyses the hydroxylation of the B-ring to produce delphinidin-derived pigments (Fig. 1). Flowers lacking this enzyme, such as roses (Mol *et al.*, 1999), do not naturally form purple or blue flowers. The presence of delphinidin implies that flavonoid 3’5’-hydroxylase is active in petals of ‘Purple Pride’ and ‘CWA Pink’. Delphinidin pigments are often associated with ‘blue’ flowers such as petunia and delphinium. As ‘Purple Pride’ and ‘CWA Pink’ can be described as pink to purple, pigment colour is also likely to be affected by the pH of the petal vacuoles (Mol *et al.*, 1998).

The *Chamelaucium uncinatum* cultivars ‘Purple Pride’ and ‘CWA Pink’ appeared to have the same anthocyanins in their petals. Differences between the cultivars in flower colour were due to the pigment concentration in the petals and relative proportions of the anthocyanins. It is likely that the same parts of the anthocyanin biosynthetic pathway are being expressed in ‘Purple Pride’ and ‘CWA Pink’ as they produced similar anthocyanins. The concentration and proportion of anthocyanins in the plant organs is determined by regulatory genes controlling expression of the enzymes in the biosynthetic pathway (Holton and Cornish, 1998). Cultivar petal colour variation is therefore likely to be dependent on the presence of regulatory genes for temporal and spatial expression of the enzymes.

‘Alba’ is a white variety that does not produce anthocyanins in the petals. White flowers are generally caused by a mutation of either one of the enzymes leading to the anthocyanidins or the regulatory genes controlling the expression of the pathway (Onozaki *et al.*, 1999). ‘Alba’ produced flavonols, generally in similar proportions to the other cultivars, indicating that the early part of the flavonoid biosynthetic pathway was active in the flower petals. The lesion causing white flowers in this variety is therefore likely to be either at an anthocyanidin specific enzymatic step or a regulatory gene that controls expression of the anthocyanin biosynthetic pathway.

The activity of flavonoid 3’5’-hydroxylase in *Chamelaucium* petals is likely to divert the majority of pigments produced to delphinidin derivatives as the activity of this gene is dominant (Honda *et al.*, 1999). Based on the results of this study, it is therefore unlikely that traditional breeding of current Waxflower cultivars will produce red flowers by introduction of an alternate enzyme to produce pelargonidin derived pigments, through hybridisation. Screening of other *Chamelaucium* cultivars and related species may reveal lines deficient in flavonoid-3’5’-hydroxylase which could then be targeted in the breeding program as likely to produce novel colours.

Molecular breeding techniques are an alternative method to develop cultivars lacking in flavonoid-3’5’-hydroxylase activity. Recent work on the *Torenia* variety ‘Shockwave Blue’ produced pink flowers from a blue line by reducing the level of flavonoid-3’5’-hydroxylase activity, using an antisense suppression method, which reduced the proportion of malvidin pigments (Fig.1) from 80% to not detectable, with the remaining peonidin pigments providing the colour (Suzuki *et al.*, 2000).

Further work in this project will aim to define vacuolar pH and some of the anthocyanin and flavonol structures. Molecular analysis of the expression of genes involved in anthocyanin biosynthesis could be correlated to the chemical data to allow a greater understanding of the biochemistry of waxflower petal colour. Elucidation of the
pigments in waxflower cultivars will assist in selection strategies for the breeding
program and direct development of molecular breeding programs in the future.

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Table 1. Thin layer chromatography of ‘Purple Pride’ petal extracts from mature flowers with full colour development. Hydrolysed blueberry sample contains delphinidin (Dp), petunidin (Pt) and malvidin (Mv) while hydrolysed strawberry sample contains cyanidin (Cy) and pelargonidin (Pg) (Curtright *et al.*, 1996)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Rf values (anthocyanidin)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blueberry</td>
<td>0.21 0.32 0.45</td>
</tr>
<tr>
<td>- hydrolysed</td>
<td>(Dp) (Pt) (Mv)</td>
</tr>
<tr>
<td>Strawberry</td>
<td>0.31 0.48</td>
</tr>
<tr>
<td>- hydrolysed</td>
<td>(Cy) (Pg)</td>
</tr>
<tr>
<td>‘Purple Pride’</td>
<td></td>
</tr>
<tr>
<td>‘Purple Pride’</td>
<td>0.71 0.77 0.86</td>
</tr>
<tr>
<td>- partially hydrolysed</td>
<td>0.21 0.31 0.49 0.61 0.71 0.77 0.86</td>
</tr>
<tr>
<td>‘Purple Pride’</td>
<td></td>
</tr>
<tr>
<td>- fully hydrolysed</td>
<td>0.21 0.31</td>
</tr>
</tbody>
</table>

Table 2: Major flavonols (measured as percentage area) in *Chamelaucium uncinatum* cultivars at 350nm. (Data are mean ± se)

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Peak A % Area</th>
<th>Peak B % Area</th>
<th>Peak C % Area</th>
<th>Other peaks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retention time</td>
<td>55 min</td>
<td>62 min</td>
<td>68 min</td>
<td></td>
</tr>
<tr>
<td>‘Purple Pride’</td>
<td>25.5 ± 0.3</td>
<td>48.4 ± 0.7</td>
<td>13.3 ± 0.2</td>
<td>12.8 ± 0.8</td>
</tr>
<tr>
<td>‘CWA Pink’</td>
<td>21.9 ± 0.3</td>
<td>62.3 ± 0.5</td>
<td>13.4 ± 0.2</td>
<td>2.6 ± 0.4</td>
</tr>
<tr>
<td>‘Alba’</td>
<td>-</td>
<td>55.0 ± 1.0</td>
<td>17.6 ± 0.2</td>
<td>27.4 ± 1.1</td>
</tr>
</tbody>
</table>
Fig. 1 The anthocyanin biosynthesis pathway, showing structures of the six most common anthocyanidins and numbering convention.
Fig. 2. HPLC profiles of pigments (relative absorbance units) at 520nm for (A) ‘Purple Pride’ petals and (B) ‘CWA Pink’ petals.

Fig. 3 Comparison of average peak areas in the cultivars ‘Purple Pride’, ‘CWA Pink’ and ‘Alba’