Effects of Salinity on the Growth and Survival of the Seedlings of Mangrove, *Rhizophora stylosa*

Hiromi Kanai[1], Mitsuki Tajima[2] and Atsushi Sakai[2*]

[1]*Department of Biological Sciences, Graduate School of Humanities and Sciences, Nara Women's University, Nara, 630-8506, Japan.*
[2]*Department of Biological Sciences, Faculty of Science, Nara Women's University, Kitauoya-nishi-machi, Nara, 630-8506, Japan.*

Authors' contributions

This work was carried out in collaboration between all authors. Author HK conducted most part of the experiment. Author MT conducted some additional culturing experiments for confirmation. Author AS designed the study and wrote the first draft of the manuscript. All authors read and approved the final manuscript.

Original Research Article

ABSTRACT

Aims: There are contradictory views on whether mangroves are obligate halophytes or facultative halophytes. In this study, we examined: (1) if seedlings of a mangrove, *Rhizophora stylosa*, require NaCl for their survival and (2) whether the growth response of *R. stylosa* seedlings under varying salinities was explained from the context of carbon economics.
Methodology: Seedlings of *R. stylosa* were hydroponically grown under varying salinities (0 - 480 mMNaCl) and their growth, mortality, photosynthetic and respiration rates were analyzed.
Results: Most of the seedlings grown under NaCl-free condition died during the 34-week culture, demonstrating their salt dependency. The best growth was accomplished under moderate salinity (240 mMNaCl) with highest stem elongation, maximum biomass gain and lowest leaf mortality. Whole-plant photosynthetic production was highest under the moderate salinity and declined towards high and low salinity ranges whereas whole-plant respiration did not increase towards high and low salinity ranges. The lower photosynthetic production under high salinity involved reductions in both leaf area and

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*Corresponding author: E-mail: sakai@cc.nara-wu.ac.jp;

photosynthetic potential per area while the lower photosynthetic production under low-salinity involved reduction in leaf area only.

Conclusion: *Rhizophora stylosa* appeared to require salt for survival. The maximal growth under moderate salinity might be explained by reduced photosynthetic production under low and high salinity ranges. The reduced photosynthetic potential and leaf area causing lower photosynthetic production under high salinity might be ultimately explained by the accumulation of excessive salt in leaf cells. In contrast, the ultimate causes for the reduced leaf area and increased mortality under low salinity remained unclear. Several possible mechanisms are discussed in relation to ion metabolism.

1. INTRODUCTION

Mangroves are woody plants dominating the coastal areas of tropical and subtropical regions. Major mangrove species inhabiting Japan include *Bruguiera gymnorhiza*, *Rhizophora stylosa*, and *Kandelia obovata*. Usually, mangrove species constitute mangrove forests forming obvious zonation [1]. Among several candidates, the salinity appears one of the most likely factors affecting the physiology and zonal distributions of mangrove species [2,3,4,5].

High salinity is generally harmful to plants but mangroves have developed various mechanisms for salt tolerance e.g., salt exclusion (ultrafiltration by roots and/or hypocotyls), salt excretion (pumping salt out of leaves via salt glands), salt accumulation and compartmentalization (transport of ions to the vacuoles of old leaves, which will be eventually lost by leaf falling) and accumulation of compatible solutes in the cytosol for osmotic balance [6,7,8,9,10,11,12,13]. The degree of salt-tolerance, as well as underlying mechanisms, differs among mangrove species. Among the three Japanese mangrove species mentioned above, *R. stylosa* appeared most highly salt-tolerant because it grows on soil with high salinity (3.3%) whereas *B. gymnorhiza* and *K. obovata* grow in lower salinities (2.9% and 1.5%, respectively) under natural conditions [2]. The results of cultivation experiment also suggested that *R. stylosa* had the highest salt-tolerance among the three [2].

Salt-enhanced growth and high mortality in the absence of NaCl have been reported for some mangrove species [2,14], suggesting that at least some mangroves are "obligate halophytes" i.e., plants that absolutely require salts for growth and survival. However, other researchers have regarded mangroves as "facultative halophytes" with no absolute requirement for salts [15]. Whether some mangrove species are indeed obligate halophytes or not, as well as the mechanisms for their salt-requirement and salt-enhanced growth, remains unclear at present.

In the present study, we examined the effects of various concentrations of NaCl (including its absence) on the growth and survival of seedlings of a mangrove, *R. stylosa*. We chose this species as an experimental material because (i) it exhibits high salt-tolerance, (ii) this and closely related species reportedly show salt-enhanced seedling growth [2,14], (iii) it is one of the most representative mangrove species in Japan and (iv) its seedlings are commercially available from market, being sold as souvenirs and/or interior plants. We then examined

whether the observed growth responses could be explained in the context of carbon economics. Finally, possible mechanisms underlying the observed growth/survival responses were discussed from the viewpoint of ion metabolism.

