J. Environ. Eng. Manage., 19(1), 21-27 (2009)

BIOMARKERS OF GREEN ROOF VEGETATION: ANTHOCYANIN AND CHLOROPHYLL AS STRESS MARKER PIGMENTS FOR PLANT STRESSES OF ROOF ENVIRONMENTS

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Key Words: *Sedum*, green roof, biomarker, anthocyanin, chlorosis

ABSTRACT

Sedum plants are broadly utilized in green roofs. To establish biomarkers for stress evaluation of green roof vegetation, anthocyanin and chlorophyll contents in *Sedum* plants grown on the green roof were examined. Total anthocyanin content in shoots was 12- to 17-fold higher in the stressed group than in the less-stressed group of *Sedum acre* L., *S. makinoi* Maxim., and *S. uniflorum* Hook. et Arnot subsp. *Japonicum* (Sieb. ex Miq.) H. Oba. The stressed group of another *Sedum* species, *S. polytrichoides* Hemsl. subsp. *yabeanum* (Makino) var. *setouchiense* (Murata et Yuasa) H. Oba. accumulated anthocyanin only 3 times as much as the less-stressed one. Total chlorophyll content was reduced in the stressed group of *S. acre*, *S. uniflorum* and *S. polytrichoides*, but not in *S. makinoi*. Chlorophyll *a*/*b* ratio was lower in the stressed group of *S. makinoi* and *S. uniflorum*, but remained the same level in *S. acre* and *S. polytrichoides*. The results indicate that anthocyanin accumulation and chlorosis can be plant biomarkers for rooftop environmental stresses. Difference in anthocyanin accumulation and chlorosis among species suggests that the rooftop environmental stress consists of multiple stress factors and each species possess different sensitivities to each stress factor.

INTRODUCTION

Roof greening is a widely recognized environmental friendly technology that moderates the urban heat island and contributes to energy conservation of buildings [1]. Green roofs also play more roles such as protection the roof materials resulting in a long life span, sound insulation, amenity space, filtration of airborne particles, temporal delay of storm water runoff, and ecological function as “stepping stones” or “island” habitats that connect natural habitats of birds and insects [2]. On the other hand, the vegetation on roofs are suffered from severe rooftop environmental factors that may include heat, drought, high light, ultraviolet irradiation, temperature difference and etc. However, actual stress factors suffering the plants on rooftops have not been understood at all.

Roof vegetation technology on such severe rooftop conditions has been developed in many aspects, mostly in construction engineering, such as low weight, high water holding capacity and low cost planting materials. Characterization and evaluation of rooftop environmental stresses from which plants were suffered has not been well examined, most likely due to lack of suitable biomarkers. Biomarkers that indicate plant stresses on a roof have not been examined, while biomarkers are beneficial to assess the stresses and impacts to organisms, such as pollutions, environmental quality, pharmaceuticals and medical examinations [3-9]. Establishment of biomarkers is a prerequisite to characterize rooftop stress factors and hence is advantageous for green roof horticultural technologies.

Plants belonging to the genus *Sedum* possess the CAM metabolism that allows the plants to survive in the condition with very high or low temperatures, and extremely low humidity, such as a desert or on-rocks. As a matter of fact, *Sedum acre* L. and *S. mexicanum* Britt are widely utilized for green roofs. In a previous study, we proposed usage of domestic and endangered *Sedum* plants for the rooftop vegetation, instead of naturalized species [10].

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In this study, accumulation of anthocyanin and reduction of chlorophyll content (chlorosis) in shoots of several *Sedum* plants grown on a green roof were studied, aiming at development of simple colorimetric biomarkers of rooftop environmental stresses to the vegetation.

MATERIALS AND METHODS

1. Green Roof and Sampling of Plants

Sedum species used in this study were *S. acre* L., *S. makinoi* Maxim., *S. polytrichoides* Hemsl. subsp. *yabeanum* (Makino) var. *setouchiense* (Murata et Yuasa) H. Oba, and *S. uniflorum* Hook. et Arnot subsp. *japonicum* (Sieb. ex Miq.) H. Oba. These plants naturally distribute in Japan as domestic plants, except *S. acre* L. that was a naturalized plant to Japan and had been globally used in typical green roofs. All plants, originally collected from the natural habitat in the Okayama prefecture, Japan, were grown on a green roof settled on the Research Institute for Bioresources, Okayama University, Kurashiki, Japan (34°35'2" N, 133°46'2" E) from April to September in 2007 (Fig. 1). In April 2007, nursery plants of those species were planted on matted soil (Ekuseru Soil, 5 cm thickness, Minoru Sangyou, Akaiwa-shi, Japan) with Styrofoam basements and were watered daily before the dawn with 100% water holding capacity of the soils. Vegetations were fertilized every 3 weeks with the granular fertilizer, Nagaoka Yuuki No. 4 (approximately 20 g m^{-2}; Nagaoka Kasei Co., Amagasaki, Japan).

