Artificial Shading and Temperature Influence on Anthocyanin Compositions in Sweetpotato Leaves

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Abstract. Sweetpotato leaves contain biologically active anthocyanins that have significant medicinal value for certain human diseases and may also be used as natural food colorants. Foliar anthocyanins and their relative abundance were investigated in leaves of sweetpotato cultivars ‘Shimon-1’, ‘Kyushu-119’, and ‘Elegant Summer’ grown under artificial shading and different temperature conditions. High-performance liquid chromatography profiles of the cultivars tested showed similar peaks but with peak areas differing with cultivar, temperature and shading. The relative quantity of individual anthocyanin was YGM (Yamagawamurashaki)-1a> YGM-4b> YGM-1b> YGM-5a> YGM-0d> YGM-0a> YGM-2> YGM-0c> YGM-3> YGM-6> YGM-5b> YGM-0b> YGM-0f> YGM-0e> YGM-0g. Seven were peonidin and eight cyanidin derivatives. The highest anthocyanin contents were found in plants grown at a moderate temperature (20 °C) with lower levels at 25 and 30 °C. The leaves of plants grown in full sun accumulated significantly more total as well as the major individual anthocyanins than plants grown in 40% and 80% shade. The results indicate that growing sweetpotatoes at moderate temperatures and without shading facilitates the accumulation of anthocyanins in the leaves. The anthocyanin composition of the leaves is discussed relative to their physiological function in human health.

The sweetpotato [*Ipomoea batatas* (L.) Lam.] is the seventh most important food crop in the world (FAO, 1997), and is among the crops selected by the U. S. National Aeronautics and Space Administration to be grown in a controlled ecological life support system as a primary food source (Hoff et al., 1982). The utilization of the sweetpotato leaves as a vegetable in addition to the storage roots could significantly increase food availability in countries with reoccurring food shortages. Currently sweetpotato greens are consumed to a limited extent as a fresh vegetable in some parts of the world (Nwinyi, 1992; Villareal et al., 1982). Faced with increasing food shortages, horticulturists and food scientists are becoming increasingly interested in previously neglected tropical green leafy vegetables such as sweetpotato greens. Low yield is a common deficit for tropical greens, however, the sweetpotato has an advantage in that it can be harvested several times during the year thus giving yields that are substantially higher than other greens. In addition, it is one of the few vegetables that can be grown during the monsoon season of the tropics such that sweetpotato leaves are often the only greens available in some countries after flood or a typhoon. They are rich in vitamin B, β -carotene, iron, calcium, zinc and protein, and as a crop are more tolerant of diseases, pests, and high moisture than many

other leafy vegetables grown in the tropics (AVRDC, 1985; Woolfe, 1992; Yoshimoto et al., 2003). We previously reported that sweetpotato leaves were an excellent source of antioxidative polyphenolics, superior in this regard to other commercial vegetables (Islam et al., 2002a, 2002b, 2003a, 2003b; Yoshimoto et al., 2003). In addition to use as a vegetable, sweetpotato leaves can also be used in noodles, breads, drinks, and confectionery.

The anthocyanins are the predominant group of visible polyphenols and comprise the red, purple and blue pigmentation of many plants. They represent a diverse group of secondary metabolites (Meyer et al., 1998) among which there are important natural antioxidants and food colorants. As a food additive, anthocyanins are rich in color compared to cochineal pigments. Furthermore, anthocyanins have been shown to have some positive therapeutic and physiologic effects as antineoplastic agents (Kamei et al., 1995), radiation-protective agents (Akhmadieva et al., 1993), chemoprotective agents against platinum toxicity in anticancer therapy (Karaivanova et al., 1990), hepatoprotective agents against carbon tetrachloride damage (Mitcheva et al., 1993), and possible other beneficial effects due to their interaction with various enzymes and metabolic processes (Costantino et al., 1995; Gibb et al., 1987). No adverse effects were observed in animals fed a grape color extract containing principally anthocyanins (Becci et al., 1983). Likewise, there did not appear to be any adverse effects on humans consuming a

grape skin extract that has been approved by the Food and Drug Administration as a food colorant (Timberlake et al., 1988).