2. MATERIALS AND METHODS

2.1 Plant Material, Culture Conditions and Chronological Flow of the Experiments

The chronological order of the experiments is shown in (Fig. 1), together with the outlook of the culture setting. Seedlings of *Rhizophora stylosa*, sold as pot-grown hydro-cultured interior plants, were purchased from Ishigaki-jima Shopping Plaza (Naha-City, Okinawa, Japan). Each pot (9 cm in diameter, 7.6 cm in depth, harboring three seedlings) was filled with expanded-clay for hydroponic plant culture (hydrocorn, Jongkind Ground BV, Aalsmeer, Netherlands). Two pots were set in one box container (14.5 cm × 23.5 cm × 7 cm, L × W × H) and immersed in 1 l of nutrient solution, to the lower half of their depth. The composition of nutrient solution has been reported in [14]: 4 mM $Ca(NO_3)_2$, 1 mM KNO_3, 1 mM $MgSO_4$, 1 mM $(NH_4)H_2PO_4$, 1 mM $(NH_4)_2HPO_4$, 50 μM KCl, 2.5 μM $MnSO_4$, 20 μM NaFe-EDTA, 2 μM $ZnSO_4$, 1 μM KI, 0.5 μM $CuSO_4$, 0.5 μM Na_2MoO_4 and varying concentrations of NaCl (0, 30, 60, 120, 240, 360, and 480 mM), pH 6.4-6.9. Air temperature was 25°C, relative humidity was 60% and photoperiod was 14-h light (80 μmol quanta m^{-2} s^{-1} from fluorescent tubes, light on at 05:00) and 10-h dark. The seedlings were initially grown NaCl-free for 1 week, divided into seven groups (for 0 to 480 mMNaCl) each containing 12 seedlings (4 pots, 2 containers), and the step-wise increase in NaCl concentration (maximum 60 mM per week) was started (designated as day 0). The raise in NaCl concentration by weekly medium renewal was continued until target concentrations of respective groups were reached. After all groups had reached their respective target concentrations, the medium was renewed once per 2 weeks.
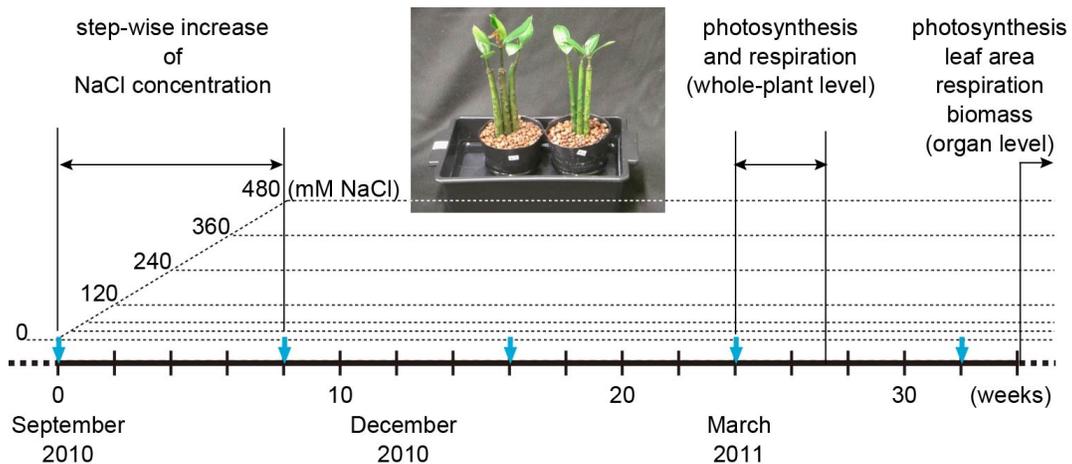

Fig. 1. Culture setting and experimental design. A photograph on the top center depicts the culture settings. Thick vertical ticks represent the timing of medium exchange and measurements of leaf number. Climbing and horizontal dotted lines represent the changes in NaCl concentration within the medium. Vertical arrows represent the timing of measurement of stem length

Stem lengths of the seedlings were measured at 8-week intervals while the number of leaves per seedling and the number of dead seedlings were monitored at 2-week intervals. The whole-plant photosynthesis and respiration rates were measured 24-27 weeks after the start of the experiment, as described later. At the end of the experiment (34 weeks after the start), seedlings were carefully removed from the pot and separated into respective organs. Then, photosynthetic and respiratory rates of respective organs were measured as described later, and total leaf area was measured by pixel counting using photocopied images of the leaves. Finally, separated organs were dried at 70ºC for 3 days and their weights measured.

