Temperature dependence of O$_2$ respiration in mangrove leaves and roots: implications for seedling dispersal phenology

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Summary

- Seasonal differences in diaspore dispersal of three mangrove species, *Kandelia obovata*, *Bruguiera gymnorrhiza* and *Rhizophora stylosa*, suggest that respiratory energy production and demand may differ as a result of interspecific differences in temperature dependence of growth and maintenance processes during seedling establishment.
- We analyzed growth, temperature dependencies of respiratory O$_2$ consumption and amounts of respiratory chain enzymes in seedlings of these species grown at various temperatures.
- Respiration rates measured at the low reference temperature, R_{REF}, were highest in leaves of 15°C-grown *K. obovata*, whose dispersal occurs in the cold season, while root R_{REF} of 15°C-grown *R. stylosa* was 60% those of the other species, possibly because of warm conditions during its establishment phase. In leaves and roots of *K. obovata* and leaves of *R. stylosa*, the overall activation energy, E_o, changed with growth temperature associated with changes in the ratios of the amount of protein in the two respiratory pathways. However, E_o of seedlings of *B. gymnorrhiza*, which has a long dispersal phase, were constant and independent of growth temperature.
- The different temperature responses of seedling respiration and growth among these three species may reflect the seasonal temperature range of seedling dispersal and establishment in each species.

Introduction

Mangrove plants grow mostly on tropical and subtropical tidal flats (Tomlinson, 1986). Their habitat temperature fluctuates daily and seasonally in the range of *c.* 10–35°C (Tomlinson, 1986; Quisthoudt *et al.*, 2012). *Kandelia obovata* Sheue, *Bruguiera gymnorrhiza* (L.) Lam. and *Rhizophora stylosa* Griff. are typical mangrove species in the Indo-Pacific region (Fig. S1). They differ in their reproduction phenology in Japan, which is near the northern latitudinal limit for these species (Kamruzzaman *et al.*, 2013, 2016a,b). *Kandelia obovata* starts to disperse its diaspores in winter, with dispersal continuing from January to May (temperature range: 11–30°C, Fig. S2). Beginning 2 months later, *B. gymnorrhiza* disperses its diaspores from March to July (12–34°C, Fig. S2). Finally, dispersal of *R. stylosa* occurs in summer from July to September (23–34°C, Fig. S2). The three mangroves release their diaspores as viviparous seedlings, which have no period of dormancy. The seasonally different photoperiod and temperature therefore may affect energy demands owing to the physiological processes of growth and maintenance of the seedlings as they are becoming established, which raises the question of how the respiration rates of the species' diaspores respond to the changes in energy demand in these different seasons.

Respiratory ATP is used for maintenance and growth requirements such as protein turnover, maintenance of solute concentration gradients across membranes, synthesis of new structures, phloem loading, nutrient uptake and nitrogen (N) assimilation (de Wit *et al.*, 1970; Amthor, 1989; Thornley & Johnson, 1990; O'Leary *et al.*, 2019). Plant respiration rates are often acclimated to growth temperatures. This temperature acclimation of respiration rates has been divided into two types, Type I and Type II (Atkin & Tjoelker, 2003). In Type I acclimation, the temperature sensitivity of the respiration rate differs between plants grown at different temperatures. The temperature sensitivity of the respiration rate has been expressed in terms of E_o, the overall activation energy of respiration processes, which can be obtained from the slope of an Arrhenius plot, a graph of the empirical linear relationship between the logarithm of respiration rate and the reciprocal of measurement temperature (Lloyd & Taylor, 1994; Atkin & Tjoelker, 2003):

$$\log_e(R) = \log_e(A) - \frac{E_o}{rT} \qquad \text{Eqn 1}$$

where R is the O$_2$ consumption rate, A is the preexponential factor (often called the frequency factor), r is the gas constant (8.314 J mol^{-1} K^{-1}), T is the absolute temperature (K) at which

R was measured and E_o is the activation energy (J mol^{-1}). Although E_o changes in Type I acclimation, an overall shift of the temperature response curve occurs in Type II acclimation. In many cases, Type II acclimation results in an increase of the respiration rate at a low reference temperature (R_{REF}) when the growth temperature is low. Noguchi *et al.* (2015) reported that there is a positive correlation between R_{REF} and respiratory capacity, Cap(R), quantified as the integral of $\log_e(R)$ over a distinct temperature interval. The positive correlation found between R_{REF} and N concentration in a variety of plant species (Reich *et al.*, 1998, 2008; Inoue *et al.*, 2022b) indicates that there is a close link between respiratory ATP production and energy demands for biosynthesis, phloem loading and protein maintenance in plants.

Some studies have compared changes in the temperature sensitivity of respiration rates and R_{REF} to growth temperature among plant species and ecotypes/varieties. Atkin *et al.* (2015) and Crous *et al.* (2022) showed that R_{REF} tends to decrease with increasing growth temperatures across latitudes from boreal/tundra to tropical regions. Criddle *et al.* (1994) found that tree species from warm sites tend to have higher E_o values than those from cold sites. These findings suggest that plants grown under warm conditions often acclimate to those temperatures by enhancing E_o and/or decreasing R_{REF}. Mangroves grow in warm habitats that are likely to become warmer as the global climate warms. Knowledge of the responses of the respiration rate to temperature-dependent, energy-demanding processes and how it differs among mangrove species will enhance our understanding of how mangrove species have adapted to warm habitats and how they may adapt to warmer climates in the future.

During plant respiration, O_2 is consumed mainly by the two terminal oxidases in the mitochondrial respiratory chain: cytochrome c oxidase (COX) and alternative oxidase (AOX) (Millar *et al.*, 2011). These two oxidases are components of two pathways, the cytochrome pathway (CP) and the alternative pathway (AP), respectively. Electron flow to the CP creates a proton electrochemical gradient that is accompanied by respiratory ATP production, whereas partitioning electrons to the AP does not contribute to a proton electrochemical gradient and respiratory ATP production (Millar *et al.*, 2011; Del-Saz *et al.*, 2018). According to the kinetic model of the temperature dependence of respiration formulated by Inoue & Noguchi (2021), E_o is determined by electron partitioning between the CP and AP and/or the values of the activation energy of the CP and AP. Do the R_{REF} and E_o of the mangrove species with different seedling dispersal and growth seasons respond differently to growth temperature? Are the responses of these respiratory parameters related to leaf characteristics such as N concentrations and amount of protein in the respiratory chain?

The seedling dispersal season of the three Rhizophoraceae species used in this study differs. Our goal was to answer the following questions: Does *K. obovata*, whose seedlings become established in the cold season, have a high R_{REF} that contributes to its ability to adjust to low growth temperatures? Does *R. stylosa*, whose seedlings become established in the warm season, have a low R_{REF} and change its E_o in response to

growth temperature? Does *B. gymnorrhiza*, whose seedlings become established in a season with a wide temperature range, have a constant E_o that is independent of growth temperature? Do the responses of R_{REF} and E_o to growth temperature differ between leaves and roots? Are the species-specific responses of R_{REF} and E_o related to the N concentration and amounts of terminal oxidases of the CP and AP, respectively? To answer these five questions, we grew seedlings of the three Rhizophoraceae species at four growth temperatures in glasshouses and measured the temperature dependencies of the O_2 consumption rates, N concentration and amounts of the terminal oxidases (COX and AOX) in the leaves and roots. Based on our analyses of the relationships among the variables, we discuss whether the species-specific characteristics of the temperature dependencies of the O_2 consumption rates could be related to growth temperatures during seedling establishment and whether the mangrove species will respond differently to future temperature changes.

