Photosynthetic Electron Transport in Seeds of a Tropical Seagrass *Enhalus koenigii* Rich.

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ABSTRACT

Enhalus koenigii Rich. (Hydrocharitaceae, Vallisnerioideae) is a widespread monospecific seagrass of the Indian Ocean and West Pacific. The seeds are photosynthetic, an important consideration in their likelihood of successful germination, but their level of photosynthetic activity is not documented. We characterized the photosynthesis and respiration of *E. koenigii* seeds. Chlorophyll *a* content of *E. koenigii* seeds considerably varied, with an average from 13.98±1.25 µg·Chl *a*·seed^{-1} or 18.6±2.17 µg Chl *a*·g^{-1} FW, while Chl *a* content of the cone-shaped top part of the seed was 66.5±7.31 mg Chl *a*·m^{-2} with Chl *b/a* ≈ 0.4171±0.0188. Photosynthetic Electron Transport Rates (ETR) of *E. koenigii* seeds were measured using PAM (Pulse Amplitude Modulation) Fluorescence Technology and the Waiting-in-Line model was used to fit the rapid light curves. The Optimum Irradiance (E_{opt}) was 366±40.9 µmol photon·m^{-2}·s^{-1}, ETR_{max} was 230±15.3 (µmol e$^-$·g^{-1} Chl *a*·s^{-1}), and photosynthetic efficiency (Alpha, α_0) was 1.707±0.222 (e$^-$·g^{-1} Chl *a*·photon^{-1}). Photosynthetic ETR was recalculated as an estimate of Gross Photosynthesis (P_g) based on 4 e$^-$ through PSII ≡ 1 O_2): $P_{g\text{-}max}$ was ≈ 57.5±3.8 µmol O_2·g^{-1} Chl *a*·s^{-1}. Respiration rate was measured by O_2 electrode in air phase to minimise diffusion difficulties, R = 18.4±3.94 µmol O_2·g^{-1} Chl *a*·s^{-1}): $P_{g\text{-}max}$/R ratio at optimum irradiance was ≈ 3.12±0.70, but diurnal net photosynthesis was calculated to be near zero on an intertidal sandflat in a typical diurnal tidal cycle. Photoinhibition of *E. koenigii* seeds is very severe at sunlight-level irradiances, resulting in photo inhibitory effects >50%.

Keywords: *Enhalus koenigii*, Maximum photosynthetic electron transport rate (ETR_{max}), Optimum irradiance (E_{opt}), PAM, Tropical seagrass, Seed photosynthesis, Seed respiration

INTRODUCTION

Enhalus koenigii (acoroides) Rich. (Hydrocharitaceae, Vallisnerioideae) is a widespread monospecific seagrass of the Indian Ocean and West Pacific (southern Japan, New Caledonia, Northern Australia) (den Hartog, 1970; den Hartog and Kuo, 2006). *E. koenigii* is an unusual seagrass in some respects of its reproductive biology (Kuo and den Hartog, 2006). Like its freshwater relative, *Vallisneria*, it is fertilised by surface-floating pollen, and after fertilisation the flower is pulled underwater by contraction of its spiral stem. Hence, *E. koenigii* is typically found growing intertidally or at shallow depths, although it can spread to depths of about 4 m by vegetative growth by rhizomes. *E. koenigii* seeds are photosynthetic, the plant typically flowers prolifically, unlike many seagrasses that only flower irregularly, and the seeds germinate readily with no dormancy. Seedlings can grow to several cm high in moist aquarium gravel, either in boxes in air protected from desiccation in the laboratory, or immersed in aquaria (Ambo-Rappe *et al*., 2019; Ambo-Rappe, 2022; Liu *et al*., 2023). Den Hartog

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(1970) commented that the distribution of this species is surprisingly restricted considering that it has relatively buoyant or semi-buoyant seeds. Only a few genera of seagrasses produce large photosynthetic seeds; seeds of *Thalassia hemiprichii* are green and photosynthetic (Soong *et al*., 2013), but their degree of photosynthetic activity does not appear to have been documented. *Posidonia oceanica*, *P. australis* and *P. sinuosa* also produce large photosynthetic seeds. Photosynthesis of *P. oceanica* seeds has been studied (Celdrán *et al*., 2015), but *P. oceanica* flowers en mass only every 8 to 10 years and so its seeds are rarely available for study (Balestri and Lardicci, 2008; Celdrán and Marín, 2013). *Posidonia australis* also flowers too irregularly (at least in NSW, Australia) for it to be practical to grow this species from seeds for restoration projects (cf. Terrados *et al*., 2013).

A seagrass with large, readily available photosynthetic seeds should be easier to cultivate, but it is important to know its environmental preferences. In this study, *E. koenigii* seeds were characterised in terms of photosynthetic light responses and respiration. Gross and net photosynthesis (P_g and P_n) are problematic in the case of rooted seedlings because the roots of seagrasses grow in anoxic marine sediment and as such, seedlings previously rooted in anoxic sediment, when washed and removed from sediment, are not likely to have a respiratory rate representative of the seedlings *in situ*. Respiration of unrooted seeds, however, is less equivocal and so P_g and P_n can be confidently calculated. Seedlings are easily grown, making them a good prospect for restoration projects once the problems involved in improving survival of seedlings are properly addressed (Ambo-Rappe *et al*., 2019; Ambo-Rappe, 2022; Liu *et al*., 2023).

MATERIALS AND METHODS

Organism and culture conditions

The Indo-Pacific tropical seagrass *Enhalus koenigii* Rich. (= *E. acoroides* [L.F.] Royle) fruits (identified according to den Hartog [1970] and den Hartog and Kuo [2006]) were collected from seeds washed up from a healthy intertidal seagrass meadow at Thala Beach, Thalang District, Phuket Province, Thailand (8°01'17.7"N 98°24'53.4"E) on 16 May 2022, and the seeds were extracted from the seed pods and kept in aerated seawater in the laboratory at the campus of Prince of Songkla University – Phuket, in Kathu, Phuket, Thailand. Seagrass beds are only found intertidally in Phuket but extend to greater depths in other locations (such as Trang Province: Rattanachot *et al*., 2016) due to less turbid conditions (Dennison *et al*., 1993; Kirk 1994). *E. koenigii* is a widespread species (den Hartog, 1970; den Hartog and Kuo 2006) that can be kept in the laboratory (Ambo-Rappe *et al*., 2019; Ambo-Rappe, 2022; Liu *et al*., 2023).

Phuket has an equatorial maritime monsoon climate with a wet season from April to November (rainfall>200 mm per month, ≈ 20 rainy days per month) and a short dry season from December to March (rainfall ≈ 50 mm per month, ≈ 7 rainy days per month). The dry season is not as severe as in more inland areas due to the maritime location of Phuket. Average daily temperature throughout the year is about 28 °C and sunshine averages about 7 to 9 h per day.

