

RESEARCH ARTICLE

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Sand supplementation favors tropical seagrass *Thalassia hemprichii* in eutrophic bay: implications for seagrass restoration and management

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Abstract

Background: Sediment is crucial for the unique marine angiosperm seagrass growth and successful restoration. Sediment modification induced by eutrophication also exacerbates seagrass decline and reduces plantation and transplantation survival rates. However, we lack information regarding the influence of sediment on seagrass photosynthesis and the metabolics, especially regarding the key secondary metabolic flavone. Meanwhile, sulfation of flavonoids in seagrass may mitigate sulfide intrusion, but limited evidence is available.

Results: We cultured the seagrass *Thalassia hemprichii* under controlled laboratory conditions in three sediment types by combining different ratios of in-situ eutrophic sediment and coarse beach sand. We examined the effects of beach sand mixed with natural eutrophic sediments on seagrass using photobiology, metabolomics and isotope labelling approaches. Seagrasses grown in eutrophic sediments mixed with beach sand exhibited significantly higher photosynthetic activity, with a larger relative maximum electron transport rate and minimum saturating irradiance. Simultaneously, considerably greater belowground amino acid and flavonoid concentrations were observed to counteract anoxic stress in eutrophic sediments without mixed beach sand. This led to more positive belowground stable sulfur isotope ratios in eutrophic sediments with a lower Eh.

Conclusions: These results indicated that coarse beach sand indirectly enhanced photosynthesis in *T. hemprichii* by reducing sulfide intrusion with lower amino acid and flavonoid concentrations. This could explain why *T. hemprichii* often grows better on coarse sand substrates. Therefore, it is imperative to consider adding beach sand to sediments to improve the environmental conditions for seagrass and restore seagrass in eutrophic ecosystems.

Keywords: Eutrophic sediment, Sandy sediment, Seagrass, Photosynthesis, Metabolomics, Stable sulfur isotope

Introduction

Seagrasses are marine ecosystem engineers that provide important ecological services including nutrient uptake, carbon sequestration, food and habitat for marine animals, and shoreline and sediment stabilization [1–3]. However, global climate change and sustained pressures from coastal development, including dredging and eutrophication (nutrient enrichment), have weakened

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the capacity of seagrass meadows to support coastal productivity [4, 5]. Eutrophication affects the structure of primary producers in seagrass beds [6–8], modifying sediment origin, grain size and nutrient availability [7, 9]. An increase in sediment clay and silt fractions and high organic matter content might lead to anoxic conditions [7]. Sediment anoxia inhibits respiration and other metabolic functions in seagrass roots, resulting in reduced photosynthesis, leaf number, and the shoot-to-root ratio [10–12]. Meanwhile, sediment nutrient toxicity might induce an imbalanced carbon-nitrogen ratio due to increased carbon demand [13]. Elevated nutrient levels, respiration, and anoxic conditions also enhance sediment sulphide concentrations [14]. This causes sulphide intrusion in seagrasses, as assessed by stable sulphur isotope signals, leading to adverse effects [14]. Sulphide intruding into seagrass interferes with cytochromes in the electron transport chain, leading to a negative energy balance, which eventually results in seagrass mortality [10, 15]. To date, physiological indicators have largely failed to monitor seagrass health and prevent its decline [16]. The alarming decline highlights the urgent need to implement effective seagrass management strategies to prevent habitat decline [17].

Recently, omics-based systems biology (transcriptomics, proteomics and metabolomics) has emerged as a new frontier in seagrass research and has deepened our understanding of their stress tolerance mechanisms and accurately identified biomarkers of their phenotypic plasticity to environmental stress [18, 19]. Metabolomics has been instrumental in connecting the genotype and phenotype of vascular plants under adverse environmental conditions, and has been applied in seagrass research [19–21] providing new insights into diverse cellular pathways to identify stress tolerance biomarkers. Much is known about the effect of environmental stress on the primary metabolites of seagrass [22, 23] and the total content of key secondary metabolites [24]. However, little is known about the response of key secondary metabolite compositions by applying targeted metabolomics techniques.

The seagrass *Thalassia hemprichii* is a dominant tropical species, growing mainly in sandy sediment or coral substrate [25, 26]. Over the past decade, nutrient inputs into seagrass beds in Xincun Bay, Hainan Island, South China Sea, have increased immensely, leading to high eutrophication [4]. Cage farming and shrimp pond cultures produce large quantities of food debris, which modifies the sediment particle sizes [4]. The sediment particle size decreased from coarse to fine. Hypoxic conditions in sediments occur frequently, and the emergence of red tides has been observed in these areas [27]. Overall, these adverse environmental conditions have induced

a decline in seagrass beds, resulting in an approximately 50 ha loss [28]. Interestingly, according to our continuous observations, *T. hemprichii* occurrence in Xincun Bay has declined dramatically, especially in the high intertidal zones. Moreover, we observed a relatively low success rate in transplanting and restoring *T. hemprichii* in this bay (personal observation). This failure might be attributed to the desiccation exposure during low tide and sediment composition (mud vs. sand) [29]. Nevertheless, limited studies have considered the effect of sediment type on seagrass physiology [22], especially flavonoids, which are the key secondary metabolites. Flavonoids have been implicated in plant resistance to many stress factors [30]. The ecological plant strategy theory indicates that stressed plants containing high levels of protective flavonoids tend to show low levels of constitutive productivity [30, 31]. Sulfation of flavonoids in seagrass might also mitigate the sulphide intrusion [32], but limited evidence is available.

Therefore, it is imperative to investigate the effect of sediment type on the physiological responses of the dominant tropical seagrass, *T. hemprichii*. We performed a laboratory manipulative experiment by growing *T. hemprichii* under three sediment types (by combining different ratios of in-situ eutrophic sediment and coarse beach sand) and assessed its growth performance by evaluating photosynthetic performance, flavonoid and amino acid profiling, and stable sulfur isotope and elemental composition analysis. Measurements of seagrass photosynthesis, nitrogen and amino acids contents were used to evaluate the plant growth, whereas measurements of $\delta^{34}\text{S}$ and flavonoids were used to assess the extent of sulphide intrusion in seagrass and the role of flavonoids in mitigating sulphide intrusion, respectively. We examined leaf fluorescence parameters to assess the continuous photosynthetic characteristics of seagrasses in the same leaf in a non-destructive manner [33, 34], without disturbing sediments. The results obtained in this study provide new insights that will aid in understanding the mechanisms controlling seagrass physiological responses to sediment types. This information is critical for strengthening knowledge on improving the success rate of seagrass planting and transplantation in eutrophic coastal areas or in the process of eutrophication and to implementing effective seagrass management strategies to prevent their decline.

Results

Sediment physiochemical parameters

The Eh in the sediment type of 1:0, 1:1 and 1:2 were -177.0 ± 29.4 mV, -148.7 ± 24.2 mV and -53.3 ± 17.1 mV, respectively, and the corresponding sediment sulphur contents were $(0.020 \pm 0.001)\%$,

Table 1 Sediment physiochemical parameters at the end of the experiment

Sediment type	Eh (mV)	S (%)	Organic matter (%)
1:0	-177.0 ± 29.4 ^a	0.020 ± 0.001 ^c	1.14 ± 0.09 ^b
1:1	-148.7 ± 24.2 ^a	0.011 ± 0.001 ^b	0.75 ± 0.22 ^a
1:2	-53.3 ± 17.1 ^b	0.006 ± 0.002 ^a	0.58 ± 0.04 ^a

The different lower case letters indicated significant differences among treatments

(0.011 ± 0.001)%, and (0.006 ± 0.002)%, respectively (Table 1). Meanwhile, sediment organic matter also exhibited a decreasing trend with increasing sediment particle size.

Photosynthesis

The effects of sediment type on photosynthetic parameters at the two stages were depicted in Fig. 1. No significant difference was observed in Y (II) (effective quantum yield) on days 6 and 21 (at the end of the experiment) (Additional file 1). Indeed, the differences among treatments on day 6 were not significant, considering the relative maximum electron transport rate ($rETR_{max}$), minimum saturating irradiance (Ek_{ETR}) and initial slope of the light-limited relationship (α_{ETR}). $rETR_{max}$ and Ek_{ETR} were slightly higher in sediments with larger particle size. However, markedly difference was found at day 21 for both $rETR_{max}$ and Ek_{ETR} , with much higher values in the sediment with larger particle sizes (Fig. 1).

