Black Flower Coloration in Wild *Lisianthius nigrescens*: Its Chemistry and Ecological Consequences

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The major pigments responsible for the flower color within the black flowered Gentianaceae, *Lisianthius nigrescens,* were characterized by HPLC and chemical analyses. HPLC analysis showed one major and one minor anthocyanin and 3 major and 3 minor flavone glycosides. The anthocyanins [delphinidin-3-*O*-rhamnol(1-6)galactoside and its 5-*O*-glucoside] comprised an extraordinary 24% of the dry weight of wild collected *L. nigrescens* corallas, and were accompanied in a 1:1 ratio by a range of apigenin and luteolin 8-*C*-glucosides and their 7-*O*-methyl ethers. The high levels of anthocyanins and flavones (and their copigmentation) is thought to account for the almost complete absorption of both UV and visible wavebands observed by reflectance photography.

Key words: Anthocyanins, *Lisianthius nigrescens*, Spectral Reflectance Analysis

Introduction

The coevolution of color signals and receptor-organism responses remains one of the most fascinating aspects of animal and plant interaction. Color is an interaction between plant pigments, light and the receptor organs of various animals (Kevan and Backhaus, 1998). In this paper we report on preliminary investigations into an unusual black-flowered plant, *Lisianthius nigrescens* of the Gentianaceae.

The Gentianaceae is a cosmopolitan family of herbaceous annuals and perennials and includes 80 genera with 900 species. Economic uses have been varied, yielding ornamentals such as *Eustoma, Gentiana, Sabatia* and *Lisianthius*, medicinal principles from *Gentiana, Swertia*, *Halenia, Lisianthius*, and *Chironia* and dyes from *Blackstonia* and *Gentiana.* The genus *Lisianthius*, as recently monographed by Weaver (1972), includes 27 species and has a New World distribution of the Greater Antilles and southcentral Mexico to northwestern Colombia.

The name *Lisianthius* was first published by P. Browne in 1756 but was published again in 1767 as *Lisianthus* by Linnaeus , which became more prevalent. Both names have been used and for this paper we will use *Lisianthius* as this reflects the most current taxonomic monograph. *Lisianthius nigrescens* is found in the Mexican states of Hidalgo, Veracruz, Oaxaca and Chiapas and also in Guatemala. It is a herbaceous perennial to 2 m tall found on road embankments and in pine and/or oak forests from 400–1800 m altitude. It is notable for its striking black tubular blossoms, up to 5 cm long, one of the blackest flowers known in the plant kingdom. Weaver (1972) considered it to be "by far the most common of the Mexican species ... especially in Chiapas". He described two varieties, var. *nigrescens* with shorter corolla lobes and a more northerly distribution, and var. *chiapensis* with larger flowers and a range of only Chiapas State and Guatemala.

Ethnobotanical records are provided by Schultes (1941), who collected this plant from ten sites in Oaxaca State, Mexico during 1938 and 1939. His doctoral dissertation details the medicinal usage of the plant by indigenous peoples of the region.

Black coloration, as perceived by the human eye, can be seen in a variety of plant parts; flowers, fruits, leaves, seeds and pollen. Species tulips such as *Tulipa julia* have a prominent black portion on the lower portion of the flower petal and horticultural hybrid tulips such as 'Queen of the Night' are a highly saturated violet which can appear black under certain light conditions. Shibata and Ishikura (1960), in a study of pigments in 107 tulip cultivars, investigated five selections with black flowers. The constituent pigments were predominantly delphinidin based, with the average anthocyanin levels in the five varieties being: delphinidin 50%, cyanidin 29% and pelargonidin 21%. For the black tulip cultivar 'Queen of the Night' they stated tulipanin (delphinidin-3-glucorhamnoside) was the dominant delphinidin glycoside but gave no indication of total dry weight percentage.