The study was started in the wet season of Phuket. Seeds for experiments were kept on a 12/12 light-dark cycle in the laboratory. The light intensity was about 162 μmol photon·m^{-2}·s^{-1} (PPFD Photon Irradiance, 400–700 nm) measured using a MQ-200 Quantum Meter, Apogee Instruments, Logan, Utah, USA. The laboratory temperature was 25 to 30 °C. When extracted from the seed pods washed up on the beachfront, the seeds are already green (as found in *Posidonia* species, Celdrán and Marín, 2011; Celdrán *et al*., 2015). Photosynthetic seeds are common in angiosperms (Shackira *et al*., 2022), but seagrasses *E. koenigii*, various *Posidonia* species, and *Thalassia hemiprichii* are noted for having large photosynthetic seeds. In the laboratory, *E. koenigii* seeds quickly started to produce a primary root and shoots (within 2 to 3 weeks). We used unshooted materials for the present study to focus on the physiology of the seeds and not on seedling growth, although seedlings grew well in coarse aquarium gravel in pots in aquaria kept in the laboratory.

Chemicals

DMSO (Dimethylsulphoxide, dimethyl sulfoxide, $(CH_3)_2SO$) was from WINNEX (Thailand) Co. Ltd, Bangkok, Thailand.

Scanning dual beam spectrophotometer

A Shimadzu UV-1601 UV-visible double-beam spectrophotometer (Shimadzu Corporation, Kyoto, Japan) was used for routine chlorophyll determinations using standard scanning settings (1 nm bandwidth and 1 nm sampling interval); Chl *a* and Chl *b/a* ratio were determined using the algorithms developed for DMSO using 1 cm quartz cuvettes (Ritchie *et al*., 2021a).

Chlorophyll extraction

It was difficult to properly extract chlorophyll from *Enhalus koenigii* seeds by simply placing samples in DMSO (dimethyl sulphoxamine) even after heating at 60 °C. Cutting up the seeds was necessary for proper extraction, but grinding was counter-productive because the seeds released a stiff gel-like material that made centrifugal separation difficult. Extracts were cleared by centrifuge at 5,000 rpm (3914 rcf) in a standard swing-bucket bench centrifuge for 5 min (Hermle Z323K, Hermle Labortechnik, Wehingen, Germany) and the supernatant removed for spectroscopy. 850 nm was used as the standard blank for all equations (Larkum *et al*., 2018; Ritchie *et al*., 2021a).

Chlorophyll determinations

PAM Machines (Pulse Amplitude Modulation Fluorometers) measure the photosynthetic Electron Transport Rate (ETR) as mol $e^{-}\cdot m^{-2}\cdot s^{-1}$, and so a Chlorophyll *a* per unit surface area determination is needed to convert them to mol $e^{-}\cdot g^{-1}$ Chl $a\cdot s^{-1}$. It is a standard practice to express photosynthetic rates on a chlorophyll *a* basis (the primary photosynthetic pigment) (Table 1; Figures 2,3,5,6,7) including on seagrasses (Björkman and Demmig, 1987; Figueroa *et al*., 2003; Ritchie, 2008; 2010; 2012; Ritchie and Bunthawin, 2010; Celdrán and Marín, 2011; 2013; Celdrán *et al*., 2015; Apichatmeta *et al*., 2017; Quinnell *et al*., 2017; Larkum *et al*., 2018; Ritchie and Sma-Air, 2023), and it is difficult to make cross comparisons where Chl *a* bases were not used (Rattanachot *et al*., 2016; Liu *et al*., 2023). Hence, in this study photosynthetic electron transport rates were expressed on both surface area and Chl *a* bases. The amount of chlorophyll *a+b* in *Enhalus koenigii* seeds was determined on a per seed basis and a geometric model was used to estimate chlorophyll per unit surface area. *E. koenigii* seeds are shaped like a conical top with a narrow ring base, but only the conical top is green and photosynthetic (Figure 1). Hence, only a part of the total surface area of a seed is actually photosynthetic. The Chl *a* per unit surface area of the top cone of each seed was easily calculated from standard geometric formulae for the curved surface of a cone of base width (2r) and a height (h), and the seeds were quite uniform experimental material.

The PAM software calculates relative ETR (rETR) as μmol $e^{-}\cdot m^{-2}\cdot s^{-1}$, but if the absorptance (Abt_{445} nm) of *E. koenigii* seeds (Ritchie and Runcie, 2014; Ritchie *et al*., 2021a) and the Chl *a* content of the top cone part of the seed ($mg\cdot m^{-2}$) are both known, the rETR could then be recalculated as μmol $e^{-}\cdot g^{-1}$ Chl $a\cdot s^{-1}$ (as presented in this paper).

PAM (Pulse Amplitude Modulation) fluorometry and absorptance of Enhalus koenigii seeds

We used a blue-diode-based portable chlorophyll fluorometer (Junior PAM) made under license by Gademann Instruments, Würzburg, Germany: it uses WINCONTROL software (v2.08 and v2.13; Heinz Walz Gmbh, Effeltrich, Germany) (Ritchie, 2008; Ritchie *et al*., 2021b; Ritchie and Sma-Air, 2023) and we used its standard default settings (Ralph and Gademann, 2005). The *Enhalus koenigii* seeds had an experimentally measureable Chl $a\cdot m^{-2}$ for the photosynthetic part of the seed (Figure 1). Already sprouted seeds were avoided for physiological measurements. The full protocol describing the calculation of Yield (Y) and Photosynthetic Electron Transport Rate (ETR) was based on the most recent published version (Ritchie *et al*., 2021b; Ritchie and Sma-Air, 2023).

The Walz software uses a default absorptance of 0.84 (Abt_F) (McCree, 1972; Björkman and Demmig, 1987) and so calculates relative ETR (rETR): if absorptance (Abt) is measured experimentally the actual ETR can be calculated (Ritchie and Runcie, 2014). In the present study we measured the blue-diode absorptance ($Abt_{445\ nm}$) to be about 0.98 using a Reflectance-Absorptance-Transmission (RAT) device as previously described (Ritchie and Runcie, 2014). Hence, the actual absorptance of *E. koenigii* seeds was about 17% above the default value and hence rETR had to be corrected to actual ETR by a factor of about 1.17 because the seeds were essentially optically black at the wavelength used by the PAM machine. This is a commonly found result in higher plant material (Ritchie and Runcie, 2014; Ritchie *et al*., 2021a; Ritchie and Sma-Air, 2023) because the often-quoted Abt_F value is based on white light, not monochromatic light (McCree, 1972; Björkman and Demmig, 1987). Photosynthetic Electron Transport Rate (ETR) ($\mu mol\ e^{-}\cdot g^{-1}\ Chl\ a\cdot s^{-1}$) is an *approximate estimate* of gross photosynthesis (P_g) (Figueroa *et al*., 2003).