Shoots of *Sedum* plants grown on the building roof were collected three times independently over a period from August to September in 2007. Reddish strongly stressed and green less-stressed plants were collected separately to compare the biomarkers between two groups. Since a biomarker for green roof environments had not been established, stressed and less-stressed plants were chosen from the green roof by eye due to degree of wilting, color (red or green) and size of plants. Average of surface temperatures of the vegetation and the adjacent bare concrete during the experimental period were 27.1 and 32.1 °C, respectively. Highest and lowest temperatures were 50.5 and 15.0 °C for the vegetation and 56.5 and 15.5 °C for the bare concrete surface, respectively. The average atmospheric temperature during sampling period at close proximity (34°35'4" N, 133°46'1" E) in the recent 10 years (1998-2007) and in year 2007 were 26.3 and 27.6 °C, respectively, suggesting the summer in the experimental year is not extremely different from an ordinal year. The average solar radiation during the examination period was 17.7 MJ m^{-2} in August and 13.8 MJ m^{-2} in September in 2007, which was comparable to the average of recent 10 years: 15.6 and 14.0 MJ m^{-2} for August and September, respectively. Maximum solar radiation was 21.6 MJ m^{-2} during the

Fig. 1. *Sedum* species grown on the rooftop of the study. A part of green roof vegetation of the Research Institute for Bioresources is shown. The most near plants are *S. polytrichoides* Hemsl. subsp. *yabeanum* (Makino) var. *setouchiense* (Murata et Yuasa) H. Oba. The second and third lanes from this side are *S. sarmentosum* Bunge and *S. lineare* Thunb. (not used in this study). The most distant plants are *S. acre* L. *S. makinoi* Maxim. and S. *uniflorum* Hook. et Arnot subsp. *Japonicum* (Sieb. ex Miq.) H. Oba are planted on the left-hand side of the shown vegetation (not seen on the photo).

examination. The average wind speed during the examination was 1.8 m s^{-1}, which was comparable to the average in the recent 10 years (1.6), and the maximum wind speed was 8.0 m s^{-1}.

2. Determination of Anthocyanin Content

Extraction and determination of total anthocyanin content was carried out essentially the same as described [11]. In brief, 1 g of shoots of *Sedum* plants was homogenized in 5 mL of methanol containing 1.0 N HCl and kept at 4 °C for 4 h, followed by clearing with a centrifugation and a filtration. Absorbance of the supernatant was measured at 530 nm with a spectrophotometer (UV-160, Shimadzu Corporation, Kyoto). Anthocyanin content was expressed as optical density (OD_{530}) per fresh weight (FW, g) of the tissue. Detection limit of the extracted antocyanin in the solution with the method was 0.13 OD_{530} g^{-1} FW.

3. Determination of Chlorophyll Content

Excised shoots were weighed and cut into approximately 5 mm length, followed by extraction of chlorophyll with 10-fold weight of *N,N*-dimethylformamide at 4 °C for 2 d in the dark with intermittent gentle agitations. Chlorophyll content in the *N,N*-dimethylforamide solution was quantified spectrophotometrically (UV-160, Shimadzu Corporation, Kyoto) after a filtration through 30 μm nylon mesh.

Wavelength and extinction coefficients for chlorophyll *a* and *b* are described elsewhere [12]. Chlorophyll contents were standardized due to fresh tissue weight. Detection limit of chlorophyll *a* and *b* with this method was 0.13 and 0.037 μg g^{-1}, respectively.

RESULTS AND DISCUSSION

1. Accumulation of Anthocyanin

Contents of anthocyanin in the excised shoot of the rooftop-grown *Sedum* plants were determined in two groups, which were distinguishable by apparent stress levels, as described in Materials and Methods; i.e. strongly-stressed and less-stressed.

As expected, absorbance at 530 nm of extracts from the reddish stressed *Sedum* group was higher than that from the less-stressed group, indicating anthocyanins were accumulated in the stressed group (Fig. 2). Anthocyanin consists of a group of pigments that have red to blue color predominantly depending on pH. In order to simplify the measurement of red coloration of *Sedum* species, we employed measurement of absorption at 530 nm. Determination of total anthocyanin content using absorption at 530 nm has been carried out by numbers of researches [11,13-17]. In this study, chemical species of anthocyanins of *Sedum* species were not identified. It should be also noticed that absorbance at 530 nm does not necessarily mean free of contamination of other reddish pigments in the extracted sample.

