Original Paper Plant Biotechnology, **21** (3), 177-182 (2004)

Different effects of four salts and pHs on protoplast cultures of a mangrove, *Bruguiera sexangula* suspension cells, *Populus alba* leaves and tobacco BY-2 cells

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Received 22 April 2004; accepted 12 May 2004

Abstract

Effects of a wide range of concentrations of four salts (NaCl, KCl, $MgCl_2$, $CaCl_2$) and different pHs (pH 4.5, 5.0, 5.7, 6.2) on colony formation, were investigated in protoplast culture of mangrove, *Bruguiera sexangula*, suspension cells. The results were compared to those obtained with protoplasts of tobacco BY-2 cells and leaf protoplasts of *Populus alba*. A 96-multiwell culture plate method was used and only 250 to 2500 protoplasts in 55 μl medium were sufficient to test the effect of each treatment. The effects of four salts were always found to inhibit colony formation of *Populus* and tobacco protoplasts at pH 5.7. In contrast, $MgCl_2$ and $CaCl_2$ stimulated further development of *B. sexangula* protoplasts, while KCl had an inhibitory effect. The highest number of colony formation was obtained at pH 5.7 without salt additions. However, at lower pHs, some stimulatory effects were found at low concentrations of all four salts tested. Depending on the pHs, different patterns of stimulation and inhibition of four salts were obtained in *B. sexangula* protoplasts.

Introduction

Mangrove forests are distributed along the coastal tropical and subtropical areas. Tolerance to high salinity is a unique character of mangrove plants and the incorporation of this character to crop plants is considered as a target for breeding using biotechnological methods. Prior to the utilization of mangrove species for breeding and molecular studies (Yamada *et al.*, 2002), it is imperative that we have a thorough understanding of the physiological and biochemical mechanisms (Ashihara *et al.*, 2003) of the salt tolerance process. The cell and tissue culture approach is one of the effective means to study aspects of salts tolerance in mangrove species. At present, except for a few successful species (Kura-Hotta *et al.*, 2001; Yasumoto *et al.*, 1999), many mangrove species remain very recalcitrant for rapid proliferation of cells in culture. *Bruguiera sexangula* is one of mangrove trees grown in Thailand and suspension cultures could be obtained from leaves. Stimulatory effects of NaCl on the growth of *B. sexangula* were reported (Mimura *et al.*, 2003).

The objective of this investigation is to develop a protoplast to plant regeneration system using *B. sexangula* as a potential model system to study mechanism of salt tolerance in plants. Once established, genetic engineering methods, *e.g.* cell fusion, could be applied to study the genetic aspect of salt tolerance as well. In this study, a multi-well culture plate method for isolation and culture of protoplasts of broad leaved tree species (Wakita *et al.*, 1996a) was used on the suspension cells of *B. sexangula*. This method allows the optimization of various factors in the medium, *e.g.* mannitol, sucrose, plant growth regulators, and cell density, and their effects on the proliferation of colonies could be investigated (Sasamoto *et al.*, 2000a).

In this report, using the multi-well protoplast culture system, the effects of four salts (NaCl, KCl, $MgCl_2$, $CaCl_2$) and four different pHs on colony formation in *B. sexangula* protoplasts were inves-

tigated. Their effects were compared to those obtained from protoplasts of tobacco BY-2 cell and *Populus alba*. BY-2 cells were chosen as tobacco is a herbaceous plant and the cells are often used in basic research of cell division. *P. alba* represents a forest tree species in which the protoplast to plant regeneration system has been established. Furthermore, efficient plant regeneration after electric cell fusion is also successful (Sasamoto *et al.*, 2000b).