2.2 Measurement of Photosynthesis and Respiration Rates at Whole-plant Level

The whole-plant photosynthesis and respiration rates were measured with a closed circulation system equipped with an infra-red gas analyzer (IRGA; LI-800, Li-Cor). Culture pots, each harboring three seedlings, were set in an air-tight, transparent (acrylic) assimilation box (15 cm × 15 cm × 30 cm, L × W × H) equipped with an electrical fan (to stir the air within the box) and two tubing adapters (air inlet and outlet). The air within the assimilation box was withdrawn, introduced into an IRGA, and then returned back to the assimilation box at the rate of 200 $cm^3 min^{-1}$ with the aid of a high-speed peristaltic pump. The whole-plant net photosynthesis rates were estimated under the usual culture condition (25ºC and 80 μmol quanta m^{-2} s^{-1}), based on the time required to lower the CO_2 concentration within the closed system (7350 cm^3) by 10 ppm (from 470 ppm to 460 ppm). The whole-plant dark respiration rates were estimated in a similar way, except that the plants were maintained in the dark with a black-out curtain and that the time necessary to elevate the CO_2 concentration within the circulation system by 10 ppm (irrespective of the absolute values) was measured. Although respiration was measured 6 times within a day (00:00, 04:00, 08:00, 12:00, 16:00, and 20:00) and photosynthesis measured 3 times (08:00, 12:00, and 16:00), no obvious diurnal changes were found. Thus, the results obtained at different time were averaged to obtain daily mean values for individual pots.

2.3 Measurements of Photosynthesis and Respiration at Organ Levels

Gross photosynthetic potentials of the leaves were measured with a closed chamber system equipped with a gas-phase O_2 electrode (LD 2/2, Hansatech) as described previously [16]. Leaf discs (1.1 to 2.7 cm^2 depending on leaf size) cut out from healthy young leaves were set in the leaf chamber and their O_2 generation and O_2 consumption rates were measured under illumination (1300 $\square$mol quanta m^{-2} s^{-1}) and in the dark, respectively, at 25ºC in air containing 5% CO_2. Dark respiration rates were measured with the same equipment at 25ºC in the dark and in ambient air using the leaf discs, stem cuttings (cut into 2-3 segments, each about 4 cm in length), hypocotyl sections (4 slices, total 1 cm in thickness), or fine root cuttings (about 0.2 g in fresh weight) as specimen.

2.4 Statistical Analysis

Since responses of growth and physiological parameters to salinity levels were expected to have some optimal values along the salinity gradient, they were statistically analyzed with polynomial regressions up to the quadratic term using JMP 8.0 (SAS institute, Cary, NC, USA). In this analysis, salinity level and its quadratic term were designated as independent variables while stem elongation, emerging leaf number, leaf mortality, biomass, gross

photosynthesis rate and dark respiration rate of seedlings were each designated as a dependent variable. Normality of the residuals was checked for each analysis. If the assumption was not met, the data were log_{10}-transformed after adding 0.5. The results of the regression analyses are summarized in (Table 1). For comparisons of total leaf area, photosynthetic potential and respiration rate among high, low and moderate salinities, Tukey-Kramer's multiple comparison test following ANOVA was conducted using JMP 8.0.

3. RESULTS AND DISCUSSION

3.1 Effects of Varying Salinities on the Growth and Mortality of *Rhizophorastylosa* Seedlings

As shown in (Fig. 2a), *R. stylosa* seedlings exhibited high mortality under extremely low salinities (0 or 30 mMNaCl). In the absence of NaCl, they began to die around week 16 (data not shown), and their mortality reached 0.75 at the end of the experiment (week 34), and continued to increase thereafter. In the presence of 30 mMNaCl, the seedlings also died but they began to die later (week 20, data not shown) and the final mortality was lower (0.17).

The relationship between salinity and stem elongation during experiment (Fig. 2b) demonstrated that optimal growth was accomplished under moderate salinity. Optimum NaCl concentration for stem elongation calculated from the regression equation (Table 1) was 298 mM.

In the analysis of final seedling biomass (Figs 2c and 2d), data derived from containers harboring dead individuals were excluded from data analysis to avoid possible secondary effects of dead individual(s) on others. Total biomass of the seedlings was not represented as a quadratic polynomial of salinity level (Table 1). Although the highest average value was obtained at moderate salinity (240 mMNaCl), the peak was unclear (Fig. 2c). We then excluded the weight of hypocotyls from the data analysis (Fig. 2d) because the hypocotyls occupied large proportion (about 80%) of the seedling biomass but should not reflect the biomass gained after seedling settlement when viviparous nature of *R. stylosa*was taken into account. Total biomass of the organs emerging after seedling settlement (i.e., leaves, stems, and roots) showed a clear peak at moderate salinity and the optimal salinity calculated from the regression equation (Table 1) was 270 mMNaCl.

The number of leaves that newly opened during the experimental period showed a broad peak around moderate salinity (Fig. 3a) and the optimum salinity calculated from the regression equation (Table 1) was 270 mMNaCl. The number of leaves lost during the culture period exhibited a complicated behavior according to the salinity change (Fig. 3a) and could not be represented as a quadratic polynomial of salinity level (Table 1). However, leaf mortality calculated by dividing the number of lost leaves with the total number of leaves that respective seedlings had harbored during the experiment took the lowest values under the moderate salinity (Fig. 3b), and the NaCl concentration that gave the lowest leaf mortality was calculated from the regression equation (Table 1) as 288 mM.