Materials and Methods

Plant materials and growth conditions

Two hundred fully ripe diaspores of each species were collected from more than 30 mature trees on Iriomote Island, one of the southernmost islands of Japan, during the fruiting seasons (16 January 2016 for *K. obovata*, 20 March 2018 for *B. gymnorrhiza* and 12 July 2017 for *R. stylosa*). We assumed that the diaspores were adapted to the growth temperature at the northern latitudinal limit for each species (Fig. S2a). The diaspores were planted in a tray filled with sand (Imai Co. Ltd, Tsukuba, Japan) in a glasshouse (air temperature, 25°C; humidity, 70%) and watered twice daily. After *c.* 6 months, 100 seedlings were randomly selected and transplanted individually to a polycarbonate pot (diameter, 159 mm; depth, 246 mm) containing sand. We then started fertilization with a 1 : 2000-diluted HYPONeX nutrient solution (NO_3^-, 2.34 mM; NH_4^+, 0.62 mM; PO_4^-, 0.50 mM; K^+, 2.86 mM; Ca^{2+}, 1.04 mM; Na^+, 0.13 mM; Mg^{2+}, 0.42 mM; Fe^{2+}, 0.01 mM; Hyponex Japan, Osaka, Japan) twice daily until the study ended. One week after transplantation, 48 seedlings were randomly selected and set in four sunlit growth chambers (12 seedlings for each chamber) (Koito Electric Industries, Shizuoka, Japan) that were maintained at 70% relative humidity and air temperatures of 15, 20, 25 or 30°C. The photosynthetic photon flux density (PPFD) of sunlight was as high as 1400 μmol m^{-2} s^{-1} during a sunny day. The air temperature near the leaves was not significantly different from the soil temperature near the roots (Fig. S3). Plant fresh weights on the initial day of cultivation were 27.28 ± 3.69 g (mean $\pm$ SD) for *K. obovata*, 36.08 ± 5.68 g for *B. gymnorrhiza* and 40.28 ± 4.08 g for *R. stylosa*. The temperature treatment was continued for 56 d, and then plant variables associated with respiration and N concentration of five seedlings were measured at each growth temperature. The experiment lasted 33 wk from diaspore sampling to the end of the cultivation experiment.

Measurement of O_2 respiration rates

For each seedling, leaf or root samples were detached into separate 50 ml glass vials, and the O_2 consumption rate (R, nmol O_2 g DW^{-1} s^{-1}) was measured with a fluorescence oxygen sensor (FDO 925; Xylem Analytics, Freistaat Bayern, Germany). The measurements were conducted with entire leaves in air and half portions of roots in water. For the root measurements, the vial was filled with an air-saturated nutrient solution (1 : 2000-diluted HYPONeX in 50 mM 2-(N-morpholino) containing an MES buffer; pH 6.5). For each growth temperature, leaf and root measurements were conducted in the order of 15, 20, 25 and 30°C. After the measurement at 30°C, another measurement was made at 15°C to determine whether the first and the last measurement at 15°C differed. After a constant rate of O_2 consumption was recorded for 15 min at each temperature, the sample was retrieved from the vial and dried at 80°C until the weight did not change. The final weight was recorded. The O_2 consumption rate at 15°C (R_{15}, nmol O_2 g DW^{-1} s^{-1}) was treated as R_{REF} and was regarded as an indicator of respiratory capacity. Linear regression of $\log_e(R)$ vs the inverse of temperature was used to derive the instantaneous temperature dependence of the O_2 consumption rate, E_o, in Eqn 1.

Determination of N and C concentrations

After measurement of R, dried samples were ground to a fine powder. Three 10 mg replicate samples were each wrapped in tin foil, and the N and C concentrations were measured with an elemental analyzer (Flash EA 1112; Thermo Electron Corp., Minneapolis, MN, USA). The mean value of the triplicate measurements was recorded.

Protein extraction and determination of the amounts of protein in COX and AOX

At the end of the 56 d experiment, we collected samples of *c.* 200 mg fresh weight (FW) of leaves and roots of each seedling and stored them at −80°C for protein analysis. Each frozen sample was placed in a buffer containing 2% (w/v) sodium dodecyl sulfate, 62.5 mM Tris(hydroxymethyl)aminomethane HCl (pH 6.8), 50 mM dithiothreitol, 7.5% (v/v) glycerol, 0.01% (w/v) bromophenol blue and a protease inhibitor tablet (Roche Diagnostics, Mannheim, Germany). The samples were then ground with a mortar and pestle. Warming was prevented by cooling with liquid N_2. The homogenate was heated at 100°C for 5 min and then centrifuged at 15 000 ***g*** for 10 min. Potato tuber mitochondria, which were used to verify the accuracy of the quantitative analysis of mitochondrial proteins, were isolated according to Millar *et al.* (1998). The protein content of the supernatant was measured by the modified Lowry method (Lowry *et al.*, 1951; Peterson, 1977). The amounts of mitochondrial proteins (i.e. subunit II of COX (COXII) and AOX) in the extracted samples were determined via a protein autoanalyzer (Wes, Bio-Techne, Minneapolis, MN, USA). The primary antibodies were diluted 50-fold for use as polyclonal antibodies

against the COXII protein (AS04 053A; Agrisera, Vännäs, Sweden) and 250-fold for use as polyclonal antibodies against the AOX protein (AS04 054; Agrisera). Horseradish peroxidase-conjugated antirabbit IgG (DM 001A; Bio-Techne) was used as a secondary antibody. The determination of bands and quantitative accuracy were verified in advance by using a dilution series of the isolated mitochondrial fractions (Fig. S4). The sizes of the COXII and AOX proteins were 28–32 and 36–40 kDa, respectively. The relative amount of each protein in each sample was quantified by measuring the intensity of the band relative to that of the corresponding band of a leaf of *B. gymnorrhiza* grown at 25°C. The relative amount of each protein on a sample fresh weight basis was equated to the relative amount of each protein on a total protein basis times the amount of total protein on a sample fresh weight basis. We used the mean value of triplicate protein autoanalyzer measurements of each extracted sample in subsequent calculations.

Estimation of growth and maintenance respiration rates and rates of O_2 respiration and growth under field conditions

Temperature dependencies of relative growth rate (RGR, g g^{-1} d^{-1}) as well as the O_2 respiration rates for growth (R_g) and maintenance (R_m) of *B. gymnorrhiza* and *R. stylosa* were obtained from Inoue *et al.* (2022a). The corresponding data for *K. obovata* were calculated using the methods of Inoue *et al.* (2022a) (detailed methods are described in Method S1).

Seasonal changes of leaf and root R and RGR under field condition for each species were calculated by using the estimated coefficients and y-intercepts of the equations that describe the dependencies of E_o, $\log_e(A)$ and RGR on the growth temperature (Table S1) and mean daily air temperature at Iriomote Island during 2016–2020. The latter was obtained from the Japan Meteorological Agency (https://www.data.jma.go.jp/obd/stats/data/mdrr/index.html). First, growth temperature (T_g, °C) was equated to the 1 wk moving average of daily air temperature. Second, E_o and $\log_e(A)$ at relevant T_g were determined from the equations that describe the dependence of E_o or $\log_e(A)$ on T_g. Third, R at each daily temperature (T_m, °C) was derived by using the T_m and the obtained E_o and $\log_e(A)$ in Eqn 1. The RGR at each T_g was determined using the equation that describes the dependence of RGR on T_g. The rates were estimated for 1 yr.

Statistical analysis

Three-way ANOVA was conducted to determine whether there were direct effects and interactive effects of species, measurement temperature and growth temperature on leaf and root R. Two-way ANOVA was used to determine whether there were direct effects and interactive effects of species and growth temperature on the leaf-to-root ratio of R, R_{15}, N concentration, E_o and the amounts of terminal oxidases. One-way ANOVA was used to determine whether there were differences between species of estimated annual mean R and RGR as well as mean R and RGR during seedling dispersal seasons. All ANOVAs were conducted after

a normality test on the raw data (Fig. S5). Comparisons of the variables among the treatments were conducted by using Tukey's multiple comparisons test. Leaf and root AOX/COXII and COXII as well as root AOX were analyzed after logarithmic transformations. Comparisons of R_{15}, N concentration, E_o and the amounts of terminal oxidases in leaves and roots were conducted with paired t-tests. Relationships between variables were described with second-order polynomials. We calculated the 95% confidence intervals of the coefficients and conducted likelihood tests based on a null hypothesis that the coefficients were zero. All statistical analyses were conducted in the STATS package of R v.3.6.2 software (R Core Team, 2019). A type I error rate (P) < 0.05 was considered significant.

Results

Synchronized responses of leaf and root O_2 consumption rates and their temperature dependencies

Leaf R at a given measurement temperature changed with growth temperature, and the patterns of changes differed among species (Fig. 1a; $P < 0.001$ for species × growth temperature interactions in Table S2). Leaf R of all species and root R of $K.$ $obovata$, whose diaspores are dispersed in the cold season, tended to be higher at a given measurement temperature in plants grown at low temperatures (15 and 20°C) than those grown at high temperatures (Fig. 1a; $P < 0.05$ in Table S3). By contrast, root R of $B.$ $gymnorrhiza$ and $R.$ $stylosa$ tended to be lower in plants grown at 15°C than at other temperatures: the mean root R of plants grown at 15°C was 65% and 49% of that of $B.$ $gymnorrhiza$ and $R.$ $stylosa$, respectively, grown at other temperatures (Fig. 1a; $P < 0.001$ for species × growth temperature interactions in Table S2 and $P < 0.05$ in Table S3).