Seagrass nitrogen and compositions of amino acids and flavonoids

Among the amino acids, proline, sarcosine and lysine were the three main components in the aboveground tissue of *T. hemprichii*, whereas sarcosine, proline and asparagic acid were the three main components in the belowground tissue. The amino acid content in the aboveground tissue was lower than that in the belowground tissue in the sediment 1:0 type, whereas similar concentrations were observed between aboveground and belowground tissue for *T. hemprichii* in both 1:1 and 1:2 sediment types. Significant effects were observed for 11 of the 20 amino acids in the aboveground tissue, whereas effects were observed in 18 amino acids in belowground tissue. Amino acid contents in both above- and belowground tissues in the 1:0 treatment were significantly higher than those in the 1:1 and 1:2 treatments. Sarcosine, proline and alanine in both above- and belowground tissues also showed the same trend. The nitrogen content in the aboveground tissue was significantly higher in the larger

sediment particle sizes, whereas the ratio of amino acids to nitrogen in the same tissue showed a contrasting trend (Fig. 2, Table 2, and Table 4).

Among the flavonoids, galuteolin, luteolin and isoquercitrin were the three most abundant components in the aboveground tissues of *T. hemprichii* in the three sediment types. For belowground tissue, catechin, isoquercitrin, and epicatechin were the three major components in the sediment 1:0 type, whereas catechin, isoquercitrin and luteolin were the leading three components in the sediment 1:1 and 1:2 types. Flavonoid concentrations in the aboveground tissue were lower than those in the belowground tissue in sediment 1:0 and 1:1 types, whereas similar concentrations were observed between above- and belowground tissue for *T. hemprichii* in the 1:2 sediment type. Flavonoids in both above- and belowground tissues were higher in sediment type of 1:0 than in 1:1 and 1:2 sediment types (Table 3 and Table 4).

The relationships between amino acids and flavonoids in the above- and belowground tissues were significantly positive (Fig. 3). Meanwhile, linear regression tests were performed between the concentrations of total flavonoid and amino acid and sediment sand composition. The results showed that the amino acids in both above- and belowground tissues and total flavonoids in the belowground tissue were significantly negatively correlated with the sediment sand composition (Table 5).

$\delta^{34}S$ content

The effects of sediment type on the $\delta^{34}S$ content in the belowground tissue of *T. hemprichii* were depicted in Fig. 4. A significant difference was observed in the $\delta^{34}S$ content, with higher values in the belowground tissue in the sediment with smaller particle sizes.

Discussion

Decreased sediment particle size induced by increased inputs of fish food debris undoubtedly leads to anoxic conditions. Seagrass growth and survival may be constrained by anoxic sediment conditions. Anoxia is regarded as one of the most harmful factors for plants because of the accumulation of toxic end products [23]. Furthermore, sulphide toxicity is considered one of the main contributing factors to the global decline of seagrass beds [32]. Under such circumstances, seagrass photosynthesis would be directly affected and could regulate responses through changes in primary and secondary metabolites. To the best of our knowledge, this was the first report on the response of seagrass secondary metabolic to environmental stress using targeted metabolomics.

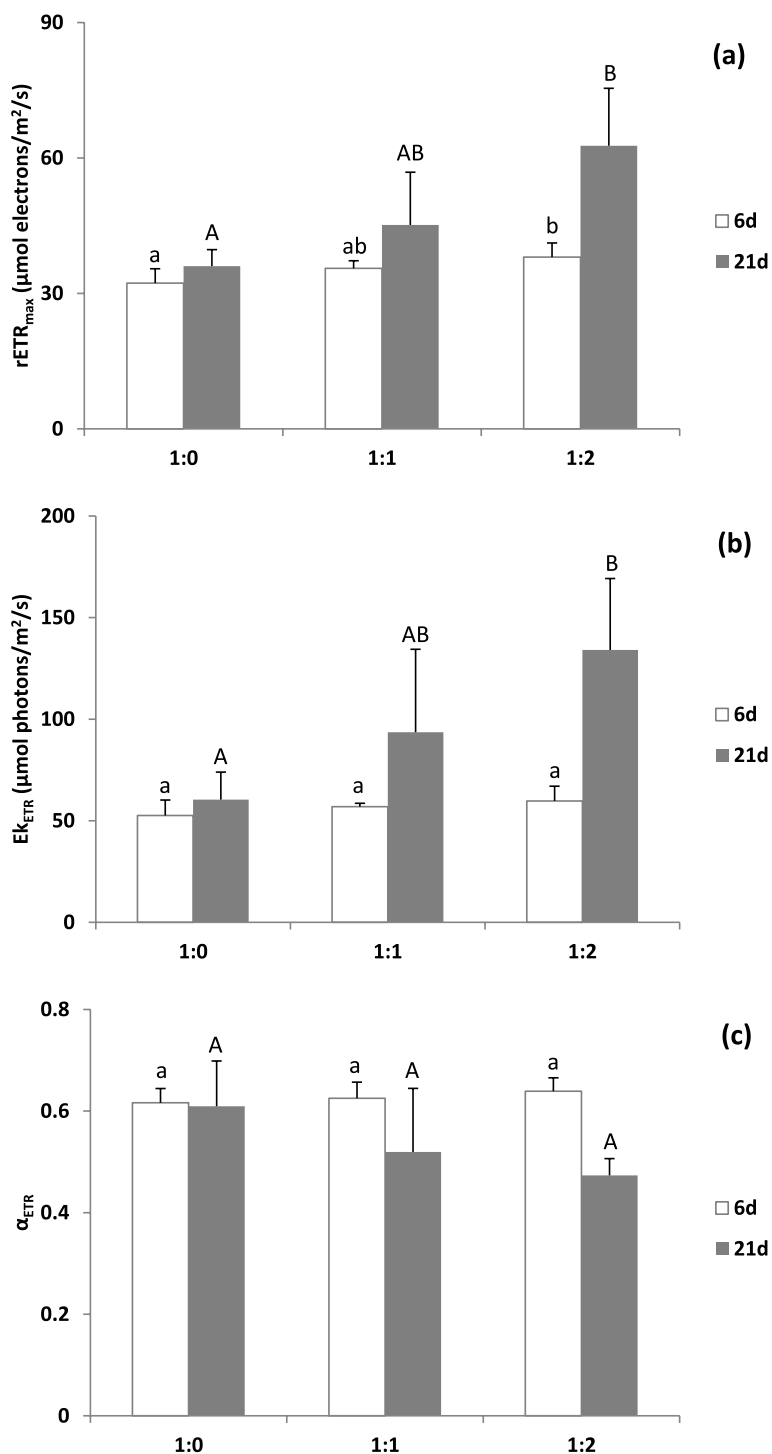


Fig. 1 Photosynthetic parameters of *Thalassia hemprichii* including $rETR_{max}$ (the relative maximum electron transport rate, **a**), $E_{k_{ETR}}$ (the minimum saturating irradiance, **b**) and α_{ETR} (the initial slope of the light limited relationship, **c**) derived from rapid light curve cultured in different sediment types. The lowercase and uppercase letters indicate significant difference at day 6 and day 21, respectively ($P < 0.05$). 1:0, the in-situ sediment without combining with coarse beach sand was added in the tank; 1:1, the combination of half in-situ sediment and half coarse beach sand was added in the tank; 1:2, the combination of 1/3 in-situ sediment and 2/3 coarse beach sand was added in the tank

