Flower colours and pigments in *Disa* hybrid (Orchidaceae)

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Abstract

Flower colours and the composition of pigments in the perianths of five cultivars of *Disa* orchids were analyzed. Carotenoids were major pigment components in the orange-red flowers of 'Dawn Angel'. We identified two types of pigment composition in the red flowered cultivars: 'San Francisco' contained more carotenoids and less anthocyanins, while 'Marlene' contained more anthocyanins than carotenoids. The red-purple flowered cultivars, only contained slight amounts of carotenoids, and the red-purple colour was attributed to the relatively high density of a cyanidin-based anthocyanin. The importance of the characterization of pigments in the perianths of orchid has been discussed in both breeding for flower colour improvement and chemotaxonomy.

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1. Introduction

The genus *Disa* Berg. (Orchidaceae) is comprised of about 130 species that are mainly distributed in tropical Africa and South Africa. *Disa uniflora* Berg. has large orange-red flowers and is highly valuable to produce as a floral crop. Hybrids with improved characteristics were developed (La Croix and La Croix, 1997) and interspecific hybridization between *D. uniflora* and related species has been achieved to produce cultivars with improved flower colours. Many varieties with various flower colours, including white, orange-red, red, pink and red-purple have been developed. However, there is limited information about the relationships between flower colour and pigment compositions in this genus (Vogelpoel, 1995; Bytebier et al., 2004). A better understanding of these relationships would aid selection processes of novel flower colours effectively *Disa* breeding programs in the future.

In general, flower colouration in the range orange-red to red is determined by a combination of anthocyanins and/or carotenoids, as in the genera *Chrysanthemum* (Kishimoto et al., 2007) and *Rosa* (Yokoi and Saito, 1973). The same tendency with respect to flower colour and pigmentation has also been reported in orchidaceous plants. In *Cattleya* and its alliance, the flower colours in the yellow to orange range are determined by carotenoids, in the orange to red range by combination of anthocyanins and carotenoids, and in the red to purple range by anthocyanins (Yokoi, 1975; Matsui, 1988; Matsui and

Nakamura, 1988; Tatsuzawa et al., 1994, 1996a, 1998).

In the present study, we investigated the relationship between flower colour and pigment compositions, in particular the combinations of anthocyanins and carotenoids, in five cultivars of *Disa* orchids displaying three categories of flower colours, namely orange-red, red and red-purple. The significant role for the examination of pigment in the perianths for the breeding of flower colour in this orchid and for the chemotaxonomy was discussed.

2. Materials and methods

2.1. Plant materials

Plants of five *Disa* cultivars, namely *D.* Child Safety Transvaal 'Dawn Angel', *D.* Foam 'San Francisco', *D.* Sid Cywes 'Marlene', *D.* Santa Rosa 'Purple Taffy', and *D.* Unilangley 'Pink Tourmaline' were purchased from Hokkaisankyo Co. Ltd. (Kitahiroshima City, Hokkaido, Japan). Ten plants of each cultivars were grown in a greenhouse with 40% shade in Fukagawa City, Hokkaido (43°43'N, 142°01'E). Maximum and minimum temperatures were below approx. 30 °C and 15 °C, respectively, in summer time. The majority of plants bloomed from early April to late May, and perianths were collected from the flowers at the time of full bloom. The collected perianths were dried in an air flow incubator at 40 °C for 2 days, and then kept in an air tight plastic container with silica gel at room temperature, until used.

2.2. Analysis of flower colour

Flower colour of the fresh outer perianths from three flowers of each cultivar were evaluated by comparison with an RHS Colour Chart (RHS CC; The Royal Horticultural Society, UK). A colourimetric instrument (CM-2002 Minolta Japan) was also used to measure the colours of fresh perianths from three flowers of each cultivar, immediately after collection. Colours were expressed according to the Commission International de l'Eclairage (CIE) L*a*b* system. Three measurements were averaged to obtain hue (b*/a*) data for each cultivar.

