RESEARCH ARTICLE

Effect of different time of salt stress on growth and some physiological processes of *Avicennia marina* seedlings

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Abstract Growth and physiological characters of *Avicennia marina* seedlings cultured under different levels of salinity were compared at 45 and 100 days after sowing. Based on the growth and physiological responses, the levels of salinity were grouped into two kinds, moderate (5–30‰) and extreme (40 and 50‰ as well as 0‰). Root and shoot length, leaf area, biomass of different organs, and net photosynthesis rate all showed a similar trend: the seedlings grew better at moderate levels of salinity but were adversely affected by extreme levels. Longer exposure (100 days) to salinity markedly enhanced the difference between the effects of the two levels on growth. By 45th day, the cotyledons had withered and fallen off. The concentration of ions (K^+, Na^+, Ca^{2+}, Mg^{2+}, Cl^-) and ash content of the cotyledons were determined before sowing and 45 days later. Ion concentrations and ash content of cotyledons were markedly lower at 45 days—lower than the initial levels—in seedlings irrigated with water at 0‰ salinity level. This suggested that the poor growth of these seedlings at 100 days may be due to lack of ions provided by the cotyledons. The high ion concentrations in the cotyledons grown at moderate salinity levels suggest that these organs may function as ion sinks at this stage, reducing the concentration of ions and consequent toxicity caused by excessive concentrations. Root biomass was higher than shoot biomass 45 days after sowing, whereas after 100 days, shoot biomass was higher. At the early stage of growth (45 days), the rate of photosynthesis at lower levels of salinity (0–30‰) was limited mainly by stomatal closure but at higher levels of salinity (40–50‰), other factors came into play. Later, at 100 days, the causes of reduced photosynthetic rate were other than stomatal closure at both low and high levels of salinity. This indicates that photosynthesis is affected by prolonged exposure to salt stress—including that caused by 0‰ salinity, as shown by poor growth of the seedlings.

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Introduction

Although mangroves are a kind of halophyte, their seedlings are sensitive to salt stress (Tomlinson 1986; Lin 1997); a saline substrate affects many aspects of their growth and physiology (Ball and Farquhar 1984a, b; Wang and Lin 1999; Clough 1984; Downton 1982). As is the case with many other halophytes, which grow poorly in a culture medium that lacks sodium chloride (Flower et al. 1977), most studies have found that whereas seedlings grow best at low salinity (25% of sea water), higher salinity (50 or 75% of sea water) or total lack dissolved salts (0‰ salinity, i.e. fresh water) affect growth (Downton 1982; Clough 1984; Wang and Lin 1999). Slow growth in fresh

water is often ascribed to the inability of halophytes to accumulate inorganic ions in quantities sufficient for osmoregulation when the substrate is lacking in sodium chloride (Clough 1984; Jennings 1976; Greenway and Munns 1980; Yeo and Flower 1980). Some authors consider this phenomenon to be the expression of a physiological trait of mangroves that demands salt (Wang and Lin 1999), but few studies have attempted to explain the mechanism.

Seedlings of halophytes can obtain the elements that their growth demands from soil or from the propagules themselves in the case of viviparous mangroves (Tomlinson 1986; Farnsworth 2000). The remarkable propagules accumulate ions and nutrients from the mother tree before being released (Zheng et al. 1999). *Avicennia marina* is a typical salt-secreting cryptoviviparous mangrove, a pioneer species widespread around the world (Tomlinson 1986; Lin 1997) which is regarded as one of the most salt-tolerant species of mangrove (Clough 1984; Tomlinson 1986; Lin 1997). Its cotyledons gain ions and nutrients during propagule development and may act as a sink for ions and nutrients (Wang et al. 2002) that support the seedlings in their early growth in the saline culture medium. But the function of the ions and nutrients accumulated during the propagule's development poses a few questions. After a few days of early growth, the cotyledons of *Avicennia marina* wither and fall off, an event that may mark the watershed between two growth stages. It is known that salt tolerance of a plant is related to the stage of growth (Munns 1986; Zhao and Wang 1990; Wang and Lin 2000). Generally speaking, halophytes are sensitive to salt stress at the seedling stage and the flowering stage but gradually increase their tolerance the longer they are exposed to salt stress (Zhao and Wang 1990; Wang and Lin 2000; Wang et al. 2001). The loss of cotyledons may affect plant growth. We measured some characteristics of growth and physiology of *Avicennia marina* seedlings at different stages of growth, i.e. following shorter or longer duration of exposure to salinity, at different levels of salinity to identify the role cotyledons play in a seedling's early growth.

