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Short Communication

Stimulatory effects of sea salts on cell growth in liquid culture of Avicenniaceae mangrove

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Abstract The effects of salts on cell proliferation in suspension cultures of *Avicennia alba* and on callus induction from the leaf-tissue of *A. marina* were investigated using a small-scale liquid culture method. The effects of the seawater components, NaCl, KCl, $CaCl_2$, $MgCl_2$, and $MgSO_4$ were examined separately. In both *Avicennia* species, the cell growth was increased in the presence of a low concentration, 10 mM, of $MgCl_2$. Even in the presence of 100 mM NaCl, growth was stimulated in *A. marina* and there was no inhibition of growth in *A. alba*. $CaCl_2$ was the most inhibitory and completely inhibited growth at 100 mM in both species. Similarities and differences in the effects of sea salts among *Avicennia* species and *Sonneratia alba* and *Bruguiera sexangula* of different mangrove families are discussed. This is the first report on establishing cell suspension culture of the mangrove plant *A. alba* belonging to the Avicenniaceae.

Key words: *Avicennia alba*, *A. marina*, halophilic, salt tolerance.

Mangrove plants survive extreme conditions in tropical and subtropical brackish waters. In mangrove forests more than 100 species from different families can be found in or near the coastal areas in the world (Tomlinson 1986). The sea water and soil where mangroves grow contain high concentrations of Na^+, Cl^-, K^+, Mg^{2+}, Ca^{2+}, and SO_4^{2-} (Dagar et al. 1993). Mangroves possess unique properties that allow them to tolerate such severe, high-salt conditions. Elucidation of the salt-tolerance mechanisms of mangrove plants and introducing these characteristics to herbaceous crops, such as rice and biomass producing tree crops, such as poplar will be a significant contribution to agriculture.

Cell cultures are excellent experimental systems to study cellular, physiological and biochemical processes of salt-tolerance (Kawana et al. 2008). However, mangrove plants are very recalcitrant in cell culture with the exception of a few species in the families Rhizophoraceae (*Bruguiera sexangula*) (Mimura et al. 1997, Kura-Hotta et al. 2001) and Sonneratiaceae (*Sonneratia alba* and *S. caseolaris*) (Kawana et al. 2007, Yamamoto et al. 2009) which are amenable to suspension culture studies. The effects of sea salts on the growth of mangrove have been investigated using suspension cultures of *B. sexangula* from leaves and of *S. alba* from cotyledons. Research on the former has revealed the Na^+-tolerant nature of mangroves, and that on the latter has revealed the Na^+-, Mg^{2+}-halophilic nature (Kawana and Sasamoto 2008).

Avicennia alba Blume and *Avicennia marina* (Forsk.) Vierh. are two dominant species in mangrove forests in the tidal coasts of Myanmar and Thailand. The latter species is also found in most seaside areas of Iriomote Island, Okinawa, Japan. In the reforestation, *A. alba* can grow in the most seaside area (Robertson et al. 1992). Suspension culture of Avicenniaceae has been unsuccessful, although nodular structures from axillary buds cultured in Murashige and Skoog's (MS) (Murshige and Skoog 1962) basal medium, have been successfully used for micropropagation of *A. marina* (Saenger 2002, Al-Bahrany and Al-Khayri 2003). In this study, we established cell suspension cultures of *A. alba*. Then, we studied the effects of sea salts on the proliferation of *A. alba* cell suspension culture and on callus induction in leaf segments of *A. marina* in liquid culture. We also discuss the similarities and the differences in the reactivity to salts of these and other mangrove species from different families. All cultures in the present study were incubated on a rotary shaker at 100 rpm in the dark at 30°C, and all the data for the effects of salts were described as the average of the percent difference from the control. A student t-test ($P<0.05$) was used to evaluate the difference of mean values between the control and each treatment.