We examined the relationships between leaf and root R as a function of growth temperature to clarify whether the R of leaves and roots responded similarly to growth temperatures and whether the relationships differed among the three species. In all three species, the correlations were positive between leaf and root R (Fig. 1b; Table S4 shows R^2 for the fitted models). The ratio of leaf to root R changed with growth temperature, and the patterns of change differed among species (Fig. 1b; $P < 0.001$ for the interaction between species and growth temperature in Table S2). In $K.$ $obovata$ and $B.$ $gymnorrhiza$, R was higher in leaves than in roots at all growth temperatures. In $R.$ $stylosa$, R was higher in leaves than in roots of plants grown at 15 and 20°C, but it was similar in plants grown at 25 and 30°C.

Increment of leaf R_{REF} in plants grown at low temperature

R_{REF} can be an indicator of respiratory capacity (Noguchi et $al.$, 2015). The leaf R_{15} of $B.$ $gymnorrhiza$ and $R.$ $stylosa$ increased only slightly as the growth temperature decreased, but the corresponding increase was very pronounced in $K.$ $obovata$, whose diaspores are dispersed in the cold season (Fig. 2): mean leaf R_{15}
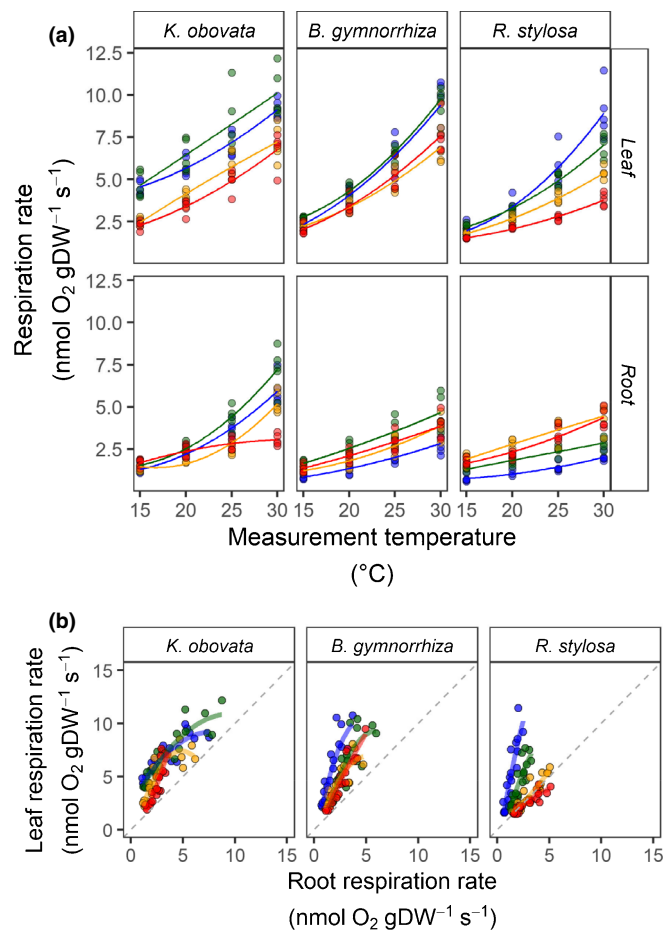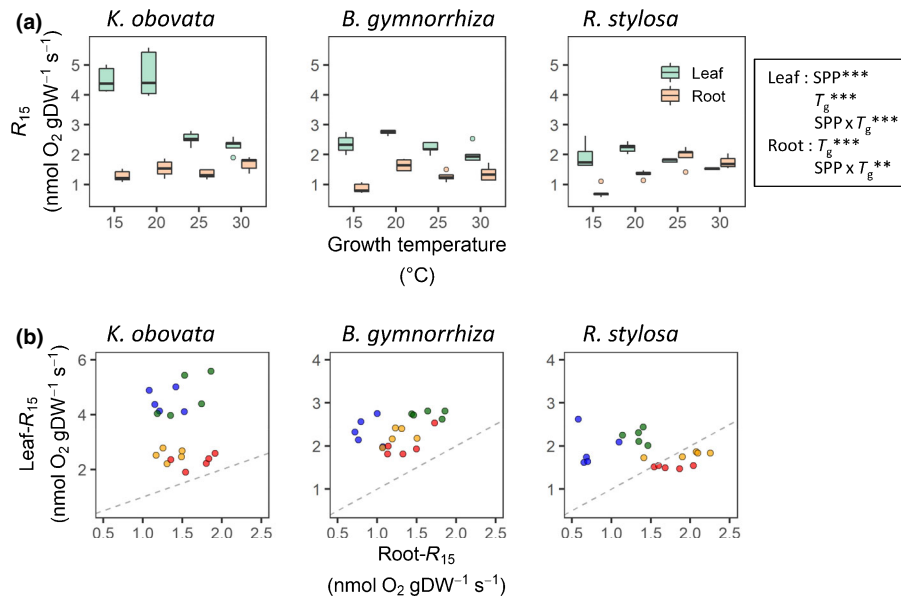

Fig. 1 (a) Temperature dependencies of leaf and root O_2 consumption rate (nmol O_2 g DW^{-1} s^{-1}) in three Rhizophoraceae species ($Kandelia$ $obovata$, $Bruguiera$ $gymnorrhiza$ and $Rhizophora$ $stylosa$) grown at 15–30°C. (b) Relationships between leaf and root O_2 consumption rates in the three species. Curves fitted with a second-order polynomial regression model are shown. Growth temperature is indicated by color: blue, 15°C; green, 20°C; yellow, 25°C; red, 30°C. DW, dry weight. The dashed lines in (b) indicate $y = x$.

of plants grown at 15°C was 2.0, 1.2 and 1.3 times mean leaf R_{15} of $K.$ $obovata$, $B.$ $gymnorrhiza$ and $R.$ $stylosa$, respectively, grown at 30°C (Fig. 2a; $P < 0.001$ for species × growth temperature interactions in Table S2 and $P < 0.05$ in Table S3).
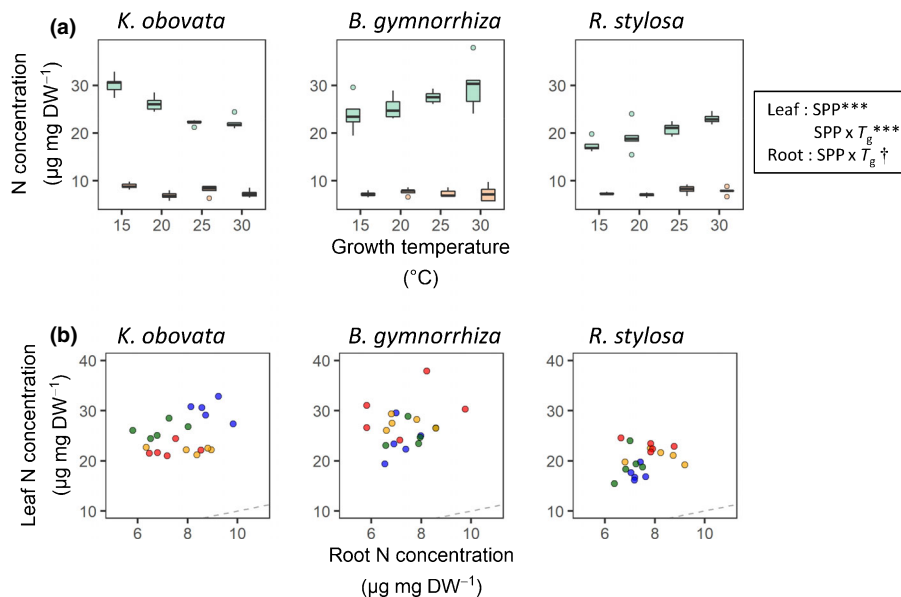
Root R_{15} decreased as growth temperature decreased in all species (Fig. 2a). The mean root R_{15} of species grown at 15°C was higher in $K.$ $obovata$, whose diaspores are dispersed in the cold season, than in other species: 1.5 and 1.7 times the corresponding rates of $B.$ $gymnorrhiza$ and $R.$ $stylosa$, respectively (Fig. 2a; $P < 0.01$ for species × growth temperature interactions in Table S2 and $P < 0.05$ in Table S3).