Materials and methods

Sampling and cultivation

Propagules of *Avicennia marina* were collected from Haimen Island (24°30′N, 117°55′E), Longhai city, Fujian province of China, on 1 October 2003. The collected propagules were more or less uniform in size (2.1 ± 0.3 cm in diameter and weighing 1.8 ± 0.5 g) and free of holes or other signs of damage by insects.

The propagules were raised in a greenhouse in plastic pots (35 cm in diameter and 14 cm deep), each filled with 4 kg of coarse beach sand (particles 2–3 mm in diameter) collected from Xiamen harbor and washed with fresh water before use. The treatments comprised eight levels of salinity, each was replicated three times (0, 5, 10, 15, 20, 30, 40, 50‰). The saline solutions were prepared by mixing fresh water with either sea water (for 0, 5, 10, 15, or 20‰ solutions) or sea salt (for 30, 40, or 50‰ solutions). The salinity level of sea water was 27‰. While the pots were watered with the saline solutions every 15 days throughout the experiment, fresh water was added daily to make up for losses by evaporation or transpiration. The experiment lasted 100 days (October 2003 to January 2004).

Methods of analysis

Six seedlings were collected from each pot on the 45th day and on the 100th day. The length of the stem, mean root length, and leaf area were measured in three seedlings and the other three seedlings were used for determining the biomass: the roots, stems, and leaves were separated, washed with distilled water, and weighed after drying at 75°C for 48 h. The cotyledons, collected on the 45th day, were also dried at 75°C for 48 h and pulverized in a mill until the powder was fine enough to pass through a 1-mm sieve. The powder was stored for analyzing the ion content.

Cl^- was determined by $AgNO_3$ titration. The subsamples (about 0.2 g each) were incinerated at 550°C for 3 h. To avoid the loss of Cl^-, about 0.05 g of calcium oxide was added and mixed with the sample beforehand. The ash was dissolved in distilled water and then filtered, and the filtrate was used to determine Cl^-. Another sample (about 0.1 g) was incinerated at 550°C for 5 h, the ash dissolved in 50 ml hydrochloric acid (1 N), and the solution used in an atomic absorption spectrophotometer (AAnalyst 800, Perkin Elmer Instrument, USA) for determining the concentrations of K^+, Na^+, Ca^{2+}, and Mg^{2+}. Dry powder of the cotyledons was collected on the day the experiment began and on the 45th day to calculate the gross calorific value by using a bomb calorimeter (PARR 1266, USA).

Three seedlings were selected from each pot on 50th and 90th day and nine or more leaves from each treatment were chosen to measure the net photosynthesis rate (P_n, µmol m^2 s^{-1}), stomatal conductance (G_s, µmol m^2 s^{-1}), intercellular concentration of CO_2 (C_i, ppm) and evaporation rate (T_r, µmol m^2 s^{-1}) by the Photosynthesis System (Model CIRAS-1, PPsystem, UK). Photosynthetic photon flux density was maintained at 800 µmol photons m^{-2} s^{-1} on the cuvette surface by a portable light unit. All measurements were carried out between 9 a.m. and 12 noon, when ambient relative humidity was about 80%, the leaf

temperature ranged from 29 to 32°C, and ambient CO_2 concentration was about 355 ppm.

