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Colored and White Sectors From Star-Patterned Petunia Flowers Display Differential Resistance to Corn Earworm and Cabbage Looper Larvae

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Abstract Anthocyanins are likely a visual aid that attract pollinators. However, there is also the possibility that anthocyanins are present in some flowers as defensive molecules that protect them from excess light, pathogens, or herbivores. In this study, resistance due to anthocyanins from commercial petunia flowers (*Petunia hybrida*) was examined for insecticide/antifeedant activity against corn earworm (CEW, *Helicoverpa zea*) and cabbage looper (CL, *Trichoplusia ni*). The petunia flowers studied contained a star pattern, with colored and white sectors. CEW larvae ate significantly less colored sectors than white sectors in no-choice bioassays in most cases. All CEW larvae feeding on blue sectors weighed significantly less after 2 days than larvae feeding on white sectors, which was negatively correlated with total anthocyanin levels. CL larvae ate less of blue sectors than white sectors, and blue sectors from one petunia cultivar caused significantly higher CL mortality than white sectors. Partially purified anthocyanin mixtures isolated from petunia flowers, when added to insect diet discs at approximately natural concentrations, reduced both CEW and CL larva weights compared to the controls. These studies demonstrate that the colored sectors of these petunia cultivars slow the development of these lepidopteran larvae and indicate that anthocyanins play some part in flower defense in petunia.

Keywords Anthocyanins · Petunia · Insect · Resistance

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Introduction

Insect herbivores cause crop losses by physically damaging tissue but can also contribute to the colonization of crops by fungi, some of which synthesize toxins (e.g., mycotoxins) that can harm livestock and humans (Dowd 1998). Strengthening plant resistance to herbivore damage is an important strategy of crop management (Dowd et al. 2005). Plants are capable of synthesizing a large variety of biochemicals that serve as defensive molecules (Sadasivam and Thayumanavan 2003), mediate biotic and abiotic stresses (Arasimowicz and Floryszak-Wieczorek 2007; Dixon and Paiva 1995; Horváth et al. 2007; Korkina 2007; Wasternack 2007), and potentially contribute to human health (Sampson et al. 2002). One class of plant secondary biochemicals, the flavonoids, exhibit a wide range of functions that include protection against UV-light and pathogens, signaling during nodulation, male fertility, and auxin transport (Koes et al. 2005). Humans consume substantial quantities of flavonoids (estimated to be several hundred milligrams daily) in diets rich in fruits and vegetables (Hollman and Katan 1999). Blue and red flavonoids in flowers, fruits, and vegetables, called anthocyanins, are free-radical scavengers and antioxidants (Wang et al. 1997; Tsuda et al. 2000) that may have a role in preventing carcinogenesis and heart disease (Omenn 1995). Anthocyanins are thought to function primarily in nature as pollinator attractors

(Koes et al. 2005). Anthocyanins possibly protect leaves from excess light, water stress, or herbivores (Manetas 2006; Schaefer and Rolshausen 2006). Studies on two different types of tree species suggest that leaf anthocyanins play a role in herbivore protection (Karageorgou and Manetas 2006; Schlindwein et al. 2006). Some anthocyanins from cotton flowers can inhibit the development of insect larvae (Hedin et al. 1983; Jenkins et al. 1983).

Petunia can synthesize more than three dozen steroidal compounds that are involved in resistance to lepidopteran larvae; however, these compounds appear to be made in the leaves but not in flowers (Elliger and Waiss 1991). Petunia has served as a model species for identifying many of the regulator genes and enzymes of anthocyanin biosynthesis (Koes et al. 2005). Many anthocyanin structures from garden and wild petunias have been determined (Wiering and De Vlaming 1984; Ando et al. 1999). Blue or violet petunia flowers generally contain delphinidin-type anthocyanins, which have three hydroxyl groups on the B ring of the anthocyanin, due to the presence of flavonoid 3′,5′-hydroxylase (Holton et al. 1993). Methylation of the 3′ hydroxyl group of delphinidin results in petunidin derivatives, while an additional methylation on the 5′ hydroxyl group of petunidin results in malvidin derivatives. A large percentage of commercial petunias available contain petunidin or malvidin derivatives (Ando et al. 2004).