Table 1. Summary table for the regression analyses

Dependent variables	Figure number	Linear term		Quadratic term		Data transform	Equation
		t	*P*	*t*	*P*		
stem elongation (mm)	Fig 2a	8.28	<0.001	—5.73	<0.001	Log_{10}	$Log_{10}(Y+0.5) = 17.5 + 0.0585 X - 0.000312 (X-184)^2$
biomass incl. hypocotyl (g per plant)	Fig 2c	0.06	0.955	—1.43	0.156	No	$Y = 4.24 + 0.000049 X - 0.0000097 (X-232)^2$
biomass w/o hypocotyl (g per plant)	Fig 2d	0.78	0.438	—2.61	0.011	Log_{10}	$Log_{10}(Y+0.5) = -0.059 + 0.00015 X - 0.0000038 (X-232)^2$
leaf emergence (per plant)	Fig 3a	3.13	0.003	—2.86	0.005	No	$Y = 4.10 + 0.0048 X - 0.000028 (X-184)^2$
leaf loss (per plant)	Fig 3a	1.16	0.249	0.22	0.828	No	$Y = 1.89 + 0.0019 X - 0.0000023 (X-184)^2$
leaf mortality	Fig 3b	—3.64	0.001	3.02	0.003	Log_{10}	$Log_{10}(Y+0.5) = -0.287 - 0.00031 X + 0.0000016 (X-184)^2$
gross photosynthesis ($pmol\ s^{-1}$ per plant)	Fig 4	0.42	0.680	—2.06	0.053	No	$Y = 99.1 + 0.0200 X - 0.000763 (X-232)^2$
dark respiration ($pmol\ s^{-1}$ per plant)	Fig 4	0.20	0.845	—1.64	0.118	No	$Y = 24.5 + 0.0024 X - 0.000155 (X-232)^2$

Responses of growth- and physiological parameters to salinity levels were statistically analyzed with polynomial regressions up to the quadratic term where salinity level (X) and its quadratic term were designated as independent variables while stem elongation, emerging leaf number, leaf mortality, biomass, gross photosynthesis rate and dark respiration rate of seedlings were each designated as a dependent variable (Y). Normality of the residuals was checked for each analysis. If the assumption was not met, the data were log10-transformed after adding 0.5
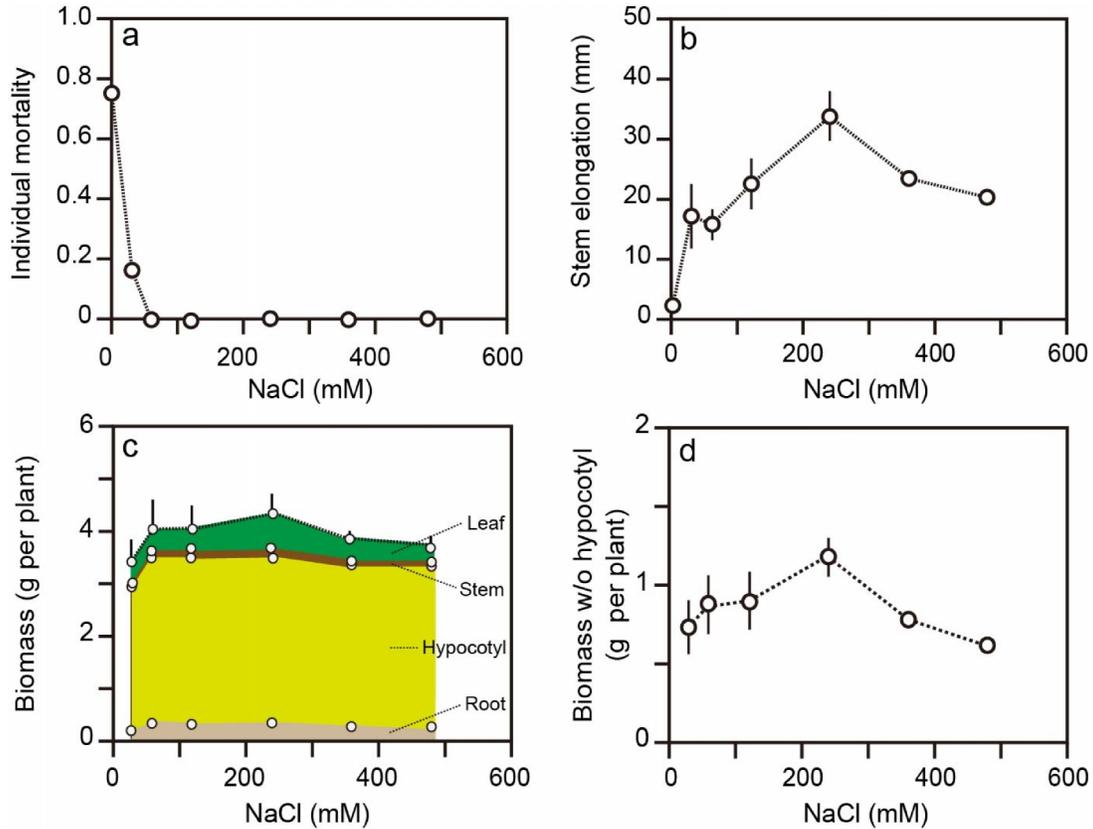
Fig. 2. Effects of NaCl concentrations on the growth and mortality of *R. stylosa* seedlings. Mortality (a), stem elongation (b), total biomass and biomass distribution among respective organs (c) and total biomass of non-hypocotyl organs emerging after seedling settlement (d) measured at the end of the 34-week culture experiment, are represented as a function of salinity. In (b), each data represents average from 12 seedlings including dead individuals. In (c) and (d), containers harboring dead individual(s) were excluded from data analysis and each data represents average from 6 to 12 individuals. Vertical bars represent standard errors