The Waiting-in-Line equation was used as a model for rapid light curves of ETR vs. Irradiance (Ralph and Gademann, 2005; Ritchie, 2008; Ritchie *et al*., 2021a; Ritchie and Sma-Air, 2023). The Waiting-in-Line equation has the form $y = x.e^{-x}$ with a maximum at $x = 1$ and is >50% of maximum in the range $0.232<x<2.678$. Effectively, this means that photosynthesis is >50% at irradiances as low as about ¼ of the optimum irradiance (E_{opt}) to irradiances as high as 270% of the optimum irradiance. The measured parameter is the photosynthetic Electron Transport Rate ($\mu mol\ e^{-}\cdot m^{-2}\cdot s^{-1}$); E is the irradiance ($\mu mol\ photon\cdot m^{-2}\cdot s^{-1}$ 400–700 nm PPFD). In the Waiting-in-Line equation, expressed in a form suitable for fitting photosynthetic rapid light curves, E_{opt} is the least squares fitted optimum irradiance and ETR_{max} is the fitted maximum photosynthetic Electron Transport Rate ($\mu mol\ e^{-}\cdot m^{-2}\cdot s^{-1}$). The asymptotic Photosynthetic Efficiency (α_0) ($e^{-}\cdot photon^{-1}$) was converted into $e^{-}\cdot g^{-1}\cdot Chl\ a\cdot photon^{-1}$ using the measured $Chl\ a\cdot m^{-2}$ of *E. koenigii* seeds.

Non-photochemical quenching (NPQ), as defined by Brestic and Zivcak (2013), was calculated by the Walz software and has more to do with photoprotection mechanisms for PSII than as a stress indicator (Alboresi *et al*., 2019). In most plants NPQ *vs*. irradiance can be fitted to a simple exponential saturation curve of the form $NPQ = NPQ_{max}\times(1-e^{-kE})$, where NPQ_{max} is the maximum NPQ and k is an exponential constant. The irradiance at which ½ of the NPQ_{max} is achieved is $E_{½\text{-}NPQmax} = -\ln2/k$).

Seed volume

The volume of seeds was measured experimentally by an Archimedean method. Three to five seeds were blotted dry and placed in a 25-mL measuring cylinder with 10 mL of seawater. The displacement of the seeds was measured and the number of seeds used was recorded, measured and weighed and in most cases their chlorophyll content was also measured.

Oxygen electrode experiments

A Rank Brothers Oxygen Electrode (Rank Bros Ltd, Cambridge, CB25 9DA) was used to measure respiration of *Enhalus koenigii* seeds in air (Curran, 1985; Ritchie and Sma-Air, 2023). Measurements in air-phase were chosen because of the bulky geometry of the seeds (Figure 1) and substantial volume (≈ 1 $mL\cdot g^{-1}$ FW). Cutting the seeds into small pieces was avoided to avoid wounding artefacts that would have led to misleading respiration determinations. Care was taken not to allow the seeds to dry out and the chamber had 100 µL of seawater to cover the membrane and the stirrer flea. The volume of the electrode chamber was sealed with a glass slide. The amount of oxygen in the volume of air in the chamber (7.933 mL) was calculated from the Universal Gas Law ($n = PV/RT$) and 20.95% O_2 in standard air and allowing for the volume of the seeds (≈ 0.8 mL). Incubations were run for about 1 h (3,600 s) at 25 °C. Sodium dithionite (0.5%) was used for zero calibration using methylene blue indicator. Oxygen fluxes were calculated as $mol\ O_2\cdot s^{-1}$, then using fresh weights of the seeds and Chl *a* assays, the oxygen fluxes were converted into $mol\ O_2\cdot g^{-1}\ FW\cdot s^{-1}$ or $mol\ O_2\cdot g^{-1}\ Chl\ a\cdot s^{-1}$.

Estimates of irradiance in intertidal Phuket

The SMARTS algorithm and software were used to estimate representative daily irradiance in Phuket (Ritchie, 2010; Ritchie *et al*., 2017; Larkum *et al*., 2018). This approach was recently used for modelling photosynthesis *vs*. field irradiance in a littoral herb (Ritchie *et al*., 2021a). Irradiance *vs*. seawater depth was estimated as outlined by Ritchie *et al*. (2017) but the limitation of this approach is that it takes no account of the turbidity of shallow intertidal habitats (Dennison *et al*., 1993; Kirk, 1994).

Statistics

The standard statistical reference text was Zar (2014). All spectrophotometric scans for chlorophyll determinations were based on means of at least four replicates. All PAM experiments had six or eight replicates. Where two numbers are quoted for n (n = a,b), the first value (a) is the number of separate experiments and the second (b) is the total number of data points. All data are quoted as means±95% confidence limits. Yield, ETR and NPQ data were fitted using least squares fitting routines with asymptotic errors calculated by matrix inversion (Ritchie, 2008). The EXCEL routine for the Waiting-in-Line Model has been published previously (latest version, Ritchie and Sma-Air, 2023) and is available upon request.

RESULTS

Figure 1 shows the model used to calculate the approximate surface area of the *Enhalus koenigii* seeds and approximate dimensions. Only the top conically shaped part of the seed (cotyledon) is photosynthetic. The base is not photosynthetic and its area was not included in the calculation of the mean photosynthetic surface area of seeds (217± 7.99×10^{-6} $m^2\cdot seed^{-1}$ n = 50). The average seed was 0.8338±0.0457 g FW (n = 81). Based on a sample of 45 seeds, the average Chl *a* content was 13.98± 1.25 µg Chl *a*·$seed^{-1}$ or 18.62±2.17 µg Chl *a*·g^{-1} FW; the Chl *a* content of the top cone-shaped part of the seed was 66.5±7.32 (30) mg Chl *a*·m^{-2} (Figure 1) with a Chl *b/a* ratio of 0.4171±0.0188. Only about 51.8±0.95% of the surface area of the seed is actually photosynthetic. The measured volume of seeds using an Archimedean method was 0.815± 0.0255 mL·$seed^{-1}$ (11, 46). The calculated density of seeds was 1.04±0.0255 g·mL^{-1}, very near neutral buoyancy in seawater at 25 °C (1.024 g·mL^{-1}). Only a few seeds were positively buoyant, most had a slight negative buoyancy ($D_{25\,^{o}C} \approx$ 1.04± 0.0255 g·mL^{-1}). *E. koenigii* seeds were essentially optically black at the 445 nm wavelengths used by the PAM machine used in the present study (Abt_{445nm} = 98.3±1.5%, reflectance ≈ 1.7±1.5%, n = 24). Hence, the actual ETR was about 17% higher than rETR (ETR = (1.17±0.018)×rETR).