S. polytrichoides plants even in the less-stressed group showed red stems and relatively dark-colored leaves. Ones in the stressed group had strongly red stems and leaves. Content of anthocyanin in “less-stressed” *S. polytrichoides* shoots was 1.6- to 3.0-fold higher than that in the other 3 species (Fig. 2, Student’s t-test, $P < 0.05$, n = 3). This might indicate that *S. polytrichoides* is more sensitive to a rooftop stress factor(s), and thus exploits the stress response under a weak stress such that other plants do not response to. Note that high sensitivity to the stress does not necessarily indicate less tolerance of the plant to the stress and vice versa; i.e. an early stress response possibly save plants from the condition, oppositely the plants possibly exhibit emergency response even in a relatively weak stress.

In the stressed *S. polytrichoides* plants, anthocyanin contents were increased 3 times as much as the less-stressed plants (Fig. 2, $P < 0.01$, n = 3). The achieved anthocyanin level was not significantly different from that in stressed *S. makinoi* and *S. uniflorum* ($P > 0.05$, n = 3). This observation supports the hypothesis that *S. polytrichoides* is more sensitive to the stresses. Most likely it does not have ability of constitutive anthocyanin accumulation. *S. polytrichoides* did not show visible red coloration, when it was cultured in a glass house (OD_{530} g^{-1} FW = 2.65, n = 2).

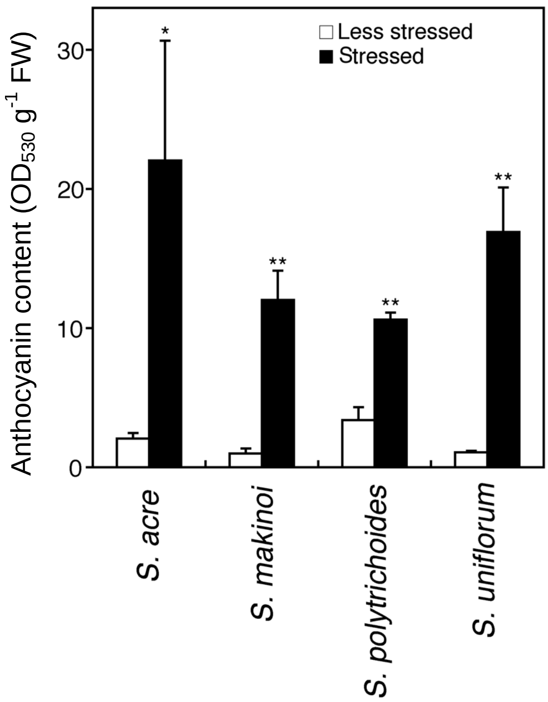

Fig. 2. Anthocyanin content of stressed and less-stressed *Sedum* plants on green roof.
Total anthocyanin was extracted from 4 *Sedum* species, *S. acre* L., *S. makinoi* Maxim., *S. polytrichoides* Hemsl. subsp. *yabeanum* (Makino) var. *setouchiense* (Murata et Yuasa) H. Oba and *S. uniflorum* Hook. et Arnot subsp. *Japonicum* (Sieb. ex Miq.) H. Oba, which were grown and maintained on the roof, as described in Materials and Methods. White and black bars represent less-stressed and stressed groups. Error bars indicate standard errors of the mean (n = 3). Single and double asterisks indicate significant difference at $P < 0.05$ and $P < 0.01$, respectively, between stressed and less-stressed groups.

Shoots of stressed groups of *S. acre*, *S. makinoi* and *S. uniflorum* contained 12- to 17-fold anthocyanin than that of less-stressed groups (Fig. 2, $P < 0.01$, n = 3), indicating that some rooftop stresses induced anthocyanin accumulation in *Sedums* on the roof. Similar pigmentation was not observed in a glass house (OD_{530} g^{-1} FW = 1.38 and 2.15 for *S. makinoi* and *S. uniflorum*, respectively). It should be noted that not the entire vegetation colored evenly on the roof. This indicates that the rooftop stress was not uniform even on the same roof, might due to micro environmental divergence.