We compared the responses of R_{15} to growth temperature in leaves vs roots. In $K.$ $obovata$ and $B.$ $gymnorrhiza$, leaf R_{15} was higher than root R_{15} over a wide range of growth temperatures (Fig. 2b; $P < 0.05$ in Table S5). In $R.$ $stylosa$, leaf R_{15} was higher than root R_{15} in plants grown at 15 and 20°C (Fig. 2b; $P < 0.05$ in Table S5), whereas leaf R_{15} was slightly lower than root R_{15} in plants grown at higher temperatures.

Fig. 2 Leaf and root O_2 consumption rate at 15°C (R_{15}: nmol O_2 g DW^{-1} s^{-1}) in three Rhizophoraceae species (*Kandelia obovata*, *Bruguiera gymnorrhiza* and *Rhizophora stylosa*) grown at 15–30°C. (a) Box plots of leaf and root R_{15} in the three species. Boxes indicate interquartile range, horizontal lines within the boxes indicate the medians of the data, whiskers above and below the boxes indicate the highest and lowest values, and the points above and below the whiskers indicate potential outliers. **, $P < 0.01$; ***, $P < 0.001$ for two-way ANOVA with species and growth temperature as independent variables. Results of ANOVA are shown in Table S2. (b) Relationships between leaf and root R_{15} in the three species. The dashed lines indicate $y = x$. Growth temperature is indicated by color: blue, 15°C; green, 20°C; yellow, 25°C; red, 30°C. Comparisons between leaf and root by paired t-test are shown in Table S5. DW, dry weight; SPP, species; T_g, growth temperature.
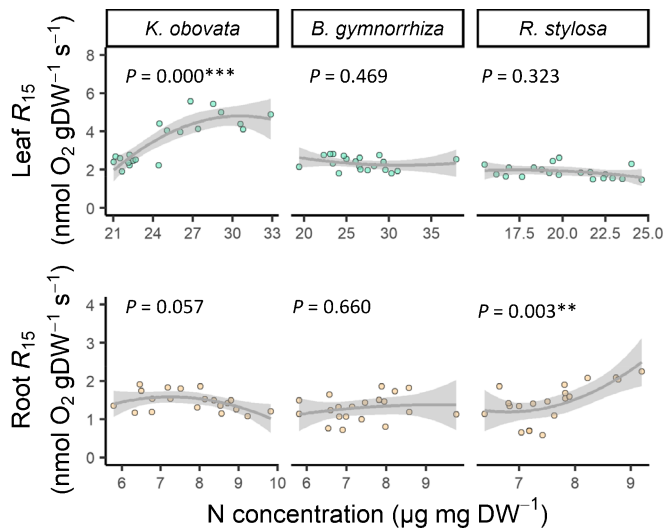

Fig. 3 Leaf and root nitrogen concentration (N concentration: μg mg DW^{-1}) in three Rhizophoraceae species (*Kandelia obovata*, *Bruguiera gymnorrhiza* and *Rhizophora stylosa*) grown at 15–30°C. (a) Box plots of leaf and root N concentration in the three species. Boxes indicate interquartile range, horizontal lines within the boxes indicate the medians of the data, whiskers above and below the boxes indicate the highest and lowest values, and the points above and below the whiskers indicate potential outliers. †, $P < 0.1$; ***, $P < 0.001$ for two-way ANOVA with species and growth temperature as independent variables. Results of ANOVA are shown in Table S2. (b) Relationships between leaf and root N concentration in the three species. The dashed lines indicate $y = x$. Growth temperature is indicated by color: blue, 15°C; green, 20°C; yellow, 25°C; red, 30°C. Comparisons between leaf and root by paired t-test are shown in Table S5. DW, dry weight; SPP, species; T_g, growth temperature.

Positive relationship between N concentration and R_{REF} was observed in certain cases

We examined whether the changes of R_{15} were related to changes of N concentration. The leaf N concentration of *B. gymnorrhiza*

and *R. stylosa* increased as the growth temperature increased (Fig. 3a; $P < 0.05$ in Table S3). By contrast, the leaf N concentration of *K. obovata* increased significantly as the growth temperature decreased (Fig. 3a; $P < 0.001$ for species × growth temperature interactions in Table S2 and $P < 0.05$ in Table S3).

Fig. 4 Relationships between O_2 consumption rate at 15°C (R_{15}: nmol O_2 g DW^{-1} s^{-1}) and nitrogen concentration (N concentration: μg mg DW^{-1}) in leaves (green) and roots (yellow) of *Kandelia obovata*, *Bruguiera gymnorrhiza* and *Rhizophora stylosa*. Data for all growth temperatures were combined. Curves fitted with a second-order polynomial regression model are shown; gray shading indicates 95% confidence interval. *P*-values of likelihood ratio test in comparison with null model are shown. **, $P < 0.01$; ***, $P < 0.001$. DW, dry weight.

Although the changes of root N concentration with growth temperature were small in all three species, the root N concentration was higher in *K. obovata* grown at 15°C vs other temperatures (Fig. 3a; $P < 0.05$ in Table S3). The ratio of leaf to root N concentration changed with growth temperature, and the

patterns of change differed among species ($P < 0.01$ for species × growth temperature interactions Table S2).

There was a positive correlation between leaf R_{15} and leaf N concentration of *K. obovata* when data for all growth temperatures were combined (Fig. 4; Table S1; $P < 0.001$, $R^2 = 0.75$). A positive correlation existed between root R_{15} and root N concentration of *R. stylosa* (Fig. 4; Table S1; $P < 0.05$, $R^2 = 0.49$) but not of other species.

Distinct responses of E_o to growth temperatures among the three species

The linear relationships apparent in Arrhenius plots based on these measurements were very strong for leaves and roots of all groups ($R^2 > 0.95$ in all cases, Fig. S6). The leaf E_o of *K. obovata* and *R. stylosa*, but not of *B. gymnorrhiza*, changed with growth temperature in opposite directions (Fig. 5a; $P < 0.001$ for species × growth temperature interactions in Table S2). In *K. obovata*, the lowest leaf E_o (33.9 kJ mol^{-1}) was observed in plants grown at 15°C, whereas the lowest E_o (43.1 kJ mol^{-1}) of *R. stylosa* was observed in plants grown at 30°C (Fig. 5a; $P < 0.05$ in Table S3).

The root E_o of *K. obovata*, but not that of *B. gymnorrhiza* or *R. stylosa*, changed significantly with growth temperature (Fig. 5a; $P < 0.001$ for species × growth temperature interactions in Table S2). In the roots of *K. obovata*, the lowest E_o (28.4 kJ mol^{-1}) was observed in plants grown at 30°C (Fig. 5a; $P < 0.05$ in Table S3).

We compared the responses of E_o to growth temperature in leaves vs roots. In *K. obovata*, leaf E_o was higher than root E_o in
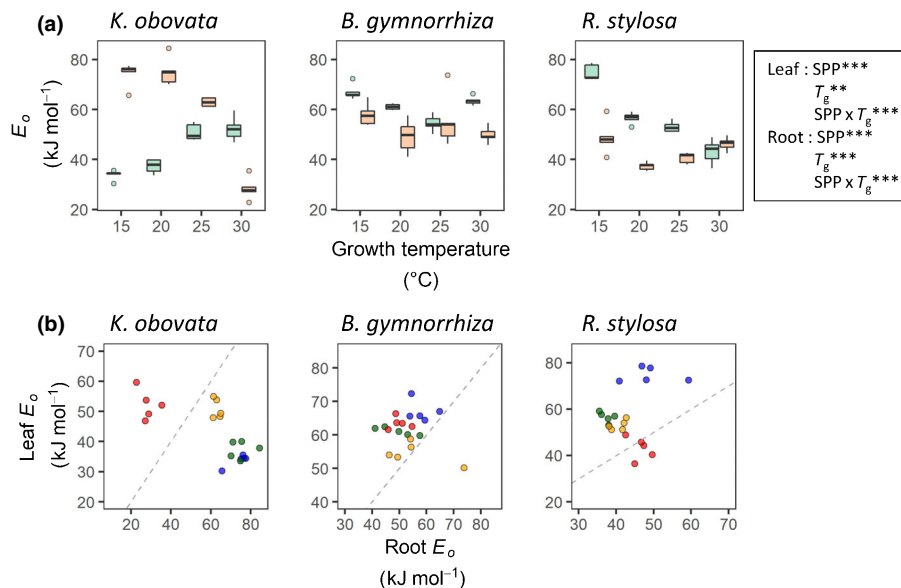
Fig. 5 Leaf and root overall activation energy (E_o: kJ mol^{-1}) in three Rhizophoraceae species (*Kandelia obovata*, *Bruguiera gymnorrhiza* and *Rhizophora stylosa*) grown at 15–30°C. (a) Box plots of leaf and root E_o in the three species. Boxes indicate interquartile range, horizontal lines within the boxes indicate the medians of the data, whiskers above and below the boxes indicate the highest and lowest values, and the points above and below the whiskers indicate potential outliers. **, $P < 0.01$; ***, $P < 0.001$ for two-way ANOVA with species and growth temperature as independent variables. Results of ANOVA are shown in Table S2. (b) Relationships between leaf and root E_o of the three species. The dashed lines indicate $y = x$. Growth temperature is indicated by color: blue, 15°C; green, 20°C; yellow, 25°C; red, 30°C. Comparisons between leaf and root by paired *t*-test are shown in Table S5. SPP, species; T_g, growth temperature.