There is a growing demand in the food industry for use of natural food colorants and purple-fleshed sweetpotatoes are considered a good source of stable anthocyanins that can be used as a red food colorant (Otake et al., 1994, 1998). Sweetpotato anthocyanins are equal in stability to light and heat as those derived from red cabbage and have a superior shelf-life (Otake et al., 1998). A high anthocyanin producing cell line (APL) has been established from the storage roots of sweetpotato cultivar ‘Ayamurasaki’ (K-Islam et al., 2000, 2003). The anthocyanins from the APL are superior as a natural food additive to those from red cabbage, elderberry, and purple corn (Otake et al., 1994, 1998) due to their free radical scavenging and antimutagenic activity (Furuta et al., 1998; Yoshimoto et al., 1999, 2002). Furthermore, anthocyanin composition in sweetpotato affects the quality of food colorants (Otake et al., 1994) and paste color (Yoshinaga et al., 1999). The major coloring constituents in sweetpotato have been identified as acylated anthocyanins (Zulin et al., 1992) and the chemical structure of major storage root anthocyanins determined (Goda et al., 1997; Otake et al., 1992; Terahara et al., 1999, 2000). Their base structure is either cyanidin or peonidin (3-sophoroside-5-glucosides) which are acylated with caffeic, ferulic and *p*-hydroxybenzoic acid.

We report the chemical compositions of sweetpotato leaf anthocyanins. To begin to determine environmental factors modulating anthocyanin synthesis in the sweetpotato, we investigated the effect of temperature and shading on total and individual anthocyanins in the leaves, focusing on those with known positive medicinal attributes and value as food colorants.

Materials and Methods

Plant materials and cultural methods. The experiments were conducted at the National Agricultural Research Center for Kyushu Okinawa, Japan, using three sweetpotato cultivars: ‘Shimon-1’ (S-1) (introduced from Brazil; the leaves have been commercially used for medicinal purpose); ‘Kyushu-119’ (K-119) (a breeding line for processing use program especially for anthocyanin pigment extraction, and also selected for its edible leaves) and ‘Elegant Summer’ (ES) (variety, developed for use as a leafy vegetable). The average air temperature during the growing period (May to October) was 25 °C. Cuttings were planted in 12-cm vinyl pots of sterilized soil in a glasshouse. Six weeks after planting, pots were transferred to a greenhouse having a constant temperature of 25 $\pm$ 2 °C. There were five pots per treatment and the five replications were arranged in a completely randomized design. After 7 d, the pots were transferred to greenhouses with temperatures 20 $\pm$ 1.5 °C, 25 $\pm$ 2 °C, and 30 $\pm$ 2.5 °C. The shading experiment was conducted using 80%, 40% and 0% shading (control). Shading was achieved using commercial shading material

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purchased from Nippon Wide Cloth Co. Ltd., Osaka, Japan (for 40% shading material) and Dio Kasei Co., Ltd., Tokyo, Japan (for 80% shading material). The light intensity was checked using an illuminance meter (model T-1H; Minolta Co. Ltd., Osaka, Japan). After seven days of continuous treatment, all the leaves (20 to 25) were harvested, washed gently, put into pre-labeled individual vinyl bags, and immediately frozen at -85°C . The following day the samples were freeze-dried for 48 h in a vacuum freeze dryer (model TR-PK-3-80; Trio Sciences Co. Ltd., Tokyo, Japan) with a plate temperature of 27 to 30°C . The freeze-dried samples were powdered using a blender for subsequent analysis.

Extraction and measurement of total anthocyanin. Powdered leaf samples (500 mg) had 10 mL of 0.5% H_2SO_4 solution added and were steeped overnight at room temperature. The crude extracts were then centrifuged for 15 min and filtered through a $0.2\text{-}\mu\text{m}$ filter membrane (Advantec, Tokyo, Japan) and the supernatant collected. The supernatant was diluted 4x with a McIlvaine's buffer solution (Hodgman, 1954) (consisting of citric acid and Na_2HPO_4) and the pH adjusted to 3.0 before the optical density was determined at 530 nm using a spectrophotometer (CS-9300PC; Shimadzu Co., Kyoto, Japan). A color value (CV) for the pigment extract, which is a commercial indicator of total anthocyanin, was calculated using the following formula: $\text{CV} = 0.1 \times \text{OD}_{530} \times 4 \times 20/\text{g dry weight (DW)}$, where OD_{530} is a spectrophotometric reading at 530 nm, 4 corrects for the dilution, and 20 g DW represents the dry weight of the sample (Shimizu and Nakamura, 1993).