Myriads of abiotic stresses have been reported to induce anthocyanin accumulation, such as high light [15], wounding and the wound-relating plant hormone, methyl jasmonate [13,18], ultraviolet A and B [14,17,19,20], blue light [21], cold [11,22], drought and the drought-relating plant hormone, abscisic acid [23,24], and nitrogen starvation [25]. Among those stresses, cold stress should not be considered to affect on the anthocyanin accumulation in this study as the experiment was done in a high temperature season (See Materials and Methods). Besides summer, in winter the *Sedum* plants used in this study exhibit a high extent of anthocyanin accumulation that may due to a cold stress on the same green roof (data not shown). Identification of each stress factor that induced anthocyanin accumulation would be a future task. As seen in the ultraviolet stress, sensitivity to light quality as well as quantity can be diverse in specie to specie [14,17,19]. Utilization of a series of plants that possess different sensitivities to each stress factors might allow us to predict the stress factors in detail.

The extraction and determination method of anthocyanin employed in this study was very simple (See Materials and Methods). Less than 1 g plant sample is enough to examine. The result suggests that identification of chemical species of anthocyanin using a more complicated chromatography technique may not be essential in order to estimate plant stress. Examination of total anthocyanin using a simple colorimetric analysis may serve as a useful method to evaluate the plant stress response to a rooftop environment at a field research.

2. Chlorosis

Chlorosis is the term that means insufficient chlorophyll accumulation in plants. As a stress can induce chlorosis, we examined total chlorophyll content and chlorophyll *a*/*b* ratio in the shoot of the same plants from which anthocyanin was extracted (Fig. 3).

Total chlorophyll content of *S. polytrichoides* decreased in the stressed plants. On the other hand chlorophyll *a*/*b* ratio did not change significantly (Student's t-test, $P > 0.05$, $n = 3$). Essentially the same result was observed in *S. acre*. This indicates possible similarity of stress sensitivity between *S. polytrichoides* and *S. acre*. We have proposed substitution of naturalized species with domestic species in green roofs [10]. According to chlorosis (this study) and transpiration property [10], *S. polytrichoides* is a good candidate taking the place of *S. acre* in green roof in the Setonaikai area of Japan. Total chlorophyll content of the stressed *S. makinoi* was not significantly different from less-stressed one ($P > 0.05$, $n = 3$), while all the other species showed chlorosis in this study. However, ratio of chlorophyll *a*/*b* decreased significantly in *S. makinoi* ($P < 0.01$, $n = 3$). Uniquely, in *S. uniflorum* both total chlorophyll content and *a*/*b* ratio decreased. Interestingly, the response of chlorophyll was not uniform even within the same genus. Each plant grew and was maintained on the same green roof for 6 months in a side-by-side manner (a few meter distance, Fig. 1), and was sampled at almost the same timing. The stress suffering the plants was supposed essentially the same, although the response of chlorophyll contents was divergent. This may be caused from existence of multiple rooftop stress factors in green roof, and each plant possesses different sensitivity to each stress factor.

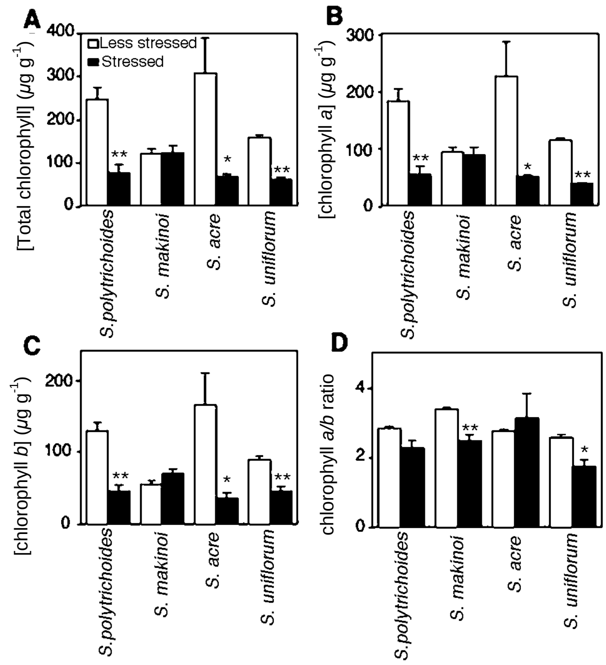

Fig. 3. Chlorosis in stressed and less-stressed *Sedum* plants on green roof.
Four *Sedum* species were examined: *Sedum polytrichoides* Hemsl. subsp. *yabeanum* (Makino) var. *setouchiense* (Murata et Yuasa) H. Oba, *S. makinoi* Maxim., *Sedum acre* L, and *S. uniflorum* Hook. et Arnot subsp. *Japonicum* (Sieb. ex Miq.) H. Oba. (A), Total chlorophyll contents; (B), Chlorophyll *a*; (C), Chlorophyll *b*; (D) Chlorophyll *a*/*b* ratio. White and black bars represent less-stressed and stressed groups. Error bars indicate standard errors of the mean ($n = 3$). Single and double asterisks indicate significant difference at $P < 0.05$ and $P < 0.01$, respectively, between stressed and less-stressed groups.