Takeda and Hayashi (1965) investigated pigmentation in Viola 'Jet Black' finding a *p*-coumaroyltriglycoside of delphinidin to be the responsible pigment. More recently the structure of the anthocyanin in the black pansy, *Viola tricolor,* has been identified as delphinidin-5-*O*-glucoside-3-*O*-[4-*p*-coumaroylrhamnosyl(1-6)glucoside], violanin, by Goto *et al.* (1978). *Lisianthius nigrescens* is a naturally occurring plant species (as opposed to a horticultural selection or hybrid) and is perhaps unique for its solid black corolla. Floral coloration results from the complex interactions of floral pigments and epidermal structures. Most flowers are colored to human beings and birds with trichromatic (blue, green, and red) vision, to insects with mostly trichromatic (ultraviolet, blue, green) color vision, and to tetrachromats such as some birds and insects with UV, blue, green, red colour receptors (Kevan and Backhaus, 1998). The red flowers that have been examined may be more or less black to most insects if they do not reflect ultraviolet (*e.g.* flowers pollinated mostly by birds, especially hummingbirds). Flowers that appear white to human beings mostly absorb strongly in the ultraviolet part of the spectrum so that they are coloured to insects. Flowers that are uniformly reflective across the trichromatic insect visual spectrum would be difficult for the insects to detect because of the colour similarity with more or less uniformly reflecting backdrops of leaves and soil, the differences in brightness not withstanding (Kevan *et al*., 1973). In fact, such white flowers are rare in nature (Kevan *et al*., 1996).

Chittka *et al.* (1994) analyzed the reflectance spectra of flowers of 573 plant species, yielding data for 1063 colors. Their system of 10 types of spectral reflectance functions in flowers was based on whether or not a petal absorbed or reflected wavelengths within four broad domains; 300–400 nm (UV), 400–500 nm, 500–600 nm or 600–700 nm. They did not produce any results for black petals, those of which would absorb wavelengths in all of these spectral domains. Their lack of data reflects the relative rarity of black as a corolla color.

Materials and Methods

Field collections

Plant material was collected in 1998 at the site of Schultes' collection #889, the village of Santo Domingo Latani, in Oaxaca State. One small stand consisting of a few dozen plants was located in a strip of ruderal vegetation between the roadside and a recently burnt field. Dried specimens of flowers were made by use of a herbarium press and specimens were also collected and preserved in 70% EtOH. Seedlings were also collected.

A second field expedition in 2001 resulted in the relocation of an additional Schultes population (collection #772) at Huatla de Jimenez, Oaxaca State and also a previously unrecorded population on the southern slopes of the Mazatec Mountains (Sierra Mazateca), 5 km west of the Uluapan River. At this latter stand we observed insects working the flowers. Insects were captured, preserved in 70% EtOH and later keyed out at the University of Kansas Entomolgy Deptartment with voucher specimens deposited there.

Pigment and copigment analyses

Petal material for analysis was freeze-dried, weighed (18 mg), ground and extracted at room temperature with 4 ml 3 N HCl/H_2O/MeOH (1:7:17; v/v/v). The optical density (at 538 nm) of the diluted (20 ×) extract was measured, and anthocyanin levels calculated in cyanidin-3-rutinoside equivalents using the published molecular extinction coefficient of 7,000 M^{-1} cm^{-1} for cyanidin-3-rutinoside (Figueiredo *et al*., 1996). Flavone levels in this extract were calculated in luteolin-monoglucoside equivalents from the optical density at 346 nm using the molecular extinction coefficient of 14,800 M^{-1} cm^{-1} for luteolin. Two-dimensional paper chromatographies (2D-PCs) were run

