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Purple Coloration in Leaves of Seagrass, *Halophila* (Hydrocharitaceae)

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ABSTRACT

Purple coloration in leaves of *Halophila* from Merambong shoal of Sungai Pulai estuary, Johore and Merchang, Terengganu, Malaysia were investigated using a simple detection method based on chemical properties of anthocyanin that changes from red in acids to blue in bases. In addition the presence of anthocyanin was examined for the absorption maxima at wavelengths between 505 to 535 nm and phycoerythrin absorption maxima at wavelengths 496, 540 and 656 nm. Based on responses of color change in acid and base, *Halophila* from Merambong shoal (green big-leaved *Halophila ovalis*, green small-leaved *Halophila ovalis*, green-leaved *Halophila* sp.) lacked anthocyanins. Purple-tinged-leaved *Halophila* sp. from Merambong, purple-blotched-leaved *Halophila* sp. from Merchang and purple-tinged-leaved *Halophila* sp. (explant from Merambong shoal) from culture tank showed dramatic changes from purple-tinged or-blotched to blue-green tinged or -blotched respectively in 2 M NaOH or reddish coloration appeared exactly on blotches and tinges of leaves after dipping in 2 M HCL. The results for the latter three seagrasses agreed with the response of anthocyanins in base (NaOH) or acid (HCL) and the presence of anthocyanin was further confirmed with the results of absorption spectra maxima obtained at 535 nm. The detection of phycoerythrin with absorption maxima at 496, 540 and 656 nm in the purple leaves *Halophila* indicated that they lack this pigment. Seaweed e.g., *Jania* sp. showed positive detection for phycoerythrin with absorption maxima at 495 and 565 nm.

Key words: Anthocyanin, *Halophila*, phycoerythrin, purple coloration, seagrass

INTRODUCTION

Blue, red and purple pigmentation always occurred in terrestrial plants such as bromeliads, carnivorous plants and berries. Similarly, several seagrasses also possessed pigments: reddish-brown bands on *Cymodocea serrulata* (Lam *et al.*, 2004) and purplish tinges on *Halophila* (Den Hartog, 1970; Hillman *et al.*, 1995). Abal *et al.* (1994) also reported the presence of anthocyanin-like pigmentation on the intertidal leaves of *Zostera capricorni* and *Halophila ovalis* in Moreton Bay.

Plant species possess pigments adaptations that differ considerably in their ability to tolerate UV radiation (Tevini and Teramura, 1989). The majority of research involving the effect of UV radiation on plants has been focused on terrestrial plants. In general, plant sensitive to UV-B radiation exhibit various adaptive mechanisms which include epidermal blistering and deformation, increased leaf thickness, reduced leaf area (Cline and Salisbury, 1966; Jackson, 1987; Barnes *et al.*, 1990) as well as the production of UV-absorbing compounds such a UV-blocking pigments and anthocyanins (Tevini *et al.*, 1981; Lovelock *et al.*, 1992). Additionally, the work of Robberecht and Caldwell (1978) on terrestrial angiosperms has shown epidermal transmission of UV-B to be less than 10% while flavonoids and related pigments were reported to account for a large percentage of attenuation, providing a UV screen for the underlying tissue. Besides, the observations of Trocine *et al.* (1981) suggested anthocyanin or other flavonoids synthesis as an adaptation of seagrasses to long term UV-B radiation. In a recent survey of the world's six seagrass bioregions (Novak and Short, 2010) documented leaf reddening in 12 seagrass species from intertidal and shallow subtidal waters at 25 locations in the tropical Atlantic and tropical Indo-Pacific Oceans including additional observations of seagrasses with reddened leaves from Australia. The phenomenon is now documented in 15 seagrass species at 29 locations worldwide. Similar to terrestrial angiosperms, leaf reddening in seagrasses may relate to enhanced production and accumulation of anthocyanins, water soluble flavonoid pigments (Lee and Gould, 2002) after exposure to one or more stressors (Novak and Short, 2010). In this present study, the purple coloration in leaves of *Halophila* in two areas; Merambong shoal, Johore and Merchang, Terengganu, Malaysia were examined and compared with the normal green leaves of the same species and two other seaweeds.