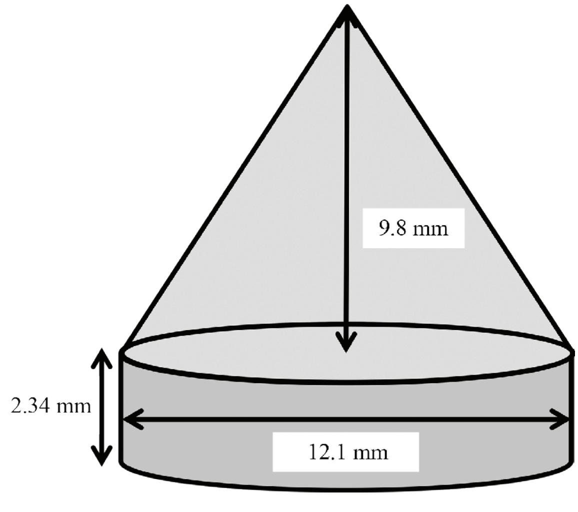

Figure 1. Geometric model for the seeds of *Enhalus koenigii*.

Figures 2 and 3 show the combined results for Rapid Light Curves on recently field-collected *E. koenigii* seeds (Figure 1) as an XYY graph showing Yield vs. irradiance and ETR vs. irradiance (Chl *a* basis). Full sunlight is about 2,200 µmol photon·m^{-2}·s^{-1}. Five irradiance ranges were used in 9 increments: 13 → 352, 26 → 552, 39 → 845, 65 → 1,300 and 91 → 1,950 µmol photon·m^{-2}·s^{-1} (PPFD). Figure 2 shows ETR on a surface area basis (the area of the seed actually photosynthetic, Figure 1). Figure 3 shows the ETR calculated on a Chl *a* basis, again only for the top cone of the seed (Figure 1). The data were fitted to the Waiting-in-Line model which we use routinely (Ritchie, 2008) and are tabulated in Table 1. Fitted to the simple exponential decay model, the maximum Yield (Y_{max}) was 0.4165±0.0184, the half point for the decay of Yield ($E_{½\text{-}Ymax}$) *vs.* irradiance was at 110.3± 11.2 µmol photon·m^{-2}·s^{-1}, r = 0.8637, n = 24, 208). Note that the statistical fit is very good at low and high irradiances but the simple exponential decay curves model is not a very satisfactory fit to the data above the Optimum Irradiance ($E_{opt} \approx 400$ µmol photon·m^{-2}·s^{-1}). The fitted Waiting-in-line ETR vs. Irradiance was first calculated on the projected surface area data (Figure 2) of the curved cone of the seed that was photosynthetic and then the ETR data was converted to Chl *a* units and then refitted (Figure 3). The Optimum irradiance (E_{opt}) was 366±40.9 µmol photon·m^{-2}·s^{-1}, ETR_{max} was 16.0± 1.07 µmol e^{-}·m^{-2}·s^{-1} (Figure 2) for the photosynthetic part of the seed or 230±15.3 µmol e^{-}·g^{-1} Chl *a*·s^{-1}, r = 0.8487, n = 24, 208 (Figure 3). The asymptotic photosynthetic efficiency (α_0) was 0.1190±0.0155 e^{-} photon^{-1} or 1.707±0.222 e^{-}·g^{-1} Chl *a*·photon^{-1}, the photosynthetic efficiency at optimum irradiance (α_{opt}) was 0.0438±0.00569 e^{-}·photon^{-1} or 0.6278± 0.0816 e^{-}·g^{-1} Chl *a*·photon^{-1}.

Figure 4 shows the calculated Photochemical (qP) and Non-photochemical quenching (NPQ) based on the rapid light curves on *E. koenigii* presented in Figures 2 and 3. Photochemical quenching followed a simple exponential decay curve with an asymptote at 1 and a $E_{½point}$ at an irradiance of 104±12.1 µmol photon·m^{-2}·s^{-1} PPFD. The tabulated data for the qP and NPQ curve fits are shown in Table 1: qP r = 0.8826, p<<0.001, NPQ r = 0.6280, p<<0.001, n = 208, spurious values given by the Walz software were omitted.

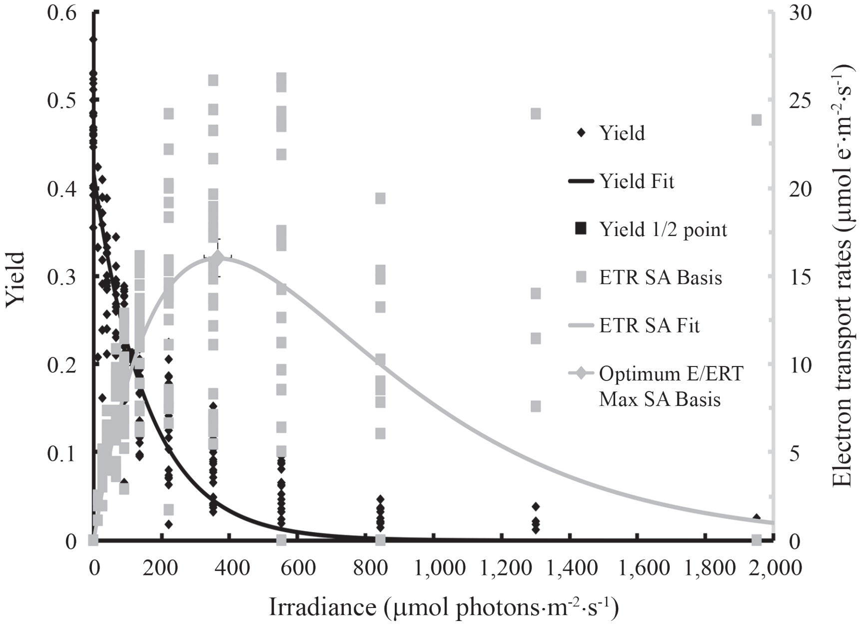

Figure 2. Rapid light curves on *Enhalus koenigii* seeds as an XYY graph showing Yield vs. irradiance and ETR vs. irradiance (photosynthetic surface area of seed basis) based on several days-worth of results. The tabulated data for the Waiting-in-Line curve fits shown on Figure 2 are shown in Table 1, spurious values given by the Walz software (error signal code) were omitted.