Preliminary experiments indicated that a variety of lepidopteran larvae can eat most but not all commercial petunia flowers (Johnson and Dowd 2003). In addition, some purified anthocyanins reduce the growth of lepidopteran larvae (Johnson and Dowd 2003, 2004). Thus, a search for petunia material that displayed differential anthocyanin production in the flowers was initiated. Extensive breeding of *Petunia hybrida* has generated a number of unique flower phenotypes that include star patterns, which exhibit alternating colored and white sectors. Since the colored and white sectors of star-patterned flowers should be genetically identical, studies that compare the feeding activities of corn earworm (CEW) larvae (*Helicoverpa zea*) and cabbage looper larvae (*Trichoplusia ni*) on these floral sectors were investigated. These insect species can feed on members of the Solanaceae family, such as potato (CL), tomato (CL and CEW), and tobacco (CEW; Metcalf et al. 1951). While these insect larvae are not natural pests of *P. hybrida*, the potential effectiveness of *P. hybrida* anthocyanins as resistance molecules against these larvae that damage important crops may ultimately improve crop protection. For example, genetic regulation of anthocyanins in maize has been well studied (Koes et al. 2005), and it may be possible to induce production of petunia-like anthocyanins in certain maize tissues for insect resistance. On the other hand, the potential insect resistance of *P. hybrida* anthocyanins may be evidence of a molecular mechanism whereby petunia flowers both attract pollinators and defend the tissue from a wide range of potential herbivores.

Methods and Materials

Plant Material Seeds were purchased from the following commercial vendors: W. Atlee Burpee & Co., Warminster, PA, USA (Razzle Dazzle Mixed); Harris® Seeds, Rochester, NY, USA (F1 Ultra Blue Star, UBS); Ball Seed Co., West Chicago, IL, USA (Carpet Blue Star). Seeds were sown in moistened Metro Mix 350 (Scotts-Sierra Horticultural Products Company, Marysville, OH, USA) and germinated under a 14:10 L/D photoperiod at 25°C. Plantlets were transferred to larger pots and placed in a walk-in plant growth facility that contained 1,000 W sodium and halide lighting. Plants were kept at 24°C±2°C day and 18°C±2°C night temperatures and 50%±10% relative humidity. Plants were fertilized weekly with Peters Professional 20–20–20 general purpose fertilizer (Scotts Company, Marysville, OH, USA) at a concentration of 1 g/l or by the placement of indoor plant food spikes (Scotts Miracle-Gro Products, Marysville, OH, USA) into the soil every 30 days.

Insects Corn earworms (*H. zea*) were reared at 27°C±1°C, 40%±10% relative humidity, 14:10 L/D photoperiod as described previously (Dowd et al. 2003). CL larvae obtained from Dr. Robert Behle (USDA-Peoria) were reared under similar conditions (Behle et al. 2003). First instars were used in bioassays. The CEW and CL colonies have been propagated for approximately 180 and 120 generations, respectively.

No-Choice Bioassays Using Flower Sectors Bioassays with CEW larvae were begun soon after initial flowering of each petunia cultivar. Bioassays with CL larvae were begun 6–31 months after initial flowering. Petunia corollas of the same developmental stage (limb as flat as possible) were sectioned by color and placed separately into 5-cm diameter Petri plates with tight fitting lids (Falcon 351006, Becton Dickinson Labware, Franklin Lakes, NJ, USA). Each plate contained two (for large flowers) or four (for small flowers) sectors. Punches (7 mm) were removed from the remaining colored and white sectors and were frozen for subsequent chemical analysis. Several flowers of each cultivar were tested. Ten first instar *H. zea* or *T. ni* were added to each dish and placed in the dark at 27°C±1°C and 40%±10% relative humidity for 48 h. Corolla sectors were rated for feeding on day 2 by counting 1-mm^2 holes (or equivalent areas) with a dissecting scope as described previously (Dowd et al. 2003). Surviving larvae were frozen to weigh

later with an analytical balance (Mettler-Toledo AE163 or AX105DR, Columbus, OH, USA). Multiple bioassays were performed for each petunia cultivar and are indicated on the tables.