MATERIALS AND METHODS

Plant material: Seagrasses and seaweeds were collected randomly during low spring tides in May and August 2008 at Merambong shoal (1°19' 00" N, 103°36' 45" E) of Sungai Pulai estuary, Johore, Malaysia. Four *Halophila* based on leaf coloration were collected: purple-leaved *Halophila* sp., green-leaved *Halophila* sp., green small-leaved *Halophila ovalis* and green big-leaved *Halophila ovalis* (determined based on number of paired cross veins and leaves sizes-characteristics of *Halophila* as reported by Den Hartog (1970). Purple-tinged leaved *Halophila* sp., green-leaved *Halophila* sp. and green small-leaved *Halophila ovalis* were collected from pure patches while green big-leaved *Halophila ovalis* was under the shade of *Enhalus acoroides*. Two other samples namely purple-blotched *Halophila* sp. from Merchang lagoon (5°02' 15.0" N, 103°17' 53.0" E) and purple-tinged *Halophila* sp. plucked directly from our *Halophila* (explant from Merambong shoal) culture tank were also included for pigmentation analyses. For comparison purposes seaweeds, *Gracilaria* sp. and *Jania* sp. were also collected from Merambong shoal. Samples collected were washed in a sieve, rinsed in seawater, placed in plastic bags and kept in ice chest before transporting to laboratory.

Pigment identification: Based on the blue, red and purple color in plants similar to the coloration in seagrasses, anthocyanins are suggested to play role on the coloration, thus experiments were performed following the chemical properties of anthocyanin that changes from red in acids to blue in bases (Harbone, 1984). Hence seagrass leaves for all *Halophila* collected were dipped in 2 M NaOH and 2 M HCl to detect any color changes. As a comparison, similar procedure was conducted for two red seaweeds from the division of Rhodophyta.

In order to reconfirm the presence of anthocyanin, 1 g of leaves of *Halophila* sp. (from Merambong shoal, Merchang lagoon, culture tank-explant from Merambong shoal) was ground in

1% HCl in absolute methanol. The total extract was centrifuged and the absorbance of the supernatant was analysed by spectrophotometer (Shimadzu model UV-160A) to obtain absorption spectrum to detect any absorption maxima at specific wavelengths (Harbone, 1984).

Phycoerythrin detection: Beside anthocyanins, phycoerythrin also changes from red in acids to blue in bases. Therefore, phycoerythrin detection was also included in this study. The method for pigment detection was modified from Dawes (1981) whereby 1 g of leaves of *Halophila* sp. (from Merambong shoal, Merchang lagoon, culture tank-explant from Merambong shoal) was ground in 0.1 M cold phosphate buffer (pH 6.5) using mortar and pestle. The homogenate was centrifuged at 6000 rpm 10 min at 4°C. The absorbance of the supernatant was measured by spectrophotometer (Shimadzu model UV-160A) to detect absorption maxima peaks at specific wavelengths (Dawes, 1981). As a comparison, similar procedure and measurement were performed on a red algae, *Jania* sp. from the division of Rhodophyta.

Data analysis: The presence of anthocyanins in seagrass and seaweed were confirmed with detection of absorption maxima at wavelengths between 505 to 535 nm (Harbone, 1984) obtained from the absorption spectrum produced after analysis through the spectrophotometer (Shimadzu model UV-160A).

The presence of phycoerythrin in seagrass and seaweed were confirmed with detection of absorption maxima at wavelengths 496, 540 and 565 nm (Dawes, 1981) obtained from the absorption spectrum produced after analysis through the spectrophotometer (Shimadzu model UV-160A).