plants grown at 30°C, whereas leaf E_o was lower than root E_o in plants grown at lower temperatures (Fig. 5b; $P < 0.05$ in Table S5). In *B. gymnorrhiza*, leaf E_o was higher than root E_o in plants grown at 15, 20 and 30°C (Fig. 5b; $P < 0.05$ in Table S5), whereas leaf and root E_o were similar in plants grown at 25°C. In *R. stylosa*, leaf E_o was higher than root E_o in plants grown at 15, 20 and 25°C (Fig. 5b; $P < 0.05$ in Table S5), but it was similar to root E_o in plants grown at 30°C.

Distinct responses of amounts of respiratory proteins to growth temperatures among the three species

The value of E_o can be affected by electron partitioning between the CP and AP and by the values of the activation energies of both pathways (Inoue & Noguchi, 2021). Electron partitioning between the two pathways can be affected by the ratio of the amounts of the two terminal oxidases, although the activation state of AOX and adenylate greatly affect electron portioning between the pathways. We therefore examined the amounts of protein in the terminal oxidases of the two pathways, COXII and AOX.

As growth temperature decreased, the amount of COXII increased significantly in leaves of *K. obovata* and *B. gymnorrhiza*, but it decreased significantly in *R. stylosa* leaves (Table 1; $P < 0.001$ for species × growth temperature interactions in Table S2 and $P < 0.05$ in Table S3). In *K. obovata* and *R. stylosa*, the response of the amount of COXII to growth temperature was

Table 1 Relative amounts of protein in subunit II of cytochrome *c* oxidase (COXII) and alternative oxidase (AOX) in leaves and roots of three Rhizophoraceae species.

	Growth temperature (°C)			
	15	20	25	30
COXII				
Kandelia obovata				
Leaf	4.14 ± 2.70	2.33 ± 1.08	1.41 ± 0.51	1.10 ± 0.53
Root	0.28 ± 0.12	0.17 ± 0.08	0.27 ± 0.12	0.31 ± 0.13
Bruguiera gymnorrhiza				
Leaf	1.29 ± 0.54	1.05 ± 0.16	0.65 ± 0.20	0.63 ± 0.19
Root	0.32 ± 0.26	0.43 ± 0.09	0.52 ± 0.15	0.46 ± 0.13
Rhizophora stylosa				
Leaf	1.68 ± 0.44	1.84 ± 0.43	2.43 ± 0.72	3.52 ± 1.41
Root	0.66 ± 0.36	0.68 ± 0.11	0.73 ± 0.18	0.96 ± 0.13
AOX				
Kandelia obovata				
Leaf	0.70 ± 0.35	0.66 ± 0.20	0.74 ± 0.40	0.58 ± 0.33
Root	0.56 ± 0.13	0.30 ± 0.11	0.48 ± 0.18	0.29 ± 0.08
Bruguiera gymnorrhiza				
Leaf	1.08 ± 0.30	0.90 ± 0.13	0.88 ± 0.16	0.94 ± 0.29
Root	0.12 ± 0.02	0.16 ± 0.06	0.26 ± 0.25	0.30 ± 0.09
Rhizophora stylosa				
Leaf	0.99 ± 0.22	0.73 ± 0.22	0.76 ± 0.16	1.28 ± 0.23
Root	0.33 ± 0.22	0.37 ± 0.18	0.31 ± 0.13	0.52 ± 0.23

Values are mean ± SD. $N = 5$. The relative amount of each protein in each sample was quantified by measuring the intensity of the band relative to that of the corresponding band of a leaf of *B. gymnorrhiza* grown at 25°C.

similar to that of the N concentration to growth temperature, and there was a significant positive correlation between the amount of COXII and the N concentration of the leaves (Fig. S7, Table S1; $P < 0.01$, $R^2 = 0.52$ in *K. obovata* and $P < 0.001$, $R^2 = 0.59$ in *R. stylosa*). The amount of root COXII did not differ significantly with growth temperature in any species (Tables 1, S3).

The amount of leaf AOX did not differ significantly with growth temperature in any species (Tables 1, S2). The amount of root AOX changed with growth temperature, and the patterns of change differed among the species ($P < 0.01$ for species × growth temperature interactions in Table S2). The mean amount of root AOX was highest at a growth temperature of 15°C in *K. obovata* (Table 1). The amounts of COXII and AOX were, as with the results for N concentration, in most cases lower in roots than in leaves at all growth temperatures in all three species (Table 1).

We calculated the ratio of the amounts of AOX to COXII (AOX : COXII) to determine whether both pathways responded similarly to growth temperatures in the three mangrove species. AOX : COXII decreased significantly as growth temperature decreased in leaves of *K. obovata*, but in leaves of *R. stylosa* the ratio was highest in plants grown at 15°C (Fig. 6a; $P < 0.001$ for species × growth temperature interactions in Table S2 and $P < 0.05$ in Table S3). We attributed the changes in leaf AOX : COXII mainly to changes in the amounts of COXII in all species. Root AOX : COXII did not differ among growth temperatures in any species (Fig. 6a; Tables S2, S3).

We compared the responses of AOX : COXII to growth temperature in leaves vs roots. In *K. obovata*, AOX : COXII was lower in leaves than in roots at growth temperatures of 15, 25 and 30°C, whereas the AOX : COXII of *B. gymnorrhiza* was higher in leaves than in roots at growth temperatures of 20, 25 and 30°C (Fig. 6b; $P < 0.05$ in Table S5). In *R. stylosa*, AOX : COXII was similar in leaves and roots.

Positive relationship between E_o and AOX : COXII in Type I acclimation

E_o changed with growth temperature in the leaves of *K. obovata* and *R. stylosa* (Fig. 5a). Because electron partitioning between the two pathways affects E_o, we examined the relationship between E_o and AOX : COXII (Fig. 7). The positive correlation between E_o and AOX : COXII in the leaves of both species (Fig. 7; Table S1; $P < 0.1$, $R^2 = 0.25$ in *K. obovata* and $P < 0.05$, $R^2 = 0.37$ in *R. stylosa*) suggested that the leaf E_o in both species was affected by the ratio of the amounts of the two enzymes.