Materials and Methods

Materials

Suspension culture of *Bruguiera sexangula* was induced from leaf-derived callus (Kura-Hotta *et al.*, 2001) and sub-cultured every three weeks in AA (Thompson *et al.*, 1986) medium containing 0.02 μM of 2,4-dichlorophenoxyacetic acid (2,4-D), 2 μM of N-(2-chloro-4-pyridyl)-N'-phenylurea (CPPU) and 20 mM of KCl. Medium pH was adjusted to 6.2 with NaOH before autoclaving. Tobacco BY-2 cells were maintained in a liquid modified MS medium (Murashige and Skoog, 1962) supplemented with 1.3 mM of KH_2PO_4, 1 mg l^{-1} of thiamine-HCl and 1 μM of 2,4-D (Nagata and Kumagai, 1999; Suzuki *et al.*, 2003). Medium pH was adjusted to 5.7 with KOH. One ml of suspension cells was transferred to 50 ml of fresh medium in a 300 ml flask every week. Five day-old cells were used for protoplast isolation. Shoot culture of *Populus alba* was sub-cultured every two to three months in MS agar medium (pH 5.8) containing 4 μM of indolebutyric acid (Sasamoto *et al.*, 2000b).

Protoplast isolation and culture

Suspension cells of *B. sexangula* was first filtered and washed with 0.6 M mannitol solution on 40 μm nylon mesh. These cells were then placed on a rotary shaker at 60-80 rpm for 5-6 h in 0.6 M mannitol solution containing 1% of Cellulase RS (Yakult Co. Ltd.) and 0.25% of Pectolyase Y-23 (Kyowa Chemical Products Co. Ltd.) at room temp. (25℃). For the isolation of protoplasts from BY-2 suspension cells, 0.4 M mannitol solution was used with the same enzyme combination for *B. sexangula*. A 94 μm nylon mesh was used for the filtering of protoplasts. After passing through the mesh, protoplasts were purified by washing three times with mannitol solution followed by centrifugation at 100*g* for 5 min. Leaves of *P. alba* were cut into 2-5 mm wide sections and incubated in the same enzyme solution as for *B. sexangula* in 0.6 M mannitol for 2 h at room temperature under static condition. Protoplasts were purified by washing three times with the mannitol solution followed by centrifugation at 100*g* for 3 min only.

In the culture of *B. sexangula* protoplasts, MS basic medium containing 0.3 M of mannitol, 0.02 μM of 2,4-D and 2 μM of CPPU was used. Ammonium nitrate-free MS basic medium containing 0.3 M mannitol and 1 μM of 2,4-D and 0.1 μM of 6-benzyladenine (BA) was used for protoplast culture of *P. alba*. For BY-2 protoplast culture, the same basic medium for BY-2 cell culture was used with addition of 1 μM of 2,4-D and 0.4 M mannitol. All media contained 3% sucrose. Media pHs were adjusted to 3.5, 4.5, 5.0, 5.7, 6.2, using HCl or KOH, before autoclaving at 121℃, 20 min. The effects of 4 salts (NaCl, KCl, $MgCl_2$, $CaCl_2$) at the following concentrations (10, 25, 50, 100, 200, 300 and 400 mM) were tested. The control media without the addition of salts contained 20 mM of KCl, 0.2 mM of NaCl, 1.5 mM of $MgCl_2$ and 3 mM of $CaCl_2$, respectively. At the time of culture, the cell density was adjusted to $5x10^3$ to $5x10^4$ ml^{-1} and 55 μl of the protoplast suspension was used per treatment. Autoclaved water, 100 to 125 μl was supplied between wells for maintaining the humidity within the plate. Culture was tightly sealed with Parafilm® and incubated in a CO_2-incubator at 28℃ without the supply of CO_2.

Numbers of colonies were counted using an inverted microscope after 45 days of culture in *B. sexangula* and after 15 days of culture in *P. alba*. Numbers of elongated BY-2 cells were counted after five days of culture. Experiments were repeated at least once to eight times.

Results

The effects of four salts on protoplast cultures of B. sexangula, P. alba and tobacco BY-2 cells at pH 5.7.

