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Short research contribution

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PROTECTIVE FUNCTION OF FOLIAR ANTHOCYANINS: IN SITU EXPERIMENTS ON A SUN-EXPOSED POPULATION OF *IRIS PUMILA* L. (IRIDACEAE)

ABSTRACT: Anthocyanins are a group of water-soluble flavonoids known for their protective role against photoinhibitory and photooxidative damage to leaf cells under environmental stress. The effects of variation in light quantity on rates of anthocyanin production in foliage of *Iris pumila* were evaluated spectrophotometrically in a field experimental setting accomplished by shielding one half of each examined plant with a 65% neutral-density shade, whereas the other half experienced full sunlight. In unshaded leaves, the average anthocyanin level increased by 55.3% compared to their shaded counterparts. Because there was no a significant difference in the average level of pheophytin (a breakdown product of chlorophyll) between unshaded and shaded leaves, the results suggested that the elevated anthocyanin concentrations in sun-exposed foliage of *I. pumila* could act as a light attenuator, protecting its chloroplasts from excess high-energy quanta that would otherwise be intercepted by the chlorophylls.

KEY WORDS: anthocyanins, chlorophylls, *Iris pumila*, light intensity, pheophytin

There has been significant progress over the last several years in elucidating the possible ecophysiological functions of foliar

anthocyanins. Anthocyanins are a group of water-soluble pigmented flavonoids that may appear red, purple, or blue depending on pH (Harborne 1988). They occur in all tissues of higher plants, including flowers, fruits, stems, leaves, and roots. In reproductive plant structures (flowers and fruits) anthocyanins serve to attract animal vectors for pollination and/or seed dispersal, whereas in vegetative structures (leaves and occasionally stems) their physiological and ecological functions are still far from being fully understood (Steyn *et al.* 2002, Gould *et al.* 2002, Close and Beadle 2003, Hughes *et al.* 2005, Hatier and Gould 2008). A large number of ecophysiologicalists support the hypothesis that anthocyanins serve to protect plant cells against a multitude of abiotic stresses, including strong light, UV radiation, temperature extremes, water deficit, and heavy metals (Chalker-Scott 1999, Neill and Gould 2003, Steyn *et al.* 2002, Hughes *et al.* 2005). Conversely, there are many researchers who believe that the principal function of these pigments would be to assist in plant defense by repelling opportunistic herbivores (Lev-Yadun *et al.* 2004, Schaefer and Rolshausen 2006, Lev-Yadun and Gould 2007). Recently, Hatier and Gould (2008) pro-

moted a 'signal-modulation hypothesis' for the possible role of anthocyanins in vegetative tissues. According to the Hatier-Gould hypothesis: "Anthocyanins reduce production of reactive oxygen species (ROS), increase rates of ROS scavenging, and interact with soluble sugars, thereby protecting antioxidant enzymes and facilitating efficient oxidative signaling following biotic or abiotic stress" (Hatier and Gould 2008, pp. 626).

The expression of foliar anthocyanins differ considerable among the plant species. In some plant taxa, anthocyanins are permanently present in all leaves, whereas in others they are synthesized only during juvenile and/or senescing stages of leaf ontogeny, or in stressed leaves (Chalker-Scott 1999, Steyn *et al.* 2002, Merzlyak *et al.* 2008). Anthocyanins generally accumulate in photosynthetic tissues exposed to high irradiance such as adaxial epidermis and/or outermost mesophyll layers of leaves (Gould *et al.* 2000, 2002, Neill and Gould 2003, Hughes *et al.* 2005), but occasionally are localized in the abaxial tissues (Lee and Graham 1986, Hughes and Smith 2007). Anthocyanins are synthesized in the cytoplasm and transported into the plant cell vacuole where they are stored in the form of spherical pigmented inclusions (Conn *et al.* 2003). Since anthocyanin biosynthesis requires light, it frequently co-occurs with periods of high excitation pressure and, consequently, an increased potential for oxidative damage. Recent studies have demonstrated that anthocyanins accumulated in the plant cell vacuoles play a photoprotective role in vegetative tissues predisposed to photoinhibition by acting as light attenuators and/or antioxidants (Smillie and Hetherington 1999, Gould *et al.* 2000, 2002, Neill and Gould 2003, Hughes *et al.* 2005).

The objective of this study was to explore the effect of light intensity on anthocyanin level in vegetative tissue of *Iris pumila* (L.) plants naturally growing under direct sunlight in the field. We tested the possibility that anthocyanins accumulated in sun-exposed leaves of *I. pumila* function as a darkening filter that shields the mesophyll from light stress.