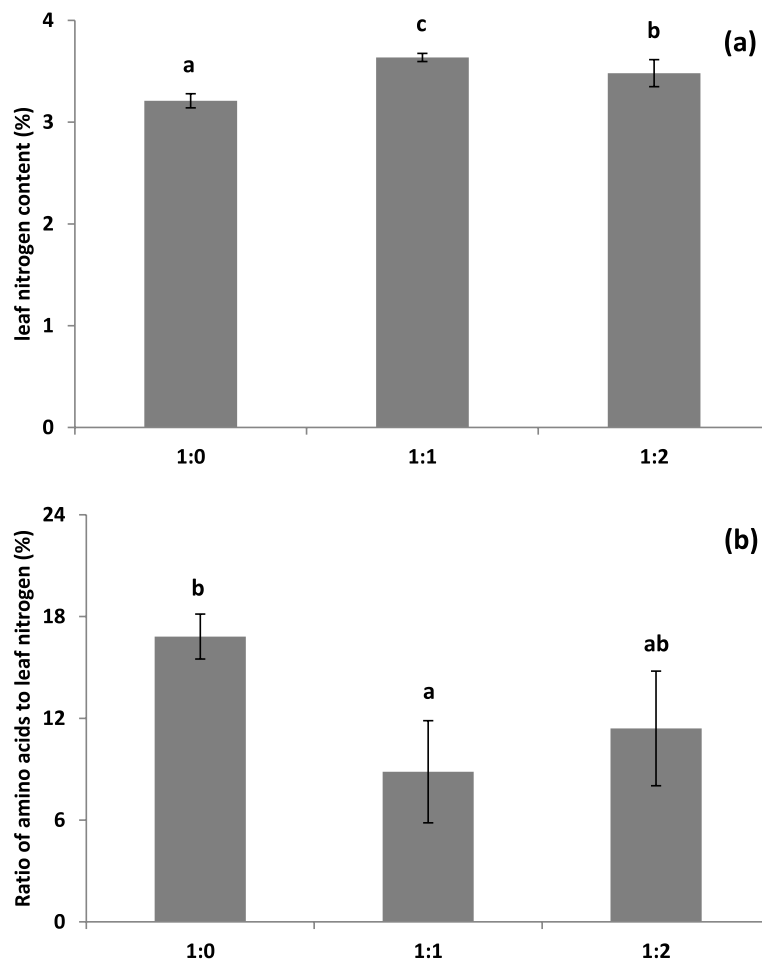


Fig. 2 Effect of sediment type on leaf nitrogen (a) and the ratio of amino acids to nitrogen (b) in seagrass *Thalassia hemprichii*. Different letters on column indicate significant difference ($P < 0.05$). 1:0, the in-situ sediment without combining with coarse beach sand was added in the tank; 1:1, the combination of half in-situ sediment and half coarse beach sand was added in the tank; 1:2, the combination of 1/3 in-situ sediment and 2/3 coarse beach sand was added in the tank

Seagrass photosynthesis was indirectly enhanced by adding coarse beach sand

Seagrass-sediment interactions are dynamic [7]. The present results showed that $Y (II)$ was not significantly different at either stage among the treatments, implying that this parameter was not a good indicator of stress. Meanwhile, there was almost little change in the parameters of the rapid light curve on day 6 among the treatments, indicating that this effect was not obvious at the initial stage. However, a noticeable enhancement in $rETR_{max}$ and Ek_{ETR} was observed on day 21 (at the end of the experiment) under the coarse beach sand addition treatment, suggesting the ability to transfer more electrons and larger energy investment for CO_2 fixation, scavenging reactive oxygen species [35], photosystem photoprotection [36], nitrogen assimilation and redox signalling pathways [37] in *T. hemprichii*. Similarly, a higher sand

composition induced a higher Eh, indicating that coarse beach sand addition increased sediment porosity, therefore benefiting oxygen permeation. This favourable condition might benefit seagrass growth by enhancing photosynthesis. Conversely, higher photosynthesis also induced a positive effect on the sediment redox potential [38]. High photosynthesis resulted in greater pools of O_2 in belowground tissue, enhancing the radial O_2 loss and the oxic shield [39, 40]. Furthermore, O_2 consumption by seagrass roots increased with increasing shoot-to-root mass ratio, which was dominated by root mass and disrupted by sulphide [41]. The effect of the sediment might show a specific difference. Some seagrass species such as *Zostera marina* and *Cymodocea nodosa* showed greater tolerance to reducing conditions in sediments than *T. hemprichii* [23]. Muddy sediments might be more favourable for *Z. marina* than sandy sediments, although they

Table 2 Effects of sediment type on the amino acids in the aboveground and belowground tissues of seagrass *Thalassia hemprichii*. 1:0, the in-situ sediment without combining with coarse beach sand was added in the tank; 1:1, the combination of half in-situ sediment and half coarse beach sand was added in the tank; 1:2, the combination of 1/3 in-situ sediment and 2/3 coarse beach sand was added in the tank

Amino acid	Aboveground tissue						Belowground tissue					
	1:0		1:1		1:2		1:0		1:1		1:2	
	content (µg/g)	ratio (%)	content (µg/g)	ratio (%)	content (µg/g)	ratio (%)	content (µg/g)	ratio (%)	content (µg/g)	ratio (%)	content (µg/g)	ratio (%)
glycine	38.2 ± 5.6 ^a	0.71	17.9 ± 4.2 ^b	0.56	21.4 ± 5.9 ^b	0.54	125.8 ± 22.5 ^A	1.32	27.7 ± 4.1 ^B	0.80	36.1 ± 1.9 ^B	0.92
sarcosine	927.1 ± 194.8 ^a	17.18	182.8 ± 98.5 ^b	5.69	457.9 ± 286.4 ^b	11.57	2393.1 ± 618.4 ^A	25.15	305.0 ± 38.6 ^B	8.83	511.8 ± 254.0 ^B	13.07
alanine	296.4 ± 40.0 ^a	5.49	74.7 ± 31.4 ^b	2.33	155.0 ± 100.4 ^b	3.91	605.9 ± 120.9 ^A	6.37	103.0 ± 7.3 ^B	2.98	149.2 ± 45.9 ^B	3.81
valine	93.2 ± 17.9 ^a	1.73	27.9 ± 13.9 ^b	0.87	51.0 ± 21.1 ^b	1.29	147.2 ± 9.0 ^A	1.55	31.4 ± 1.7 ^B	0.91	35.4 ± 10.3 ^B	0.90
proline	1234.0 ± 54.6 ^a	22.87	860.8 ± 267.9 ^a	26.81	979.6 ± 201.1 ^a	24.74	1532.4 ± 225.5 ^A	16.11	711.5 ± 44.9 ^C	20.60	930.2 ± 65.2 ^B	23.75
threonine	55.5 ± 4.0 ^a	1.03	37.3 ± 14.4 ^a	1.16	39.0 ± 13.8 ^a	0.98	288.4 ± 39.8 ^A	3.03	92.6 ± 10.8 ^B	2.68	65.3 ± 5.1 ^C	1.67
isoleucine	41.9 ± 9.5 ^a	0.78	11.6 ± 6.2 ^b	0.36	22.8 ± 8.2 ^b	0.58	62.6 ± 11.3 ^A	0.66	12.2 ± 1.5 ^B	0.35	13.2 ± 3.2 ^B	0.34
leucine	32.9 ± 6.9 ^a	0.61	5.5 ± 2.1 ^b	0.17	15.3 ± 9.6 ^b	0.39	25.3 ± 5.3 ^A	0.27	4.8 ± 1.0 ^B	0.14	6.2 ± 0.8 ^B	0.16
ornithine	59.4 ± 4.1 ^a	1.10	53.5 ± 15.5 ^a	1.67	52.2 ± 6.6 ^a	1.32	208.6 ± 17.0 ^A	2.19	115.8 ± 8.1 ^B	3.35	99.6 ± 19.5 ^B	2.54
methionine	2.5 ± 0.5 ^a	0.05	1.2 ± 0.1 ^b	0.04	1.2 ± 0.1 ^b	0.03	12.7 ± 0.9 ^A	0.13	2.1 ± 0.3 ^B	0.06	1.8 ± 0.4 ^B	0.05
histidine	20.3 ± 5.4 ^a	0.38	12.4 ± 1.1 ^a	0.39	21.1 ± 5.8 ^a	0.53	49.8 ± 5.1 ^A	0.52	13.3 ± 2.3 ^B	0.39	11.4 ± 1.8 ^B	0.29
phenylalanine	23.6 ± 5.9 ^a	0.44	6.8 ± 2.0 ^b	0.21	12.9 ± 7.6 ^{ab}	0.33	13.4 ± 4.4 ^A	0.14	3.7 ± 0.9 ^B	0.11	3.8 ± 0.7 ^B	0.10
arginine	14.6 ± 1.7 ^a	0.27	6.7 ± 1.1 ^b	0.21	9.7 ± 4.5 ^{ab}	0.25	116.9 ± 74.2 ^A	1.23	221.7 ± 27.2 ^B	6.42	329.8 ± 27.7 ^B	8.42
tyrosine	16.5 ± 3.1 ^a	0.31	5.9 ± 1.8 ^b	0.19	10.5 ± 4.3 ^{ab}	0.27	20.1 ± 2.5 ^A	0.21	4.0 ± 1.3 ^B	0.12	3.8 ± 0.6 ^B	0.10
asparagic acid	350.5 ± 62.3 ^a	6.50	339.5 ± 92.3 ^a	10.57	299.4 ± 16.4 ^a	7.56	1224.5 ± 86.9 ^A	12.87	729.9 ± 49.4 ^B	21.14	618.1 ± 117.4 ^B	15.78
tryptophan	409.0 ± 23.2 ^a	7.58	350.4 ± 114.9 ^a	10.91	378.1 ± 47.6 ^a	9.55	443.7 ± 192.7 ^A	4.66	229.4 ± 24.7 ^{AB}	6.64	200.0 ± 60.3 ^B	5.11
4-aminobutyric acid	555.5 ± 57.8 ^a	10.29	182.0 ± 98.9 ^b	5.67	334.2 ± 206.1 ^{ab}	8.44	792.5 ± 112.1 ^A	8.33	162.9 ± 47.3 ^B	4.72	287.5 ± 51.2 ^B	7.34
serine	73.7 ± 4.9 ^a	1.36	47.1 ± 8.7 ^b	1.47	69.6 ± 16.8 ^{ab}	1.76	176.9 ± 40.1 ^A	1.86	50.6 ± 3.0 ^B	1.47	51.8 ± 3.0 ^B	1.32
lysine	643.5 ± 50.1 ^a	11.92	574.8 ± 195.1 ^a	17.90	577.8 ± 119.7 ^a	14.59	717.3 ± 417.3 ^A	7.54	316.5 ± 35.3 ^A	9.17	285.2 ± 96.1 ^A	7.28
glutamate	508.3 ± 30.6 ^a	9.42	412.6 ± 103.2 ^a	12.85	450.7 ± 56.1 ^a	11.38	557.6 ± 213.6 ^A	5.86	315.0 ± 33.4 ^{AB}	9.12	277.1 ± 77.2 ^B	7.07
sum	5396.7 ± 378.0 ^a	100	3211.5 ± 1064.1 ^b	100	3959.5 ± 1108.6 ^{ab}	100	9514.5 ± 1890.8 ^A	100	3453.3 ± 247.6 ^B	100	3917.3 ± 145.0 ^B	100