The effects of each salt on colony formation from protoplasts culture of *B. sexangula* and *P. alba* at $5x10^4$ ml^{-1} and on elongation of cells of tobacco protoplasts at $5x10^3$ ml^{-1} at pH 5.7 were examined (**Fig. 1A** (KCl), **Fig. 1B** (NaCl), **Fig. 1C** ($MgCl_2$), and **Fig. 1D** ($CaCl_2$)). The number of elongated cells counted after 5 days of culture without the addition of salts was 577 ± 11 (standard error). The number of elongated tobacco cells was a good marker for cell proliferation after one month of culture. The proliferation tendency of tobacco protoplasts at high cell density, $5x10^4$ ml^{-1}, was similar to **Fig. 1**, though the counting of cells was difficult. All values shown in **Fig. 1** were percentages of the control without the addition of salts. As shown in **Figs. 1A** and **1B**, both KCl and NaCl were inhibitory to cell growth and colony formation of all

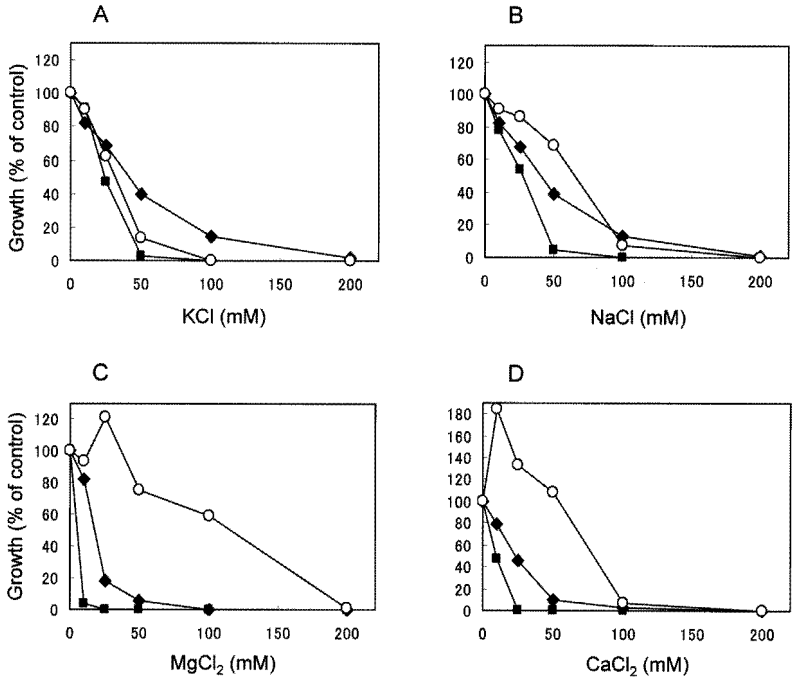

Fig. 1 Effects of different concentrations of salts (A: KCl, B: NaCl, C: $MgCl_2$, D: $CaCl_2$) on numbers of colonies of *B. sexangula* (open circle) and *P. alba* (closed square) and numbers of elongated cells of tobacco BY2 cells (closed diamond) in protoplast cultures at pH 5.7. Data were described as the percentage of the average value without the addition of salts, *i. e.* 95 ± 5 (*B. sexangula*), 42 ± 4 (*P. alba*), 577 ± 37 (BY-2).

three species studied. The addition of 50 mM KCl was more inhibitory to *B. sexangula* and *P. alba* than tobacco protoplasts. NaCl had an inhibitory effect on *P. alba* and tobacco protoplasts but less drastic on *B. sexangula* (**Fig. 1B**). Colony proliferation could still occur at 50 mM NaCl in *B. sexangula*. However, NaCl at 100 mM inhibited growth on all three materials.

As shown in **Fig. 1C**, $MgCl_2$ severely affected colony formation of *P. alba* and tobacco. The addition of 10 mM $MgCl_2$ inhibited colony formation of *P. alba*. In tobacco BY-2 protoplasts, 25 mM of $MgCl_2$ had an 80% inhibitory effect on cell elongation and $MgCl_2$ was the most inhibitory when compared to other salts tested (KCl, NaCl, $CaCl_2$). In contrast, $MgCl_2$ promoted colony formation in *B. sexangula* at low concentration. An increase in colony formation was obtained at 25 mM. At 100 mM $MgCl_2$, the number of colony was 60% of the control treatment.