Quantification of anthocyanin compositions by RP-HPLC. The crude extract was vacuum dried. The residue was redissolved in 15% acetic acid and filtered through a $0.2\text{-}\mu\text{m}$ filter membrane (Advantec, Tokyo, Japan) and used for anthocyanin identification and quality analysis. The HPLC analysis was performed according to the method of Odake et al. (1992) with slight modification using a liquid chromatograph (LC-10ADvp) with a detector (SPD-M10Avp), column oven (CTO-10Avp), and autosampler (SIL-10ADvp) (all from Shimadzu Co., Tokyo, Japan). Analytical HPLC was run on a $3\text{-}\mu\text{m}$, $100 \times 4.60\text{-mm}$ column [Luna C18(2); Phenomenex, Torrance, Calif.] at 35°C with $120\text{ kg}\cdot\text{cm}^{-2}$ pump pressure and monitored at 520 nm. The following solvents in water with a flow rate of $1\text{ mL}\cdot\text{min}^{-1}$ were used: A) 1.5% phosphoric acid, and B) 1.5% phosphoric acid, 20% acetic acid, 25% acetonitrile. The elution profile was a linear gradient elution for B of 15% to 50% during 90 min solvent A. The chromatograms were recorded and the relative concentration of pigments was calculated from the peak areas. In all samples, $20\text{ }\mu\text{L}$ of the leaf extract was injected. Identification of anthocyanins as carried out by comparing the peaks with authentic standard peaks of APL cell line and purple-fleshed sweetpotato anthocyanins named YGM. The anthocyanins were YGM-0a, [cyanidin 3-sophoroside-5-glucoside]; YGM-0b, [peonidin 3-sophoroside-5-glucoside]; YGM-1a, [cyanidin 3-(6,6'-caffeoyl-*p*-hydroxybenzoyl-

Table 1. Effect of Temperature on the Anthocyanin pigments (color value per gram dry weight) in leaves of different sweetpotato cultivars

Treatment temp ($^{\circ}\text{C}$)	Simon-1	Kyushu-119	Elegant Summer
20	$3.68 \pm 0.28\text{ a}^{\dagger}$	$16.75 \pm 2.27\text{ a}$	$23.12 \pm 1.34\text{ a}$
25	$2.84 \pm 0.23\text{ ab}$	$10.61 \pm 1.31\text{ b}$	$18.62 \pm 1.31\text{ b}$
30	$2.27 \pm 0.33\text{ b}$	$7.39 \pm 0.68\text{ b}$	$16.86 \pm 1.24\text{ b}$

[†]Each value is the mean of five replications $\pm$ standard error. Values within each column followed by the same letter are not significantly different ($p < 0.05$).

Table 2. Effect of Shading on the anthocyanin pigments (color value per gram dry weight) in leaves of different sweetpotato cultivars

Treatment (% shading)	Simon-1	Kyushu-119	Elegant Summer
0	$3.15 \pm 0.28\text{ a}^{\dagger}$	$12.95 \pm 0.65\text{ a}$	$19.16 \pm 1.24\text{ a}$
40	$2.32 \pm 0.14\text{ b}$	$7.05 \pm 0.34\text{ b}$	$10.61 \pm 1.25\text{ b}$
80	$2.04 \pm 0.13\text{ b}$	$4.19 \pm 0.35\text{ c}$	$4.26 \pm 0.30\text{ c}$

[†]Each value is the mean of five replications $\pm$ standard error. Values within each column followed by the same letter are not significantly different ($p < 0.05$).

sophoroside)-5-glucoside]; YGM-1b, [cyanidin 3-(6,6'-dicaffeoylsophoroside)-5-glucoside]; YGM-2, [cyanidin 3-(6-caffeoylsophoroside)-5-glucoside]; YGM-3, [cyanidin 3-(6,6'-caffeoylferuloylsophoroside)-5-glucoside]; YGM-4b, [peonidin 3-(6,6'-dicaffeoylsophoroside)-5-glucoside]; YGM-5a, [peonidin 3-(6,6'-caffeoyl-*p*-hydroxybenzoylsophoroside)-5-glucoside]; YGM-5b, [cyanidin 3-(6-caffeoylsophoroside)-5-glucoside] and YGM-6, [peonidin 3-(6,6'-caffeoylferuloylsophoroside)-5-glucoside] (Odake et al., 1992; Goda et al., 1997; Terahara et al., 1999, 2000). Other related YGMs (YGM-0c, -0d, -0e, -0f, and -0g) have been tentatively identified based upon their chemical structure, chemical analyses and mass measurement as *p*-hydroxybenzoylated (cyanidin 3-sophoroside-5-glucoside), caffeoylated (cyanidin 3-sophoroside-5-glucoside), *p*-hydroxybenzoylated (peonidin 3-sophoroside-5-glucoside), caffeoylated (peonidin 3-sophoroside-5-glucoside), and feruloylated (cyanidin 3-sophoroside-5-glucoside), respectively (Terahara et al., 2000; K-Islam et al., 2000, 2003). The chemical characteristics of the sweetpotato leaves anthocyanin are presented in Fig. 1.

