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Original Paper

Stimulation effects of salts on growth in suspension culture of a mangrove plant, *Sonneratia alba*, compared with another mangrove, *Bruguiera sexangula* and non-mangrove tobacco BY-2 cells

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Abstract Effects of a wide range of concentrations of four salts (NaCl, KCl, $MgCl_2$, $CaCl_2$) on cell proliferation were investigated in suspension culture of a mangrove plant, *Sonneratia alba*. The results were compared to those obtained with suspension culture of another mangrove species, *Bruguiera sexangula* and non-mangrove tobacco BY-2 cells. Effects of salts on suspension cells were determined with both large scale (LS) method using a 100 ml culture flask, and/or a small scale (SS) method using a 24-well culture plate. Settled cell volume (SCV), packed cell volume (PCV), wet weight (WW) and dry weight (DW) were measured in LS method and PCV was in SS method. High concentrations of NaCl (25–100 mM) and $MgCl_2$ (25 mM) showed strong stimulatory effects on growth in *S. alba* suspension culture, and low concentrations of KCl, $MgCl_2$ and $CaCl_2$ (10 mM) also showed similar stimulatory effects. In contrast, a remarkable stimulatory effect was not observed in *B. sexangula* and tobacco BY-2 cells. Cationic ions are effective for the growth of suspension cells in both mangrove species *S. alba* and *B. sexangula*. The SS method using multi-well culture plate and micro-tube measurement method developed in this report, can be applied for analysis of factors for stress tolerance.

Key words: Cationic ions, halophilic, multi-well culture plate, salt tolerance, sea salts.

The mangrove plants growing in the brackish water region in the tropics and the subtropics must have very strong tolerance to salinity. In mangrove forests, more than 100 species from different families can be found (Tomlinson 1986). The necessity of micropropagation and conservation of these tree species from potential destruction of mangrove forests have been discussed (Ogita et al. 2004). A better understanding of the mechanisms of salt tolerance in mangroves may enable us to develop new methods to grow plants in a relatively saline environment. Mangrove species are favorable materials to study the salt tolerant process in plants. Physiological and biochemical mechanisms of salt tolerance were studied using callus culture of *Sonneratia alba* (Akatsu et al. 1996) and suspension culture of *Bruguiera sexangula* (Ashihara et al. 2003). Molecular biological studies and transformant production (Yamada et al. 2002) using suspension culture of *B. sexangula* (Mimura et al. 1997; Kura-Hotta et al. 2001) were performed. The cell and tissue culture approach of mangrove species is one of the effective means to study aspects of salts tolerance. However, the efficiency of plant regeneration in tissue culture of mangrove plants is very low (Rao et al. 1998; Al-Bahrany and Al-Khayri 2003) and many mangrove species remain very recalcitrant to the establishment of cell culture systems, except for a few species. No plant regeneration from callus nor suspension culture of mangrove plants was obtained. Recently, suspension cultures using the cotyledons as explants have been successfully established for *S. alba* (Kawana et al. 2007). *S. alba* is a representative of a mangrove species that is commonly found on the most seaward side while *B. sexangula* plants are located more inland of mangrove forests.

The purpose of this investigation was to elucidate the characteristics of salts tolerance at the cellular level and to develop a regeneration system of a mangrove plant, *S. alba*. In this report, the effects of a wide range of concentrations of four salts (NaCl, KCl, $MgCl_2$, $CaCl_2$), that are the main constituents of sea water, on cell proliferation were investigated in suspension cultures of *S. alba*. The results were compared to those obtained with suspension cultures of another mangrove species, *B. sexangula* and of non-mangrove plant, tobacco BY-2

Abbreviations: 2, 4-D, 2, 4-dichlorophenoxyacetic acid; DW, Dry weight; PCV, Packed cell volume; SCV, Settled cell volume; WW, Wet weight
This article can be found at http://www.jspcmb.jp/

cells. We also attempted to develop a small scale efficient method using a multi-well culture plate for the measurement of growth in a suspension culture.