Results

Influence of salt stress on growth of *Avicennia marina* seedlings

Root and shoot length, leaf area, biomass of different organs, and net photosynthesis rate all showed a similar response to salinity after short- and long-term exposure to salt stress (Figs. 1, 2, 4). The root: shoot ratio was also unaffected by salinity at day 50 up to 30‰ and then rose above this (Fig. 3). In other words, moderate levels of salinity promoted growth but high levels or total lack of it suppressed growth. The seedlings at 5–20‰ salinity grew fastest. In order to express this difference, we use the *C*-value, which can be calculated by the formula

$$C = \left(\frac{M - T}{T}\right) \times 100\%$$

where *M* is the average value of a parameter (root and shoot length, leaf area, biomass of different organs, or net photosynthesis rate) at low salinities (5–20‰) and *T* is the value at 0‰ salinity. The results, given in Table 1, show that the *C*-values on the 45th day were notably lower than those on the 100th day; root biomass, in particular, showed a value of 21% on the 45th day but of 71% on the 100th day.

The ratio of root biomass to shoot biomass (RB/SB) was also different at the two stages (Fig. 3): on the 45th day, the ratio showed a slight increase with salinity from 0 to 20‰ and a steep rise from 30 to 50‰ whereas on the 100th day, the slight increase was confined to only 0 and 5‰ salinity; as salinity increased further, the value in fact decreased slightly.

Influence of on leaf P_n, T_r, G_s, and C_i of *Avicennia marina* seedlings

The net photosynthesis rate (P_n), stomatal conductance (G_s), intercellular CO_2 concentration (C_i), and transpiration rate (T_r) were all different at the two stages (Fig. 4). On the 90th day, the net photosynthesis rate was markedly depressed at zero salinity in contrast to that at salinity levels from 5 to 20‰ ($P < 0.01$). The *C*-value was 29.8% (Table 1). On the 50th day, leaf P_n was neither depressed (*C*-value was –3.41%) nor differed among salinity levels from 0 to 15‰ ($P > 0.05$) whereas leaf C_i decreased as salinity increased from 0 to 15‰ and increased from 20 to 40‰. On the 100 day, leaf C_i increased with salinity from 0 to 10‰.

Ion contents (K^+, Na^+, Ca^{2+}, Mg^{2+}, Cl^-) of cotyledons initially and on the 45th day

On the 45th day, the contents of sodium, chloride, potassium, and ash at 0‰ salinity, and of potassium at other levels as well, had all declined (Fig. 5) whereas those of calcium and magnesium had increased with salinity levels up to 20‰ and decreased at 40 and 50‰ levels.

Discussion

Effect of salt stress on growth of *Avicennia marina* seedlings

Almost all the studies in which mangrove was grown in a laboratory under controlled conditions confirm that