The different lower case and upper case letters indicated significant differences for aboveground and belowground tissues among treatments, respectively

Table 3 Effect of sediment type on flavonoids in the aboveground and belowground tissues of seagrass *Thalassia hemprichii*. 1:0, the in-situ sediment without combining with coarse beach sand was added in the tank; 1:1, the combination of half in-situ sediment and half coarse beach sand was added in the tank; 1:2, the combination of 1/3 in-situ sediment and 2/3 coarse beach sand was added in the tank

Flavonoids	Aboveground tissue						Belowground tissue					
	1:0		1:1		1:2		1:0		1:1		1:2	
	content (µg/g)	ratio (%)	content (µg/g)	ratio (%)	content (µg/g)	ratio (%)	content (µg/g)	ratio (%)	content (µg/g)	ratio (%)	content (µg/g)	ratio (%)
catechin	0.0223 ± 0.0065 ^a	0.55	0.0090 ± 0.0041 ^b	0.33	0.0115 ± 0.0033 ^b	0.43	14.4433 ± 5.4480 ^A	67.56	1.1228 ± 0.0646 ^B	31.20	0.2845 ± 0.0281 ^C	10.20
epicatechin	nd		nd		nd		1.2517 ± 0.1782 ^A	5.85	0.0437 ± 0.0016 ^B	1.22	nd ^C	
taxifolin	nd		0.0068	0.25	nd		0.0219 ± 0.0101 ^A	0.10	0.0146 ± 0.0096 ^A	0.41	0.0234 ± 0.0119 ^A	0.84
galuteolin	1.9969 ± 0.6946 ^a	49.97	1.5255 ± 0.9832 ^a	55.46	1.6700 ± 1.0298 ^a	62.55	0.0033 ± 0.0013 ^A	0.02	0.0069 ± 0.0037 ^A	0.19	0.0078 ± 0.0029 ^A	0.28
rutin	0.0049 ± 0.0041 ^a	0.12	0.0024 ± 0.0007 ^a	0.09	0.0026 ± 0.0009 ^a	0.1	0.0116 ± 0.0057 ^A	0.05	0.0025 ± 0.0001 ^B	0.07	0.0101 ± 0.0063 ^{AB}	0.36
isoquercitrin	0.4959 ± 0.2699 ^a	12.41	0.1903 ± 0.1332 ^a	6.94	0.2901 ± 0.1066 ^a	10.87	5.2404 ± 1.1379 ^A	24.51	1.4558 ± 0.4994 ^B	40.45	2.3086 ± 1.1878 ^B	82.76
astragalin	nd		nd		nd		0.0135 ± 0.0016 ^A	0.06	0.0102 ± 0.0011 ^B	0.28	0.0119 ± 0.0016 ^{AB}	0.43
diosmin	0.0027 ± 0.0006 ^a	0.07	0.0040 ± 0.0008 ^a	0.15	0.0034 ± 0.0007 ^a	0.13	Nd		nd		nd	
quercetin	0.0439 ± 0.0166 ^a	1.10	0.0076 ± 0.0061 ^b	0.28	0.0165 ± 0.0047 ^b	0.62	0.3283 ± 0.0829 ^A	1.54	0.0544 ± 0.0068 ^B	1.51	0.0282 ± 0.0110 ^C	1.01
naringenin	0.0175 ± 0.0095 ^a	0.44	0.0087 ± 0.0021 ^a	0.32	0.0067 ± 0.0018 ^a	0.25	0.0073 ± 0.0015 ^A	0.03	0.0187 ± 0.0073 ^B	0.52	0.0076 ± 0.0035 ^A	0.27
luteolin	1.3468 ± 0.2366 ^a	33.70	0.9275 ± 0.4247 ^{ab}	33.83	0.6227 ± 0.2648 ^b	23.33	0.0581 ± 0.0246 ^A	0.27	0.8522 ± 0.6017 ^B	23.67	0.1065 ± 0.0668 ^A	3.82
apigenin	0.0628 ± 0.0223 ^a	1.57	0.0631 ± 0.0118 ^a	2.30	0.0443 ± 0.0137 ^a	1.66	0.0005 ± 0.0000 ^A	0.01	0.0153 ± 0.0131 ^C	0.43	0.0008 ± 0.0001 ^B	0.03
chrysin	0.0013 ± 0.0003 ^a	0.03	0.0006 ± 0.0001 ^b	0.02	0.0008 ± 0.0001 ^b	0.03	Nd		0.0018 ± 0.0007	0.05	nd	
kaempferide	0.0015 ± 0.0007 ^a	0.04	0.0009 ± 0.0001 ^a	0.03	0.0007 ± 0.0002 ^a	0.03	Nd		nd		nd	
sum	3.9956 ± 1.1751 ^a	100	2.7421 ± 1.5258 ^a	100	2.6691 ± 1.4033 ^a	100	21.3797 ± 6.4628 ^A	100	3.5973 ± 1.1740 ^B	100	2.7894 ± 1.2502 ^B	100

nd Not detectable (i.e. below the limit of detection); the different lower case and upper case letters indicated significant differences for aboveground and belowground tissues among treatments, respectively

Table 4 Statistical analysis for the effects of sediment type on the parameters of *Thalassia hemprichii*. There were two stages for photosynthetic parameters. $P < 0.05$ (significant); $P < 0.01$ (highly significant)