Materials and methods

Materials

Suspension culture of *Sonneratia alba* was established from cotyledons (Kawana et al. 2007) and sub-cultured every three to four weeks in Murashige and Skoog (MS) medium (Murashige and Skoog 1962) containing 0.1 μM 2,4-dichlorophenoxyacetic acid (2, 4-D). Medium pH was adjusted to 5.8 with NaOH before autoclaving. Suspension culture of *Bruguiera sexangula* was prepared from leaf-derived callus (Kura-Hotta et al. 2001) and sub-cultured every three weeks in modified AA (Thompson et al. 1986) medium containing 0.02 μM 2, 4-D, 2 μM N-(2-chloro-4-pyridyl)-N′-phenylurea (CPPU) and 20 mM KCl. The glycine content was reduced to 0.1 mM. Medium pH was adjusted to 6.2 with NaOH. Tobacco (*Nicotiana tabacum* L.) cv Bright Yellow 2 (BY-2) cells were maintained in a liquid NT medium which was a modified MS medium supplemented with 1.3 mM of KH_2PO_4, 1 mg l^{-1} of thiamine-HCl and 1 μM 2, 4-D (Nagata and Kumagai 1999). Medium pH was adjusted to 5.7 with KOH. BY-2 cells were sub-cultured every seven days by transferring 2 ml of cells into 95 ml of fresh medium in a 300-ml Erlenmeyer flask. The three kinds of cells were cultured in each medium at 28°C for BY-2 and at 30°C for *B. sexangula* and *S. alba* in the dark on a rotary shaker (BR-3000, Taitec, Saitama, Japan) at 100 rpm speed.

Experimental conditions

Four salts (NaCl, KCl, $MgCl_2$, $CaCl_2$) at the following concentrations (0, 5, 10, 25, 50, 100, 200 and 300 mM) were added to each sub-culture medium described above. Suspension cells of *S. alba* and *B. sexangula* were incubated at 30°C and of BY-2 were at 28°C in the dark.

LS method: In the large scale (LS) method, suspension culture of BY-2 and *S. alba* were cultured in a 100 ml flask on a rotary shaker at 100 rpm speed. Settled cell volume (SCV), wet weight (WW) and dry weight (DW) were measured as degree of proliferation in *S. alba* suspension cells after 26 days of culture. Packed cell volume (PCV), WW and DW were measured as degree of proliferation in BY-2 cell after 6 days of culture. After incubation, 5 ml each of cells of *S. alba* and BY-2 were transferred to a 10 ml centrifuge tube. Cell volume was measured after 15 min settling time as SCV of *S. alba* and after centrifugation at 800 *g* for 5 min as PCV of BY-2 cells. After the SCV or PCV assays, cells were transferred to a filter paper to remove excess liquid and the wet weights were then determined. In addition, these cells were subsequently dried for 18 h at 80°C for DW measurements.

SS methods: In the small scale (SS) method, 550 μl suspension culture of *S. alba*, BY-2 cells and *B. sexangula* were cultured in 24 multi-well culture plates at static conditions in a CO_2 incubator without the supply of CO_2 for the former two and on a rotary shaker (SI-300C, AS ONE Co. Ltd.) at 190 rpm speed for the latter. Cells were transferred to 1.5 ml micro-tubes after 30 days of culture with *S. alba* at static condition, after 5 days of culture with BY-2, and after 12 days of culture on a shaker with *B. sexangula*. PCV was measured after centrifugation at 100 *g* for 5 min with *S. alba* and *B. sexangula*, and at 800 *g* for 5 min for BY-2.

Cell volume calculation in a micro-tube: By measuring the height of packed cells in each micro-tube, the PCV was calculated from the height and volume relation formula. All the data were described as the % increase of the control when no additional salts was included. Data from the LS method were averages of four to eight samples from two flasks. Data from the SS method were averages of 2 samples from different wells. Experiments were repeated twice.

Results and discussion

The effects of four salts on the suspension culture of S. alba using the LS method

The effects of four salts on SCV, WW, and DW were measured as degree of proliferation in *S. alba* cells utilizing the LS method (Figure 1A, B, C). As shown in Figure 1A, addition of all salts stimulated cell