As shown in **Fig. 1D**, in *P. alba*, 10 mM of $CaCl_2$ was 50% inhibitory and 25 mM of $CaCl_2$ totally inhibited colony formation. In BY-2 protoplasts, the pattern was similar to $MgCl_2$. $CaCl_2$ at 50 mM was 90% inhibitory to BY-2 protoplasts. In contrast, in *B.sexangula* protoplasts, 10, 25 and 50 mM of $CaCl_2$ had stimulatory effects. $CaCl_2$ began to exhibit its inhibitory effect only at high concentrations.

Effect of pHs on protoplast culture of B. sexangula and P. alba.

Effects of four salts at different pHs (4.5, 5.7, 6.2) on the culture of *B. sexangula*, are shown in **Fig. 2**. The patterns of stimulation and inhibition at pH 5.0 (data not shown) were similar to those at pH 4.5 (**Fig. 2**), though the colony formation at lower concentrations (addition of 10 mM and 25 mM) of three salts (KCl, NaCl, $MgCl_2$) was higher at pH 5.0 than that at pH 4.5.

Without additional salts, the highest number of colonies obtained was at pH 5.7 in protoplast culture of *B. sexangula*. The numbers of colonies were 33 ± 3 (standard error) at pH 4.5, 55 ± 7 at pH 5.0, 95 ± 5 at pH 5.7, and 69 ± 9 at pH 6.2.

Effects of KCl at different pHs on colony formation in protoplast culture of *B. sexangula* are shown in **Fig. 2A**. At lower pHs, the inhibitory effect of 25 mM of KCl was not as prominent as those at higher pHs.

Effects of NaCl at different pHs on colony formation in protoplast culture of *B. sexangula* are shown in **Fig. 2B**. For NaCl, at pH 4.5 and 5.0, similar stimulatory (25 mM and 50 mM) and inhibitory (200 mM) patterns was found. At pH 5.7, a gradual decrease in colony formation was obtained at low concentrations up to 50 mM and more than 90% inhibition was seen at 100 mM. At pH 6.2, 10 mM and 25 mM of NaCl increased colony formation,

however, 50 mM NaCl strongly inhibited colony formation.

Effects of $MgCl_2$ at different pHs on colony formation in protoplast culture of *B. sexangula* are shown in **Fig. 2C**. At pH 4.5, a small and prolonged stimulatory effect of this salt could be observed from a concentration of 25 mM to 100 mM. Colony formation was obtained even at a concentration of 200 mM $MgCl_2$. At pH 5.0, the stimulatory effect was not as prominent as pH 4.5 (data not shown). At higher pHs, again, a stimulatory effect on colony formation at low concentrations of $MgCl_2$ was