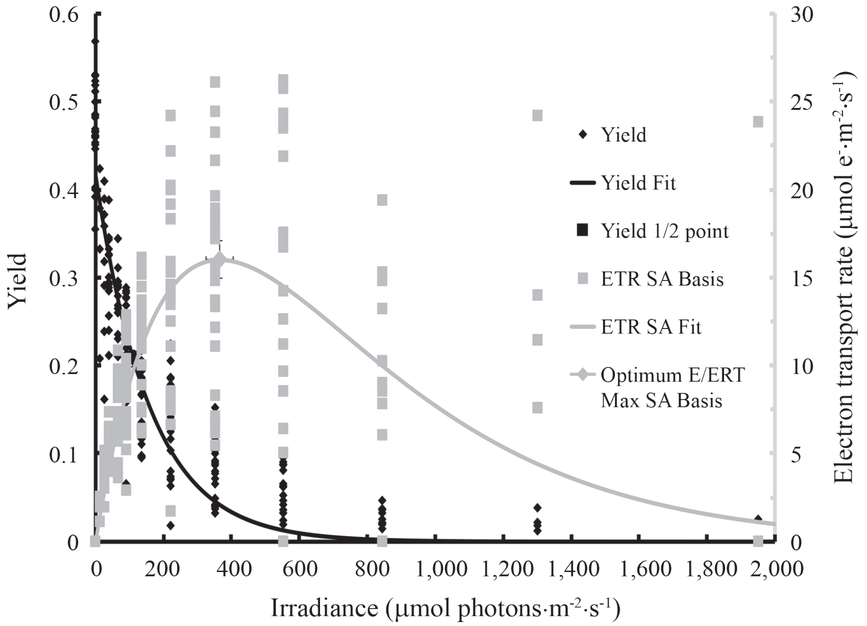

Figure 3. Rapid light curves on *Enhalus koenigii* seeds as an XYY graph showing Yield vs. irradiance and ETR vs. irradiance with ETR recalculated on a Chl *a* basis. The tabulated data for the Waiting-in-Line curve fits shown on Figure 3 are shown in Table 1, spurious values given by the Walz software were omitted.

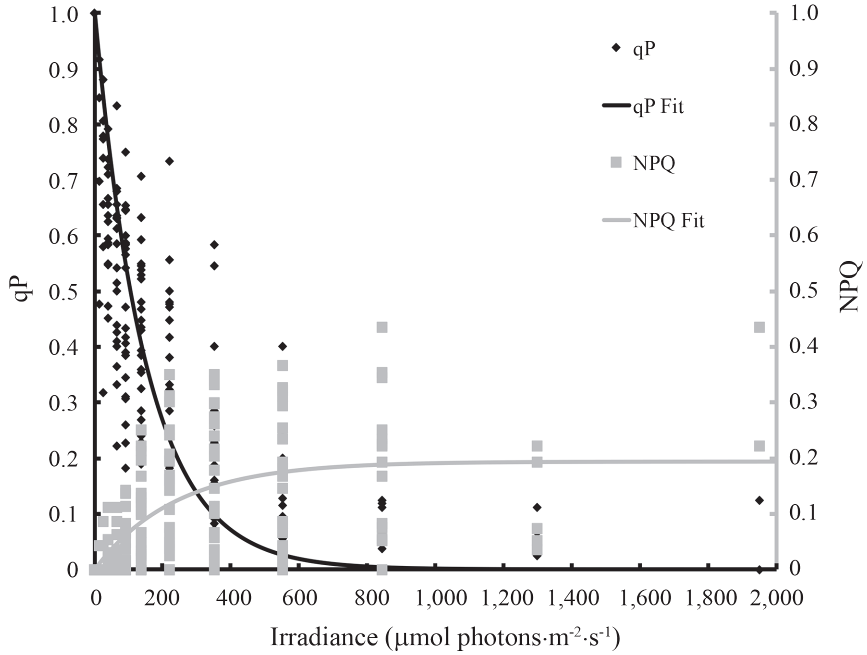

Figure 4. Non-photochemical quenching (NPQ) calculated for the rapid light curves on *Enhalus koenigii* seeds presented in Figures 2 and 3. The tabulated data for the NPQ curve fits are shown in Table 1, spurious values given by the Walz software were omitted.

The measured mean volume of seeds using the Archimedean method was 0.815±0.0354 mL·seed^{-1}. The seed volume was needed for estimations of their respiration rate because they occupied a significant volume of the oxygen electrode chamber. The respiratory oxygen consumption rate was 483±109 pmol·g^{-1} FW·s^{-1} or 18.39±3.94 µmol O_2·g^{-1} Chl *a*·s^{-1}.

Table 1 is a compendium of the photosynthetic and respiratory data found in the present study of *E. koenigii* seeds from Figures 2 to 4. Overall values are based on three separate rapid light sets of curves for *E. koenigii*. The spacing of the irradiances was determined by the WinControl software. Overall number of data points for the rapid light curves was n = 24, 208, the r value for yield was r = 0.8637 and r = 0.8487 for the fit to the ETR data and r = 0.8826 for the qP and r = 0.6281 for the NPQ data. Data sets with spurious values for NPQ were omitted on the basis of the error signal code given by the Walz software. The control Chl *a* contents, and Chl *b/a* ratios are based on 24 separate seeds.

Figure 5 shows the ETR shown in Figure 3 recalculated as an approximate Pg based upon the assumption that the flow of four electrons through PSII was equivalent to one O_2. Thus $P_{g\text{-max}}$ was ≈ 57.5±3.8 µmol O_2·g^{-1} Chl *a*·s^{-1}. The respiratory oxygen consumption rate was 424±74.2 pmol O_2·g^{-1} FW·s^{-1} or 18.39±3.94 µmol O_2·g^{-1} Chl *a*·s^{-1}. The respiratory rate could be used to estimate the compensation point for photosynthesis of the seeds and the P_g/R ratio. The $P_{g\text{-max}}$/R ratio at optimal irradiance was ≈ 3.12±0.70. Photosynthesis did not reach compensation point at irradiances below about 100 µmol photon·m^{-2}·s^{-1} PPFD or at irradiance higher than about ½ sunlight (≈ 1,100 µmol photon·m^{-2}·s^{-1} PPFD).

The low optimum irradiance of *E. koenigii* has important consequences for photosynthetic electron transport under field conditions. Figure 6 shows the estimated ETR at various solar times for Summer Solstice (June 22nd) at Phuket, based on rapid light curve data shown in Figures 2 and 3. The full tidal range in Phuket is about 2 m, which attenuates full sunlight PFFD from about 2,207 µmol photon·m^{-2}·s^{-1} to about 1,318 µmol photon·m^{-2}·s^{-1}. The intense sunlight during the middle of the day at low tide would result in very strong photoinhibition such that substantial photosynthesis only happens

Table 1. Compendium of PAM data for *Enhalus koenigii* seeds.