In the roots of *K. obovata*, but not the other two species, E_o varied significantly among growth temperatures (Fig. 5a). The positive correlation between E_o and AOX : COXII in *K. obovata* roots (Fig. 7; Table S1; $P < 0.05$, $R^2 = 0.35$) suggested that changes in the ratio of these respiratory chain proteins may affect the relationship between E_o and growth temperature in the roots of this species. In the other two species, root E_o did not differ significantly among growth temperatures (Fig. 5a), and there was no significant correlation between E_o and AOX : COXII (Fig. 7).
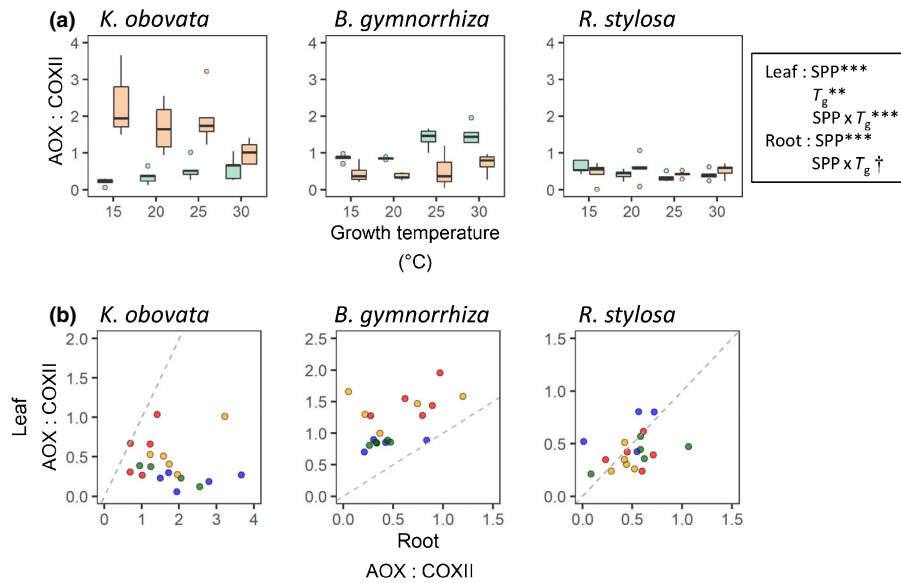
Fig. 6 Leaf and root ratio of respiratory proteins (AOX : COXII) in three Rhizophoraceae species (*Kandelia obovata*, *Bruguiera gymnorrhiza* and *Rhizophora stylosa*) grown at 15–30°C. (a) Box plots of leaf and root AOX : COXII in the three species. Boxes indicate interquartile range, horizontal lines within the boxes indicate the medians of the data, whiskers above and below the boxes indicate the highest and lowest values, and the points above and below the whiskers indicate potential outliers. †, $P < 0.1$; **, $P < 0.01$; ***, $P < 0.001$ for two-way ANOVA with species and growth temperature as independent variables. Results of ANOVA are shown in Table S2. (b) Relationships between leaf and root AOX : COXII in the three species. The dashed lines indicate $y = x$. Growth temperature is indicated by color: blue, 15°C; green, 20°C; yellow, 25°C; red, 30°C. Comparisons between leaf and root by paired t-test are shown in Table S5. SPP, species; T_g, growth temperature.
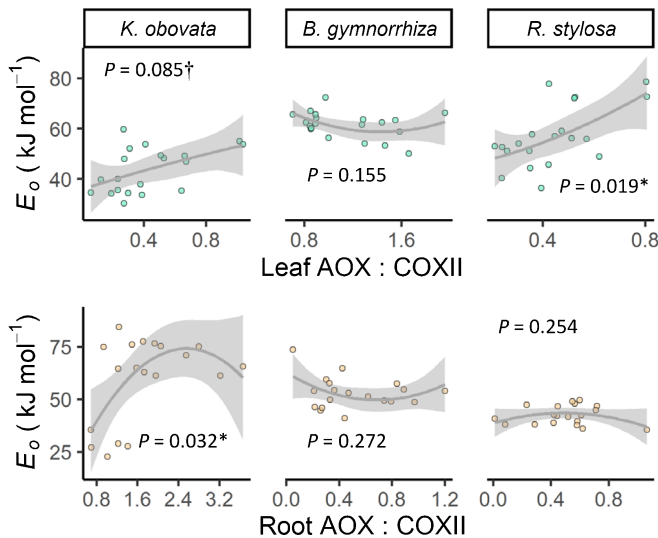
Fig. 7 Relationships between overall activation energy (E_o: kJ mol^{-1}) and the ratio of respiratory proteins AOX : COXII in leaves (green) and roots (yellow) of *Kandelia obovata*, *Bruguiera gymnorrhiza* and *Rhizophora stylosa*. Data for all growth temperatures were combined. Curves fitted with a second-order polynomial regression model are shown; gray shading indicates 95% confidence interval. P-values of likelihood ratio test in comparison with the null model are shown. †, $P < 0.1$; *, $P < 0.05$.

Occurrence of seedling dispersal during the season when the growth rate is high

To consider the practical dynamics of R under field conditions, we estimated yearly R for the three species. The mean daily temperature at Iriomote Island during 2016–2020 fluctuated seasonally from 17.2 to 29.8°C: it was coldest in January–February and warmest in July–August (Fig. 8a).

Leaf and root R at relevant growth temperatures were higher in plants grown at a higher temperature in all three species (Fig. S8). Leaf R of *K. obovata* grown at a low growth temperature was higher than that of other species because of Type II acclimation. The estimated annual leaf and root R for all species varied similarly to air temperature. Annual mean leaf and root R were highest in *K. obovata*, followed by *B. gymnorrhiza* and *R. stylosa* (Fig. 8b; $P < 0.05$). Mean leaf R during the seedling dispersal season was similar for *K. obovata* and *B. gymnorrhiza* and lowest for *R. stylosa* (Fig. 8b; $P < 0.05$). Mean root R during the seedling dispersal season was highest in *R. stylosa*, followed by *B. gymnorrhiza* and *K. obovata* (Fig. 8b; $P < 0.05$).

In all three species, a large part of R was associated with maintenance of the plant body (Fig. S8). Leaf and root R_m increased with increases of growth temperature in all three species (all $P < 0.001$). Leaf and root R_g increased with increases of growth temperature in *B. gymnorrhiza* and *R. stylosa* but decreased in *K. obovata* (all $P < 0.001$).

Total, leaf and root RGR were higher in plants grown at higher temperatures than those in plants grown at lower temperatures in *B. gymnorrhiza* and *R. stylosa* (Inoue *et al.*, 2022a). In *K. obovata*, however, total and leaf RGR were lower in plants grown at higher growth temperatures than those in plants grown at lower temperatures, and root RGR was constant among growth temperatures (Fig. S9). The estimated annual leaf and root RGR of *B. gymnorrhiza* and
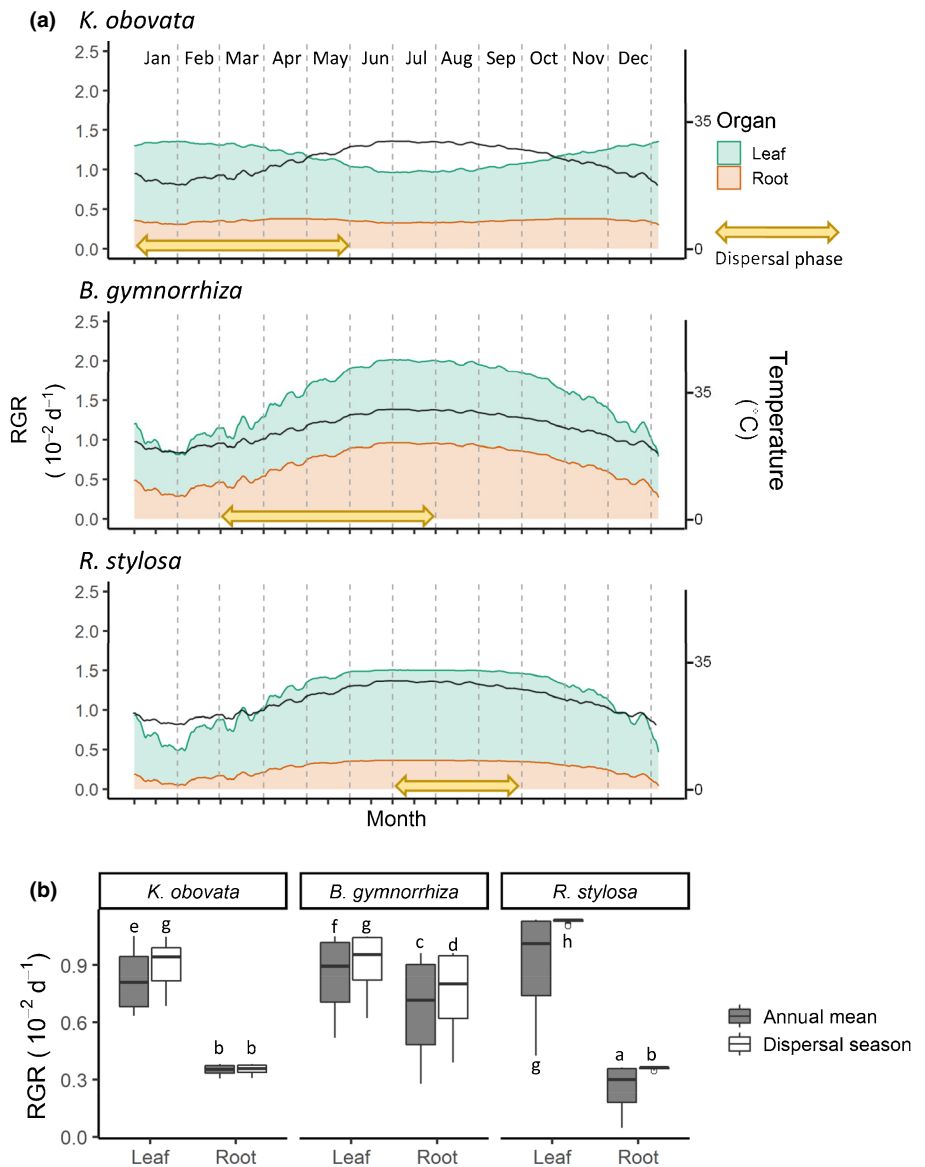
(a) *K. obovata*