Fig. 1 Biomass of leaf, stem, and root of *Avicennia marina* seedlings on 45th and 100th day at different levels of salinity. Values are means ± standard deviation ($n = 6$)
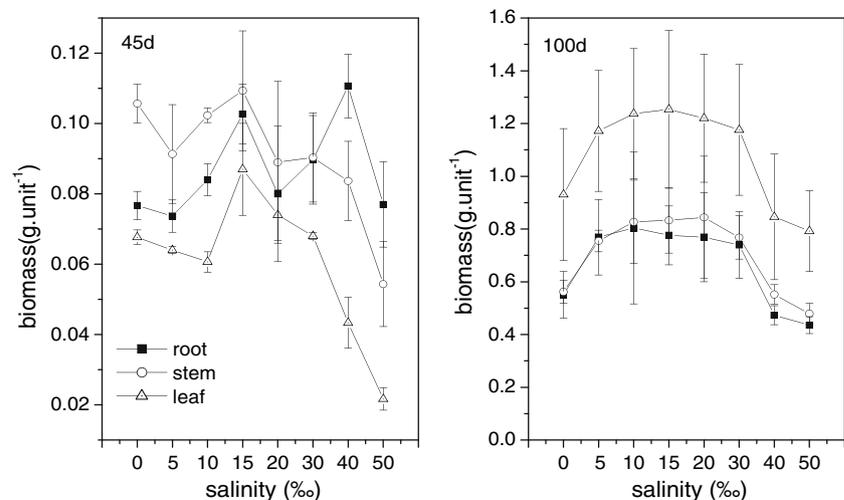
Fig. 2 Root and stem length and leaf area of *Avicennia marina* seedlings on 45th and 100th day at different levels of salinity. Values are means ± standard deviation (*n* = 6)
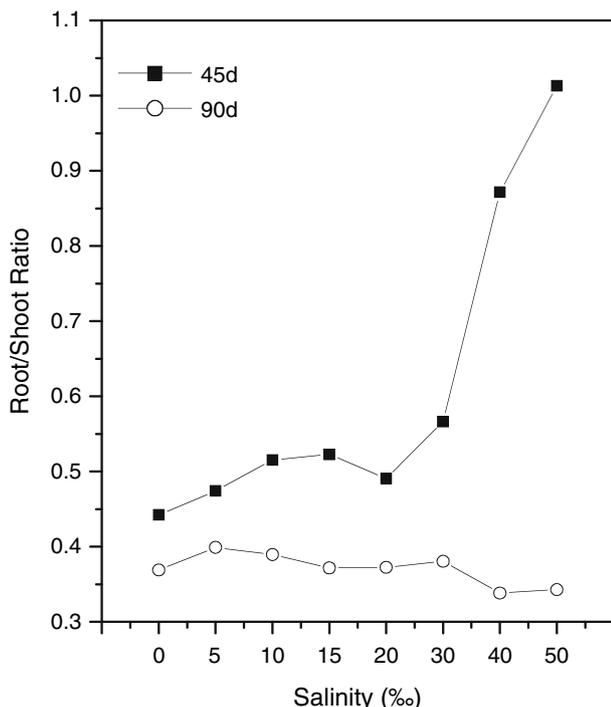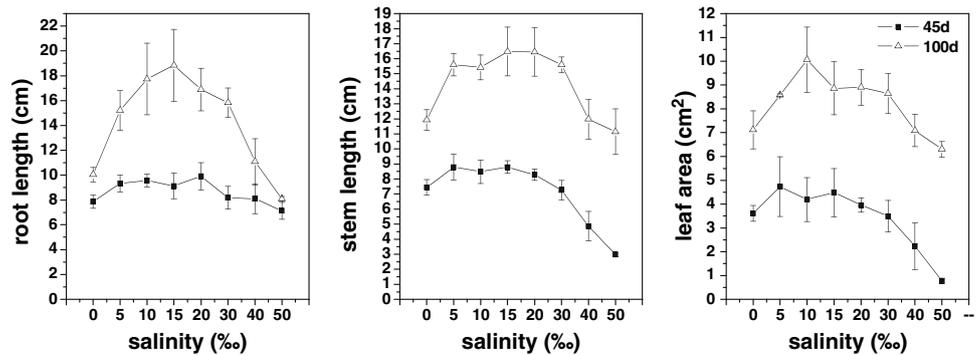

were smaller and fewer) in *Avicennia marina* and *Rhizophora stylosa* when grown in fresh water, compared with growth 25, 50, and 75% of seawater salinity. Similar results were also obtained by Pezeshki et al. (1990), who found that the total dry weight of *Laguncularia racemosa*, *Rhizophora mangle*, and *Avicennia germinans* increased under conditions of salt stress and salt stress combined with water logging. Although the net carbon assimilation rate (mmol m^{-2} s^{-1}) did not differ between the control and the treatments, the increased leaf area per plant under moderate salinity meant higher net carbon assimilation per plant. This partly explains why some halophytes grow better under moderate salinity than when salinity is lacking altogether.

The possible osmoregulatory function of cotyledons

The poor growth of seedlings exposed for a long time to the stress caused by 0‰ salinity may be explained by the depletion of ions and energy stored in cotyledons during the early growth stage. At 0‰ salinity, ash content and the concentrations of some ions (K^+, Na^+, and Cl^-) had declined by the 45th day (Fig. 5), perhaps because they had been deployed for the growth of other organs: with no supplementary source of ions—the salinity level was 0‰—the supply of ions may have been inadequate to enable maximum growth potential.