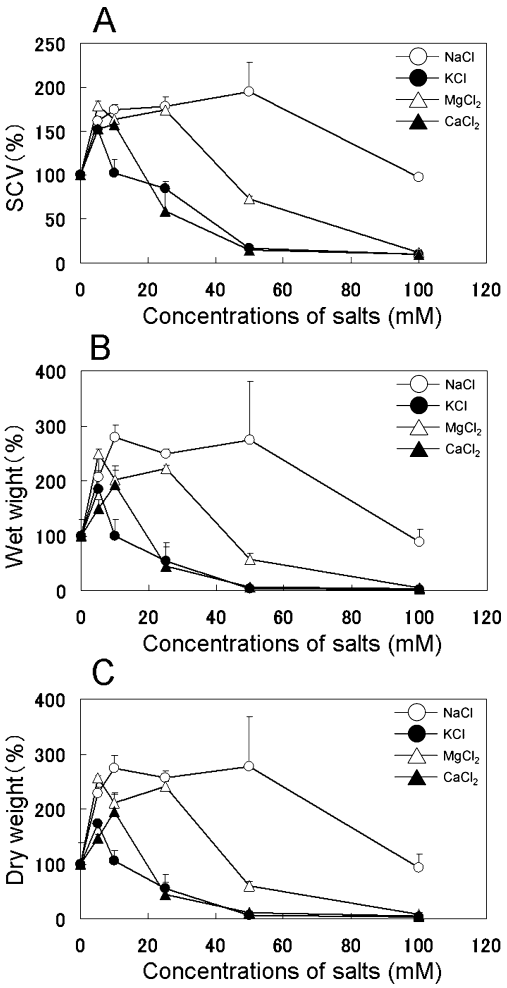

Figure 1. The effects of four salts, NaCl (open circle), KCl (closed circle), $MgCl_2$ (open triangle), and $CaCl_2$ (closed triangle) on suspension culture of *S. alba* using the LS method. SCV (A), WW (B) and DW (C) were measured as degree of proliferation after 26 days of culture. Data were an average of 4 samples from 2 flasks (error bar is s. e.).

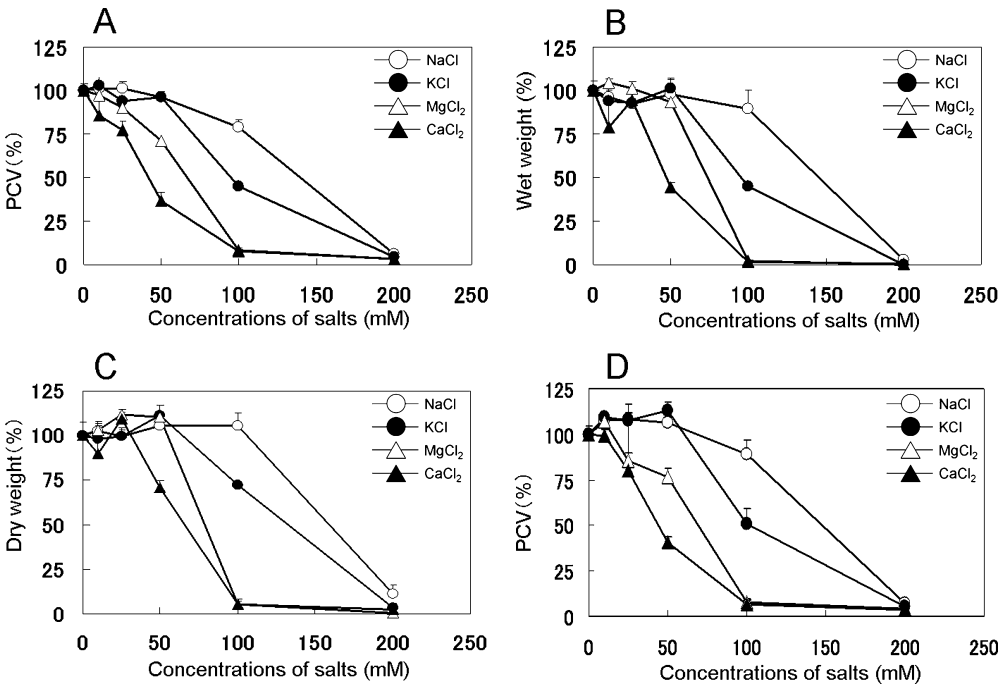

Figure 2. The effects of four salts, NaCl (open circle), KCl (closed circle), $MgCl_2$ (open triangle), and $CaCl_2$ (closed triangle) on suspension culture of BY-2 cells utilizing the LS method. PCV (A, D), WW (B) and DW (C) were measured as degree of proliferation after 6 days of culture. PCV (D) was measured using micro-tube method. Data were an average of 8 samples. Error bar is standard error.

proliferation of *S. alba*. In particular, under the conditions of 50 mM NaCl and 25 mM $MgCl_2$, cell proliferations was stimulated strongly with 195% and 173% increase of SCV, respectively. Under the condition of 200 mM NaCl, some cell growth was detected (25% SCV). Growth was completely inhibited at 300 mM NaCl (data not shown). In MS medium, concentrations of K^+ ions is about the 20 mM in the control without addition of salts, while Na^+ ions, Mg^{2+} ions and Ca^{2+} ions are 0.2 mM, 1.5 mM and 3 mM, respectively (Murashige and Skoog 1962). Our results indicated that *S. alba* suspension cells are not only salt tolerant but also halophilic. All results of SCV (Figure 1A), WW (Figure 1B) and DW (Figure 1C) indicated stimulatory effects of all four salts. This report is the first to observe the stimulatory effects of salts on the growth of *S. alba* suspension cells.