Variable	df	F	P	Variable	df	F	P
Day 6				Day 21			
Y (II)	2	1.412	0.314	Y (II)	2	1.727	0.256
rETR _{max}	2	3.346	0.106	rETR _{max}	2	5.312	< 0.05
E _k ETR	2	1.020	0.415	E _k ETR	2	3.965	0.080
α _{ETR}	2	0.466	0.649	α _{ETR}	2	1.740	0.254
leaf nitrogen	2	62.547	< 0.01	δ ³⁴ S	2	629.078	< 0.01
ratio of amino acid to leaf nitrogen	2	6.708	< 0.05	Organic matter	2	13.158	< 0.01
Eh	2	21.653	< 0.01	S	2	59.828	< 0.01
aboveground tissue				belowground tissue			
glycine	2	12.702	< 0.01	glycine	2	92.525	< 0.01
sarcosine	2	9.832	< 0.05	sarcosine	2	23.737	< 0.01
alanine	2	8.964	< 0.05	alanine	2	41.459	< 0.01
valine	2	10.335	< 0.05	valine	2	204.800	< 0.01
proline	2	2.841	0.136	proline	2	41.100	< 0.01
threonine	2	2.181	0.194	threonine	2	134.373	< 0.01
isoleucine	2	10.712	< 0.05	isoleucine	2	73.448	< 0.01
leucine	2	12.082	< 0.01	leucine	2	66.719	< 0.01
ornithine	2	0.435	0.666	ornithine	2	42.395	< 0.01
methionie	2	21.875	< 0.01	methionie	2	306.695	< 0.01
histidine	2	3.281	0.109	histidine	2	122.650	< 0.01
phenylalanine	2	6.683	< 0.05	phenylalanine	2	19.644	< 0.01
arginine	2	5.777	< 0.05	arginine	2	14.548	< 0.01
tyrosine	2	8.073	< 0.05	tyrosine	2	94.401	< 0.01
asparagic acid	2	0.737	0.517	asparagic acid	2	39.413	< 0.01
tryptophan	2	0.484	0.639	tryptophan	2	3.826	0.085
4-aminobutyric acid	2	5.708	< 0.05	4-aminobutyric acid	2	57.394	< 0.01
serine	2	4.827	0.056	serine	2	117.694	< 0.01
lysine	2	0.247	0.789	lysine	2	2.830	0.136
glutamate	2	1.419	0.313	glutamate	2	3.955	< 0.080
total amino acids	2	4.433	0.066	total amino acids	2	76.191	< 0.01
catechin	2	6.456	< 0.05	catechin	2	19.131	< 0.01
epicatechin	2	–	–	epicatechin	2	143.001	< 0.01
taxifolin	2	–	–	taxifolin	2	0.590	0.584
galuteolin	2	0.209	0.817	galuteolin	2	2.146	0.198
rutin	2	0.942	0.441	rutin	2	3.498	0.098
isoquercitrin	2	2.143	0.198	isoquercitrin	2	12.003	< 0.01
astragalinal	2	–	–	astragalinal	2	4.119	0.075
diosmin	2	2.616	0.152	diosmin	2	–	–
quercetin	2	9.660	< 0.05	quercetin	2	35.336	< 0.01
naringenin	2	3.003	0.125	naringenin	2	5.609	< 0.05
luteolin	2	3.882	0.083	luteolin	2	4.859	0.056
apigenin	2	1.279	0.345	apigenin	2	23.055	< 0.01
chrysin	2	8.173	< 0.05	chrysin	2	–	–
kaempferide	2	3.753	0.088	kaempferide	2	–	–
total flavonoids	2	0.883	0.461	total flavonoids	2	26.375	< 0.01

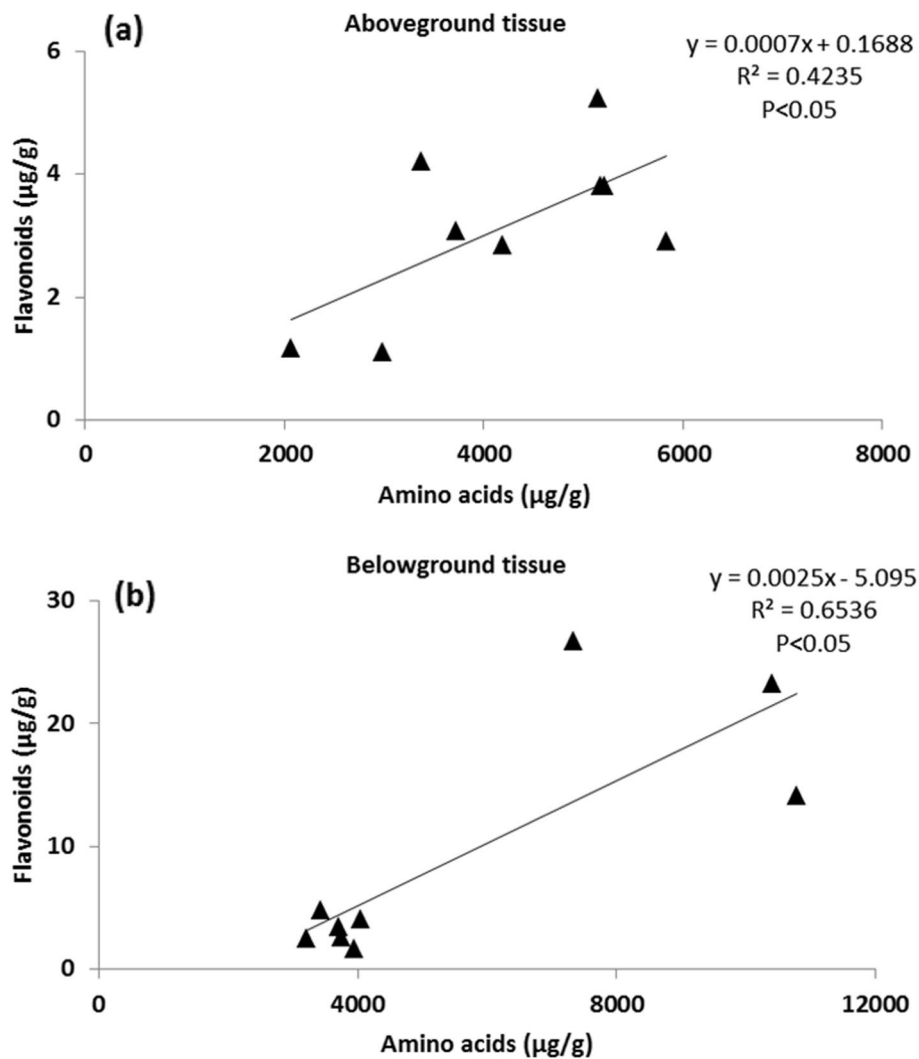


Fig. 3 Relationship of amino acids and flavonoids in the aboveground (a) and belowground (b) tissues of *Thalassia hemprichii* under different sediment types

Table 5 Correlation coefficients (r) and significance values (p) between the total flavonoids and amino acids concentration and sediment sand composition (grain size)

Parameters	r	p
Amino acids in aboveground tissue	-0.682	< 0.05
Amino acids in belowground tissue	-0.928	< 0.01
Flavonoids in aboveground tissue	-0.475	0.197
Flavonoids in belowground tissue	-0.933	< 0.01

can be grown in either sediment [29]. Sediments with high silt and clay contents could promote the successful transplantation of *Z. marina* [42].

Belowground amino acids and flavonoids were stimulated to counteract anoxic stress in sediment with smaller particle sizes

Amino acids are important for protein biosynthesis, other metabolic pathways, and signal transduction [43]. Proline and sarcosine were the main amino acids in both the above- and belowground tissues of *T. hemprichii*. Asparagic acid and proline were the main amino acids in both the above- and belowground tissues of *Posidonia oceanica* and *C. nodosa*, respectively [44]. Amino acids may change substantially in response to environmental factors. The present study indicated that the total amino acid content in the above- and belowground tissues were both higher in smaller sediment particle sizes. In particular, the belowground amino