Parameter	**Waiting-in-Line Model**
Maximum Yield (Y_{max})	0.417±0.0184
Yield half-point irradiance ($E_{½\text{-}Ymax}$)	110±11.2 (µmol photon·m^{-2}·s^{-1})
Optimum irradiance (E_{opt})	366±40.9 (µmol photon·m^{-2}·s^{-1})
ETR_{max}	16.0±1.07 (µmol e$^-$·m^{-2}·s^{-1})
(expressed on both surface area and Chl *a* bases)	230±15.3 (µmol e$^-$·g^{-1} Chl *a*·s^{-1})
Photosynthetic efficiency ($Alpha_0$) (α_0)	0.119±0.0155 (e$^-$·photon^{-1})
(expressed on both surface area and Chl *a* bases)	1.71±0.222 (e$^-$·g^{-1} Chl *a*·photon^{-1})
Photosynthetic efficiency at Optimum irradiance	0.0438±0.00569 (e$^-$·photon^{-1})
E_{opt} ($Alpha_{opt}$) (α_{opt})	0.628±0.0816 (e$^-$·g^{-1} Chl *a*·photon^{-1})
(expressed on both surface area and Chl *a* bases)	
qP half-point irradiance ($E_{½\text{-}qP}$)	104±12.1 (µmol photon·m^{-2}·s^{-1})
NPQ_{max}	0.194±0.0352
NPQ half-point irradiance ($E_{½\text{-}NPQmax}$)	164±69.4 (µmol photon·m^{-2}·s^{-1})
Chl *a* content of green part of seed (n = 24)	66.6±12.7 (mg Chl *a*·m^{-2})
Chl *b/a* ratio (n = 24)	0.406±0.0657

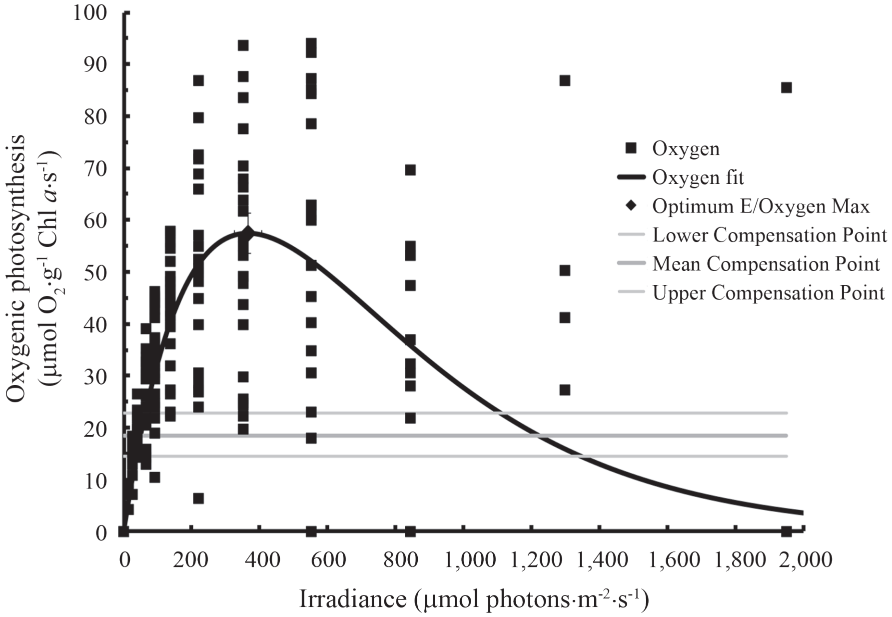

Figure 5. The ETR shown in Figure 3 was recalculated as an approximate P_g based upon the assumption that the flow of four electrons through PSII was equivalent to one O_2. The respiratory oxygen consumption rate is also shown.

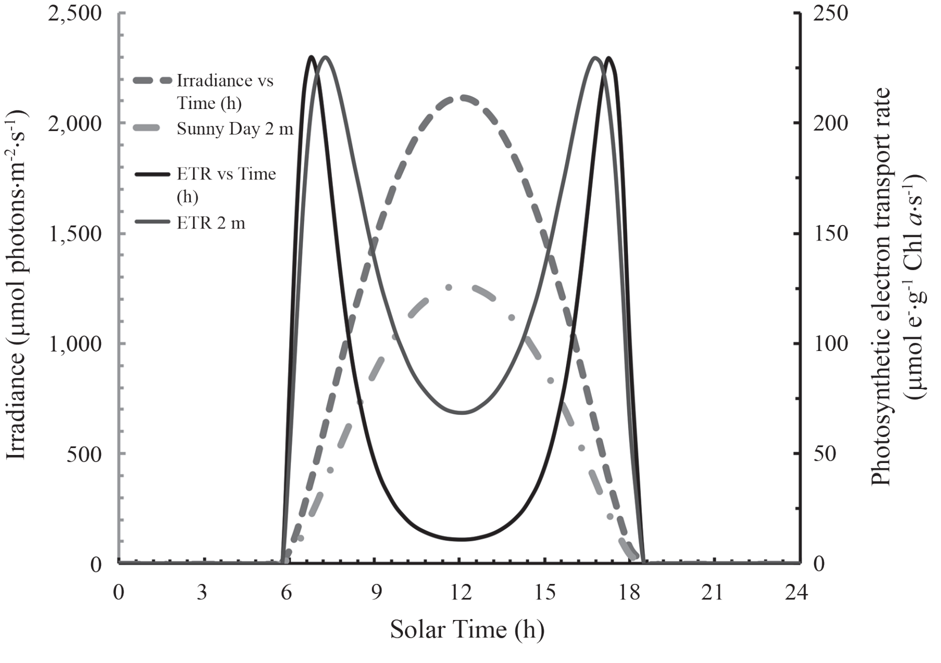

Figure 6. Estimated ETR at various Solar Times for Summer Solstice (June 22nd) at Phuket, based on the rapid light curve data shown in Figure 3.

in the early morning and late afternoon. Very high ETR would occur in the early morning and late afternoon in *E. koenigii* seeds regardless of the state of the tide: but ETR under 2 m of water is substantially improved if high tide occurs in the middle of the day. Estimates of respiration show that Net Photosynthesis (Pn) would be well above zero in the mornings and afternoons but marginal during the middle of the day under 2 m of water and would be less than zero in full sunlight. The respiratory oxygen consumption rate was 18.39± 3.94 µmol $O_2 \cdot g^{-1}$ Chl $a \cdot s^{-1}$ (from Figure 5, Table 1).