On the other hand, ash content and ion concentrations of the cotyledons increased at salinity levels from 5 to 20‰, which may indicate another function of the cotyledons: instead of being a *source* of ions when they are lacking in the culture medium (0‰ salinity), the cotyledons serve as a *store* of ions when, at higher levels of salinity (5–20‰), ions are surplus to requirements. This accumulation of ions in the cotyledons may also reduce the stress caused by high salinity. This high concentration of the ions corresponded with better growth of the seedlings at these levels of salinity. However, at even higher levels salinities, the ion concentration decreased with increasing salinity, and the concentration of Ca^{2+} and Mg^{2+} was lower than the

Fig. 3 Root:shoot ratio in *Avicennia marina* seedlings on 45th and 100th day at different levels of salinity

maximum growth does not occur in fresh water (Flower et al. 1977; Downton 1982; Clough 1984; Zheng and Lin 1992; Wang and Lin 1999), and our study was no exception. Moderate levels of salinity always facilitate seedling growth but extreme levels or total lack of it (0, 40, and 50‰) arrest growth. We consider 0‰ salinity as extreme salinity because, in a sense, it *is* a stress as far as the growth of *Avicennia marina* seedlings is concerned. With prolonged exposure to salt stress, growth was poor at the extreme levels (0, 40, and 50‰) and better growth at moderate levels (5–30‰). Downton (1982) found that the seedlings of *Avicennia marina* grew rapidly at 0‰ salinity in the first few weeks but the growth of leaf and bud was affected later and the accumulation of dry matter reduced. Clough (1984) found leaf development inhibited (leaves

Fig. 4 Influence of salinity on leaf net photosynthesis rate (P_n), stomatal conductance (G_s), intercellular CO_2 concentration (C_i), and transpiration rate (T_r) in *Avicennia marina* seedlings on 50th and 90th day. Values are means ± standard deviation ($n = 3$)
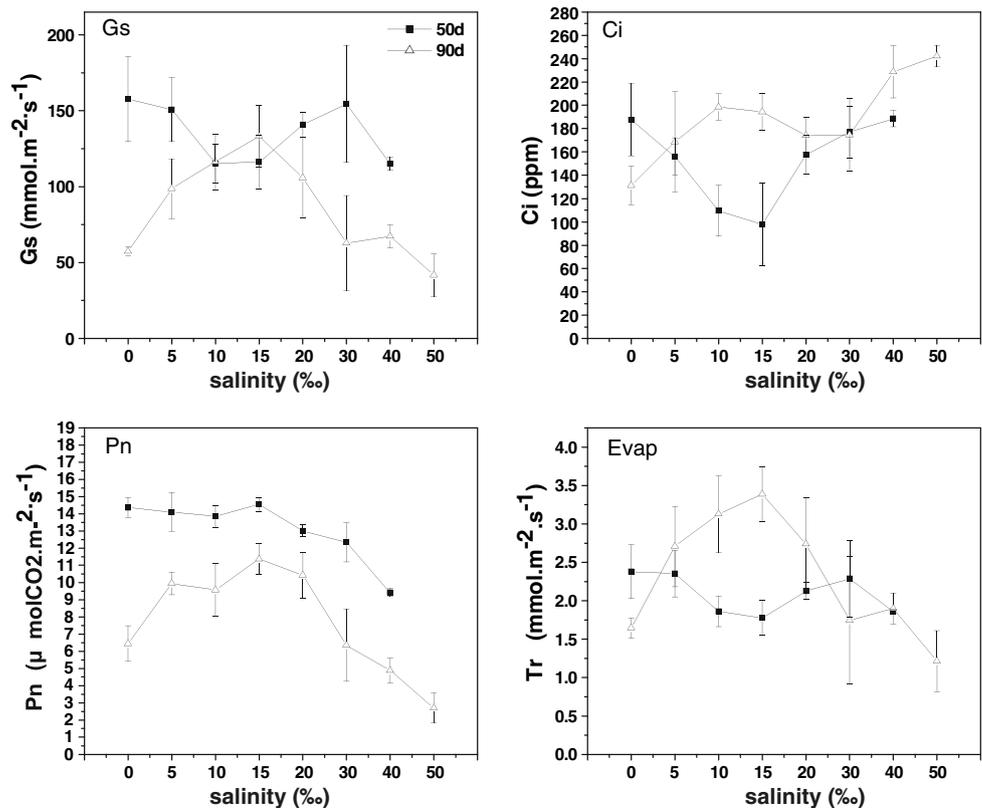
original levels, which suggests that high salinity had adversely affected the sink function. The accumulation of high concentrations of Ca^{2+} and Mg^{2+} at 0‰ salinity was observed also by Clough (1984), who found that root, stem, and leaf tissues of *Avicennia marina* seedlings accumulated markedly more Ca^{2+} and Mg^{2+} at 0‰ salinity than at other levels. Ca^{2+} is often considered an important ion in salt tolerance (Wilson et al. 2000; Bernstein 1975; Cramer et al. 1986). It can be seen from Fig. 5 that cotyledons