The effects of four salts on suspension culture of BY-2 cells utilizing the LS methods

The effects of salts on the BY-2 suspension cultures are shown in Figure 2. PCV (Figure 2A), WW (Figure 2B), DW (Figure 2C) were measured using the LS method. All four salts inhibited growth of BY-2 suspension cell culture. PCV and WW showed similar curves, however, inhibitory effects of salts were obtained at higher concentrations in DW. Inhibitory effect of NaCl on BY-2 cells was similar to that reported by Nakayama et al. (2000). No cell growth was observed at 200 mM NaCl and KCl. Cell growth was also completely inhibited at 100 mM of $MgCl_2$ and $CaCl_2$

In order to reduce the amount of suspension needed for each determination, smaller volumes of suspension were used to determine the PCV. In Figure 2D, PCV was measured after centrifugation using 1.5 ml micro-tubes. The results were comparable to that obtained using 10 ml centrifuge-tubes (compare Figures 2A, 2D). Hence measurement of PCV using 1.5 ml micro-tubes is useful in the LS culture method.

The effects of four salts on the suspension culture of S. alba, B. sexangula, and BY-2 utilizing the SS methods

Small-scale analytical methods can provide an efficient use of experimental materials as well as reduce the operating costs of the experiment. Successful small-scale methods have been established for the determination of plant growth substances (Sasamoto 2002). In order to develop small-scale methods for future experimentation for mangrove studies, we used the multi-well plates for the maintenance and growth of the suspension cultures and evaluated the outcome using PCV data.

The effects of four salts on the suspension cultures of *S. alba* (Figure 3A), BY-2 (Figure 3B), and *B. sexangula* (Figure 3C) were investigated. The PCV of all materials are described as the % of the control without addition of salts. Figure 4A shows devided cells of *S.alba* in the control condition. PCV of *S. alba* suspension using SS method indicated that the highest value obtained was at 50 mM NaCl (166%) and at 25 mM $MgCl_2$ (165%). Similar high values were obtained using LS method

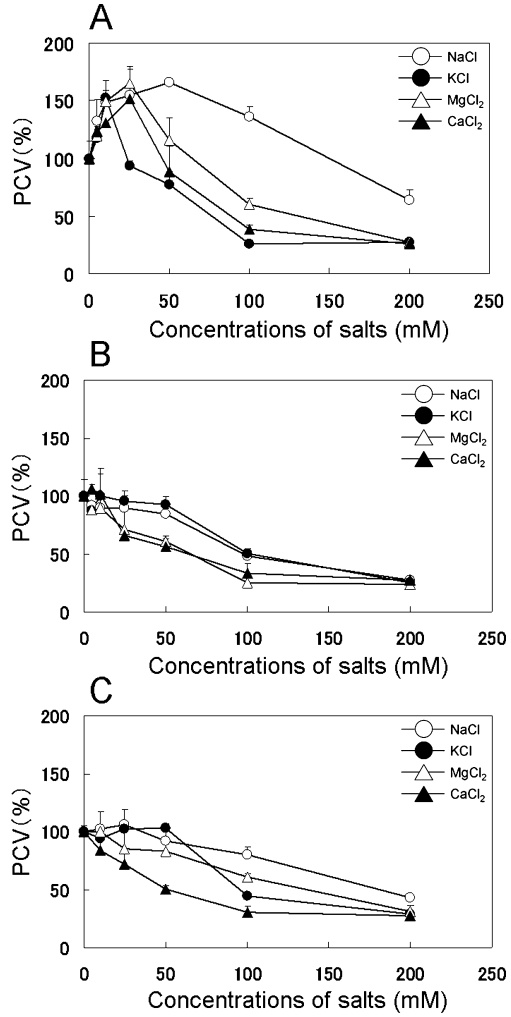

Figure 3. Effects of different concentrations of salts NaCl (open circle), KCl (closed circle), $MgCl_2$ (open triangle), and $CaCl_2$ (closed triangle) on suspension cells of *S. alba* after 30 days (A), of BY-2 after 5 days of culture (B), and of *B. sexangula* after 12 days of culture (C) utilizing the SS methods. PCV was measured as degree of proliferation. Data were taken from 2 tubes.