Quantification of Anthocyanins Anthocyanins were extracted from one or two punches overnight at 4°C in the dark with 500 μl of methanol + 0.1 M HCl (hereafter, solvent). The next day, the liquid extract was removed and the volume reduced by vacuum with an Eppendorf® Vacufuge™ Concentrator 5301 (Brinkmann Instruments, Inc. Westbury, NY, USA) at room temperature. The dried extract was suspended in 100 μl of solvent. Five hundred microliters of solvent were added to the flower punch(s), and they were ground with small plastic pestles. After another overnight extraction at 4°C in the dark, the first- and second-day extracts were combined, and volume was reduced in the Eppendorf® Vacufuge™. The residue was suspended in 200 μl of solvent and passed through a 0.45-μm syringe filter (4-mm diameter, Alltech Associates Inc. Deerfield, IL, USA). The final volume was measured with a gastight 250-μl syringe. Fifteen microliters of each sample were injected onto an Inertsil 5-μm ODS-3 column (250× 4.6 mm, Metachem Technologies Inc., Torrance, CA, USA) housed in an Agilent (Santa Clara, CA, USA) 1100 high-performance liquid chromatography (HPLC) with a diode array detector set to detect compounds at 520 nm. The mobile phases were A, 1% formic acid and B, acetonitrile and programmed to go from 0% to 39% B after 35 min, with a flow rate of 1 ml/min. The calculation for total anthocyanins consisted of all the peaks (excluding peaks that eluted within the first 5 min of the run) that had an integrated area that exceeded the lowest integrated peak area of the standard regression with malvin chloride (Fluka, Germany). The anthocyanin calculation was normalized to account for the final punch extract volume and was expressed as equivalents of malvin chloride per two flower disks.

Identification of Anthocyanins Flowers were collected approximately 3 months (UBS), 6 months (Razzle Dazzle blue), 7 months (Carpet Blue), and 23 months (Razzle Dazzle red and pink) after initial flowering and freeze dried at least overnight. Fractions were ground into a fine powder with a mortar and pestle. Typically, 100–200 g of dried flower material were needed to produce 5–25 mg of purified anthocyanins. For anthocyanin analysis, the samples were extracted with 0.12 M HCl in methanol. Fractions were sonicated for 15 min then allowed to stand overnight at room temperature. This was repeated at least twice more for each sample. Extracts from a sample were pooled, and an aliquot was removed from the vial and filtered through a 0.45-μM nylon 66 filter for liquid chromatography–electrospray ionization–mass spectrometry (LC–ESI–MS) analysis. For preparative work, the extracts were allowed to dry in a hood, then resuspended in a lesser volume of 0.12 M HCl in water. Samples were run on a ThermoFinnigan LCQ DECA XP Plus LC–MS system with a surveyor HPLC system (autoinjector, pump, degasser, and PDA detector) and a nitrogen generator, all running under the Xcaliber 1.3 software system. The MS was run with the ESI probe in the positive mode. The source inlet temperature was set at 220°C, the sheath gas rate was set at 88 arbitrary units, and the sweep (auxiliary) gas rate was set at 12 arbitrary units. The MS was optimized for the detection of the anthocyanins by using the autotune feature of the software, while infusing a solution of malvin with the effluent of the column and tuning on an atomic mass unit of 655 $[M]^+$ for malvin. The column used was an Inertsil ODS-3 reverse phase C-18 column (3 μm, 150×3 mm, with a Metaguard column, from Varian). For anthocyanin LC–MS analysis, the initial HPLC conditions were 2% acetonitrile and 0.25% acetic acid in water, at a flow rate of 0.3 ml per min. The effluent was monitored at 520 nm on the PDA.

Isolation of Anthocyanin Composites From UBS Flowers Flowers were collected over several weeks from two UBS plants that had been flowering for approximately 5 months and placed in a −20°C freezer. Once enough material was collected, the flowers were freeze dried and extracted as above. A Buchi (Newcastle, DE, USA) Sepacore flash chromatography system with dual C-605 pump modules, C-615 pump manager, C-660 fraction collector, C-635 UV photometer, with SepacoreRecord chromatography software was used. A Buchi C-670 Cartridger system was used to load 40×150 mm flash columns with approximately 90 g of preparative C18 reverse phase bulk packing material (125 Å, 55–105 μm, Waters Corp., Milford, MA, USA). The columns were installed in the flash chromatography system and equilibrated with 5% methanol and 0.5% acetic acid in water for 5 min at a flow rate of 30 ml per min. After samples (10–15 ml) were injected, the column was developed with a binary gradient to 100% methanol over 30 min. The effluent was monitored at 520 nm, and all fractions were collected in the fraction collector by the software program. Fractions were concentrated by evaporation in the hood at room temperature.