Table 1 Growth parameters and rate of photosynthesis in *Avicennia marina* seedlings on 45th and 100th day

Items	C-value (%)	
	45 day	100 day
LeafP_n	–3.41	29.80
Leaf area	20.34	27.81
Root length	20.64	71.02
Stem length	15.34	34.01
Root biomass	10.98	41.47
Stem biomass	–7.26	44.97
Leaf biomass	5.54	31.12

C-value can be calculated by the formula $C = \left(\frac{M-T}{T}\right) \times 100\%$ where M is the average value of different growth parameters at moderate levels of salinity (5–30‰) and T is that at a salinity of 0‰

accumulated more of Ca^{2+} at low salinity but lost it at very high levels (30, 40, and 50‰), suggesting that cotyledons function as a Ca^{2+} sink at low salinity and as a source at high salinity.

Effect of duration of exposure to salt stress on P_n, T_r, G_s and C_i of *Avicennia marina* seedlings
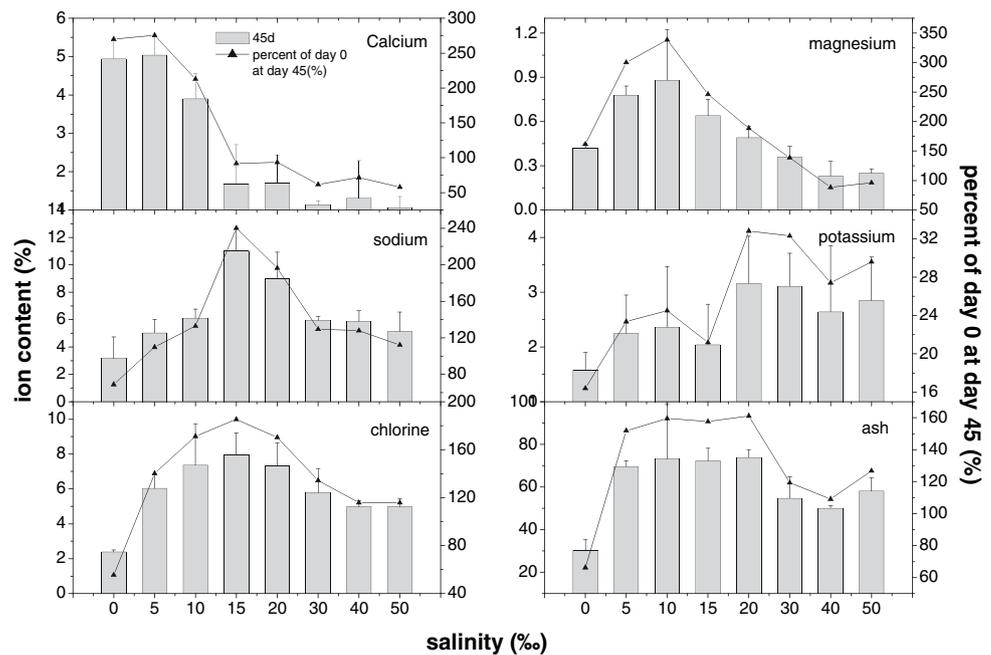
Two kinds of factors limit photosynthesis, those related to stomatal function and those unrelated (Farquhar and Sharkey 1982). The intercellular concentration of CO_2 (C_i) is useful in distinguishing between the two limiters (Xu 1997; Guan et al. 1995). If the rate of photosynthesis decreases with decrease in C_i, increased stomatal resistance is implicated; if photosynthetic rate decreases despite increase in C_i, the non-stomatal factors must be working (Xu 1997). Following the above argument, we conclude that after 50 days of moderate (5–20‰) salt stress, photosynthesis proceeds in an atmosphere saturated with CO_2 and is not limited by stomatal conductance. At higher salinity (40‰), the depressed photosynthetic rate is due to inhibition of the photosynthetic apparatus since stomatal conductance decreases only a little but C_i rises substantially. Similarly, after 90 days, it is stomatal conductance that limits photosynthesis at salinity levels up to 20‰, whereas higher salinity damages the photosynthetic apparatus itself,