on 3MM Whatman paper using *t*-BuOH/HOAc/H_2O (3:1:1; v/v/v) and 15% HOAc as solvents, according to Markham (1982). HPLC data were obtained using an end capped RP-18 column (11.9 cm × 0.4 cm I.D.; 5 m), a Waters 994 photodiode array detector and a Jasco (Tokyo, Japan) PU-980 HPLC pump. Injection volume was 20 μl and elution was performed using a flow rate of 0.8 ml min^{-1} at 30 °C with a gradient solvent system comprising solvent A (1.5% H_3PO_4) and solvent B [HOAc/CH_3CN/H_3PO_4/H_2O (20:24:1.5:54.5; v/v/v)] mixed using a linear gradient starting with 80% A, decreasing to 33% A at 30 min, 10% A at 33 min and 0% A at 39.3 min. Traces were recorded at 352 and 530 nm (see Fig. 1) and on-line absorption spectra measured for each peak. The peak retention times (RT in min) and spectra (λ_{max} in nm) of the flavones are as follows: RT 12.42, 12.80; 270, 353; 272, 351 (orientin and swertisin monoglycosides); RT 16.35; 270, 351 (isoorientin); RT 17.40; 270, 349 (swertiajaponin); RT 20.38; 271, 338 (isovitexin); RT 21.96; 271, 337 (swertisin). For the anthocyanins, equivalent data are: RT 6.98; 275, 295sh, 343, 523 (delphinidin-5-*O*-glucoside-3-*O*-rutinoside); RT 10.15; 277sh, 300sh, 347, 440sh, 523 (delphinidin-3-*O*-rutinoside). Various chemical anthocyanins were achieved with 2 N NaOH in a sealed syringe as described by Markham (1982), and partial acid hydrolyses were carried out with 1 N trifluoroacetic acid/MeOH (1:1; v/v) at 100 °C for 10 min. Attempted removal of sugars from flavone-*C*-glycosides involved treatment with 2 N HCl at 100 °C for 2 h. 7-*O*-Methylation of authentic flavone-*C*-glycosides (from the personal collection of one of the authors, KRM) was achieved using diazomethane generated from nitrosomethylurea with 70% aq. KOH. The KOH was added dropwise to solid nitrosomethylurea covered with diethyl ether in an ice-cooled test-tube. The resultant ethereal solution of diazomethane was added dropwise to each of the authentic flavone-*C*-glycosides in MeOH. After a few minutes standing, the solutions were evaporated to dryness with a stream of N_2. Each was redissolved in MeOH for HPLC analysis and co-chromatography with compounds from *L. nigrescens*. This procedure did sometimes produce dimethylated product as well as the 7-*O*-monomethyl ethers.

Reflectance methodology

The flower's reflectance in ultraviolet light was measured according to the methods outlined in Kevan (1983) by which an ultraviolet transmitting lens is used on a camera with a bellows attachment. Photographs were taken through a broadband monochromatic ultraviolet passing filter (see Fig. 2).

The equipment used was a Hasselblad 500 C/M with a Carl Zeiss UV-Sonnar 1:4.3, f 105 mm lens on a bellows extension and equipped with a Kodak 18A filter (UV-passing filter but opaque to human-visible wavelengths). Film was Kodak Tri-X black and white, ASA 400. The custom made UV reflective gray scale (Kevan *et al.,* 1973) was placed by the flower on a black velvet background to assure correct exposure (see Kevan, 1979). The light source was a double electronic flash (Balcar, Paris, France) Studio Flash Unit at 2400 Ws angled at about 45 degrees and 1.3 m above on either side of the specimen to minimize shadows and possible specular reflections.

Results and Discussion

The chemical basis of color in L. nigrescens petals

High pressure liquid chromatography (HPLC) analysis (see Fig. 1) of an acidic extract from dried

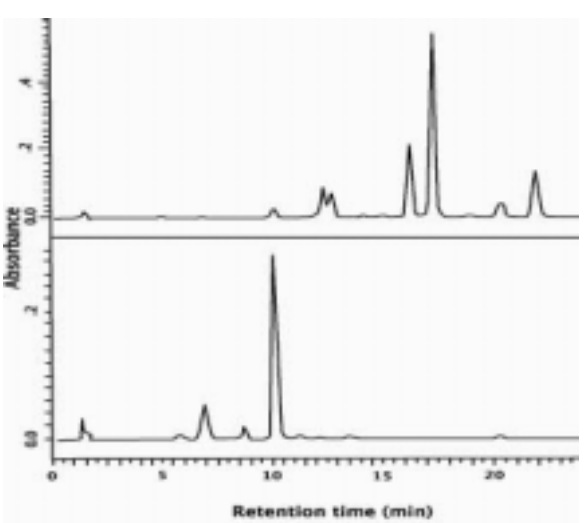

Fig. 1. HPLC chromatograms of the flavone-*C*-glycosides (upper trace, recorded at 352 nm) and the anthocyanins (lower trace, recorded at 530 nm) extracted from *Lisianthius nigrescens* petals. Retention times for peaks: (upper trace) 12.88, 13.22, 16.80, 17.83, 20.80, 22.25; (lower trace) 7.36, 10.45 min.

L. nigrescens petals revealed the presence of three major and three minor flavone glycosides (detected at 352 nm) and one major and one minor anthocyanin (detected at 530 nm). The compound types were evident from the on-line spectra obtained during chromatography. No other absorbing compounds were observed thus indicating that the petal colour is derived from one or both of these two pigment types. Involvement of the (colourless) flavones in the production of visible colour is also possible because anthocyanic petal colours can be affected by copigmentation of anthocyanins both with themselves and with other flavonoids (Brouillard and Dangles, 1994).