A Shimadzu (Columbia, MD, USA) preparative HPLC system was used with dual 8A pumps, SIL 10vp autoinjector, SPD M10Avp photodiode array detector, SCL 10Avp system controller, all operating under the Shimadzu Class VP operating system. Ten milliliters of sample aliquots in methanol were injected on a Phenominex (Torrance, CA, USA) Luna C18 (2) semi-preparative reverse-phase column (10 μm, 100 Å, 250×50 cm). The column was pre-equilibrated with 1% acetic acid, 2% acetonitrile, and 97% water at a flow rate of 50 ml per min, and the effluent was

monitored at 520 nm. The column was developed to 50% acetonitrile over 45 min. UV absorbing peaks were collected by hand. The procedure was repeated to obtain sufficient purified material. Pooled material was allowed to evaporate to remove organic solvent and then freeze-dried to recover the purified anthocyanins.

Bioassays with UBS Anthocyanin Composites Dried anthocyanin composites were dissolved in distilled water and absorbed onto small discs of pinto bean diet as previously described (Dowd et al. 2007) and kept at 4°C for several hours to overnight for full absorption. Ten first instar CEW or CL were added to the dishes and, after resealing, were kept in the rearing incubator in the dark. Mortality was recorded on each day, and the larvae were frozen on the second or third day (depending on the quantity of diet remaining in controls) and subsequently weighed with an analytical balance (Mettler-Toledo AE163 or AX105DR, Columbus OH, USA).

Statistical Analyses Statistical differences in feeding and mortality rates were determined by the paired means test (proc means), while larvae survivor weights were compared by analysis of variance (ANOVA; proc glm) using SAS version 9.1 (Cary, NC, USA). Correlation analyses were completed by using "proc reg corr" with the same SAS software.

Results

Flower Sector Studies Colored and white sectors from three different petunia star cultivars were tested for corn earworm resistance. CEW mortality was low (<10%) for all of the sectors tested, and none of the cultivars displayed significantly different CEW mortality rates between the two sector types (data not shown). The Razzle Dazzle cultivar contained star-patterned flowers with different types of anthocyanins. For Razzle Dazzle red/white flowers, the feeding ratings were significantly lower on the red compared to the white sectors, but there was no significant difference in the weights of larvae feeding on the two types (Table 1). One of the Razzle Dazzle pink/white flowered plants displayed significantly lower feeding on the colored sectors, and the weights of surviving larvae feeding on the colored sectors were lower than the weights of larvae feeding on the white sectors, but the other two Razzle Dazzle pink/white flowered plants did not display the same pattern of insect resistance (Table 1). One Razzle Dazzle blue/white flowered plant showed differential feeding on the colored and white sectors, and the weights of the larvae feeding on the colored sectors were lower than the weights of larvae feeding on the white sectors, but no additional blue/white flowering plants germinated from the Razzle Dazzle seed packets obtained. Thus, more blue/white star petunia flower cultivars from other vendors were purchased, including Carpet Blue Star and Ultra Blue Star. Both cultivars showed lower feeding rates on the colored sectors and lower larvae weights on the colored sectors, which indicated that the constituents of the blue sectors consistently inhibited CEW larvae growth.

Sectors of the blue/white flowers were also fed to CL larvae. No significant differences in feeding ratings or survivor weights (data not shown) were found in Razzle Dazzle blue/white flowers (Table 2). The mortality rate of larvae feeding on the Razzle Dazzle blue sectors was higher than that of larvae feeding on the white sectors but not significantly (P=0.11). No significant differences in the

Table 1 CEW mean feeding rates (number of 1 mm^2 holes) and survivor weights (mg ± standard error) on star patterned petunia flowers after the second day of the bioassay

Cul	Col FR (*N*)[a]	Whi FR (*N*)	Col Wt ± SE (*N*)	Whi Wt ± SE (*N*)
Red1	46 (20)[b]	62 (20)[c]	0.12±0.0090 (74)[d]	0.12±0.0084 (84)[d]
Red2	46 (19)[b]	63 (19)[c]	0.11±0.0065 (146)[d]	0.12±0.0068 (171)[d]
Pink1	40 (10)[b]	78 (10)[c]	0.14±0.0073 (170)[d]	0.18±0.010 (169)[e]
Pink2	72 (30)[b]	97 (30)[c]	0.31±0.012 (170)[d]	0.28±0.012 (168)[d]
Pink3	92 (16)[b]	56 (16)[c]	ND	ND
Blu1	58 (24)[b]	89 (24)[c]	0.18±0.0086 (128)[d]	0.23±0.0089 (143)[e]
CB1	50 (9)[b]	87 (9)[c]	0.17±0.011 (60)[d]	0.22±0.014 (61)[e]
CB2	42 (9)[b]	71 (9)[c]	0.16±0.012 (69)[d]	0.20±0.011 (72)[e]
UBS1	34 (12)[b]	62 (12)[c]	0.18±0.012 (65)[d]	0.23±0.011 (76)[e]
UBS2	46 (12)[b]	73 (12)[c]	0.19±0.010 (72)[d]	0.24±0.013 (65)[e]