2.3. Analyses of anthocyanidins

Pigments were extracted from dried perianths (1 g from each cultivar) by soaking in 20 ml of 2M HCl for 2 h at room temperature. The filtrated samples were hydrolyzed in a boiling water bath for 2 h. After cooling, the reaction products were analyzed by thin layer chromatography (TLC) and analytical high-performance liquid chromatography (HPLC), using authentic anthocyanidins as standards (Mikanagi et al., 2000). Forestal (HOAc:HCl:H_2O=30:3:10, v/v/v) was used as mobile phase for TLC. Analytical HPLC was performed on an LC-10A system (Shimadzu Co. Ltd., Kyoto, Japan), using a reverse phase C18 (4.6 x 250 mm) column (Waters Spherisorb 5 μm ODS2), at 40 °C with a flow rate of 1 ml/min monitoring at 510 nm. The solvent was applied as a linear gradient system for 40 min from 20 to 85% solvent B (25% MeCN, 20% HOAc, 1.5% H_3PO_4 in H_2O) in solvent A (1.5% H_3PO_4 in H_2O) (Tatsuzawa and Shinoda, 2005).

2.4. Quantitative analysis of anthocyanins and carotenoids

Crude anthocyanins were extracted from dried perianthes (1 mg) of each cultivar by soaking in 0.1% HCl-MeOH (1 ml) for 2 h at room temperature. UV-Vis spectra (wavelengths from 400 - 700 nm) of the extracts were recorded on a MPS-2400 spectrophotometer (Shimadzu). The absorbance at 505 nm (λ_{max}) was used for quantitative analysis of the anthocyanins (Tatsuzawa et al., 2004a).

Crude carotenoids were extracted from dried perianths (1 mg) of each cultivar by soaking in MeOH:Acetone (1:1,v/v; 1 ml) for 2 h at room temperature. UV-Vis spectra of the extracts were recorded on a MPS-2400 spectrophotometer (400 - 700 nm), and the absorbance at 445 nm (the largest λ_{max}) was used for quantitative analysis of the carotenoids (Tatsuzawa et al., 2004a).

3. Results

3.1. Analysis of flower colour

The following flower colour results were obtained for each cultivar: ‘Dawn Angel’, orange-red (Orange-Red 33A by RHS CC and a chromaticity value of b*/a* = 55.00/50.30 = 1.07); ‘San Francisco’, red (Red 44A, 65.41/69.36 = 0.92); ‘Marlene’ red (Red 46C, 54.37/63.25 = 0.86); ‘Purple Taffy’, red-purple (Red-Purple 67A, 10.31/57.22 = 0.18); ‘Pink Tourmaline’, red-purple (Red-Purple 66C, 0.00/29.14 = 0.00) (Table 1; Fig. 1).

3.2. Analyses of anthocyanidins

Acid hydrolysis of crude extracts from five cultivars yielded aglycone forms of the anthocyanins, cyanidin [Rt(min):23.9 by HPLC and Rf: 0.43 by TLC] and pelargonidin [Rt(min):28.1 by HPLC and Rf: 0.65 by TLC]. In these five cultivars, cyanidin was the major anthocyanidin (64 - 90%), and pelargonidin was detected at levels of 8%, 19%, 20%, 1% and 1% in 'Dawn Angel', 'San Francisco', 'Marlene', 'Purple Taffy' and 'Pink Tourmaline', respectively (Table 1). The concentration of pelargonidin was higher in orange-red (8%) and red flower colour cultivars (19% and 20%) than red-purple ones (1% and 1%), as assessed by TLC and HPLC analysis.

3.3. Quantitative analysis of anthocyanins and carotenoids

'Marlene' contained the highest amount of anthocyanins in perianths. 'Purple Taffy' and 'San Francisco' contained 60 - 70% of the level of anthocyanins and 'Dawn Angel' and 'Pink Toumaline' each contained 10% of the level found in 'Marlene' (Table 1; Fig. 2).

'Dawn Angel' contained the largest amount of carotenoids in perianths. 'San Francisco' and 'Marlene' contained 90% and 50% of the level of carotenoids present in 'Dawn Angel', respectively. Both 'Purple Taffy' and 'Pink Tourmaline' contained 10% of carotenoids present in 'Dawn Angel' (Table 1; Fig. 3).

4. Discussion

Both anthocyanins and carotenoids greatly influenced the flower colours of five cultivars of *Disa* orchids. Our analysis revealed that the content of anthocyanins, which was based on both cyanidin and pelargonidin, varied among the five cultivars examined. The pelargonidin contents of the orange-red (b*/a* = 1.07)- and red (b*/a* = 0.86 and 0.92)-flower cultivars differed markedly from those of the red-purple (b*/a* = 0.00 and 0.18)-flowered cultivars. On the other hand, all cultivars contained relatively high levels of cyanidin (64% or more). We could, therefore, postulate that pelargonidin-based anthocyanins might be one of the major factors determining flower colour in hybrid *Disa* orchids cultivars examined in this study.