This article can be found at http://www.jspcmb.jp/

A. alba: Seeds of *A. alba* were collected in Phang-nga, Thailand, and their cotyledons were used to obtain cell suspension cultures. The seed coat was removed and embryos were treated with a detergent for 1 min, sterilized with 2% NaClO solution for 40 min, and then washed three times with sterilized water. After keeping in sterilized water for a few days, roots and hypocotyls were removed. The remaining cotyledons were again sterilized with 1.5% NaClO solution for 20 min, washed three times with sterile water, and cut into 5–7 mm segments. Two segments were placed in a 10-ml flat-bottomed culture tube (Maruemu No. 3) covered with translucent film (BioHazard Bag 86.1199, Assist Co. Ltd.). Each tube contained 1 ml of either modified liquid AA medium (mAA, modified from Mimura et al. 1997, Thompson et al. 1986) or MS medium together with 3% sucrose and 0, 0.1, 1, or 10 μM each of 2,4-dichlorophenoxyacetic acid (2,4-D) and benzyladenine (BA). Glycine in mAA medium was reduced to one tenth of the original concentration, and pH was adjusted to 6.2 before sterilization at 121°C for 20 min. After two months of culture, the cotyledon segments with nodular structures, free cells and proliferated cell aggregates appeared (callus induction). Then, they were sub-cultured in fresh liquid medium containing 1 μM 2,4-D and BA in a 50 ml flat-bottomed tube for three months. Thereafter, the proliferated cell aggregates were sub-cultured at 3-4 week intervals in 100 ml flasks containing mAA medium to which 2 μM each of 2,4-D and BA and 3% sucrose were added. Eight months after the callus induction, BA was substituted with N-(2-chloro-4-pyridyl)-*N*′-phenylurea (CPPU) or thidiazuron (TDZ).

This is the first report on the successful establishment of cell suspension culture of *A. alba* belonging to Avicenniaceae, the third family following Rhizophoraceae and Sonneratiaceae, which are successful among recalcitrant mangrove species. Since *A. alba* plants grow in high temperature environments and their seeds are cryptoviviparous, obtaining materials for sterile culture is very difficult. Repeated decontamination with concentrated NaClO solution (1.5–2%) for 20 min was effective. Modified AA basal medium was more effective than MS medium in inducing cell proliferation in cotyledon explants of *A. alba*. Callus induction in *A. alba* was observed in mAA medium in the presence of both 2,4-D and BA at the concentration of 0.1–10 μM, but not in MS. Though the AA basal medium was more profitable than MS for callus-induction in solid culture (Mimura et al. 1997) and cell suspension culture of *B. sexangula* (Kura-Hotta et al. 2001), mAA was better than MS and AA when the 24-well culture plate method was used for suspension culture of *A. alba* (data not shown). Since the only difference between mAA and AA is the glycine content, and the total N content did not differ substantially, amino acid metabolism might be important for the suspension culture of *A. alba*.

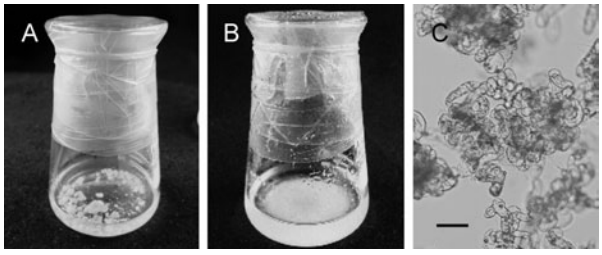

Figure 1. Suspension culture of *A. alba* sub-cultured in 100 ml flasks. Medium was mAA containing 3% sucrose and 2 μM each of 2,4-D, and BA (A) or TDZ (B, C). C, cell aggregates. Bar = 100 μm.

Like *S. alba*, which was cultured in MS basal medium (Kawana et al. 2007), *A. alba* took a long time (5 months) to develop into well-grown cells in liquid culture. Free cells obtained at the callus induction stage could not be sub-cultured; however, well-grown cell aggregates were obtained from the nodular structures of explants. After a five months culture of the cell aggregates, a cell suspension culture of *A. alba* was established using the medium containing 2 μM each of 2,4-D and BA, though large clumps were also observed (Figure 1A). Hormonal conditions for subculture were optimized by a survey experiment using BA, CPPU and TDZ as cytokinins. Judging from the results, we selected TDZ for establishing the fine cell suspension culture of *A. alba* (Figure 1B, C). Cell aggregates were sub-cultured every three weeks in mAA medium containing 2 μM 2,4-D and TDZ and 3% sucrose by adding 5 ml of the culture to 40 ml fresh medium in a 100 ml flask.