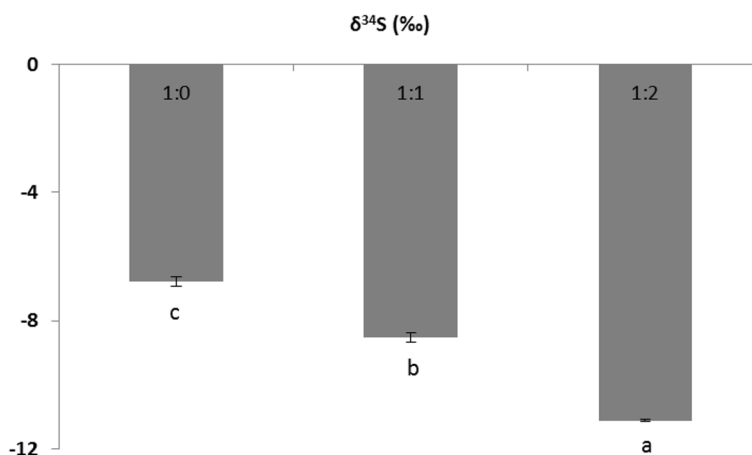


Fig. 4 Effect of sediment type on the sulfur stable isotope ($\delta^{34}\text{S}$) in the belowground tissue of seagrass *Thalassia hemprichii*. Different letters on column indicate significant difference ($P < 0.05$). 1:0, the in-situ sediment without combining with coarse beach sand was added in the tank; 1:1, the combination of half in-situ sediment and half coarse beach sand was added in the tank; 1:2, the combination of 1/3 in-situ sediment and 2/3 coarse beach sand was added in the tank

acid concentration in the smaller sediment particle sizes was more than twice that in the larger sediment particle sizes. This phenomenon could be attributed to two reasons. First, increased ammonium assimilation was induced by the higher nitrogen content in sediments with smaller particle sizes [22]. Second, the adverse effects of lower oxygen conditions. Higher alanine (an end product of anaerobic fermentation in higher plants) and proline contents in both above- and belowground tissues of *T. hemprichii* were observed in the smaller sediment particle size with lower Eh. Similarly, the alanine concentration was enhanced in *Z. marina* [12, 45] and *P. oceanica* [23] under anoxic condition. Alanine enhancement is a known phenomenon due to pyruvate accumulation in plants subjected to anoxia, which would mitigate cell acidification [21, 43] and provides support for carbon metabolism and energy homeostasis by linking glycolysis with the tricarboxylic acid cycle [46]. The increase in alanine occurs at the expense of glutamate and aspartate, and concomitantly with the GABA accumulation [47]. Furthermore, leucine and valine, the two branched-chain amino acids, were also enhanced, which could be synthesized de novo from pyruvate [43]. Proline in most higher plants often responds to an increase in concentration under environmental constraints, including salinity, drought, and anaerobiosis [43]. Increased proline content is also a factor in free radical detoxification in flooded corn plants [46]. Moreover, excess sulphate is also reduced to sulphide and incorporated into methionine, a sulphur-containing amino acid [48]. Significantly higher methionine in the belowground

tissue was observed in smaller sediment particle sizes, indicating that methionine biosynthesis might function as a detoxification agent for excess sulphate or sulphide. Similarly, *Z. marina* also detoxified gaseous sediment-derived sulphide through incorporation, and most of the detoxification occurred in the belowground tissues, where sulphide intrusion was the greatest [49].

Among phenolic compounds, flavonoids are potentially reliable biomarkers of environmental quality [50]. The present study indicated that galuteolin and luteolin were the prime flavones in the aboveground tissue of *T. hemprichii*, whereas catechin and isoquercitrin were the main components in belowground tissue. In *P. oceanica*, myricetin and isorhamnetin were the main constituents of leaf flavonols [50]. The flavonoid of *Halophila stipulacea* was dominated by apigenin-7-O- β -glucopyranoside [51]. Seagrasses with larger leaves and/or more pairs of cross-veins in the leaves contained sulfated flavonoids, whereas those with smaller leaves and/or fewer cross-veins lacked these compounds [52]. This difference might be associated with the measurement method or specific differences. Low oxygen stress changed the expression of metabolic genes, such as flavonoid biosynthesis, and induced flavonoid biosynthesis that involves methylation as a modification of compounds to accomplish activation or intracellular translocation [53]. The present study showed that lower flavonoid concentrations in belowground tissue were observed in sediments with larger particle sizes. Similarly, a decrease in the total phenolic concentration in *Z. marina* was also observed when grown in high pCO₂ waters [24]. This might be attributed to the reallocation of carbon to other pathways [54].

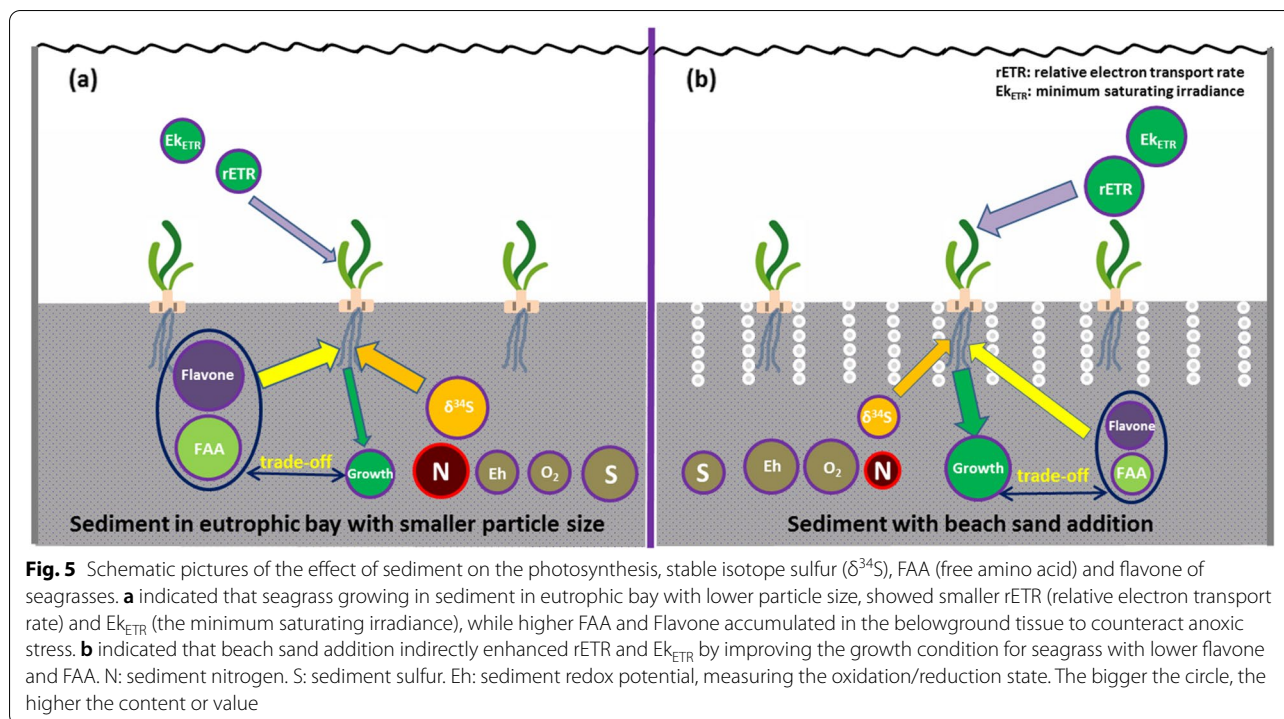
Phenolic compounds are regarded as storage compounds for carbohydrates, which are only produced when plants cannot convert carbohydrates into growth [50, 55]. Ecological plant strategy theory implies that plants investing in biochemical means of stress protection are likely to invest less carbon in constitutive productivity [31]. A trade-off mechanism between growth and secondary production for protection might occur in the present study, which required further research. Interestingly, the $\delta^{34}\text{S}$ in the belowground tissue of *T. hemprichii* was more positive in the sediment with smaller particle size, which was similar to the change in belowground flavonoids. Flavonoid sulfation might facilitate the consumption of intruded sulphide, which functions as a detoxification agent [32]. *Z. marina* and *T. testudinum*, which are rich in flavonoid sulfates, could tolerate higher sulphide intrusion than *P. oceanica*, with an almost total absence of flavonoids [32, 56–59]. Fifty percent of the radiolabelled sulfate fed to *Z. marina* was recovered from the phenolic flavonoid fraction [60]. Flavonoid sulfates might play a key role in the allelochemical relationships of seagrasses [59, 61]. In particular, catechin was extremely higher in the belowground tissue of *T. hemprichii* in the sediment with smaller particle sizes. Catechin might play a crucial role in the response to anoxic conditions. Exogenous catechin can markedly reduce waterlogging injury in roots by sufficiently enhancing the free radical scavenging system to lower hydrogen peroxide and superoxide concentrations [62].

In the present study, a strong positive correlation between flavonoids and amino acids indicated that amino acids were a good indicator of flavonoid accumulation. The available aromatic amino acids are intended for the flavonoid pathway and provided by the primary metabolism [63], which was confirmed by the fact that aromatic amino acids including phenylalanine, tryptophan, and tyrosine, were higher in the in-situ sediment without combining with coarse beach sand. Leucine and valine are precursors of plant secondary metabolites. Further research is needed to perform a cross phytochemical/phylogenetic analysis of seagrasses to correlate the phenolic fingerprint and amino acid sequences of the genes encoding the flavonoid pathway [61].