Thus it appears that a combination of pelargonidin-based anthocyanins and carotenoids resulted in orange-red and red flowers in the cultivars 'Dawn Angel', 'San Francisco', and 'Marlene' based on the relative quantities of carotenoids in the perianths ranging 0.1 - 1.0. By contrast, the expression of the red-purple flower colour in 'Purple Taffy' and 'Pink Tourmaline' was mainly due to the presence of cyanidin-based anthocyanins. Matsui and Nakamura (1988) divided *Laeliinae* into three groups with respect to both hue value and pigments component; the first group with carotenoid and hue value of higher than 0.47, the second by carotenoid and anthocyanin and hue value was between 0.47 and -0.13, and the third group by anthocyanin and hue value was between -0.31 and -1.00. On the contrary, in the *Disa* cultivars examined in the present study, hue value of the first group was 1.07, the second one was 0.86 and 0.92, and the third one was 0.00 and 0.18, respectively. In general, *Disa* showed higher hue values with a tendency of reddish flower colour compared to those of *Laeliinae* (Matsui and Nakamura, 1988). Therefore, it could be postulated that pelargonidin other than carotenoid greatly influenced expression of reddish flower colour in *Disa*.

Vogelpoel (1995) described that anthocyanins in *Disa* occur in the epidermal cells of the perianth and carotenoid are found in the mesophyll cells. The actual colour rendition of orchid flowers is the result of an interplay of the relative amounts of these pigments in their respective layers. Furthermore, we found two types of pigment composition in the red-flowered cultivars (Table 1). 'San Francisco' contained more carotenoid and less anthocyanins than 'Marlene'. Thus, the genotypes that determine pigment formation in the red-flowered cultivars could be different between these two types. The ranges of variation in flower colours in progeny from these two types are likely to be different. A better understanding of relationships between flower colour and pigments, and their inheritance, will stimulate more efficient breeding this genus.

The results of this study indicated that the relationships between flower colour and the pelargonidin- and cyanidin-based anthocyanins as well as carotenoids in *Disa* was similar to those in *Chrysanthemum morifolium* and the *Rosa* spp. (Kishimoto et al., 2007; Yokoi and Saito, 1973). On the other hand, the flower colours of *Cattleya* and its alliance (Orchidaceae) are mainly determined by the cyanidin-based anthocyanins (Tatsuzawa et al., 1994, 1996a). Thus, we could postulate that *Disa* orchids have more complex mechanisms for the expression of flower colour than *Cattleya* and its alliance.

To the best of our knowledge, the occurrence of pelargonidin in the flowers of orchidaceous plants was previously detected only in the genera *Brassotonia*, *Broughtonia*, *Cattleyopsis*, *Cattleytonia* (Arditti, 1969), and *Catasetum* (Yokoi, 1975). In the present study, pelargonidin was detected in the genus Disa. Recently, the glycosylation and acylation patterns of cyanidin, peonidin and delphinidin have been used in chemotaxonomic studies of Orchidaceous plants (Strack et al., 1986, 1989; Saito et al., 1994, 1995; Williams et al., 2002; Fossen and Øvstedal, 2003; Tatsuzawa et al., 1994, 1996a,b, 1997, 1998, 2004b, 2005,

2006). These studies included the genera *Anacamptis*, *Barlia*, *Bletilla*, *Cattleya*, *Cephalanthera*, *Cymbidium*, *Dactylorhiza*, *Dendrobium*, *Dracula*, *Epipactis*, *Gymnadenia*, *Himantoglossum*, *Laelia*, *xLaeliocattleya*, *Limodorum*, *Neottianthe*, *Nigritella*, *Ophrys*, *Orchis*, *Phalaenopsis*, *Serapias*, *Sophronitis*, *Traunsteinera* and *Vanda*.

Further analysis of the pelargonidin contents in other orchid genera may contribute to the progress of chemotaxonomic and phylogenic studies in the Orchidaceae. This work will also have relevance to our understanding of the co-evolution between flower colour and pollinators in the Orchidaceae.