B. gymnorrhiza

R. stylosa

(b)
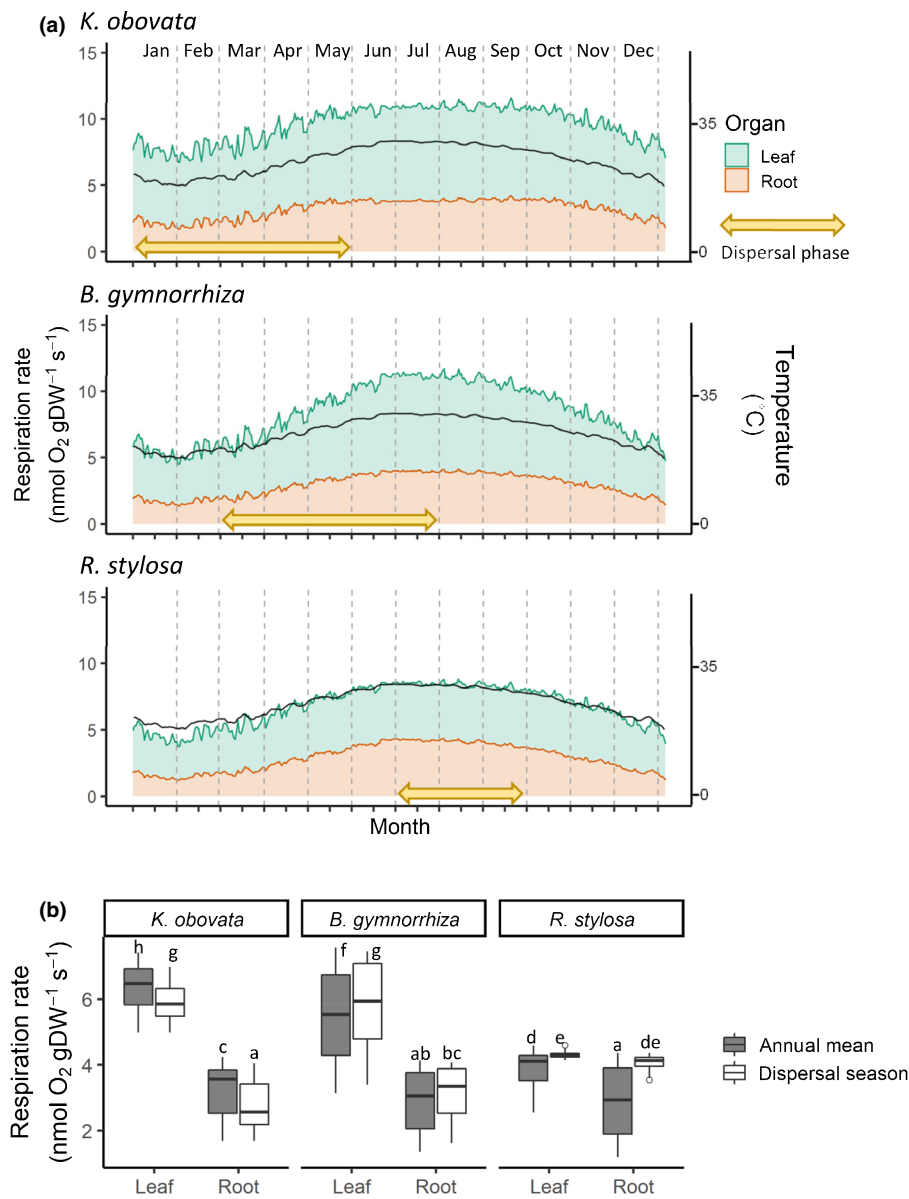
Fig. 8 Estimated O_2 respiration rates of *Kandelia obovata*, *Bruguiera gymnorrhiza* and *Rhizophora stylosa*. (a) Yearly profiles of mean daily air temperature on Iriomote Island during 2016–2020 (black solid lines) and estimated O_2 respiration rates in leaves (green) and roots (orange) of the three species. Yellow arrows indicate seedling dispersal phase for each species. (b) Annual mean O_2 respiration rate (gray) and mean O_2 respiration rate during seedling dispersal months (white) for each species (January–May for *K. obovata*, March–July for *B. gymnorrhiza* and July–September for *R. stylosa*). Different letters in (b) indicate a significant difference by multiple comparison tests ($P < 0.05$). Boxes indicate interquartile range, horizontal lines within the boxes indicate the medians of the data, whiskers above and below the boxes indicate the highest and lowest values, and the points above and below the whiskers indicate potential outliers. DW, dry weight.

R. stylosa varied similarly to air temperature (Fig. 9a). By contrast, leaf RGR was higher during the cool winter (November–February) than the warm summer (June–August), and root RGR was almost constant throughout the year in *K. obovata*. Annual mean leaf RGR was highest in *R. stylosa*, followed by *B. gymnorrhiza* and *K. obovata* (Fig. 9 b; $P < 0.05$). Annual mean root RGR was highest in *B. gymnorrhiza*, followed by *K. obovata* and *R. stylosa* (Fig. 9b; $P < 0.05$). Mean leaf RGR during the seedling dispersal season was similar between *K. obovata* and *B. gymnorrhiza*, and it was higher than those of the other two species in *R. stylosa* (Fig. 9b; $P < 0.05$). Mean root RGR during the seedling dispersal season was similar for *K. obovata* and *R. stylosa* and lower than that of *B. gymnorrhiza* (Fig. 9b; $P < 0.05$). Mean RGR during the seedling dispersal season was higher than annual mean RGR, except for RGR in *K. obovata* roots.

Discussion

Distinct responses of the respiratory chain in leaves to growth temperatures among the three mangrove species

Among the three species examined, the fact that the leaf R_{15} (an indicator of leaf respiratory capacity) of *K. obovata* increased substantially when it was grown at low temperature (15°C) suggests that homeostasis of the leaf respiration rate is likely to have occurred across growth temperatures. This result is consistent with the seedling dispersal season of *K. obovata*, which is the coldest season among the three species (Fig. S2). The high leaf R_{15} at 15 and 20°C was accompanied by a large amount of COXII, high N concentration and low E_o in *K. obovata* leaves, leading us to hypothesize that high respiratory ATP production is a response to the large energy demand for the growth and maintenance processes in *K. obovata* leaves at these low growth temperatures.

Fig. 9 Estimated relative growth rates (RGRs) of *Kandelia obovata*, *Bruguiera gymnorrhiza* and *Rhizophora stylosa*. (a) Yearly profiles of mean daily air temperature on Iriomote Island during 2016–2020 (black solid lines) and estimated RGRs in leaves (green) and roots (orange) of the three species. Yellow arrows indicate the seedling dispersal phase for each species. (b) Annual mean RGR (gray) and mean RGR during seedling dispersal months (white) for each species (January–May for *K. obovata*, March–July for *B. gymnorrhiza* and July–September for *R. stylosa*). Different letters in (b) indicate a significant difference by multiple comparison tests ($P < 0.05$). Boxes indicate interquartile range, horizontal lines within the boxes indicate the medians of the data, whiskers above and below the boxes indicate the highest and lowest values, and the points above and below the whiskers indicate potential outliers.

By contrast, the leaf R_{15} value of *R. stylosa* was not higher at low growth temperatures than at other temperatures. In *R. stylosa* leaves, the O_2 respiration rate at the high measurement temperature (30°C) was significantly lower in plants grown at high temperature than that in plants grown at lower temperatures. This suggests that strong downregulation accompanied by low E_o occurs in the respiratory system of *R. stylosa* leaves at high growth temperatures, although no significant suppression of leaf RGR was observed in this species at high growth temperatures. The E_o values of *B. gymnorrhiza* leaves were constant across the range of growth temperatures. The constant E_o of the leaves of *B. gymnorrhiza* may be required to enable it to establish seedlings over a wider range of temperatures than *R. stylosa* (Fig. S2).