3.2 Effects of Varying Salinities on the Whole-plant Photosynthesis and Respiration Rates of *Rhizophora stylosa* Seedlings

To examine the reason why *R. stylosa* seedlings grew best under moderate salinity from the viewpoint of carbon economics, we then analyzed the effects of salinity on whole-plant photosynthesis and dark respiration rates because (i) growth of a plant is determined by the balance between the gain of biomass (through photosynthetic assimilation) and its loss (through respiratory consumption and organ loss such as leaf fallings) and (ii) analyses on leaf loss and individual mortality had been conducted (Figs. 2 and 3).
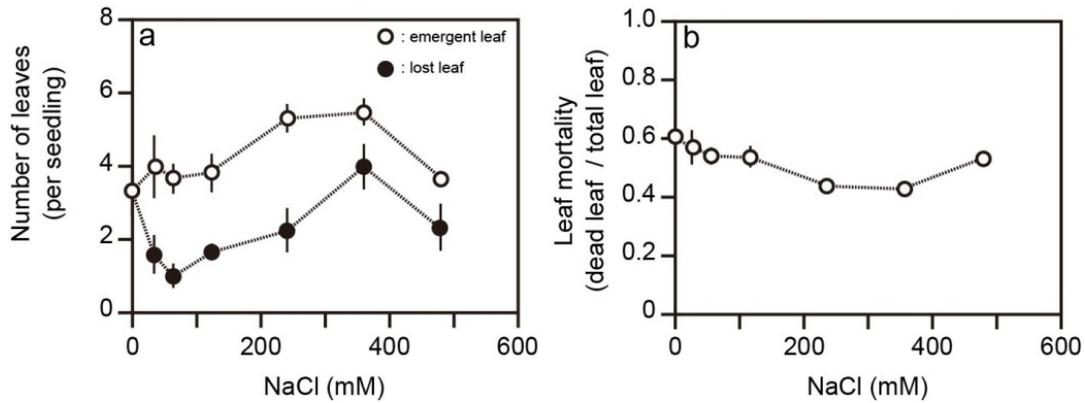
Fig. 3. Effects of NaCl concentrations on emergence and loss of leaves in *R. stylosa* seedlings: (a) Effects on the numbers of leaf emergence and leaf loss. (b) Effects on mortality of the leaves (the number of lost leaves divided by total number of leaves that the seedling has harbored during the experimental period). Each data represents average from 12 individuals with standard error

For per-plant gross photosynthetic rate, the highest average value was recorded under moderate salinity (Fig. 4). Regression analysis (Table 1) showed that the effect of the quadratic term of salinity level was marginally significant (*P* = 0.053) and that the highest average value was obtained at 259 mMNaCl, suggesting that gross photosynthesis per plant might be maximum under the moderate salinity, contributing to the optimal growth under that condition.
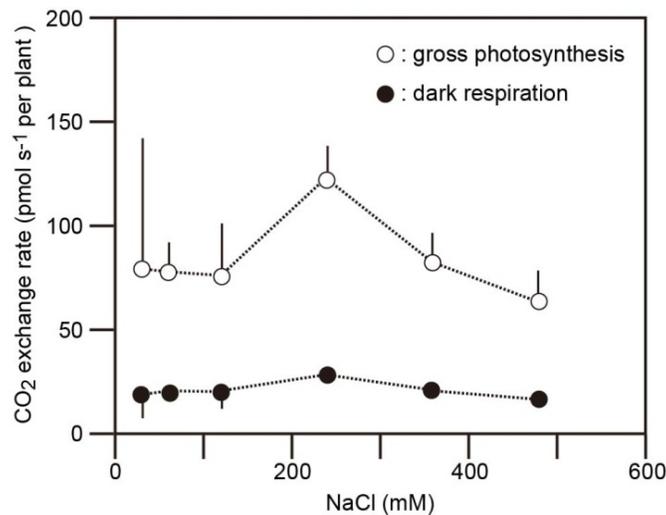

Fig. 4. Effects of NaCl concentrations on the gross photosynthetic CO_2 uptake and respiratory CO_2 release at whole-plant level. The *R. stylosa* seedlings were grown for 24 – 27 weeks under varying concentrations of NaCl and their whole-plant CO_2 exchange rates were measured under illuminated (80 μmol quanta m^{-2} s^{-1}) and dark conditions. The results are shown in per-plant basis. Each data represents average of 2 to 4 pots (each harboring three individuals) with standard error

The whole-plant dark respiration did not increase towards low- and high-salinity ranges (Fig. 4) and was not expressed as a quadratic polynomial of salinity (Table 1), indicating that the changes in the respiratory consumption could not be responsible for the growth reduction under low- and high salinity ranges.