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Figure

Figure 1. Flower colors of *Disa*

A: Orange-Red ('Dawn Angel')
B: Red ('Marlene')
C: Red ('San Francisco')
D: Red-Purple ('Purple Taffy')

Figure

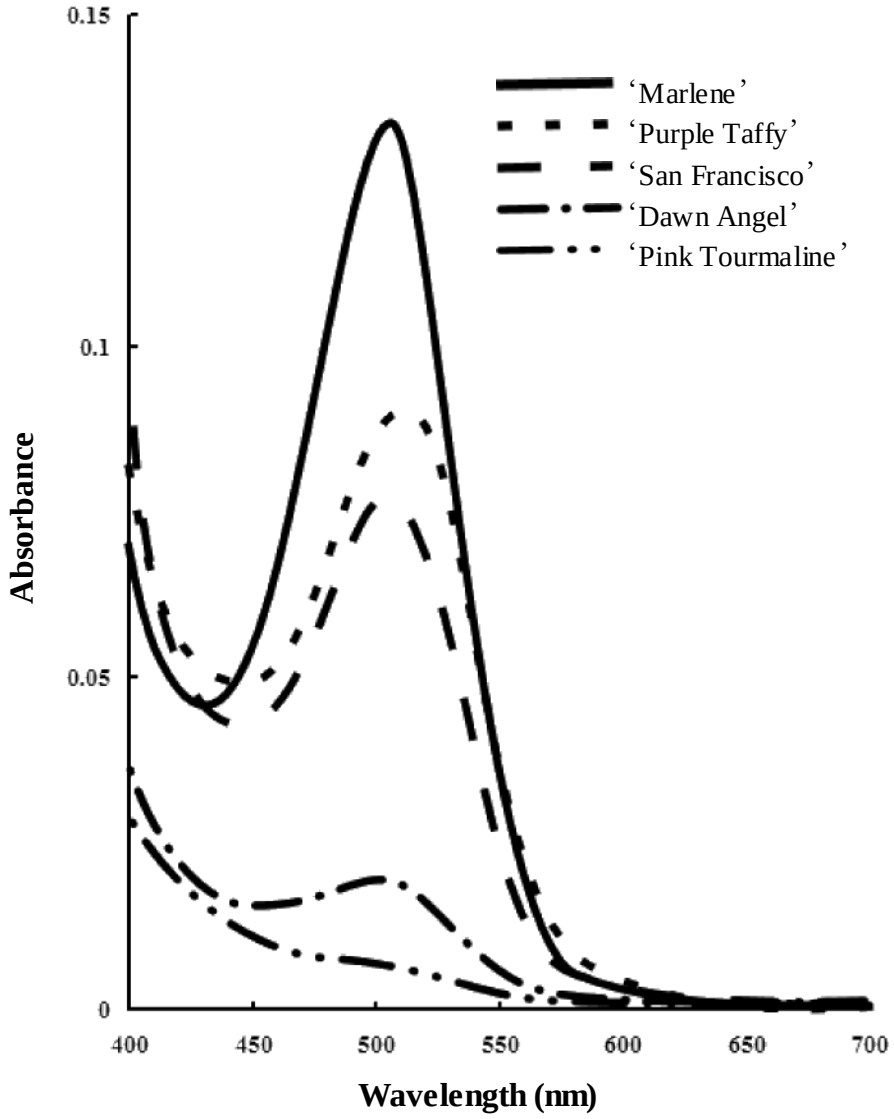

Figure 2. Absorption spectra of total anthocyanins in 0.1% HCl-MeOH of five *Disa* cultivars.