Responses of root O_2 consumption rates to growth temperatures

The O_2 consumption rates and the amounts of COXII were in most cases lower in the roots than in the leaves of the three

mangrove species. These patterns resemble those observed for the N concentration. As in other plant species, the leaves of the three mangrove species contain photosynthetic enzymes such as Rubisco that lead to higher N concentration in their leaves than roots. Although the absolute rates of O_2 consumption differed between the leaves and roots, the rates in the leaves and roots were closely correlated with each other. Such a close relationship may be important for plant growth for the following reasons: changes in root temperature affect the rates of respiration and nutrient uptake through the roots, and the nutrient demands, growth rate and respiration rate of the shoots adjust to the root nutrient uptake capacity (Chapin, 1974; Chapin & Bloom, 1976; Clarkson *et al.*, 1992). The conspicuous decrease in root R at low growth temperatures in *R. stylosa* may decrease the capacity of the roots to take up nutrients at low temperatures. This decrease may be related to the fact that the low-temperature limit for seedling dispersal of *R. stylosa* is the warmest among the three species (Fig. S2).

The failure of the roots of the three Rhizophoraceae species to increase their R_{15} at low growth temperatures suggested that the

roots were less tolerant to low temperature than the leaves in these mangrove species. This conclusion is consistent with our previous observation that the growth rate of *R. stylosa* at low temperatures is limited by the rate of N uptake by its roots (Inoue *et al.*, 2022a). Under field conditions, root temperatures may be buffered by the heat capacity of the soils; tidal flooding events, which occur repeatedly in mangrove habitats, would also buffer root temperature. These considerations may explain why the leaves were more tolerant to low temperatures than the roots of these species. Comparative measurements of respiratory responses between the leaves and roots of plants grown at a combination of different temperatures will provide more information about the different respiratory responses of the leaves and roots in these species.

Responses of respiratory chain enzymes to growth temperatures and their relationship to E_o

E_o changed significantly with growth temperature in the leaves of *K. obovata* and *R. stylosa* (Type I acclimation), and the AOX : COXII ratio in their leaves also varied with growth temperature. If the amount of an enzyme is related to the electron partitioning between two pathways, the positive correlation between E_o and AOX : COXII was consistent with the conclusion of Kruse *et al.* (2011) that the activation energies of the CP and AP differ. The E_o of *K. obovata* roots was markedly lower at 30°C than at lower growth temperatures. Increases of the AOX : COXII ratio may be accompanied by an increase of E_o in *K. obovata* roots. Such a relationship would be consistent with measurements of AOX : COXII and E_o in the leaves of *K. obovata* and *R. stylosa*. In the leaves and roots of *B. gymnorrhiza*, the AOX : COXII ratio and E_o did not change with growth temperature. Inoue & Noguchi (2021) reported that E_o is affected by electron partitioning between the CP and AP. Electron partitioning to the AP contributes to the thermogenesis of the flowers of some species (Seymour, 2001). In many nonthermogenic plants, AOX lowers the levels of reactive oxygen species when the plants are subjected to various environmental stresses (Moore *et al.*, 2002; Millenaar & Lambers, 2003; Vanlerberghe, 2013; Vishwakarma *et al.*, 2015; Dinakar *et al.*, 2016). AOX : COXII was higher in *R. stylosa* leaves at low growth temperatures and in *K. obovata* leaves at high growth temperatures than at other growth temperatures; under those conditions, the electron flow to the AP may have been increased if the amount of an enzyme is related to the electron partitioning between two pathways.

In our study, most E_o values were lower in roots than in leaves. In other terrestrial plant species, some studies have shown that respiration rates are less temperature sensitive in roots than in leaves (Drake *et al.*, 2017; Noh *et al.*, 2020). Why E_o values differ between leaves and roots is unknown. The conditions that surround leaves and roots differ. For example, the O_2 concentrations are lower in the rhizosphere of underground roots than in the air surrounding aerial leaves, and the supply of electrons differs between roots and leaves because most roots do not have NADPH-producing photosynthetic systems. Further studies that address the relationship between E_o and the partial pressure of O_2 and between E_o and the supply of NADPH may reveal the mechanisms responsible for the differences between the E_o values of leaves and roots.

Estimated yearly profiles of O_2 respiration and growth rates

Our estimation suggests that under field conditions the annual average *R* values of leaves and roots of seedlings are higher for *K. obovata* than the other two species. Furthermore, the high O_2 respiratory capacity (R_{15}) of *K. obovata* leaves at low growth temperatures is reflected in the high estimated leaf *R* during the cold seedling dispersal season of this species. This implies that the high leaf *R* of *K. obovata* seedlings can respond to the energy demands required for maintenance and growth, even at low temperatures. In contrast to the behavior of *B. gymnorrhiza* and *R. stylosa* (Inoue *et al.*, 2022a), O_2 respiration for growth (R_g) and RGR increased with decreasing growth temperature in *K. obovata* leaves. To our knowledge, there has been no study of seasonal changes in seedling RGR under field conditions. Further observations of seasonal seedling growth are required to determine the accuracy of our estimation. Because *R. stylosa* starts to disperse its seedling in the middle of summer (July), the remaining time of warm conditions for growth in the first year is shorter than for the other two species. This constraint may be related to the short seedling dispersal period of *R. stylosa*, which ends in September.

Conclusions

We examined the temperature dependencies of *R*, N concentration and the amounts of respiratory chain enzymes in the leaves and roots of the seedlings of three Rhizophoraceae species grown at different temperatures to determine whether the responses of *R* to growth temperatures were related to the temperature intervals for seedling dispersal and energy demand in establishment. The respiratory capacity of *K. obovata*, whose seedling dispersal occurs in the coldest season among the three species, was higher than that of the other species and was accompanied by increased N concentration at low growth temperatures. This response was less apparent in the roots than in the leaves, where the amount of COXII was enhanced at low growth temperatures. The estimated *R* and RGR under field conditions revealed that *K. obovata* leaves sustained their *R* and growth rate even during the cold dispersal season. By contrast, the root *R* and RGR of *R. stylosa* were the lowest among the three species during the cold season (January–February). This pattern may be related to the fact that the lower temperature limit for dispersal of its seedlings is the warmest among the three species. The E_o values changed with the growth temperature of the leaves of *K. obovata* and *R. stylosa* and the roots of *K. obovata*. These changes were accompanied by changes in the ratios of the amounts of protein in the two respiratory pathways. A constant leaf and root E_o of *B. gymnorrhiza* may be required to accommodate the wider temperature range of its establishment phase. The species-specific responses of *R* revealed in this study suggest that the seedling dispersal phenology and growth of the three mangrove species will respond differently to anticipated temperature changes associated with global warming. The seedlings of *K. obovata* may not necessarily respond to cool

conditions by enhancing R_{15}, whereas seedling establishment of *R. stylosa* may move to more northern regions in the future under a warmer climate.

Author contributions

TI designed the research. TI, YA and SB cultivated the plants and conducted the measurements. TI and KN discussed and interpreted the data and contributed to the writing of the manuscript.

Competing interests

None declared.

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Data availability

The data that support the findings of this study are available upon reasonable request to the corresponding author.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Global distribution maps of the three Rhizophoraceae species.

Fig. S2 Air temperature and seedling dispersal ranges of the three Rhizophoraceae species.

Fig. S3 Relationship between temperature at the leaf and root in the growth chambers.

Fig. S4 Typical immunoblots of subunit II of COX and AOX in leaf and root extracts of the protein of the three Rhizophoraceae species measured with a protein autoanalyzer.

Fig. S5 Normal Q–Q plot of the data.

Fig. S6 Arrhenius plots of leaf and root O_2 consumption rates in the three Rhizophoraceae species.

Fig. S7 Relationships between the amount of subunit II of COX and nitrogen concentration in leaves and roots of the three Rhizophoraceae species.

Fig. S8 Temperature dependencies of O_2 respiration rate, O_2 respiration rate for maintenance (R_m) and O_2 respiration rate for growth (R_g) in leaves and roots of the three Rhizophoraceae species.

Fig. S9 Temperature dependencies of relative growth rate (RGR) during the 56 d cultivation in total seedlings, leaves and roots of the three Rhizophoraceae species.

Method S1 Measurement of growth and maintenance respiration rates.

Table S1 Estimated coefficients and y-intercepts of the models of the relationships between variables.

Table S2 F values and P values of the ANOVAs.

Table S3 Comparison of respiration variables in Rhizophoraceae species based on Tukey's multiple comparisons tests.

Table S4 Estimated coefficients and y-intercepts of the models of the growth–temperature dependencies of leaf and root respirations (R_{leaf} and R_{root}, nmol O_2 g DW^{-1} s^{-1}) of the three Rhizophoraceae species.

Table S5 P values for paired t-test analysis.

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