3.3 Comparison of Respiration Rates of Various Organs among *R. stylosa* Seedlings Grown under Low-, Moderate-, and High Salinities

The absence of increased respiratory consumption in any organ under low and high salinity ranges was confirmed by measuring dark respiration rates of individual organs and comparing them among low, moderate and high salinities (30, 240, and 480 mMNaCl). For leaf respiration (Fig. 5a), significant difference was detected among the three salinity levels (*ANOVA, F* =3.83, *d.f.* = 22, *P* = 0.039) but the highest average value was recorded for the moderate salinity. For stems, hypocotyls and roots (Figs. 5b to 5d), differences in the respiration rates among the three salinity levels were not significant (*ANOVA*; for stems, *F* =1.81, *d.f.* = 23, *P* = 0.188; for hypocotyls, *F* =0.61, *d.f.* = 23, *P* = 0.554; for roots, *F* =0.736, *d.f.* = 23, *P* = 0.491). Those results excluded the possibility that enhanced respiration in any organ was involved in the reduced growth under low and high salinity ranges.
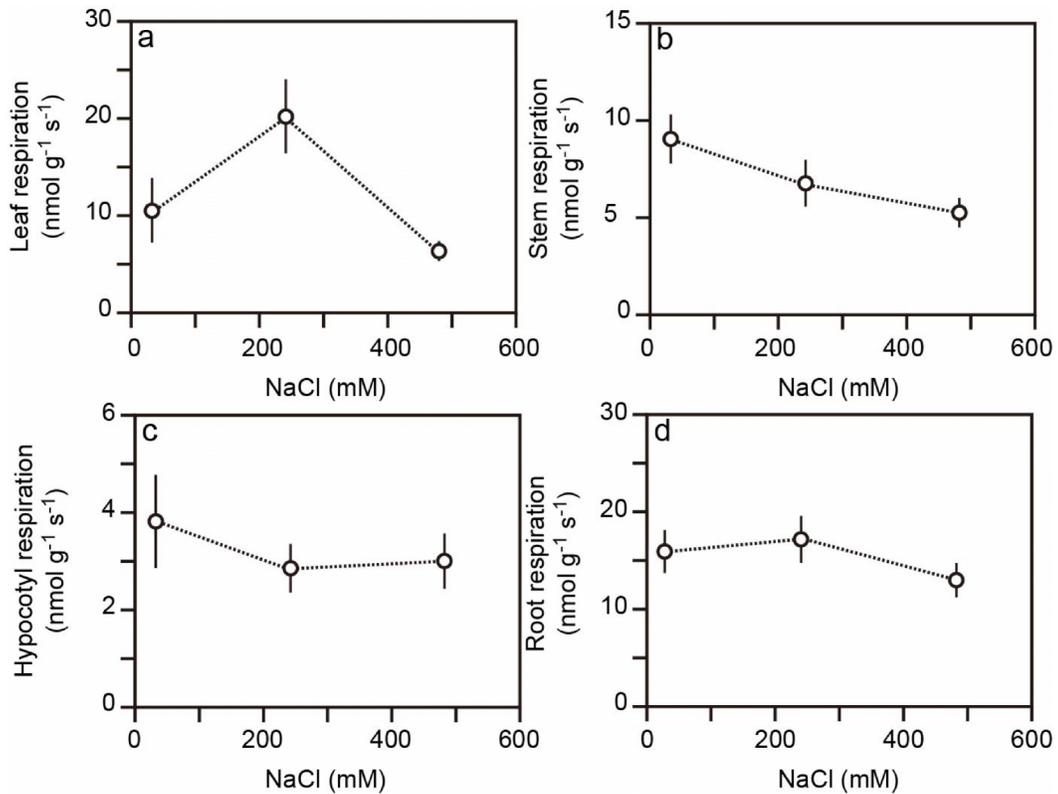

Fig. 5. Effects of low, moderate and high salinities on respiration rates in various organs. The *R. stylosa* seedlings were grown for 34 weeks in the nutrient solutions containing 30, 240 or 480 mM of NaCl and respiration rates of their leaves (a), stems (b), hypocotyls (c) and roots (d) were measured with a gas-phase O$_2$ electrode system. Each data represents average value from 6 to 12 measurements with standard error

3.4 Comparison of Total Leaf Area and Photosynthetic Potential of the Leaves among *R. stylosa* Seedlings Grown under Low, Moderate and High Salinities