Cul cultivar, *Col* colored sector, *Whi* white sector, *FR* feeding rating, *wt* weight, *Red* Razzle Dazzle red/white, *Pink* Razzle Dazzle pink/white, *Blu* Razzle Dazzle blue/white, *CB* Carpet Blue Star, *UBS* Ultra Blue Star, *ND* not determined

[a] For FR, *N* refers to the number of bioassay dishes assayed; for wt, *N* refers to the number of survivor larvae weighed.

[b,c] Means that are significantly different (P<0.05) by paired means test

[d,e] Means that are significantly different (P<0.05) by ANOVA

Table 2 CL mean feeding rates (number of 1 mm^2 holes) and mortality rates on star-patterned petunia flowers after the second day of the bioassay

Cul[a]	Col FR (*N*)[b]	Whi FR (*N*)	Col MR (*N*)	Whi MR (*N*)	Col Corr (*P*)[c]	Whi Corr (*P*)[c]
Blu1	39 (8)[d]	42 (8)[d]	23% (8)[d]	8% (8)[d]	0.21 (0.61)	−0.53 (0.18)
CB1	28 (9)[d]	36 (9)[d]	23% (9)[d]	3% (9)[d]	−0.71 (0.03)	−0.40 (0.28)
CB2	27 (7)[d]	35 (7)[d]	11% (7)[d]	9.0% (7)[d]	−0.65 (0.11)	−0.52 (0.24)
UBS[f]	34 (74)[d]	44 (74)[e]	29% (74)[d]	15% (74)[e]	−0.68 (<0.001)	−0.60 (<0.001)

[a] See Table 1 for abbreviations; *MR* mortality rate
[b] For FR and MR, *N* refers to the number of bioassay dishes assayed.
[c] Correlation between FR and MR
[d,e] Means that are significantly different ($P<0.05$) by paired means test
[f] UBS mean FR and MR based on 11 different plants tested at least once.

feeding ratings, mortality rates, and survivor weights (data not shown) were found on Carpet Blue flowers (Table 2). Colored sectors from Ultra Blue Star (UBS) flowers (11 different plants were tested) were effective against CL larvae both in feeding ratings and mortality rates (Table 2). Correlations between feeding ratings and mortality ratings were significant for CB1 colored sectors and UBS colored and white sectors (Table 2).

Qualification of Anthocyanins All petunia white flower sectors contained low to no detectable anthocyanins (Table 3). The blue sectored cultivars, which displayed good resistance to CEW larvae feeding (Table 1), generally contained more malvin equivalents than the other colored cultivars. This was not true, however, for the Razzle Dazzle plant with blue-white sectored flowers, which had less malvin equivalents than Razzle Dazzle red sectors but was more resistant to CEW larvae feeding. The level of malvin equivalents in all the blue-sectored cultivars were negatively correlated to CEW larva weight. Malvin equivalent levels of both of the Razzle Dazzle pink sectored plants were seemingly negatively correlated to CEW larva weights, but in only one plant (Pink1) was this correlation significant. Neither of the Razzle Dazzle red-white sectored plants displayed a significant correlation between malvin equivalent levels and larva weights.

Identification of Anthocyanins The pigments from the blue sectored flowers were analyzed by LC–ESI–MS and contained primarily malvidin and petunidin derivatives (Table 4 and Fig. 1). The major pigments in red sectors were derivatives of cyanidin and delphinidin, while the pink sectors contained a peonidin derivative (Table 4). Structural identities were assigned only for those anthocyanins in which the experimental molecular weight matched with previously identified anthocyanins in petunia (Ando et al. 1999). The instruments used in the current study were not able to discriminate between *cis*- and *trans*-isomers, and thus, some presumed molecules were listed as either the *cis*- or *trans*- form (e.g., anthocyanin **5**). Most of the wild petunia anthocyanins with only a single sugar molecule are attached at the 3 position (Ando et al. 1999), and thus, anthocyanins **14** and **15**, which were not detected in wild petunias (Ando et al. 1999), were assumed to have their sugar attached at the 3 position. Peonidin-type anthocyanins are not present in wild petunias (Ando et al. 1999) but are present in a number of commercial *P. hybrida* lines (Ando et al. 2004). For the compounds detected in Razzle Dazzle pink sectors, it was assumed that the sugars would be attached to the peonidin base as it would be to malvidin or petunidin base for anthocyanins **10** and **13**. Because the Razzle Dazzle pink sectors are capable of producing petunidin- or peonidin-type anthocyanins, it was not possible to tell if the 933 mass from these flowers is anthocyanin **5** or **12**.