Iris pumila is a perennial rhizomatous monocot native to the Deliblato Sands (44°47'39"N 21°20'00"E to 45°13'10"N

28°26'08"E), an isolated complex of sand masses situated in the southeastern part of Serbia. Natural populations of *I. pumila* are very abundant at sun-exposed dune sites, where individual plants form circle-shaped clones composed of tightly packed and horizontally growing rhizome segments, which spread from the center of a clone towards its margin. The species exhibits a remarkable flower color polymorphism, which enables identification and mapping of individual clone genotypes in the field (Tucić *et al.* 1988).

For the present study we selected one sun-exposed population of *I. pumila*, which inhabited the top and south-facing slope of a large dune. In April 2007, at the peak of *I. pumila* blooming phase, we randomly selected nine large clones with distinct flower color, marked each of them with a wooden peg, and covered one half of every clone with a neutral-density PVC screen, which transmitted approximately 35% of the ambient light. In July 2007, we collected leaf samples from each of the marked clones. Specifically, a fully expanded leaf from two ramets per unshaded and shaded half of each clonal plant was harvested between 15:00 and 16:00 hours during the same day, immediately frozen in liquid nitrogen, and transported to the laboratory, where the leaf samples were stored at -70°C until preparation.

For quantification of total anthocyanins, tissue extracts were prepared by pulverization of one gram of frozen leaves under liquid nitrogen, followed by homogenization in 12 ml of 1% (w/v) HCl in methanol for 2 days at 5°C with continuous shaking (Mancinelli *et al.* 1975). Absorbance of the extracts were measured at 530 and 653 nm using a UV/visible light spectrophotometer (Multiskan Spectrum, Thermo Electron Corporation, Vantaa, Finland). Anthocyanin concentrations were calculated as $A_{530} - 0.24 A_{653}$ (Murray and Hackett 1991). Chlorophyll content was determined using 0.25 mm² leaf squares obtained from the same leaves as above. Each leaf square was placed in 2 ml DMSO (dimethyl-sulfoxide) to extract for six hours at 65°C in the dark. Absorbance of the DMSO extracts was measured at 470, 647 and 663 nm to calculate concentrations of chlorophyll *a*, chlorophyll *b* and total chlorophyll accord-

ing to the equation given by Wellburn (Wellburn 1994). Photooxidative damage, estimated in term of the amount of chlorophyll converted to pheophytin (*a* form of chlorophyll *a* in which the Mg ion is replaced by two hydrogens) was determined by an increase in absorbance at 553 nm relative to absorbance at 665 nm (Gupta *et al.* 1993). All measurements were conducted in triplicate. Statistical significance of between-treatment pigment levels was evaluated applying a Student *t*-test.

Light-induced plasticity in pigment expressions of a clone was determined by calculating an index of plasticity (PI_v) (Valladares *et al.* 2006):

$$PI_v (\%) = [(|X_H - X_L|) / X_L] \times 100 \quad (1)$$

where X_H is the mean trait value of a clone in the high light treatment, while X_L is the mean trait value of the same clone in the low light treatment. This index of plasticity measures the percentage change in a trait (pigment content) from the high to the low light environment.

Table 1 shows the effects of light intensity on pigment accumulation and pheophytin production in unshaded and shaded leaves of *I. pumila* plants naturally growing in the field. As expected, leaves from the shaded clone parts exhibited an average of 55.3% less anthocyanin level than those exposed to full sunlight, and this difference was found to be highly significant ($P < 0.0001$). Because sun leaves of *I. pumila* received a greater level of

incoming radiation, and thus produced more anthocyanins than shaded leaves, this indicates that the accumulation of anthocyanins in leaf cells exposed to photoinhibitory conditions might confer a photoprotective benefit. A growing body of experimental evidence suggests that epidermal anthocyanins may act as light-attenuators in leaves by absorbing blue-green to yellow (500–600 nm) light that would otherwise be absorbed by chlorophyll *b* in the subjacent spongy mesophyll (Smillie and Hetherington 1999, Gould *et al.* 2000, Nishio 2000, Field *et al.* 2001, Gould *et al.* 2002, Neill and Gould 2003, Hughes *et al.* 2005, Hughes and Smith 2007). By intercepting these photons, anthocyanins are thought to protect the shade-adapted chloroplasts from overexcitation, reducing the requirements for non-photochemical mechanisms of excess energy dissipation and, ultimately, the formation of biologically deleterious reactive oxygen species (ROS) such as O_2^- , H_2O_2 , $ONOO^-$, and probably OH^- and 1O_2 . It has been recently reported that the exposure of red (anthocyanin-containing) leaves of *Galax urceolata* [(Poir.) Brummitt.] to green light (which anthocyanins absorb strongly) produced a significantly lower decline in maximum photosystem II efficiency (F_v/F_m) than in green (acyanic) leaves, but were similar under red light, which anthocyanins absorb poorly (Hughes *et al.* 2005, Hughes and Smith 2007). Moreover, red leaves recovered to starting F_v/F_m levels more rapidly than did green leaves, indicating a