The structures of the anthocyanins were established by HPLC comparisons with anthocyanins of known structure isolated from *Eustoma grandiflorum* and *Vitex lucens* (Markham *et al.*, 2000). On-line absorption spectra of both anthocyanins in *L. nigrescens* exhibited maxima at ca. 525 nm, which in the solvent used indicate that they are both delphinidin glycosides. The absorption spectrum of the major anthocyanin differed from that of the minor in possessing a marked shoulder at ca. 440 nm. This is indicative of the presence of glycosylation at the 3-hydroxyl only in the major anthocyanin (Harborne, 1967). Conversely, the absence of a significant 440 nm shoulder in the spectrum of the minor anthocyanin is consistent with glycosylation at the 3- and 5-hydroxyls. Both anthocyanins lacked the UV-absorbing acyl groups found in the purple prairie gentian (*Eustoma grandiflorum*) anthocyanins (Asen *et al.*, 1986). The minor anthocyanin eluted from the HPLC with a retention time (6.98 min) suggestive of it being a triglycoside, and indeed it co-chromatographed with authentic delphinidin-5-*O*-glucoside-3-*O*-rhamno(1-6)galactoside. The reference sample, obtained by alkaline deacylation of the major anthocyanin in purple *E. grandiflorum* petals, also gave an identical absorption spectrum. The slower eluting (RT 10.15 min) major anthocyanin appeared to be a related delphinidin diglycoside, and with sugars attached only to the 3-hydroxyl, it was thought likely to be delphinidin-3-*O*-rhamno(1-6)-galactoside. This was confirmed by direct comparison with an authentic sample produced from the major purple *E. grandiflorum* by alkaline deacylation followed by mild acid hydrolysis. The authentic sample was chromatographically and spectroscopically identical to the black *L. nigrescens* anthocyanin.

The accompanying flavone pigments gave on-line absorption spectra consistent with their being apigenin and luteolin based flavones (Markham, 1982), and their retention times (12–23 min) indicate that they are all glycosides (Markham and Bloor, 1998). Acid treatment however failed to remove the sugars from the three major compounds leading to the conclusion that all are flavone-*C*-glycosides. Individual compounds were isolated by 2D-PC and two of the three major flavones were shown to be derivatized at the 7-hydroxyl using shift reagents (Markham, 1982). Because acid treatment failed to remove a sugar from any of the major components, the substituent on the 7-hydroxyl was most likely to be a methyl group. Authentic 7-*O*-methyl derivatives of vitexin (apigenin-8-*C*-glucoside), isovitexin (apigenin-6-*C*-glucoside), orientin (luteolin-8-*C*-glucoside) and isoorientin (luteolin-6-*C*-glucoside) were prepared using diazomethane. When compared with the flavones in *L. nigrescens* by thin-layer chromatography (TLC), HPLC and absorption spectroscopy, it was evident that the two 7-hydroxyl derivatized flavones (RT 21.96 and 17.40 min) in black *Lisianthius* are 7-*O*-methylisovitexin (swertisin) and 7-*O*-methylisoorientin (swertiajaponin), respectively. The third major flavone was similarly identified as isoorientin.

None of the minor flavones was 7-*O*-methylated. One was positively identified, as for the majors, as isovitexin (RT 20.38 min), and the other two are considered to be *O*-glycosides of swertisin and isoorientin on the basis of their retention times (12.80 and 12.42 min, respectively) and their luteolin-like absorption spectra.

The relative levels of anthocyanins and flavone-*C*-glycosides were determined by absorption spectroscopy. A freeze-dried sample of petal tissue (18 mg) was found to contain 7.3×10^{-6} mol or 4.3 mg of anthocyanin calculated as cyanidin-3-rhamno(1-6)glucoside, an anthocyanin type with an unusually low molecular extinction coefficient of 7×10^3 M^{-1} cm^{-1} according to Figueiredo *et al.* (1996). On this basis anthocyanins would thus comprise an extraordinary 24% of the dry weight of *L. nigrescens* petals.