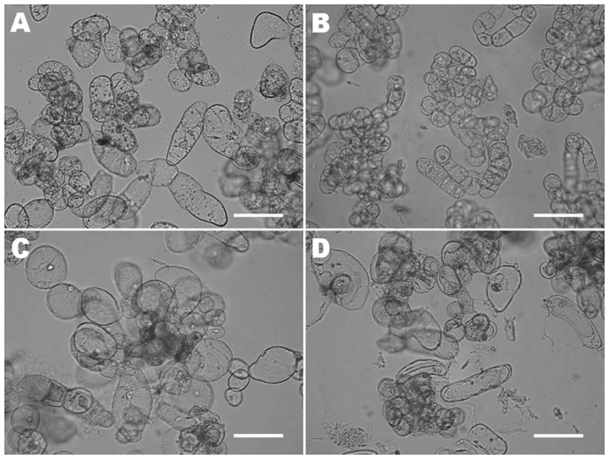

Figure 4. Suspension culture of *S. alba* after 26 days of culture utilizing the SS method under the condition without NaCl (A), with NaCl of 50 mM (B), with NaCl of 200 mM (C), and with KCl of 200 mM (D). Suspension culture of *S. alba* sub-cultured every three to four weeks for more than 6 months. Scale bar = 100 μm.

(Figure 1A) for *S. alba*. Figure 4B shows well devided cells at 50 mM NaCl. High as well as a wide range of concentrations of NaCl (10–100 mM) and $MgCl_2$ (10–25 mM) stimulated proliferation strongly in *S. alba* suspension cells and low concentrations of KCl and $CaCl_2$ also showed the same stimulatory effects (Figure 3A). A similar remarkable stimulatory effect was not observed in BY-2 and *B. sexangula* (Figures 3B, C). Some cell growth was still detected under the condition of 200 mM NaCl in both mangrove species studied, but not in BY-2. Both *S. alba* and *B. sexangula* cells were tolerant to NaCl, while BY-2 cells were not. Figure 4C shows divided small cells and viable enlarged cells of *S. alba* at 200 mM NaCl, which is consistent with the higher PCV value obtained than at 200 mM of other salts (Figure 3A). Figure 4D shows the plasmolysed dead cells at 200 mM KCl. The addition of 100–200 mM of KCl to the culture medium resulted in the complete inhibition of cell proliferation in *S. alba* (Figure 3A). However some cell proliferation was still shown at 100 mM KCl in *B. sexangula* (Figure 3C) and BY-2 (Figure 3B). Though suspension cells of *S. alba* indicate tolerance to NaCl and $MgCl_2$, there was no tolerance to KCl at 100 mM. The order of effective salts for the cell proliferation was NaCl, $MgCl_2$, $CaCl_2$, and KCl in *S. alba* (Figure 3A) and NaCl, $MgCl_2$, KCl, and $CaCl_2$ in *B. sexangula* (Figure 3C). As $MgCl_2$ or $CaCl_2$ was inhibitory at about half the concentrations of KCl or NaCl, chloride ions could be the main cause of inhibition in BY-2 cells (Figure 3B), but mangrove cells were mainly influenced by cations. Similar effects of cations and anions were suggested in protoplasts cultures of *B. sexangula* and BY-2 cells (Fukumoto et al. 2004).

Tolerance to NaCl in *B. sexangula* suspension cells shown in this report using SS method was the same as the large scale method (Kura-Hotta et al. 2001). In the protoplast culture of *B. sexangula*, some stimulative effects of NaCl and $MgCl_2$ were observed (Fukumoto et al. 2004). It would be useful to compare the effects of salts on suspension and protoplast cultures of *S. alba* and determine the potential role of the cell wall in cell proliferation.

Suspension culture cells of *S. alba* were found to be highly halophilic to not only NaCl but also low concentrations of other salts which are major components of sea water. *B. sexangula* suspension cells were tolerant but not halophilic to these salts. Highly halophilic and salts tolerance of *S. alba* cells might be related to the fact that plants of *S. alba* grow in the most seaward side of mangrove forests, while plants of *B. sexangula* grow more inland. As *S. alba* suspension cells can be sub-cultured in the medium containing high

concentrations of NaCl (Figure 4B), the effects of salts on some metabolic changes can be investigated.

In *S. alba* and BY-2, similar effects of four salts were obtained with both methods, LS and SS. Using a 24-well culture plate and micro-tube measurement method developed, that only one ml of suspension cells is enough for investigation of various factors. Observation of cells under an inverted microscope and PCV measurement in SS method are efficient procedures and can be applied to the analysis of factors for stress tolerance, *e.g.* salts, sugars, heavy metals, and plant hormones, before the large scale molecular studies at a selected condition. They also can be applied for selection of stress tolerant cells in molecular biological studies.

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