The predicted Gross Photosynthetic Rate (P_g) on *E. koenigii* seeds deposited at Tidal Gauge zero level in Phuket over the course of 22nd June 2022 is shown in Figure 7. The Local Solar Time is -29 min of standard Bangkok time (latitude plus equation of time) and so the tidal timetable needs to be corrected to Local Solar time. 22nd June 2022 was a neap tide day and so the seeds at dead low water (0 m) were inundated to some degree (> ≈ 0.4 m) throughout daylight. Irradiance was estimated from the manually interpolated tidal curve at 30 min intervals (See Supplementary Figure). For the tidal cycle on June 22nd high tide was at 5:28 solar time, 1.15 m; Low tide 11:20, 0.65 m; High tide 17:17, 1.17 m; Low tide 23:55, 0.43 m. For June 22nd maximum photosynthesis was in early morning and late afternoon. Photoinhibition would be less severe on June 22nd because most seeds would still be covered by some water during the middle of the day. On a spring-tide event in the middle of the day, severe photoinhibition would occur because many seeds would be exposed to the air under full sunlight (Figure 6). Maximum photosynthesis was nevertheless in early morning and late afternoon. The respiratory oxygen consumption rate was 18.39±3.94 µmol $O_2 \cdot g^{-1}$ Chl $a \cdot s^{-1}$ (from Figure 5, Table 1).

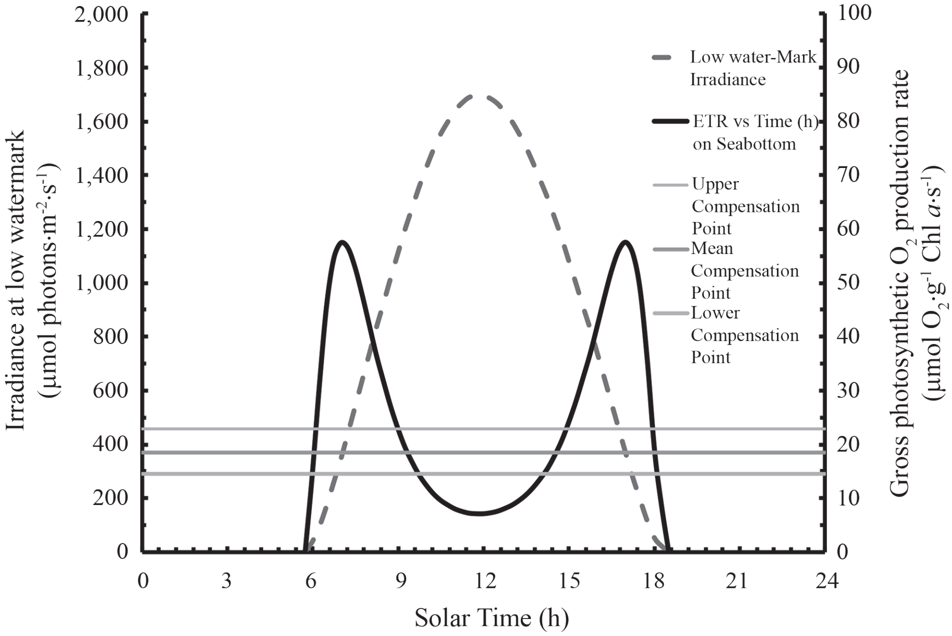

Figure 7. Predicted Gross Photosynthetic Rate (P_g) of *Enhalus koenigii* seeds if seeds were planted at Tidal Gauge zero level in Phuket on 22nd June 2022, times corrected to local solar time from local tide tables (Bangkok Standard Time).

DISCUSSION

The *Enhalus koenigii* seeds were already green when extracted from the seed pods or washed up on Thala Beach (Phuket, Thailand), as found in *Posidonia oceanica* (Celdrán and Marín, 2011; Celdrán *et al*., 2015) but *P. oceanica* seeds are uniformly green in colour. The Chl *b/a* ratio of *E. koenigii* seeds was relatively high compared to much vascular plant material (Table 1, *b/a* ≈ 0.4) but not as high as that found in *P. oceanica* (≈ 0.6, Celdrán and Marin, 2011). Figures 2 and 3 and Table 1 show that the Waiting-in-Line model can be used to model Yield and ETR of *E. koenigii* seeds. Care needs to be taken about many estimates of ETR in the literature because they are relative ETR (rETR) based on the default absorptance of 0.84 used in much PAM software and the actual absorptance has not been determined experimentally (Ritchie and Runcie, 2014). Based on our measurements of absorptance, the actual absorptance of the photosynthetic top of the seeds was about 17% higher than the default value of 0.84: this is a common observation in the leaves of vascular plants (Ritchie and Runcie, 2014; Ritchie *et al*., 2021a). The maximum Yield (Y_{max}) was about 0.4165, which is rather low for vascular plant material and lower than the value of about 0.6 found in all three *Posidonia* species studied by Celdrán *et al*. (2015).

The NPQ data for *E. koenigii* seeds had a maximum (NPQ_{max}) of only about 0.2. Some caution is appropriate not to over-interpret NPQ (Brestic and Zivcak, 2013) because it was developed for terrestrial plants. It is a measure of losses by PSII as waste heat but it is often interpreted as a stress indicator, which might not be appropriate for non-terrestrial vascular aquatic plants. Although it fitted a simple exponential saturation curve quite well, NPQ_{max} was very low compared with typical values found in leaves of terrestrial vascular plants (usually 1 to 2) (e.g., in *Launaea sarmentosa*, Ritchie *et al*., 2021a) but is similar to the seagrass *Zostera capricornii* (Ralph and Gademann, 2005) and those typically found in algae (usually<<0.5, Ritchie, 2008).

The saturating irradiances (E_{opt}) for *E. koenigii* are not unusual for terrestrial plants from shaded habitats but were lower than was expected for seeds expected to settle on open littoral and intertidal sand-flats (Figures 6 and 7) compared to terrestrial plants exposed to unshaded light such as water lilies, pineapple, oil palm, epiphytes and the littoral herb *L. sarmentosa* (Ritchie and Bunthawin, 2010; Ritchie, 2012; Apichatmeta *et al*., 2017; Quinnell *et al*., 2017; Ritchie *et al*., 2021a). However, our findings of optimal irradiances of about 366 μmol photon·m^{-2}·s^{-1} PPFD are considerably higher than found for *P. oceanica* seeds based on O_2 -electrode work (≈100–150 μmol photon·m^{-2}·s^{-1} PPFD; Celdrán and Marín, 2011). A consequence of the relatively high optimum irradiance is that *E. koenigii* seeds would have better than 50% maximum ETR over a wide range of irradiances (lower limit: 84 μmol photon·m^{-2}·s^{-1} PPFD; upper limit: 977 μmol photon·m^{-2}·s^{-1} PPFD, Figure 4 to 7). Hence, *E. koenigii* leaves covered by about 2 m of water at high tide would have about 50% of optimum photosynthesis as found by Rattanachot *et al*. (2016) in Trang Province. The P_g/R ratio at optimum irradiance of about 3 found in *E. koenigii* in the present study (Figure 5) was a considerable improvement over the findings of Celdrán and Marín (2011) and Celdrán *et al*. (2015), who only found a net O_2 production of about zero in *P. oceanica* and two Australian *Posidonia* species (*P. australis* and *P. sinuosa*), meaning a P_g/R of only about 1–1.2 under optimum irradiance (E_{opt}). Photoinhibition on an exposed sandflat during the middle of the day at low tide is so severe that there would be little photosynthesis under such conditions but would greatly improve if tidally inundated in the middle of the day (Figure 6). Maximum photosynthesis would occur in the early morning and late afternoon regardless of the state of the daily and monthly tidal cycles (Figures 6 and 7).