By comparison, the deep purple petals of purple praire gentian flowers, *Eustoma grandiflorum*, contain only 1.1 to 1.4% anthocyanins (Markham *et al.*, 2000). The anthocyanin concentration in *L. nigrescens* is much higher than could be produced in aqueous solutions at vacuolar pH (Markham

and Ofman, 1993; Markham *et al*., 2000), and in other cases where this has been observed, anthocyanin-protein binding within the vacuole has been implicated as a concentration enhancing mechanism.

Copigmentation (Brouillard and Dangles, 1994) may also be influencing the colour intensity of anthocyanins remaining in solution. The level of flavone (copigments) in the same 18 mg sample of *L. nigrescens* was 6.1×10^{-6} mol or 2.8 mg (calculated as luteolin monoglucoside), indicating a flavone to total anthocyanin ratio of about 1:1 on a molar basis. However in solution the ratio would be considerably higher than this. There are thus ample levels of flavone-*C*-glycosides in the vacuoles of *L. nigrescens* petals to enable copigmentation to occur. That this is likely to occur is indicated by the findings of Asen *et al*. (1986) who demonstrated that flavone-*C*-glycosides such as swertisin and isoorientin are highly effective copigments for the purple *Eustoma grandiflorum* anthocyanins which are related to those found in *L. nigrescens*.

On the basis of the above data we propose that the intense black colouration and UV-absorbing properties of *L. nigrescens* petals result from 1) the unusually high concentrations of anthocyanins and UV-absorbing flavone-*C*-glycosides, and 2) from the interaction/copigmentation of the anthocyanins with vacuolar protein and flavone-*C*-glycosides.

Spectral reflectance analysis and pollination

The flowers of *L. nigrescens* were scentless during the day and appear black to human beings. The floral form is reminiscent of flowers pollinated by hummingbirds, moths, or long-tongued bees, and as such was thought possibly to be reflective in ultraviolet. However, spectral reflectance photography revealed that the flower was non-reflective in the ultraviolet part of the spectrum (see Fig. 2). The analyses of the floral pigments reported here are in accord with the almost complete absorbance of all wavebands of both UV and visible light.

Field observations resulted in the capture of a suite of visiting insects, all bees. These include *Partamona bilineata* Say and *Pleibeia frontalis* Friese of the tribe Meliponini, both stingless social bees. Also identified were three separate species of *Lasioglossum*, subgenus Dialictus (tribe Halictini), one of which is an unknown species. These were the only recorded visitors during field observations at three populations.

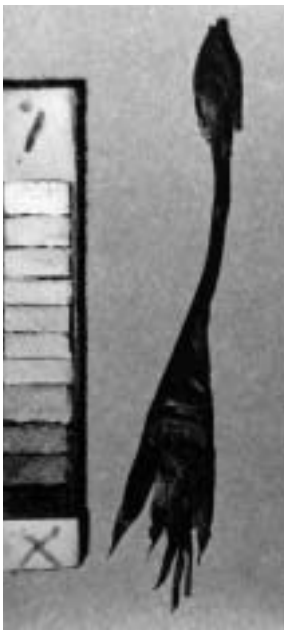

Fig. 2. Photograph of ultraviolet reflections from flower of *Lisianthius nigrescens* on a black velvet background.

The tubular floral structure and pendant presentation is consistent with flowers pollinated by hummingbirds or hovering moths but no records exist of either pollinator being attracted by black flowers. How a truly black flower of the *L. nigrescens* type might attract pollinators remains to be discovered, but it is certainly a peculiar natural anomaly awaiting explanation.

These preliminary studies confirm the highly unusual and perhaps unique floral biology and chemistry of *Lisianthius nigrescens*. Further studies are planned to examine the pigment compartmentation of fresh flower material and to determine how pollinators are attracted to its black flowers.

On the basis of the findings above, it is concluded that "the blackest flower in the world", *Lisianthius nigrescens*, achieves its blackness through the accumulation of extraordinary high levels of anthocyanin glycosides. It is thought that these levels are achieved in part through concentration of the pigments on gelatinous vacuolar inclusions. However, the intensity of their visible and UV absorption is also enhanced by the co-occurrence in the vacuole of UV-absorbing flavone-*C*-glycosides of a type known to be ideal copigments for these

anthocyanins. The problem of how this flower might attract the pollinators when it totally absorbs in both the UV and the visible remains to be explained.

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