Table 1. Pigment concentration and index of plasticity (PI_v) (formula 1) to light intensity in unshaded and shaded leaves of *Iris pumila* grown in the field. The presented values refer to the mean \pm standard error of nine leaf samples. *P* denotes significance levels from a *t*-test of observed differences between sun-exposed and shaded clone parts.

Pigment	Unshaded	Shaded	<i>P</i>	Plasticity (%)
Anthocyanin (A_{530})	0.1121 \pm 0.0089	0.0473 \pm 0.0032	0.0001	55.3 \pm 4.8
Chlorophyll <i>a</i> (mg cm ⁻²)	0.0166 \pm 0.0006	0.0199 \pm 0.0009	0.0067	22.3 \pm 5.7
Chlorophyll <i>b</i> (mg cm ⁻²)	0.0109 \pm 0.0002	0.0121 \pm 0.0003	0.0046	12.3 \pm 3.0
Total Chlorophyll (mg cm ⁻²)	0.0275 \pm 0.0008	0.0320 \pm 0.0011	0.0059	18.3 \pm 4.6
Chlorophyll <i>a/b</i> ratio	1.5289 \pm 0.0241	1.6389 \pm 0.0330	0.0159	8.6 \pm 2.3
Pheophytin (A_{553}/A_{665})	0.3463 \pm 0.0116	0.3219 \pm 0.0036	0.1096	8.4 \pm 3.6

reduced extent of photoinhibition had been incurred.

Our study provides evidence that the levels of chlorophyll *a*, chlorophyll *b*, and total chlorophyll, as well as the ratio of chlorophyll *a:b* were significantly lower in sun exposed than in shaded leaves ($P = 0.007, 0.005, 0.006,$ and $0.016,$ respectively), supposedly due a faster decline in chlorophyll *a*, since unshaded leaves produced an average of 22% less chlorophyll *a*, but only 12% less chlorophyll *b* than shaded leaves (Table 1). Such an unusual trend has been also observed between red and green leaves of *G. urceolata* (Hughes *et al.* 2005), and the tropical understory plants *Begonia pavonina* (Ridl.) and *Triolena hirsuta* [(Benth.) Triana] (Gould *et al.* 1995). According to Hughes *et al.* (2005), the tendency of red leaves to display a lower chlorophyll *a:b* ratio than green conspecifics indicates “either that anthocyanin concentration is somehow related to this change, or that chlorophyll spectrophotometric assays are affected by the presence of anthocyanin”. Interestingly, despite of a lower ratio of chlorophyll *a:b* in unshaded than in shaded leaves of *I. pumila*, the strong solar radiation did not induce a significant increase in pheophytin production in sun versus shade leaves, indicative of chlorophyll damage ($P = 0.1096$), which suggests that anthocyanins accumulated in sun-exposed foliage conferred photoprotection to leaf cells.

One additional hypothesis that has received considerable experimental attention proposes that anthocyanins may act as antioxidants as well (Mittler 2000, Neill *et al.* 2002, Hughes *et al.* 2005). For example, in chloroplast suspensions of *Lactuca sativa*, the colorless cytosolic anthocyanins at pH 7.0 removed the O_2^- generated by chloroplasts, and their effects appeared to be concentration dependent (Neill and Gould 2003). Of note, the antioxidant capacity of anthocyanins was found to be about four times greater than that of ascorbic acid and α -tocopherol (Wang *et al.* 1997). Our preliminary results on the same *I. pumila* clones as those included in this study have revealed that the specific activity of three principal ROS-scavenging enzymes, superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) was lower in unshaded than in shaded leaves (un-

published data), supporting the hypothesis that anthocyanins “reduce production of reactive oxygen species (ROS)” and “increase rates of ROS scavenging” in vegetative plant structures exposed to strong solar radiation (Hatier and Gould 2008).

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