RESULTS AND DISCUSSION

Pigment identification: Seagrasses (green big-leaved *Halophila* sp., green small-leaved *H. ovalis*, green-leaved *Halophila* sp.) leaves dipped in base (Table 1, Fig. 1) showed slight change

Table 1: Response of seagrasses and seaweeds to acid and base media

	Observation on color change after dipping in:	
	2 M NaOH	2 M HCl
Seagrass		
Merambong shoal, Sungai Pulai, Johore		
Halophila sp. (purple-tinged-leaved)	Purple tinges (Fig. 1a) to blue-green tinges (Fig. 1b)	Purple tinges (Fig. 1c) to purple-red (purple-tinges (Fig. 1d)
Halophila sp. (green-leaved)	Green to bright green	Color remained green (Color unchanged)
Halophila ovalis (green big-leaved)	Green to bright green	Color remained green (Color unchanged)
Halophila ovalis (green small-leaved)	Green to bright green	Color remained green (Color unchanged)
Merchang lagoon, Merchang, Terengganu		
Halophila sp. (purple blotched-leaved)	Purple blotches (Fig. 1e) to purple-blue blotches (Fig. 1f)	Purple blotches (Fig. 1g) to purple-red blotches (Fig. 1h)
Culture tank (explant from Merambong shoal		
Halophila sp. (purple-tinged-leaved)	Purple tinges (Fig. 1i) to blue-green tinges (Fig. 1j)	Purple-tinges (Fig. 1k) to purple-red tinges (Fig. 1l)
Seaweed		
Merambong shoal, Sungai Pulai, Johore		
Gracilaria sp.	Purple (Fig. 1m) to greenish (Fig. 1n)	Purple to purple color (Fig. 1o, p)
Jania sp.	Red (Fig. 1q) to green (Fig. 1r)	Red (Fig. 1s) to purple-red (Fig. 1t)