Chlorosis is induced by low temperature and iron deficiency [26,27]. It can also be explained by ozone, excess metals, oxidative stress, dehydration and macronutrient deficiency that might occur in thin layer soil [23,28-30]. Viral and fungal infection can be other reasons for chlorosis [31,32]. Low temperature may not be the reason of chlorosis in this study as mentioned above. Drought and associated decrease of macro- and micro-nutrition bioavailability might be chlorosis-inducing factors. Which environmental stress factor that actually induced chlorosis is still an open question. Further study may allow predicting what stress is the main factor at a green roof.

Chlorophyll *a*/*b* ratio is reported to decrease under a dim light and high temperature [33,34]. Endo et al. [35] reported a decrease of chlorophyll *a*/*b* ratio of *Zoysia matrella* (L.) Merr at the edge of the vegetation settlement on a thin layer green roof, indicating that a stress of the rooftop environment induces a reduction of the *a*/*b* ratio in plants. Their results suggested a correlation of chlorophyll *a*/*b* ratio decrease with high temperature stress [35]. In this study we showed that the stressed *S. makinoi* and *S. uniflorum* showed decreased chlorophyll *a*/*b* ratio. High temperature may be the reason of the change in chlorophyll *a*/*b* ratio on the rooftop also in our study.

3. Biomarkers for Rooftop Environments and Diverse Roof Stress

Rooftop environments have been described by physical parameters, such as temperature, humidity, light intensity, wind speed, soil moisture and heat balance. Only few studies have described characteristics of rooftop environment in terms of stresses on plants, while it is essential for the green roofing technology. Currently, selection of plant species and method of rooftop vegetation maintenance are carried out based on experience of horticultural engineers.

Even on the same rooftop, plant stress response appeared differently at least in terms of chlorosis and chlorophyll *a*/*b* ratio. It may be due to variation in stress factor in a few meter distances on the same roof, i.e. edge of the roof, near the prominence of building or the distance from the irrigation device. Such few-meter-level local environmental difference is actually an issue for maintaining healthy vegetations on rooftop. Assessment of a meter-level environmental stress variation using bioindicators that we proposed in this study would serve clues for a strategic design of green roofs.

Factors of the rooftop environment are attributed to extreme temperature, high light, ultraviolet irradiation, nutrient deficiency and/or drought. It may also be attributed to other factors: salinity, metal stress, airborne pollutants and etc. Anthocyanin accumulation and chlorosis could behave differently in some cases indicating that plants were suffered from multiple stresses that affected on anthocyanin accumulation or chlorosis. Sensitivity of plants to the same factor may be different among species. These pigment markers provide a method for fine characterization of rooftop stress factors by utilizing several appropriate plant species. An advantage of those pigment analyses is their low cost and the simple principle. Comparative studies with bio-molecular makers (gene expression) would allow a finer description of nature of rooftop stresses.

This research is a new field of plant ecological physiology and also a border science between plant physiology/phytochemistry and urban environmental science/micrometeorology, which focused on the rooftop environment. We consider that the approach of this study is one of frontier researches in roof greening studies.

SUMMARY

Anthocyanin and chlorophyll contents in four roof-top grown *Sedum* species were examined to demonstrate possibility of those natural phytopigments as biomarker of roof vegetation stresses. Stressed plants showed accumulation of anthocyanin. Chlorosis was observed in three species, while decrease of chlorophyll *a*/*b* ratio was observed in two species. The results indicate that anthocyanin and chlorophyll contents are possible biomarker of environmental stress to roof-vegetations, and plants were suffered from multiple stress factors.

ACKNOWLEDGEMENTS

This research was supported by grants from The Ohara Foundation to I.M and S.U, and to the roof greening project of the Research Institute for Bioresources, Okayama University.

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Discussions of this paper may appear in the discussion section of a future issue. All discussions should be submitted to the Editor-in-Chief within six months of publication.

Manuscript Received: April 25, 2008
Revision Received: November 13, 2008
and Accepted: November 15, 2008