We then measured total leaf area per plant and photosynthetic potential per leaf area and compared them among low, moderate and high salinities. Total leaf area (Fig. 6a) was significantly different among the three salinity levels (ANOVA, F = 11.46, $d.f.$ = 29, P< 0.001) and was smaller in low and high salinities than in moderate salinity (Tukey-Kramer's HSD, P = 0.034 and P< 0.001 for low and high salinities, respectively). Photosynthetic potential per leaf area (Fig. 6b) was also significantly different among the three (*ANOVA*, F = 8.58, $d.f.$ = 22, P = 0.002). While high salinity significantly reduced the photosynthetic potential (Tukey-Kramer's HSD, P = 0.002), low salinity did not (Tukey-Kramer's HSD, P = 0.874). Thus, reduction in the whole-plant photosynthesis under high salinity involved both smaller leaf area and lower photosynthetic potential whereas the reduction under low salinity involved smaller total leaf area only.
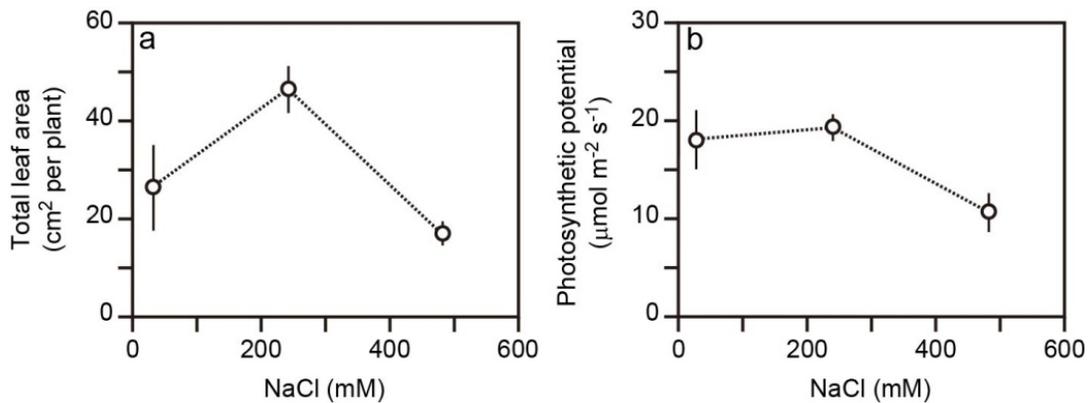

Fig. 6. Effects of low, moderate and high salinities on the total leaf area and photosynthetic potential of the mature leaves. The *R. stylosa* seedlings were grown for 34 weeks in the nutrient solutions containing 30, 240, or 480 mM of NaCl and their total leaf area per seedling (a) and photosynthetic potential of the leaf discs (b) were measured. In (a), each data represent average value from 6 to 12 seedlings with standard errors. In (b), the rates of O$_2$ generation and O$_2$ consumption by leaf discs were measured with a gas-phase O$_2$ electrode system under illuminated (1300 μmol quanta m^{-2} s^{-1}) and dark conditions to calculate gross photosynthetic potential. Each data represents average value from 5 to 12 leaf discs with standard error

3.5 Discussion

3.5.1 Response of *R. stylosa* seedlings to varying salinities

In the present study, *R. stylosa* seedlings were demonstrated to grow best under moderate salinity (around 240 mMNaCl), similarly to the case with some mangrove species [17,18,19]. Moreover, most of the seedlings grown in the absence of NaCl died during the 34-week experimental period, similarly to the case with *R. mangle* [14]. Judging from the salt dependence of effective survival, it is highly probable that *R. stylosa* fulfills the definition of obligate halophytes [15]. Thus, we can pose the following three questions:

(1) How does the high salinity reduce the growth?
(2) How does the low salinity reduce the growth?
(3) Why do *R. stylosa* seedlings require NaCl for survival?

3.5.2 Growth reduction under high salinity

Our results indicated that the growth reduction under high salinity might primarily be attributable to reduced photosynthetic assimilation (Fig. 4) and increased leaf loss (Fig. 2). We initially suspected that the respiration rate might become higher under high salinity to cause growth reduction because the construction and operation of salt-tolerance mechanisms should be energy-consuming [20]: However, it was not the case (Figs. 4 and 5). The reduction in the whole-plant photosynthesis under high salinity was accompanied by both reduced photosynthetic potential and smaller total leaf area (Fig. 6). Although both reduced leaf emergence and increased leaf mortality (Fig. 2) should cause the smaller total leaf area, reduced leaf emergence is one aspect of growth reduction itself. Thus, we tentatively propose that reduced photosynthetic potential and increased leaf mortality might be the ultimate causes for the growth reduction under high salinity.

The reduced photosynthetic potential and increased leaf mortality under high salinity might be both explained by accumulation of excessive salt in the leaf cells. *Rhizophora stylosa* is a "salt accumulator" that accumulates high concentration of salts in leaf cell vacuoles. Because accumulated salts must eventually be shed through leaf fallings [13], more frequent leaf falling and thus increased leaf mortality may be necessary under high salinity. Moreover, several enzymes derived from mangroves are as equally sensitive to salts as those from non-halophytes [6,15,21]. Thus, slight increase in cytoplasmic NaCl concentration under high-salt condition might interfere with the cellular activities including photosynthesis, as also reported for one of the most highly salt-tolerant mangrove *Avicennia marina* [22].