since the rate of photosynthesis falls despite higher C_i, although stomatal conductance is also lower.

Figure 4 shows the U-shaped curve followed by C_i after short time (the first 45 days) duration to stress of varying intensities (different levels of salinity): the values decline at salinity levels from 0 to 15‰ but begin to rise at those from 20 to 40‰, which indicates that the main limiting factors are related to stomatal function at lower levels of salinity and to other functions at higher levels of salinity. After a longer exposure (45–90 days) to salt stress, the shape of the curve is different: C_i values increase with salinity levels from 0 to 10‰ and then decrease slightly up to 30‰, only to rise sharply up to 50‰ This means that following a longer exposure to salt stress, the main limiting factors are other than stomatal functioning at both low salinity levels (0–10‰) and at very high levels (40–50‰). At intermediate salinities (15–30‰), photosynthesis is constrained by stomatal factors: the slight dip in C_i implicates the stomata, which is also consistent with the high photosynthesis rate in seedlings at these levels of salinity. In other words, when salt stress is for a shorter period, seedlings cope first by reducing transpiration (T_r) by reducing stomatal conductance (G_s) and then by 'shipping out' the ions accumulated in toxic proportions by the evaporation. That is, seedlings can adjust their physiological processes to adapt to low levels of salinity in the short term. However, as salinity increases (or is lacking altogether) and the salt stress is prolonged, the seedlings lose this adaptive response and both the rate of photosynthesis and growth of the seedlings are adversely affected, especially at 0, 40, and 50‰ salinity. The limiting factor turns out to be non-stomatal at these salinities and the photosynthesis apparatus of the leaves is damaged.

Conclusion and future prospects

This study focused on the behavior of *Avicennia marina* seedlings in response to the stress occasioned by 0‰ salinity in the short term as well as in the long term. In contrast to the growth at 5–20‰ salinity, growth at 0‰ salinity was particularly affected in the long term. Analysis of ion concentration in the cotyledons showed that cotyledons may play an important role at low salinity levels (especially at 0‰ salinity) during early growth. This is a hypothesis that originates from partial experimentation and demands more direct evidence.

In addition, *Avicennia marina* is a cryptoviviparous species: its propagules are smaller than those of true viviparous species. A review of earlier work showed that the propagule mass (weight or size) in mangrove is closely correlated with their initial growth. For example, the large propagules of *Laguncularia racemosa* represent a stronger ability to endure both waterlogging and dehydration than the small propagules of *Avicennia germinans*. Further, the survival of seedlings also varies with propagule size within species, the percentage survival being greater in seedlings from larger propagules (Rabinowitz 1978). Lin et al. (1995) also found that total leaf area and stem length of the seedlings are positively correlated with initial weight of the mature propagules. Based on these findings, we believe that because the propagules of the

truly viviparous *Kandelia candel* and the cryptoviviparous *Avicennia marina* are of different sizes, their ion contents may also be different and the two will exhibit different growth patterns at low levels of salinity. More comparative research should be carried on these two kinds of species.

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