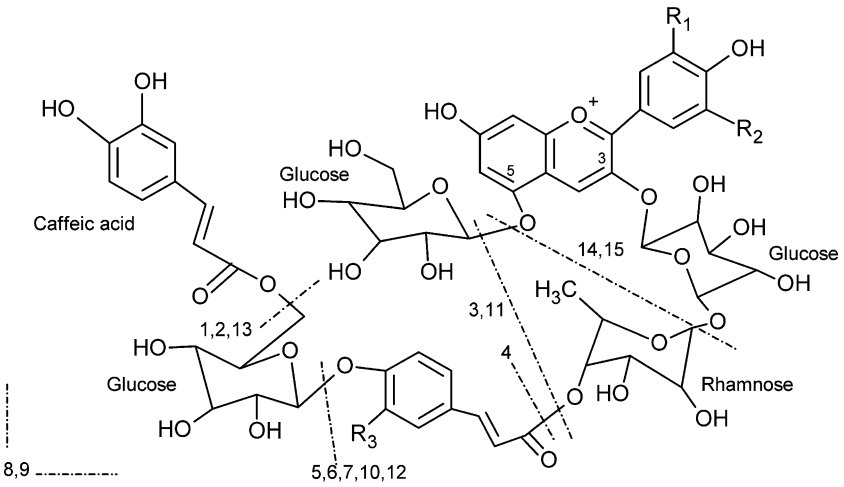
Bioassays with UBS Anthocyanin Composites Anthocyanins were partially purified from UBS flowers by using preparative chromatography. Seven different anthocyanin

Table 3 Correlation of anthocyanin levels to CEW larvae survivor weights

Cul[a]	Col Mal Eq (*N*)[b]	Whi Mal Eq (*N*)[c]	Correlation[d]	*P*[d]
Red1	183±13 (5)	0 (5)	0.06	0.46
Red2	189±14 (5)	0 (4)	−0.039	0.64
Pink1	103±8 (4)	0.7±0.7 (4)	−0.23	0.0072
Pink2	95±12 (4)	1.8±1.8 (3)	−0.17	0.072
Blu1	163±22 (8)	0 (8)	−0.24	<0.0001
CB1	343±36 (7)	0 (7)	−0.27	0.0027
CB2	377±42 (9)	0 (9)	−0.17	0.047
UBS1	266±45 (4)	0 (4)	−0.18	0.036
UBS2	311±17 (4)	0 (4)	−0.28	0.0011

See Table 1 for CEW larvae survivor weights.
[a] See Table 1 for abbreviations.
[b] Mean malvidin equivalents (μg) in two colored flower disks ± standard error
[c] Mean malvidin equivalents (μg) in two white flower disks ± standard error
[d] Correlation between mean malvidin equivalents to weights of larva survivors feeding on colored or white sectors and the resulting *P* value

Fig. 1 Structures of anthocyanins identified in this study



	R1	R2	R3
1	OH	OCH_3	H
2	OCH_3	OCH_3	H
3	OH	OH	
4	OH	OH	
5	OH	OCH_3	H
6	OCH_3	OCH_3	H
7	OCH_3	OCH_3	OH
8	OCH_3	OCH_3	H

	R1	R2	R3
9	OH	OCH_3	OH
10	OCH_3		H
11	OH	OCH_3	
12	OCH_3		OH
13	OCH_3		H
14	OH		
15	OCH_3		

composites added separately or in combination to insect diet significantly inhibited CL larvae growth (Table 5). While the combination of anthocyanin composites inhibited CEW larvae growth, only composites 1, 4A, 5A, and 5B were significantly inhibitory (Table 5). ANOVA between the following CEW means (Table 5) were not significant at $P<0.05$: 1 vs. 2, 1 vs. 3, and 5A vs. 3. No CEW or CL larvae died while feeding on the anthocyanin combinations (data not shown).