The present results show that *E. koenigii* seeds are capable of substantial photosynthesis and for an aquatic species the optimum irradiance is relatively high (≈366 μmol photon·m^{-2}·s^{-1} PPFD: Table 1). There can be little doubt that although the photosynthesis of the seeds would enhance seedling growth in *E. koenigii* as found in *P. oceanica* (Celdrán and Marín, 2013), but the net daily photosynthesis of the seeds in realistic situations would be near zero even under the favourable condition of a spring high tide at about midday (Figure 7). The photosynthetic capacity of photosynthetic seeds is likely to have more importance in the actual mobilisation of the seed reserves of the seeds (Shackira *et al*., 2022) rather than actual carbon fixation. The seeds of *E. koenigii* are relatively large for a seagrass and have a substantial carbon reserve, but only the top cone of the seed is photosynthetic in a very thin layer of cells (Kuo and den Hartog, 2006).

As pointed out in the Materials and Methods, Photosynthetic Electron Transport Rate (ETR) does not take photorespiration into account and so is a *rough estimate* of gross photosynthesis (P_g) (Figueroa *et al*., 2003). Photosynthetic ETR based on PAM technology is measured based on short (<2s) flashes of light and so there is minimal accumulation of high O_2 as does occur in oxygen electrode and IRGA (InfraRed Gas Analyser) determinations of photosynthesis, which necessarily involve much longer time courses, and so in practice PAM determinations of photosynthetic activity are based on conditions where photorespiratory effects on photosynthesis are minimised. Total daily P_g can be calculated using the trapezium rule to integrate over the period of daylight, giving a total daily P_g of 1.19 ± 0.155 mol $O_2\cdot g^{-1}$ FW$\cdot$day^{-1} under the favourable condition of a spring high tide during the middle of the day (Figure 7). This is a substantial amount of photosynthesis per day, however the seeds have a substantial respiration rate of 18.39 ± 3.94 µmol $O_2\cdot g^{-1}$ FW$\cdot$s^{-1} (Figures 5, 6 and 7) or amounting to 1.59 ± 0.34 mol $O_2\cdot g^{-1}$ FW$\cdot$day^{-1}.

Thus, until the seeds start shooting, their net photosynthesis (P_n) is about zero ($P_n \approx -0.40\pm 0.37$ mol $O_2\cdot g^{-1}$ FW$\cdot$day^{-1} on 22nd June 2022). On days where a Spring (highest) High tide occurred in the middle of the day P_n would be substantially improved (see Figure 6 and 7), perhaps reaching a clear positive value (particularly on an overcast day), but conditions for photosynthesis would be less favourable for a day where the Spring (Lowest) Low tide occurred in the middle of the day. This has important implications for the longer term viability of *E. koenigii* seeds and seedlings and the dispersal characteristics of the species. Irradiances in Figures 6 and 7 are based on transmission in clear water and so are likely to be overoptimistic overestimates, particularly during the stormy and overcast wet season (Dennison *et al*., 1993; Kirk, 1994). Light quality would be very poor during the stormy wet season and Rattanachot *et al*. (2016) noted that self-shading by mature leaves layering on one another was a major limiting factor from observations on the responses of artificially thinned out natural *E. koenigii* beds.

The respiration rate of unrooted seeds of *E. koenigii* is not an equivocal measurement, whereas the respiration of rooted seedlings removed from sediment is quite equivocal because the roots are embedded in anoxic sediment and so their respiration in situ would be likely to be quite different when removed from the anoxic sediment. Respiration of *E. koenigii* seedlings removed from sediment might not be representative of a seedling *in situ* and hence questions arise about the actual net photosynthesis of a rooted seedling (Curran, 1985). Similar questions arise in the case of other seagrasses with large photosynthetic seeds (*Thalassia hemiprichii*, Soong *et al*., 2013; *Posidonia* species, Celdrán and Marín, 2011; 2013; Celdrán *et al*., 2015).

Seagrass beds are rather notorious for being difficult to restore (Walker *et al*., 2006; Terrados *et al*., 2013), but *E. koenigii* (acoroides) beds might be an exception (Ambo-Rappe *et al*., 2019; Ambo-Rappe, 2022) because the seeding is prolific, the fruits are large and easily collected, and the seedlings grow in the laboratory; unfortunately, Celdrán and Marín (2013) and Terrados *et al*. (2013) found that although *P. oceanica* and *E. koenigii* (Ambo-Rappe, 2022; Liu *et al*., 2023) seeds can be kept and grow well in the laboratory, survival of seedlings transplanted into the field can be very low. This necessitates more information on the effects of density of planting and the best size and age for transplantation. Occasional massive natural recruitments of *P. oceanica* have been reported that were spectacular because of their rarity but suitable substrate (redox environment) appears to be critical for subsequent survival (Balestri and Lardicci, 2008).

Follow-up to determine if large-but-rare recruitment events led to colonisation of previously bare areas was outside the time frame of most studies where large recruitment events were noted. Like in the case of *P. oceanica* (Terrados *et al*., 2013), beach-cast *E. koenigii* seeds could be readily used for seagrass-bed restoration projects without damage to existing seagrass beds. Survival of transplanted *E. koenigii* seedlings in the field is heavily dependent on both substrate (only substantial in fine sand or mud) and very limited wave action; both these lines of evidence point to the redox environment of the substrate being a critical factor (Ambo-Rappe *et al*., 2019; Ambo-Rappe, 2022). Perhaps survival of larger seedlings would be higher because rooting anchorage of *E. koenigii* seedlings in the substrate in pots in the laboratory is noticeably poor in some substrates even for the relatively advanced seedlings (≈ 20 to 30 cm high) used by Ambo-Rappe (2022). It is likely that the redox potential of the substrate is critical for seedling establishment that is difficult to replicate in artificial planting operations. Perhaps it is as simple as growing seedlings in relatively large pots for planting to preserve the redox environment required by the roots of the seedlings. Rattanachot *et al*. (2016) noted that artificial thinning of *E. koenigii* beds substantially enhanced reshooting and flowering of *existing* beds. The redox demands of seagrass roots are not well documented because it is such a difficult problem to address.

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