Chemicals. All chemicals used were the highest grade available supplied by Sigma Chemical (St. Louis, Mo.), and Wako Pure Chemicals Industries Ltd., Osaka, Japan.

Statistics. A completely randomized design with five replications was used. Data are reported as mean SE. Where appropriate, treatment means were subjected to analysis of variance procedures and means separated by Duncan's multiple range test.

Results and Discussion

Effect of temperature and artificial shading on the total anthocyanin. The effect of temperature on the total anthocyanin content in the leaves is shown in Table 1. The anthocyanin content differed significantly ($P < 0.01$) among the cultivars studied. The leaves from 'ES' had the highest anthocyanin content followed by 'K-119' and 'S-1'. It was observed that moderate temperature (20°C) significantly increased

Anthocyanin	Aglycon	R1	R2	R3
YGM-0a	Cyanidin	H	H	H
YGM-0b	Peonidin	CH_3	H	H
YGM-0c	Cyanidin	H	PHB	H
YGM-0d	Cyanidin	H	Caf	H
YGM-0e	Peonidin	CH_3	PHB	H
YGM-0f	Peonidin	CH_3	Caf	H
YGM-0g	Cyanidin	H	Fer	H
YGM-1a	Cyanidin	H	PHB	Caf
YGM-1b	Cyanidin	H	Caf	Caf
YGM-2	Cyanidin	H	H	Caf
YGM-3	Cyanidin	H	Fer	Caf
YGM-4b	Peonidin	CH_3	Caf	Caf
YGM-5a	Peonidin	CH_3	PHB	Caf
YGM-5b	Peonidin	CH_3	H	Caf
YGM-6	Peonidin	CH_3	Fer	Caf

Fig. 1. Chemical structures and characteristics of the anthocyanin compositions in sweetpotato leaves.

YGM= Yamagawamurasaki, PHB= *p*-hydroxybenzoic acid, Caf= caffeic acid, Fer= ferulic acid

Fig. 2. Reverse-phase HPLC separation chromatograms for anthocyanin compositions in sweetpotato leaf extracts. The peak no. 1 is of YGM-0a, peak no. 2 is of YGM-0b, peak no. 3 is of YGM-0c, peak no. 4 is of YGM-0d, peak no. 5 is of YGM-0e, peak no. 6 is of YGM-0f, peak no. 8 is of YGM-0g, peak no. 10 is of YGM-1a, peak no. 11 is of YGM-1b, peak no. 12 is of YGM-2, peak no. 14 is of YGM-3, peak no. 15 is of YGM-4b, peak no. 16 is of YGM-5a, peak no. 17 is of YGM-5b and peak no. 18 is of YGM-6.

the total anthocyanin accumulation in sweetpotato leaves of all the cultivars as compared to higher temperatures (i.e., 25 and 30 °C), but there were no difference between the treatment 25 and 30 °C. Faragher (1983) reported that the optimum temperature for anthocyanin accumulation in ripe apple skin is 16 to 24 °C, and the maximum anthocyanin production in strawberry ('Shikinari') was obtained at 20 °C (Zhang et al., 1997). The present results indicate that moderate temperatures enhanced synthesis of sweetpotato leaves anthocyanin content. Plants grown in full sun (i.e., 0% shading) displayed a significantly higher accumulation of total anthocyanins followed by 40% and 80% shading in all cultivars except 'S-1' (Table 2). The decrease in anthocyanin content in the leaves grown under shaded conditions is indicative of the importance of light in anthocyanin synthesis. Moriyama et al. (1999) reported in tea shoot that artificial shading (i.e., 60% and 80%) decreased the total polyphenol content compared to 0% shading. Therefore sweetpotatoes grown under moderate temperatures and without shading accumulated higher leaf anthocyanin than those grown at higher temperatures and shaded conditions. The anthocyanins are ubiquitous bioactive compounds found in plant food and beverages and have received increased attention because of their potential antioxidant activities that may exert cardioprotective effects in humans (Furuta et al., 1998; Suda et al., 1998; Yoshimoto et al., 1999, 2001). Therefore sweetpotato leaves represent one of the richest and most commonly available sources of anthocyanin among fruits and vegetables.