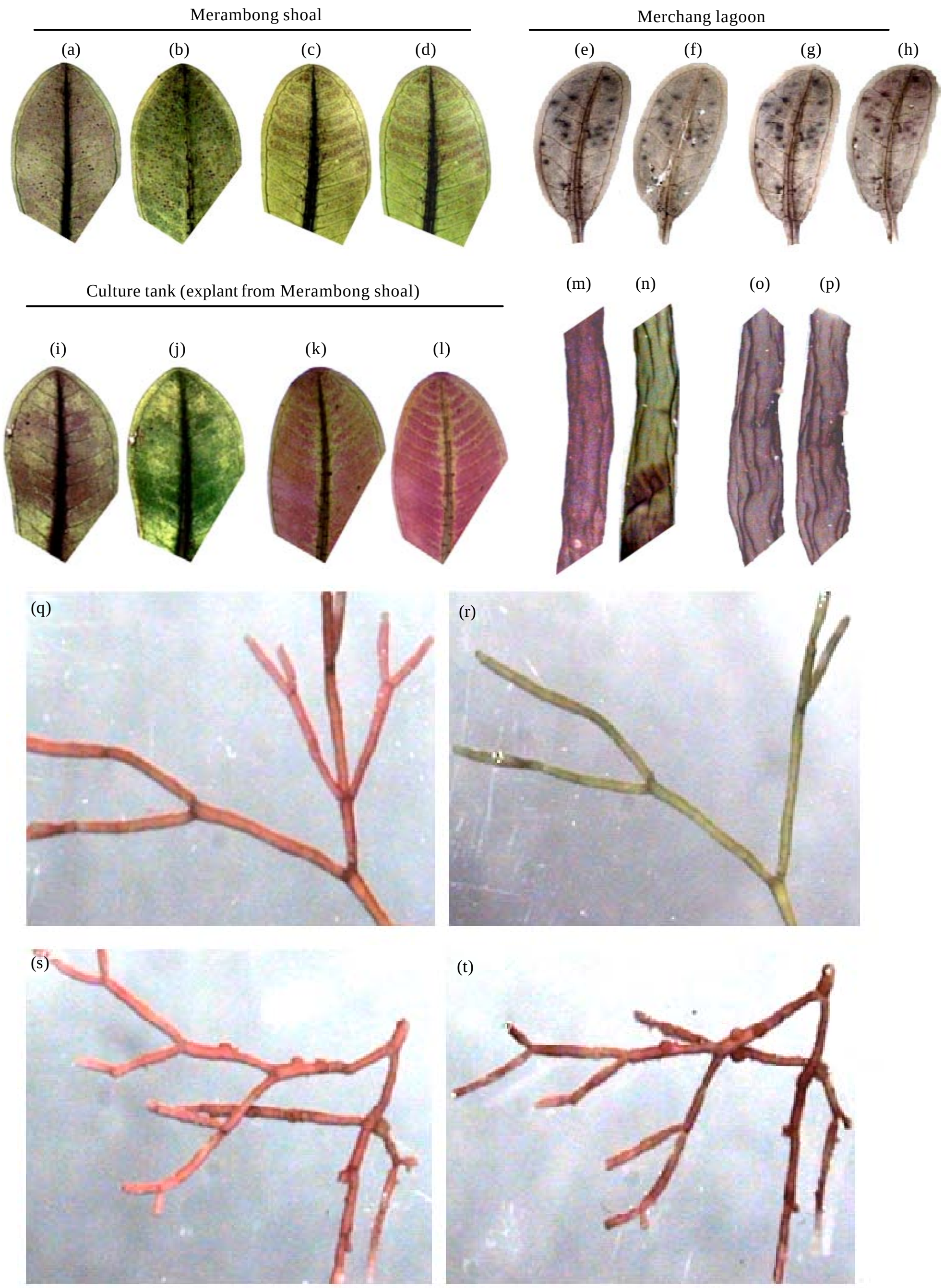

Fig. 1(a-t): Color changes in leaves of *Halophila*; Purple-tinged-leaved (a) Before and (b) After 2 M NaOH and (c) Before and (d) After 2 M HCl, Purple-blotched-leaved (e) Before and (f) After 2 M NaOH and (g) Before and (h) After 2 M HCl, Purple-tinged-leaved (i) Before and (j) After 2 M NaOH and (k) Before and (l) After 2 M HCl, *Gracilaria* sp.; (m) Before and (n) After 2 M NaOH and (o) Before and (p) After 2 M HCl and *Jania* sp. (q) Before and (r) After 2 M NaOH and (s) Before and (t) After 2 M HCl of color from green to bright green. Purple-tinged-leaved *Halophila* sp. from Merambong, purple-blotched-leaved *Halophila* sp. from Merchang and purple-tinged-leaved *Halophila* sp. from culture tank showed dramatic changes from purple-tinged or -blotched to blue green-tinged or-blotched respectively in base (2 M NaOH, Fig. 1a, b, e, f, i, j). The results for the latter three seagrasses agreed with the Harbone (1984) response of anthocyanin in base (NaOH).

Seagrasses (green big-leaved *Halophila* sp., green small-leaved *H. ovalis*, green-leaved *Halophila* sp.) leaves dipped in acid (2 M HCl), showed the leaf color remained the same or unchanged. For purple-tinged-leaved *Halophila* sp. from Merambong, purple-blotched-leaved *Halophila* sp. from Merchang and purple-tinged-leaved *Halophila* sp. from culture tank, reddish coloration appeared exactly on blotches and tinges of leaves after dipping in 2 M HCl (Fig. 1c, d, g, h, k, l). The color changes response partially agreed with Harbone (1984) where anthocyanin is stable in acid (2 M HCl). Based on observations in acid and base, the pattern of the purple coloration can be present in two forms: tinges (evenly distributed in leaves) or blotches (clumped or clustered together in leaves). The clustering of purple coloration may be a response to high intensities of visible radiation in Merchang (intertidal lagoon). Similar clumping of coloration (pigmentation) was observed in *Halophila stipulacea* (Drew, 1979; Trocine *et al*., 1981). However, leaf tissues with such clumping appeared pale but are not photosynthetically inhibited according to Drew (1979). This implied that seagrasses inhabiting marine environment are affected by physiological conditions which are considerably different from those experienced by freshwater plants and land plants. *Halophila* leaves possessing purple coloration, red blotches or spots is a form of response for protection of *Halophila* to direct exposure to strong sun-light during the low tides. It is believed that blotches are anthocyanin pigments (Low *et al*., 2005) and serves as UV-blocking pigments. Novak and Short (2010) documented leaf reddening in 12 seagrass species from intertidal and shallow subtidal waters at 25 locations in the tropical Atlantic and tropical Indo-Pacific Oceans including additional observations of seagrasses with reddened leaves from Australia. Leaf reddening in seagrasses may relate to enhanced production and accumulation of anthocyanins, water soluble flavonoid pigments (Lee and Gould, 2002) after exposure to one or more stressors (Novak and Short, 2010).