Figure

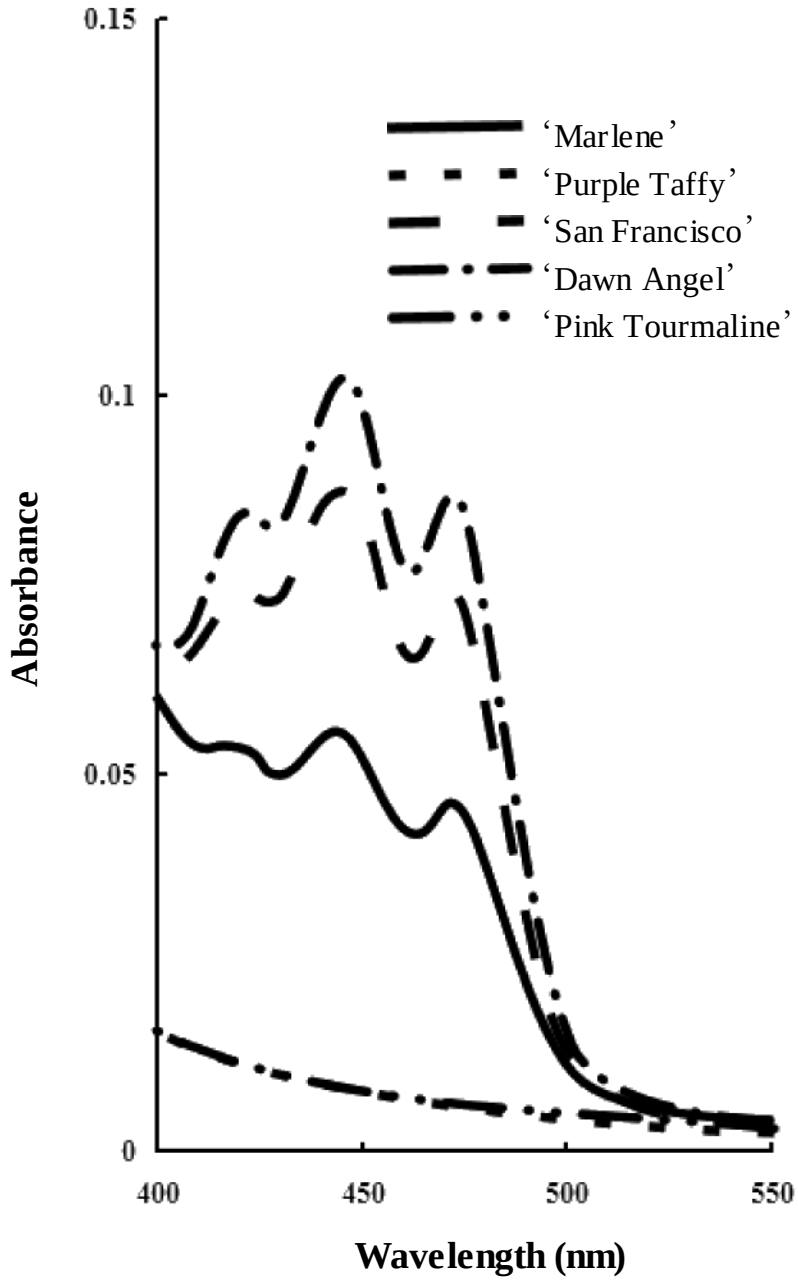

Figure 3. Absorption spectra of total carotenoids in MeOH : Acetone (1:1) of five *Disa* cultivars.

Table

Table 1. Flower colors and pigments from the flowers of *Disa* cultivars.

Cultivars	Flower colors[z]	b*/a*[y]	Anthocyanidin (%)[x]		Relative quantity	
			Pelargonidin	Cyanidin	Anthocyanin[w]	Carotenoid[v]
'Dawn Angel'	Orange-Red 33A	1.07	8%	83%	0.1 (0.0194)	1.0 (0.1026)
'San Francisco'	Red 44A	0.92	19%	72%	0.6 (0.0781)	0.9 (0.0880)
'Marlene'	Red 46C	0.86	20%	64%	1.0 (0.1340)	0.5 (0.0552)
'Purple Taffy'	Red-Purple 67A	0.18	1%	82%	0.7 (0.0901)	0.1 (0.0090)
'Pink Tourmaline'	Red-Purple 66C	0.00	1%	90%	0.1 (0.0069)	0.1 (0.0081)

[z]R.H.S. Colour Chart (The Royal Horticultural Sociaty).

[y]Hue (CIE, 1976).

[x]Percentage of total absorbance of all detected anthocyanidins at 510 nm in HPLC analysis.

[w]Relative quantity (absorbance ratio relative to 'Dawn Angel') of total anthocyanins extracts in 0.1% HCl-MeOH at 505 nm in UV-VIS spectrophotometer. Found values are in parentheses.

[v]Relative quantity (absorbance ratio relative to 'Marlene') of total carotenoids extracts in MeOH:Acetone (1:1) at 445 nm in UV-VIS spectrophotometer. Found values are in parentheses.