Anthocyanin compositions and their effect on temperature and shading. Reversed-phase HPLC analysis identified fifteen different anthocyanin compositions in sweetpotato leaves (Fig. 2). All HPLC profiles of the cultivars tested showed the peaks at the same retention times and there were quantitative but no qualitative difference among the cultivars. Quantitative differences in leaf anthocyanins were due to cultivars, temperature and shading. Anthocyanin YGM-1a is of highest concentration. Quantitatively they were found in the following order is YGM-1a> YGM-4b> YGM-1b> YGM-5a> YGM-0d> YGM-0a> YGM-2>YGM-0c>YGM-3>YGM-6>YGM-

Fig. 3. Effect of temperatures on the different anthocyanin compositions in the leaves of three sweetpotato cultivars. Each mean is based on n = 5 and the vertical bars = SE. YGM = Yamagawamurasaki.

Fig. 4. Effect of artificial shadings on the different anthocyanin compositions in the leaves of three sweetpotato cultivars. Each mean is based on $n = 5$ and the vertical bars = SE. YGM = Yamagawamurasaki.

5b>YGM-0b>YGM-0f>YGM-0e>YGM-0g. Four of the anthocyanins (YGM-0c, -0d, -0e, -0f) found in the leaves differed from those in the storage root but were identical to the APL cell line (Terahara et al., 2000; K-Islam et al., 2000). The pigments from early peak i.e., YGM-0a, YGM-0b, YGM-0c, YGM-0d, YGM-0e, YGM-0f and YGM-0g made up about 25% (average of the three cultivars at various treatment) of the total anthocyanin as calculated by peak area. All three cultivars exhibited almost the same trends of changes in anthocyanin compositions in their leaves with temperature treatments (Fig. 3). YGM-1a, YGM-1b, YGM-4b, and YGM-5a were the dominant anthocyanins at each temperature treatment and in each cultivar. The content of YGM-0a, YGM-0d and YGM-5a increased with increasing temperature. Shading also influenced the anthocyanin compositions of the sweetpotato leaves (Fig. 4). All the cultivars had their highest content of anthocyanin

compositions from the leaves grown under full sun i.e., 0% shading as compared with 40% and 80% shading. Like temperature responses, the YGM-1a, YGM-1b, YGM-4b, and YGM-5a are also the key anthocyanin under shading treatments in all the cultivars. Majority of the anthocyanin were decreased with the intensity of shading, but the YGM-0a, YGM-0c and YGM-2 were increased with more shaded conditions. The result of the present study indicates that the main anthocyanin of sweetpotato leaf extract was YGM-1a followed by YGM-4b, YGM-1b and YGM-5a. Only a few of YGM-0g, YGM-0e, YGM-0f, YGM-0b and YGM-5b pigments were detected on the chromatogram of the leaf extract, and they were present in a low amount. The sweetpotato leaves anthocyanins are classified into two groups based on the chemical structure of aromatic B-ring: YGM-0a YGM-0c, YGM-0d, YGM-0g, YGM-1a, YGM-1b, YGM-2 and YGM-3 are cyanidin derivatives, and YGM-0b, YGM-0e,

YGM-0f, YGM-4b, YGM-5a and YGM-6 are peonidin (methylated cyanidin) derivatives. Cyanidin is methylated to peonidin. Therefore, the anthocyanins of sweetpotato leaves can be evaluated with respect to the proportion of peonidin to cyanidin. Several authors indicated that cyanidin is physiologically more active (Yoshimoto et al., 1999; 2001) than pelargonidin- and delphinidin-based anthocyanins and its derivatives showed a relatively stronger antimutagenic and antioxidative activity than peonidin (Furuta et al., 1998; Yoshimoto et al., 1999).

The anthocyanins are ubiquitous bioactive compounds found in plant food and beverages, and has received increased attention because of their potential antioxidant activities that may exert cardioprotective effects (Furuta et al., 1998; Knekt et al., 1996; Suda et al., 1998; Yoshimoto et al., 1999), antidiabetic effects (Matsui et al., 2001a, 2001b, 2002) and anticancer effects (K-Islam et al., 2003). The result suggests that sweetpotato leaves contains at least fifteen different anthocyanins, which were almost identical with the anthocyanin from APL cell line. Though the production of anthocyanin from cell line is expensive and also required high technical knowledge, therefore sweetpotato leaves, which is very easy to grow with low cost serve as a potential source of anthocyanin pigment(s) of desired purposes. Furthermore, the results also indicate that sweetpotato leaves grown under moderate temperature with full sun enhanced the synthesis of anthocyanin compositions. Similar results were also found in case of polyphenolics accumulation of sweetpotato leaves in relation to shading and temperature treatments (Islam et al., 2003b). Sweetpotato leaf anthocyanins will be more important in future processed products because it makes products colorful, and is beneficial for human health when used as a food additive or the leaves consumed as a vegetable. The results may be useful for various chemical breeding programs designed to improving desired organoleptic and nutritional quality characteristics of crop plants and products useful for food colorant industries.

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