Discussion

CEW larvae generally fed less on colored than white sectors of all the petunia cultivars, which resulted in significantly lower weights in many cases (Table 1). The blue-sectored cultivars displayed superior CEW resistance compared to the red- and pink-sectored cultivars, with one exception (Razzle Dazzle Pink1). Statistically significant inverse correlations of mean CEW larva weight to mean anthocyanin levels (i.e., malvin equivalents) were found in all of the blue-sectored flower cultivars (Table 3). It may be argued that the effective CEW resistance of the blue-sectored flowers is due to a higher concentration of pigment. However, Razzle Dazzle Blu1 sectors and Razzle Dazzle Red1 sectors had statistically similar levels of malvin equivalents ($P=0.50$ by ANOVA), but the Razzle Dazzle blue sectors were more resistant to CEW feeding and caused lower CEW larva weights. Razzle Dazzle Pink1 sectors displayed good CEW resistance, while Pink2 and Pink3 colored sectors did not, perhaps due to the presence of unique allelochemicals specific to each plant. CL larvae were more sensitive to the constituents of the blue sectors because they displayed higher mortality rates than CEW larvae (compare Tables 1 and 2). While most correlations between CL mortality rates and feeding rates on Blu1 and the Carpet Blue flowers were seemingly negative (Table 2), only one was significant (CB1 on colored sectors), perhaps indicating that significant correlations would result only from more bioassays (as for UBS flowers). Correlations of CL feeding rates to mortality rates of Table 2 were −0.68 and −0.60 in UBS colored and white sectors, respectively. This suggests that anthocyanins in UBS colored sectors improves CL resistance present in the UBS white sectors but shows that other unknown defensive molecules in UBS colored and white sectors contribute to CL mortality.

Based on LC–ESI–MS analysis (not shown), the predominant anthocyanins of Razzle Dazzle blue/white and UBS flowers were anthocyanins **1** and **2**. While anthocyanin **2** was present in Carpet Blue Star flowers, the major anthocyanin was anthocyanin **1**. *P. hybrida* or garden petunias are likely the result of crosses of *P. axillaris* subsp. *axillaris* or subsp. *parodii* and *P. integrifolia* subsp. *integrifolia* or *P. inflata* (Chen et al. 2007). Anthocyanins **1** and **2** predominant in the blue star *P. hybrida* lines of this study do not occur in *P. integrifolia*, *P. inflata*, or the white flowers of *P. axillaris* subsp. *axillaris*

Table 4 Anthocyanins in petunia flower cultivars identified by LC–ESI–MS

Anthocyanins
Razzle Dazzle blue/white
Predominant anthocyanins
1095, petunidin 3-glucosyl *p*-coumaroylrutinoside-5-glucoside **1**
1109, malvidin 3-glucosyl *p*-coumaroylrutinoside-5-glucoside **2**
Other anthocyanins
611, delphinidin 3-rutinoside **3**
773, delphinidin 3-rutinoside-5-glucoside **4**
933, petunidin 3-*cis/trans*-*p*-coumaroylrutinoside-5-glucoside **5**
947, malvidin 3-*cis/trans*-*p*-coumaroylrutinoside-5-glucoside **6**
963, malvidin 3-trans-caffeoylrutinoside-5-glucoside **7**
1271, malvidin 3-caffeoylglucosyl *p*-coumaroylrutinoside-5-glucoside **8**
1273, petunidin 3-caffeoylglucosylcaffeoylrutinoside-5-glucoside **9**
Razzle Dazzle pink/white
Predominant anthocyanins
917, peonidin 3-*cis/trans*-*p*-coumaroylrutinoside 5-glucoside **10**
Other anthocyanins
625, petunidin 3-rutinoside **11**
933, peonidin 3-*cis/trans*-caffeoylrutinoside-5-glucoside **12** or **5**
1079, peonidin 3-glucosyl *p*-coumaroylrutinoside-5-glucoside **13**
Predominant anthocyanins
Razzle Dazzle red/white
449, Cyanidin 3-glucoside **14**, **3**
Ultra Blue Star
1, **2**
Carpet Blue Star
1
Other anthocyanins
Razzle Dazzle red/white
463, peonidin 3-glucoside **15**
Ultra Blue Star
3, **5**, **6**, **7**, **8**
Carpet Blue Star
2, **5**

Number(s) in bold refer to the anthocyanin structure described in Fig. 1.

and subsp. *parodii* (Ando et al. 1999). The major anthocyanin in *P. inflata* and *P. integrifolia* subsp. *integrifolia* is malvidin 3-*trans*-*p*-coumaroylrutinoside-5-glucoside (Ando et al. 1999). At some point in the breeding history of the *P. hybrida* blue star lines used in this study, an enzyme that places glucose on the coumaric acid group of the anthocyanins became more active.