3.5.3 Growth reduction under low salinity

The growth reduction under low salinity was also attributable to reduced photosynthetic assimilation and increased leaf loss (Figs. 4 and 3), rather than enhanced respiration (Figs. 4 and 5). The reduced photosynthetic assimilation under low salinity was almost fully attributable to the smaller total leaf area because there was no detectable decline in the photosynthetic potential. The reduction in the total leaf area should come from reduced leaf emergence and increased leaf mortality. However, their ultimate cause(s) cannot be easily explained. When high mortality of *R. stylosa* seedlings under NaCl-deficient condition is also taken into consideration, the reduced growth under low salinity might be better understood from mechanism of salt requirement, rather than carbon economics.

3.5.4 *Rhizophora stylosa* seedlings require salt for high survivability

In the present study, *R. stylosa* seedlings clearly required salt (NaCl) for their normal growth and high survivability. As has been mentioned above, there are contradictory views on whether mangroves are facultative halophytes or obligate halophytes [15]. However, "mangroves" are taxonomically so diverse (including 54 species in 20 genera belonging to 16 families; [1]) that the response to salt might be different among species. For example, *B. gymnorhiza* that prefers low salinity [2] can grow well in freshwater [23,24], whereas *A marina* and *R. stylosa* that can tolerate high salinity [2] require NaCl for normal growth [17, 18 and this study]. We thus propose that the obligate halophytic nature might be more

obvious in mangroves with high salt-tolerance; the high-salt environment may facilitate the evolution of certain physiological mechanism(s) dependent on salt.

In addition, the viviparous nature of mangroves as well as relatively short periods of laboratory culture experiments might cause false salt-independence and, at least partly, cause the contradiction. The propagules of mangroves can store large amounts of materials supplied from mother plants, including Na^+/Cl^- ions, in their hypocotyls or cotyledons [17]. Therefore, even a salt-dependent mangroves can grow and survive in fresh water for a while (until those reserves are exhausted). The small hypocotyl sizes (about 12 cm in length, probably preferred for use as a souvenir) of *R. stylosa* seedlings used in the present study might cause earlier exhaustion of reserves, resulting in drastic and earlier response to NaCl-shortage.

Nonetheless, the conclusion that *R. stylosa* is an obligate halophyte awaits further experimental testing. First, the culture should be continued until all of the seedlings at zero-salinity died to demonstrate that survival is impossible for any individual. Second, our culture conditions (hydro-culture in standing water) might have introduced additional stressor for the seedlings because daily fluctuations between water-logged and drained states are normal under natural conditions [25]. As to the effects of "reserves" on the longevity of the seedlings' survival, not only the sizes of the propagules but also the salinity that their mother plants had experienced should be taken into account. Future experiments to demonstrate obligate halophytic nature of this species should be conducted under artificial tiding and continued to perdition of the seedlings grown in the absence of salt, taking possible influences of the parent hydrology (including salinity) into account.

3.5.5 Possible mechanisms for salt requirement

The molecular and physiological basis for the growth reduction in the absence of NaCl observed for some mangroves (including *R. stylosa*) is not well understood [26]. One possible explanation is that they use Na^+/Cl^- ions for osmoregulation, as reported in succulent, non-mangrove halophytes [27,28,29,30] and that shortage of Na^+/Cl^- ions causes incorporation of other essential ions (K^+, for example) into vacuoles for osmoregulation, resulting in shortage of the essential ions in the cytoplasm. In the absence of NaCl, *R. stylosa* seedlings showed symptoms similar to K^+-deficiency (e.g., chlorosis and necrosis at the leaf margins and between leaf veins and narrow stems with short internodes) and their growth reduction in the absence of NaCl was ameliorated by raising KCl concentrations in the culture medium (pers. obs.), consistent with the "shortage of essential ion" hypothesis.

Another possibility is that some essential physiological processes in *R. stylosa* may require Na^+. Because Cl^- was included in our culture media in the form of KCl even in the NaCl-free condition, Na^+ is more likely candidate for the required ion. Although it has long been believed that higher plants do not require Na^+ for their physiology, several sodium-dependent mechanisms such as sodium-dependent pyruvate transporter [31,32,33] are now known to widespread among higher plants. More significantly, a seagrass *Zostera marina* utilizes Na^+ ion as a co-transport ion in incorporating phosphate- and nitrate ions across plasma membrane [34,35] because the use of Na^+ ion (instead of ordinary H^+ ion) is cost-effective under the saline conditions. It is possible that some mangroves living in high-salt conditions (including *R. stylosa*) may also have evolved similar Na^+-dependent system and thus have become salt-dependent. This "sodium-dependent mechanism" hypothesis should be examined in a future work.

4. CONCLUSION

Rhizophora stylosa is probably an obligate halophyte that requires salt for survival. They grew best in the presence of moderate concentrations of NaCl (about half the strength of seawater), and the growth reductions under the low and high salinities were accompanied by reductions in photosynthetic production but not by enhancement of respiratory consumption. While growth reductions under high salinity conditions might be ultimately explained by accumulation of excessive salts in leaf cells, those under low-salinity conditions await clarification of salt-dependent mechanism(s) in *R. stylosa*. We propose that salt-dependency differs among mangrove species and that some highly salt-tolerant mangroves might have evolved some salt-dependent mechanisms to develop obligate halophytic nature.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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