Ecological significance

Sediment type is a key factor influencing seagrass growth and success rate of transplantation [7, 64]. Recently, modifications of sediment structure and composition by removing polluted sediment and adding exogenous matrices have often been applied to better protect submerged plants and ecological restoration projects of rivers and lakes [65–67]. However, sediment type

modification has been less considered and applied in the ecological restoration of coastal zones, especially in seagrass beds suffering from eutrophication. Seagrass *T. hemprichii* in the sediment with smaller particle sizes exhibited lower $r\text{ETR}_{\text{max}}$ and $E_{k\text{ETR}}$, indicating a decrease in light tolerance (Fig. 5). Organic matter input from shrimp pond cultures along the Xincun bay coastline resulted in smaller sediment particle sizes. This induced that *T. hemprichii* in the high intertidal area suffered more from high light stress during air exposure, causing a faster decline in the high than lower intertidal area. The present study proved that adding coarse beach sand would reduce sediment total nitrogen, organic matter, and sulphur content and enhance oxygen permeability in the hypoxic/anoxic sediment, leading to less synthesis of amino acids and flavonoids. This would benefit seagrass photosynthesis and allocate more carbon to growth. The sediment particle sizes in the eutrophic area could also be modified into the same sediment of *T. hemprichii* growing in offshore and low-impact areas, with corresponding sand, silt, and clay compositions as $(97.60 \pm 1.70)\%$, $(2.40 \pm 1.70)\%$, and $(0.00 \pm 0.00)\%$, respectively [68]. Furthermore, stimulated photosynthesis also led to less toxic substance accumulation by increasing oxygenated conditions in the rhizosphere [38], and seagrasses do not have to transfer photosynthetic products, such as carbohydrates and secondary metabolites, to overcome the toxic effects of sulfide. This would benefit and accelerate seagrass growth. The enhancement of $r\text{ETR}_{\text{max}}$ and $E_{k\text{ETR}}$ may partially offset the negative effects of reduced light irradiance on C balance and improve high light tolerance. In particular, seagrass beds worldwide have faced increased eutrophication caused by a large input of nutrients from anthropogenic activity [4, 22, 69]. Considering the large variation of seagrass leaf light absorption [70, 71], the leaf light absorption needs to be measured. Field observations concerning seagrass response to sediment type by applying chlorophyll fluorescence and oxygen evolution [72–74], are needed at an ecosystem level to determine the operable habitat requirements of seagrasses [64]. It is also very important to change the sediment type to improve the growth conditions of seagrass and enhance the success rate of planting and transplanting seagrass shoots in eutrophic ecosystems. Cage farming and shrimp pond cultures in *T. hemprichii* beds should also be reduced or prohibited to decrease the input of food debris. Furthermore, *T. hemprichii* is gradually being replaced by *Enhalus acoroides* owing to a decrease in sediment particle size. Meanwhile, sediment type also affected interspecific competition between salt marsh plants [75]. Thus, further studies on the effect of changing sediment on interspecific competition and community succession in seagrasses are needed.



Conclusion

Together, our results indicated that coarse beach sand addition could indirectly enhance the photosynthesis of *T. hemprichii* by improving sediment conditions with lower total nitrogen, organic matter and sulphide intrusion. Meanwhile, considerably greater belowground amino acids and flavonoids counteracted anoxic stress in sediments with smaller particle sizes, leading to more positive belowground $\delta^{34}S$. Consequently, the sediment could be modified in the eutrophic bay to improve the growth conditions for dominant tropical seagrass *T. hemprichii*. However, more detailed analyses and field experiments on the response of seagrass to different sediment types are required. Further studies are needed to examine the metabolic pathways of key primary and secondary metabolites of seagrass and trade-off mechanisms between growth and defence, under sediment modification.

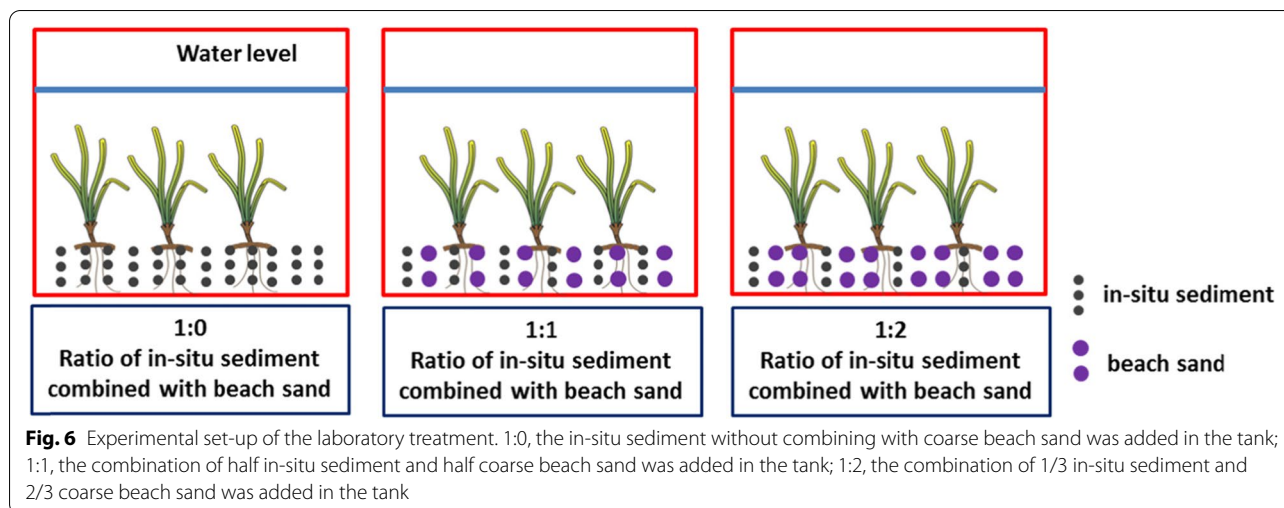
Material and methods

Approximately 250 intact shoots of healthy *T. hemprichii* (the identification was undertaken by Dr. Tan, and related voucher specimen was shown in Additional file 2) were collected in the same patch to avoid patch differences. It is a sand-clay site with a water depth of ~2 m in Xincun Bay (18°24'34"N -18°24'42"N, 109°57'42"E-109°57'58"E), located southeast of Hainan Island, Southern China

(Additional file 3). The seagrass density was between 208 and 340 shoots/m², and the biomass ratio of aboveground to belowground tissue was between 0.15 and 0.20. Plants were collected carefully to keep belowground structures intact and immediately transported to the laboratory in covered buckets containing seawater. Two boxes of in-situ sediment below *T. hemprichii* and one box of coarse beach sand without sieving from the coastline were also collected. Plants were gently washed with in-situ seawater, separated into single shoots, and then cultured in an aquarium with in-situ seawater and sediment for 7 d prior to the start of the experiments. The light intensity at the surface of the seagrass leaves was 150 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$, and the temperature was maintained at 25°C using air conditioning. The light was applied with 400 W metal-halide lamps and was set on a 12 h cycle.

Experimental design

T. hemprichii was cultured in three sediment types with in-situ sediment combinations with different ratios of coarse beach sand. Each sediment type treatment had three replicates. There were nine glass tanks (270 × 220 × 250 mm) with 20 shoots in each tank (Fig. 6). The sediment thickness was 8 cm and the overlying seawater was 8.91 L. The seawater pH, salinity and dissolved inorganic nitrogen were 8.08 ± 0.04 , 30.45 ± 0.92 , $7.45 \pm 0.74 \mu\text{mol L}^{-1}$, respectively. Seawater was aerated without replicating tides, as the seagrasses collected were



in the lower intertidal zone with little air exposure. 1:0 represented the in-situ sediment without combining with coarse beach sand added to the tank; 1:1 represented the combination of half (volume) in-situ sediment and a half (volume) coarse beach sand added to the tank; 1:2 represented the combination of 1/3 in-situ sediment and 2/3 coarse beach sand added to in the tank. The physiochemical parameters of the sediments were showed in Table 6. The concentrations of sediment organic carbon, total nitrogen, organic matter, and sulfur under the 1:0 treatment were higher than those of the other two treatments, whereas the $\delta^{34}\text{S}$ value exhibited a contrasting trend. For the particle sizes, an increasing trend was observed for sand composition from 1:0, 1:1, and 1:2, whereas the clay composition showed an inverse trend (Table 6). For the sand composition, a decreasing trend was found for the coarse sand composition from 1:0, 1:1, and 1:2, whereas fine sand showed a contrasting trend. The plants were maintained under these conditions for 21 d.