As for the seaweed samples, the color changes were detected for both *Gracilaria* sp. (Fig. 1m-p) and *Jania* sp. (Fig. 1q-t) for the presence of phycoerythrins which give the reddish coloration. The results obtained suggest phycoerythrins are unstable in base (NaOH, Fig. 1m, n, q, r) and stable in acid (HCl, Fig. 1o, p, s, t), thus responsible for the changes of colors. There is limited information concerning the flavonoids on seaweeds and most studies on seaweeds focused on the chlorophylls, carotenoids and phycobilins, therefore, further research into the effects of UV radiation on marine plants could be directed towards investigating their responses and their pigments compositions and distribution.

The absorption spectrum, e.g., given here for purple-tinged-leaved *Halophila* sp. from Merambong shoal (Fig. 2) showed the presence of a peak at 535 nm. Based on the reference of Harbone (1984), anthocyanins in Methanol-HCl extract have absorption maxima between the wavelengths of 505 to 535 nm; hence, the peak could be identified as anthocyanin. This suggests the presence of anthocyanin although at present, the flavonoid content of seagrasses was not specifically examined. Nevertheless, the survey conducted by Robberecht and Caldwell (1978) on 25 species of plants, in which the epidermis attenuated 95 to 99% of ultraviolet radiation, flavonoids and related pigments were reported to account for a large percentage of attenuation. Additionally, the studies of Tevini *et al*. (1981); Lovelock *et al*. (1992) revealed that the primary adaptive

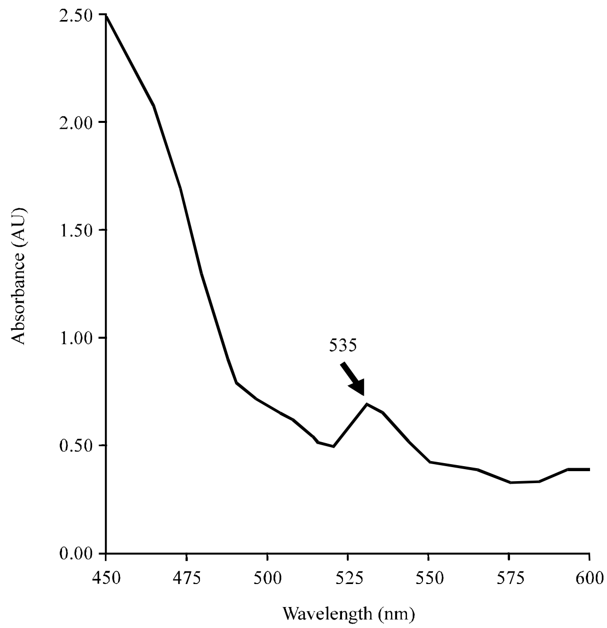

Fig. 2: Absorption spectrum of purple extract from seagrass, purple-tinged-leaved *Halophila* sp. from Merambong shoal

mechanisms employed by plants to cope with increased levels of UV radiation are by the production of UV-absorbing compounds such as UV-blocking pigments and anthocyanins, the accumulation of these compounds occur predominantly in the epidermis, providing a UV screen for underlying tissue. Similarly, Murali and Teramura (1985) reported that plants deficient in phosphorus were sensitive to UV-B irradiation with the accumulation of flavonoids in response to nutrient deficiency and increased plants' tolerance to UV-radiation. In addition, since calcareous, saline soils are particularly prone to phosphorus deficiency and the present sampling site, Merambong shoal comprised substratum characterized by sandy with calcareous fragments of shells (Japar *et al.*, 1996), hence, the purple-tinged-leaved *Halophila* sp. may accumulate UV-blocking pigments in response to their nutrient deficiency status, whereby increasing UV-tolerance and hence increasing their survival when exposed to the sun during low tides.