To determine whether the established cell suspension culture of *A. alba* is as halophilic as that of *S. alba* (Kawana and Sasamoto 2008), we investigated the effects of sea salts on cell growth. A small scale 24 well culture plate method was used to examine cell growth as described in Kawana & Sasamoto (2008). NaCl, KCl, $MgCl_2$, $CaCl_2$, and $MgSO_4$ were added to 1 ml of the suspension culture in each well at the concentrations of 10, 100, 200 or 400 mM. After 40 days of culture, the packed cell volume (PCV), which is the volume of sediment after centrifugation at 100 g for 5 min, was measured using 1.5 ml micro tube, to estimate cell growth. At the start of culture, PCV was adjusted to 2.5 or 5%. As shown in Figure 2, low concentrations (10 mM) of KCl and $MgCl_2$ stimulated the cell growth in cell suspension culture of *A. alba*, which is a halophilic characteristic. Cell growth of *A. alba* was not inhibited by NaCl at concentrations up to 100 mM, which is a characteristic of tolerance. $CaCl_2$ inhibited the growth even at 10 mM. Inhibition by $MgSO_4$ at high concentrations was slightly less than that by $MgCl_2$.

A. marina: Seeds of *A. marina* were collected on Iriomote-island, Okinawa, Japan. After imbibition,

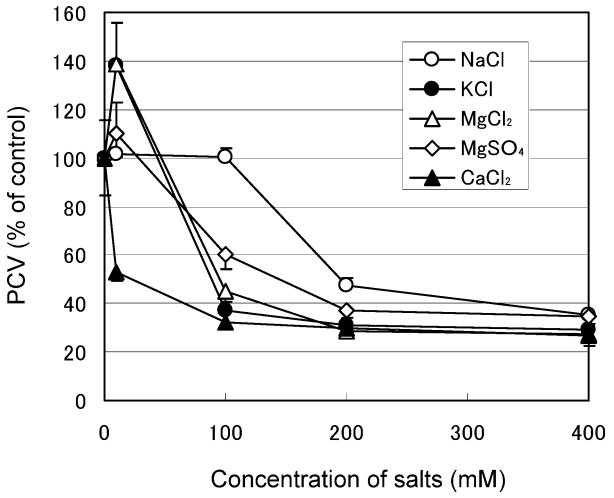

Figure 2. Effects of five salts on growth of suspension cells of *A. alba* after 40 days of culture at 30°C. Packed cell volume (PCV) was measured after centrifugation at 100 g for 5 min, using 1.5 ml micro tube. Medium was mAA containing 2 μM each of 2,4-D and TDZ and 3% sucrose. Data are averages of the percent differences from the control in three wells, with SE.

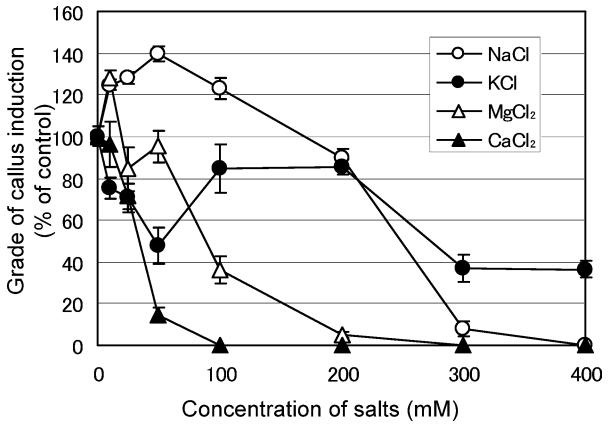

Figure 3. Effects of four sea salts on the callus induction in leaf-tissue of liquid culture of *A. marina*. The grade of callus induction was determined after 19 days of culture. Medium was MS containing 1 μM of 2,4-D and 10 μM of BA and 3% sucrose. Data are averages of the percent differences from the control in four leaf-segments with SE.