Interestingly, the flowers with the most CEW resistance in their colored sectors (blue stars) are more similar in their anthocyanin profiles to their presumed parents (*P. axillaris* and *P. integrifolia*) than those with lower CEW resistance in their colored sectors (red and pink stars). Anthocyanin **3**, detected in Razzle Dazzle red sectors, is the predominate anthocyanin in only one wild petunia species, *P. exserta* (Ando et al. 1999). However, anthocyanin **14**, also detected abundantly in Razzle Dazzle red sectors, is not present in any wild petunias, but cyanindin 3-rutinoside is present in *P. exserta* (Ando et al. 1999). The major anthocyanin found in Razzle Dazzle pink sectors was anthocyanin **10**, which is not found in any wild petunia lines (Ando et al. 1999). The limited distribution of anthocyanin **3** and absence of anthocyanins **10** and **14** in wild petunia taxa (Ando et al. 1999) suggest that these anthocyanins are not involved in pollinator attraction and/or floral defense.

When all seven of the anthocyanin composites from UBS flowers were added to insect diet at approximately natural concentrations, they significantly reduced both CL and CEW weights compared to the controls (bottom of Table 5). This may be due to antibiosis or feeding deterrence. The reduction of CEW weights observed in the UBS colored sectors (Table 1) can be attributed partially to anthocyanins from UBS flowers (bottom of Table 5). However, CL larvae feeding on UBS colored sectors exhibited significant mortality (Table 2), while no CL mortality was observed with the combination of anthocyanin composites in insect diet (data not shown). As mentioned above, this indicates that unknown compounds beside anthocyanins contribute to CL mortality. Each of the seven individual UBS anthocyanin composites resulted in significantly reduced CL weights compared to the control (Table 5). This suggests that each of the UBS anthocyanin composites causes CL antiobiosis or feeding deterrence. However, not all of the anthocyanin composites reduced CEW weights compared to the control, which suggest that the mixture of compounds in composites 2, 3, and 4B were not as inhibitory or deterrent as the others.

Optimal defense theory predicts that flower tissue should be protected from damage to ensure fitness and that secondary

Table 5 Survivor weights (mg ± standard error) of bioassays using anthocyanin composites (3%) from UBS flowers

Composite	Anthocyanins[a]	CL Weight ± SE (*N*)	CEW Weight ± SE (*N*)
Control	–	1.7±0.14 (18)[b]	1.6±0.31 (11)[b]
1	**1**, 1113	1.1±0.092 (17)[c]	0.8±0.14 (15)[c]
2	**2**, 1126	1.0±0.083 (19)[c]	1.2±0.28 (9)[b]
3	**2**, 1127, 1149	1.1±0.080 (18)[c]	1.3±0.25 (11)[b]
4A	**5**, 951, 973	1.0±0.097 (18)[c]	0.8±0.12 (14)[c]
4B	**5**, 1271, 1288	1.1±0.12 (19)[c]	1.0±0.26 (10)[b]
5A	722, 730, 872, **6**	1.0±0.11 (18)[c]	0.8±0.12 (13)[c]
5B	**6**	1.2±0.076 (19)[c]	0.8±0.13 (10)[c]
Control	–	1.6±0.13 (19)[b]	1.1±0.17 (15)[b]
All 7[d]	All above	1.2±0.097 (18)[c]	0.6±0.064 (13)[c]

[a] Known anthocyanins and unknown anthocyanin masses

[b,c] Means of the control and experiment within a column were significantly different ($P<0.05$) by ANOVA.

[d] Each composite was added at 0.3% to make a total of 2.1% anthocyanins.

chemicals should be present constitutively rather than induced upon attack (McKey 1979). Petunia petals synthesize high levels of anthocyanins: based on Table 3, anthocyanin levels in some flower tissues (e.g., Carpet Blue) are ~6% fresh weight (punches weigh ~2.9 mg). If anthocyanin production is metabolically costly, it would be advantageous for petunia to produce petal compounds that both attract pollinators and defend the flower against herbivores. The data of this study indicate that petunia anthocyanins do inhibit growth or deter feeding of both CEW and CL larvae. This suggests that the petunia anthocyanins inhibit or deter a broad range of herbivores, but CEW and CL are likely not natural enemies of petunia. More studies that use pure wild petunia anthocyanins, wild petunia lines, and a number of their indigenous pests are needed to test fully the possible dual function of anthocyanins.

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