Phycoerythrin detection: The absorption spectrum for seagrass (e.g., purple-tinged-leaved *Halophila* sp. from Merambong shoal, Fig. 2) showed almost a linear line instead of peak after 500 nm (Fig. 3), while for seaweed (e.g., *Jania* sp.), the absorption spectrum (Fig. 4) showed two peaks at 495 and 565 nm. This indicated the absence of phycoerythrin in purple-tinged-leaved *Halophila* sp. For *Jania* sp., the two peaks at 495 nm and 565 nm showed the characteristic of phycoerythrin. Dawes (1981) stated phycoerythrin peaks occurred at 496, 540 and 565 nm although the first peak varied slightly from the wavelength as noted by MacColl and Guard-Friar (1987). Additionally, phycoerythrin, a class of phycobilins (Sharma, 1986) is commonly found in seaweeds. Based on our previous reports, *Halophila* mentioned in this study from Merambong shoal, possessed chlorophyll a, b and carotenoids (Low *et al.*, 2005). Dennison (1990) reported seagrasses do not contain chlorophyll c and d, though epiphytes growing on seagrass leaves may contain the phycobilins. Similarly, several previous studies focused mainly on the chlorophyll a, chlorophyll b, carotenoids of seagrasses and ignored the phycobilins (Drew, 1979;

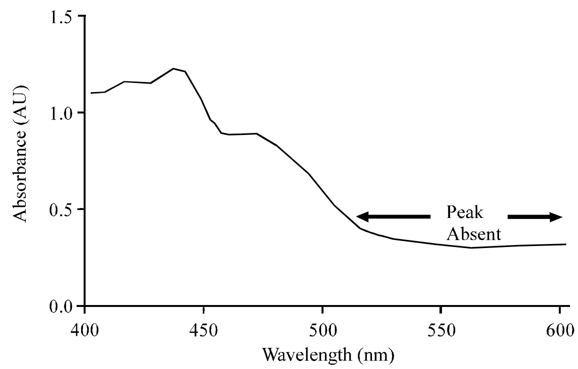

Fig. 3: Absorption spectrum of purple extract from purple-tinged-leaved *Halophila* sp. from Merambong shoal

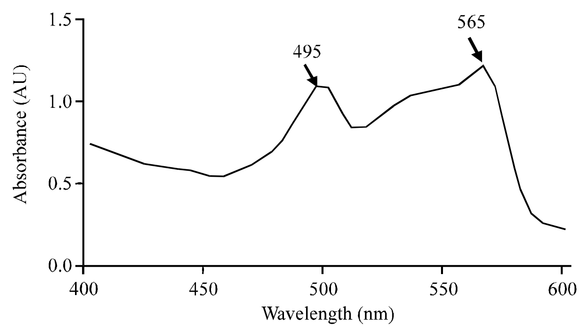

Fig. 4: Absorption spectrum of extract from seaweed, *Jania* species

Wiginton and McMillan, 1979; Jimenez *et al*., 1987) may suggest the absence of this particular pigment since phycobilins are the light-harvesting pigments commonly present in seaweeds besides the chlorophylls and carotenoids (Sharma, 1986).

CONCLUSION

The presence of anthocyanins in seagrasses can be detected easily by employing the chemical properties of anthocyanin that changes from red in acids to blue in bases. Beside anthocyanins, phycoerythrin also changes from red in acids to blue in bases. Absorption maxima between 505 to 535 nm for anthocyanins and at 496, 540 and 565 nm for phycoerythrin obtained from absorption spectrum produced after analysis through the spectrophotometer can be used to confirm the two pigments. This present study suggests the purple coloration in purple-leaved seagrasses is attributed to the presence of anthocyanin and not phycoerythrin. The coloration can be distributed in leaves in two forms, tinges and blotches; the former is evenly distributed while the latter may be due to the clustering of pigments in response to high intensities of visible radiation.

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