Photosynthetic performance and biochemical analysis

A PAM fluorometer (Mini-PAM, WALZ GmbH) was used to generate effective quantum yield (Y (II)) and rapid light curves (RLCs). Y (II) measured in light-adapted leaves indicates the amount of energy used in photochemistry [76]. Photosynthetic performance was measured in the same shoots in each tank on days 6 and day 21. Each sediment type treatment had three replicates of RLC. Y (II) was measured after the application of a saturating pulse of light (measuring intensity of $<0.15 \mu\text{mol photons m}^{-2}\text{s}^{-1}$, saturating intensity of $>4000 \mu\text{mol photons m}^{-2}\text{s}^{-1}$, saturation width of 0.8s). In the absence of dark acclimation, an effective quantum yield measurement was taken at the beginning of each rapid light curve, before the actinic light from the Mini-PAM, and

at the end of each 10s irradiance step, resulting in nine effective quantum yield measurements for each RLC [76]. The illumination time for RLCs might be short. This type of determination cannot be used to estimate the equivalent descriptors derived from the classic photosynthetic response curve to irradiance. However, the present study took standardized measurements on the RLCs, which might provide useful information for the description of relative changes in photosynthetic activity [73, 74, 77]. The process of measuring RLCs and the determination of the relative maximum electron transport rate ($r\text{ETR}_{\text{max}}$), minimum saturating irradiance ($E_{k\text{ETR}}$) and α_{ETR} (the initial slope of the light-limited relationship) by curve-fitting were according to Ralph and Gademann [76] and Jiang et al. [78].

At the end of the experiment, the plants were carefully retrieved and separated into above- and belowground tissues. Subsamples were oven-dried (60°C) and individually powdered with a grinder to pass through an 80-mesh sieve (with a mesh diameter of 0.18 mm) for measuring nutrients and stable sulfur isotopes, whereas the other subsamples were sent for measuring compositions of free amino acids and flavonoids with dry ice. The concentration of tissue nitrogen was determined using a CHN analyzer (Elementar, Vario EL-III, Germany). The stable isotope sulfur and sulfur contents were measured with a DELTA V Advantage isotope mass spectrometer and an EA-HT elemental analyzer. Amino acids were measured using Waters Quattro Premier XE, whereas flavonoids were measured using Waters ACQUITY UPLC and Triple quadrupole mass spectrometer (AB 4000).

Sediment analysis

The particle sizes of the sediment samples, divided into three groups ($<4 \mu\text{m}$ (clay), $4\text{--}63 \mu\text{m}$ (silt), and $>63 \mu\text{m}$

Table 6 The sediment physiochemical parameters including pH ($n = 9$), nitrogen ($n = 9$), carbon ($n = 9$), organic matter ($n = 9$), sulfur ($n = 9$), $\delta^{34}\text{S}$ ($n = 9$) and particle sizes ($n = 3$) in the initial stage of the three sediment types with in-situ sediment combination with different ratio of coarse beach sand. 1:0, the in-situ sediment without combining with coarse beach sand was added in the tank; 1:1, the combination of half in-situ sediment and half coarse beach sand was added in the tank; 1:2, the combination of 1/3 in-situ sediment and 2/3 coarse beach sand was added in the tank

Sediment type	pH	N (%)	C (%)	Organic matter (%)	S (%)	$\delta^{34}\text{S}$ (‰)	Particle sizes					
							Coarse sand (%)	Medium sand (%)	Fine sand (%)	Very fine sand (%)	Silt (%)	Clay (%)
1:0	8.83 ± 0.04 ^a	0.034 ± 0.005 ^b	0.344 ± 0.059 ^b	1.38 ± 0.11 ^a	0.017 ± 0.001 ^b	-0.80 ± 0.49 ^a	0.254	31.237	58.067	3.628	5.244	1.570
1:1	8.64 ± 0.02 ^b	0.025 ± 0.002 ^a	0.253 ± 0.029 ^{ab}	1.11 ± 0.17 ^b	0.013 ± 0.005 ^{ab}	1.09 ± 1.14 ^{ab}	6.274	44.017	41.076	3.842	3.369	1.422
1:2	8.92 ± 0.04 ^c	0.020 ± 0.003 ^a	0.216 ± 0.045 ^a	0.90 ± 0.03 ^b	0.009 ± 0.001 ^a	2.60 ± 1.19 ^b	11.185	44.667	35.584	4.149	3.842	0.573

The different lower case letters indicated significant differences among treatments

(sand)), were analyzed using a laser diffractometer (Malvern Mastersizer 2000) [79]. Sediment samples were processed according to Jiang et al. [26] before measuring sediment organic carbon and total nitrogen concentrations using a CHN analyzer (Elementar, Vario EL-III, Germany). Sediment organic matter content was analyzed by sediment calcination in a muffle furnace (550 °C for 4 h) [80]. Sediment pH was measured in distilled water with a 1:2.5 sediment/solution ratio using a portable pH acidometer (PHB-4).

At the end of the experiment, sediment redox potential (Eh, measuring the oxidation/reduction state) was measured using an oxidation-reduction potentiometer (Mettler Toledo, Seven 2 Go).

Statistical analysis

The means and standard errors of all variables were calculated, and all data were first tested to determine whether the assumptions of homogeneity of variance and normality were met. Where these assumptions were not met, the raw data were transformed, and further statistical analysis was conducted using the dataset that fulfilled the assumptions. The effect of sediment type was analyzed by one-way analysis of variance using SPSS for Windows version 18. Treatment means were compared and separated using the least significant difference at $P < 0.05$. A multiple comparison test that did not assume equal variances was Dunnett's T3 (Additional files 4, 5, 6).

Abbreviations

$rETR_{max}$: Relative maximum electron transport rate; $E_{k_{ETR}}$: Minimum saturating irradiance; α_{ETR} : The initial slope of the light-limited relationship; 1:0: The in-situ sediment without coarse beach sand was added to the tank; 1:1: The combination of half in-situ sediment and half coarse beach sand was added to the tank; 1:2: The combination of 1/3 in-situ sediment and 2/3 coarse beach sand was added to the tank.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12870-022-03647-0>.

Additional file 1: Figure S1. Change trend of Y(II) (effective quantum yield).

Additional file 2: Figure S2. Voucher specimen of *Thalassia hemprichii*.

Additional file 3: Figure S3. The *Thalassia hemprichii* bed in Xincun Bay.

Additional file 4: Table S1. Results of Levene's test of homogeneity of photosynthesis and nutrient.

Additional file 5: Table S2. Results of Levene's test of homogeneity of amino acids.

Additional file 6: Table S3. Results of Levene's test of homogeneity of flavonoid.

Acknowledgments

We would like to thank the BioNovoGene and the Huake Jingxin Stable Isotope Laboratory for measuring metabolics (amino acids and flavonoids), sulfur content and its stable isotope, respectively.

Authors' contributions

ZJ and XH designed the study. ZJ, SL, LC, JH, YF, CP, LL and YW performed the experiments or analyzed the data. ZJ, XH and MK wrote the manuscript. All authors contributed to the manuscript and approved the submitted version.

Funding

This research was supported by the National Natural Science Foundation of China (nos. 42176158, 41730529, 41976144), Key Special Project for Introduced Talents Team of Southern Marine Science and Engineering Guangdong Laboratory (Guangzhou)(GML2019ZD0405), the Key Research and Development Project of Hainan Province (ZDYF2021SHFZ254) and the Innovation Academy of South China Sea Ecology and Environmental Engineering, Chinese Academy of Sciences (ISEE2021PY06).

Availability of data and materials

The data generated or analyzed in this study are included in this article and its supplementary information files. Other materials that support the findings of this study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

All methods including plant sample collection and field studies were in compliance with relevant institutional, national, and international guidelines and legislation.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests. The funding bodies took part in the design of the study and collection, analysis, and interpretation of data, and the writing of the manuscript.

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Received: 13 June 2021 Accepted: 11 May 2022

Published online: 16 June 2022

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