germinated seeds were planted in a mixture of vermiculite and sand in a pot placed in a watered container. Nutrient (N : P : K = 5 : 10 : 5) and NaCl solutions were supplied occasionally. After a four-year culture, the plants were grown in the same solution but without NaCl for at least one year before its use. Then the leaves were treated with detergent for 1 min, washed with tap water, sterilized with 1% NaClO solution for 45 min, and washed three times with sterile water. A small-scale liquid culture method similar to that used for *Sonneratia* seedlings in previous studies (Kawana et al. 2007, Yamamoto et al. 2009) was used to evaluate the effects of salts on the callus induction in the leaf segments. Two 2×7 mm leaf segments were suspended in 1 ml liquid medium in 10 ml flat-bottomed culture tube, and cultured on a rotating shaker. Medium was MS basal medium containing 1 μM 2,4-D, 10 μM BA, and 3% sucrose together with 10, 25, 50, 100, 200, 300, 400 or 500 mM of NaCl, KCl, $MgCl_2$, and $CaCl_2$. The pH was adjusted to 5.8. The leaf segments were observed through translucent film under an inverted microscope (Olympus CK40), and callus induction on the segments was graded of 0 to 4 separately for two adjacent cut surfaces. Data were averaged for each segment and described as the percent difference from the control.

Figure 3 shows the effects of NaCl, KCl, $MgCl_2$ and $CaCl_2$ on callus induction in the leaf-tissue culture of *A. marina*. The grade of callus induction in the control solution was 5.1±0.45 (SE). NaCl stimulated the callus induction at 10–100 mM, and did not significantly inhibit at 200 mM. $MgCl_2$ was stimulatory at 10 mM, and had only a slight effect at 10–50 mM, though it inhibited at higher concentrations. KCl had only a slight effect on the callus induction at 100–200 mM, although it had a significant inhibitory effect at 10–50 mM. $CaCl_2$ had a strong inhibitory effect and completely inhibited callus induction at 100 mM.

$MgCl_2$ at low concentration (10 mM) stimulated cell proliferation in *A. alba* and callus induction in *A. marina*. $CaCl_2$ strongly inhibited at 100 mM in both *Avicennia* species. Tolerance to high concentrations (100 or 200 mM) of NaCl was found in both species, though stimulation by a low concentration of NaCl was observed only in callus induction of *A. marina*. KCl stimulated cell proliferation in *A. alba* at a low concentration, and scarcely inhibited callus induction of *A. marina* even at 200 mM.

Growth stimulation and tolerance by low concentrations of the four sea salts, and stimulation by a high concentration of NaCl was observed in cell suspension culture of mangrove *S. alba* (Kawana and Sasamoto 2008), which grows in seaside coasts of mangrove forests similarly to *A. marina* and *A. alba*. $CaCl_2$ stimulation was different from *Avicennia* species. In the previous report, tolerance but not stimulation by low concentrations of NaCl, KCl and $MgCl_2$, was observed in suspension cultures of mangrove *B. sexangula*, which grows in the inland area of mangrove forests (Kawana and Sasamoto 2008). $CaCl_2$ was inhibitory as it was in *Avicennia* species. However, all four salts were inhibitory at high concentrations. In *B. sexangula*, callus induction in solid culture in leaf tissue was less tolerant to NaCl than that in viviparous seedlings (Mimura et al. 1997), and it is important to note that the above-mentioned cell suspension culture of *B. sexangula* was obtained from leaf-derived calluses (Kura-Hotta et al. 2001). In *B. sexangula*, cell suspension culture has been used for biochemical (Suzuki-Yamamoto et al. 2006) and molecular biological studies

on salt (NaCl) tolerance mechanisms (Yamada et al. 2002). Little information has been available concerning the halophilic nature and the cellular mechanisms of the suspension cultures of Avicenniaceae species. One of the reasons is the difficulty in obtaining suspension cultures in this plant group. In the present study, we established a protocol for generating cell suspension cultures of *A. alba*, and the system is expected to be useful in experimental studies for further investigation in mangrove cell biology. In this study, we used a small-scale culture method using 10-ml flat-bottomed tubes and 24-well culture plates. These methods were found to be effective for callus induction in liquid culture and monitoring the effect of different salts on cell growth.

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