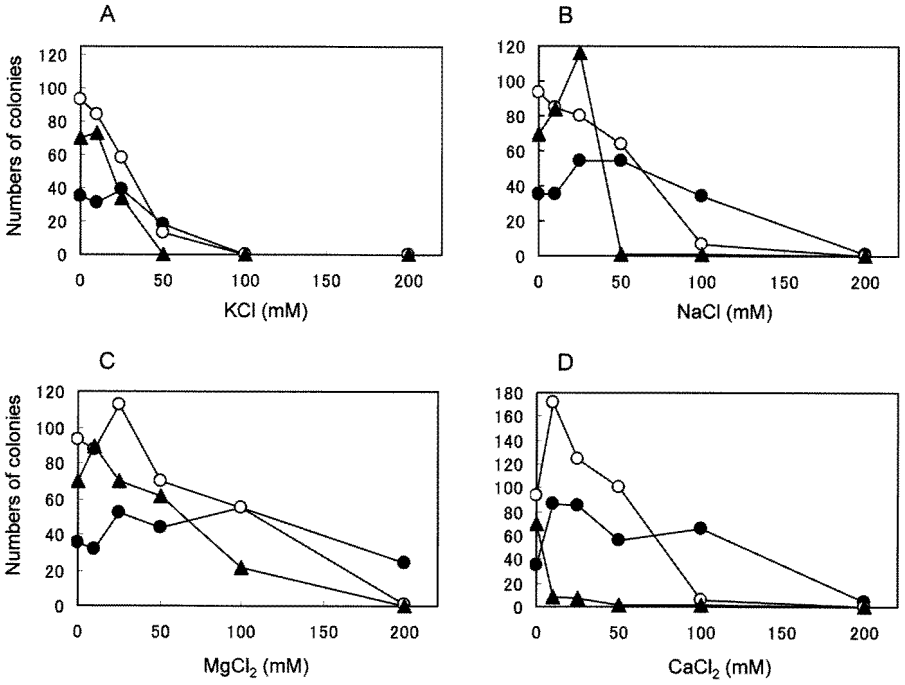

Fig. 2 Effects of pHs, 4.5 (closed circle), 5.7 (open circle), 6.2 (closed triangle), and salts (A: KCl, B: NaCl, C: $MgCl_2$, D: $CaCl_2$) on numbers of colonies after 45 days of protoplast culture of *B. sexangula.*

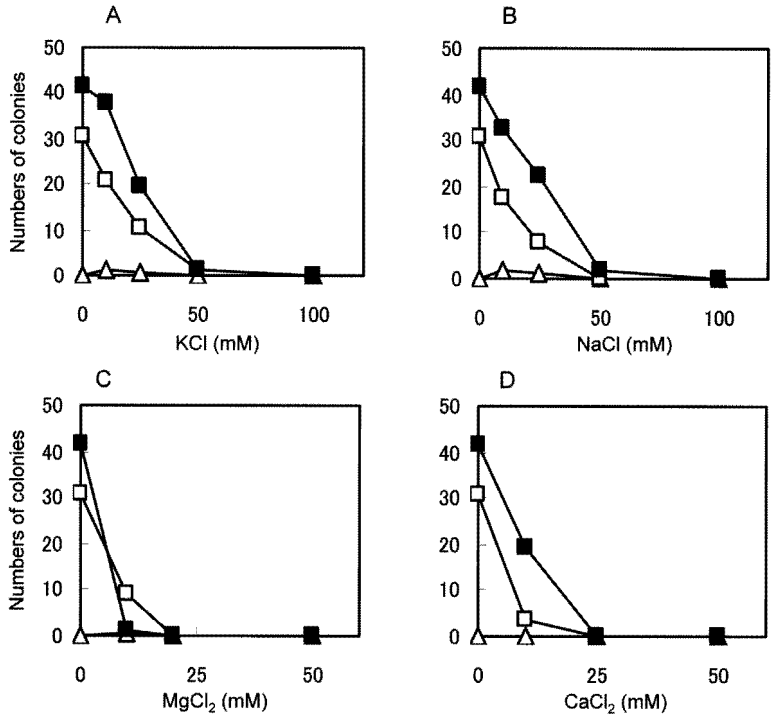

Fig. 3 Effects of pHs, 3.5 (open triangle), 4.5 (open square), 5.7 (closed square), and salts (A: KCl, B: NaCl, C: $MgCl_2$, D: $CaCl_2$) on numbers of colonies after 15 days of protoplast culture of *P. alba*.

obtained. It is important to note that division of protoplasts and colony formation could be observed even at such a high concentration of salt, *i.e.* 100 mM. Such an observation was not found with the other three salts tested.

Effects of $CaCl_2$ at different pHs on colony formation in protoplast culture of *B. sexangula* are shown in **Fig. 2D**. Stimulatory percentage by $CaCl_2$ was much higher when compared to those of NaCl and $MgCl_2$. When compared with the control treatment without the addition of salt, more than 100% increase was obtained at pH 4.5 (10 mM and 25 mM) and 5.7 (10 mM). At lower pHs, colony formation was obtained even at a high concentration of 100 mM $CaCl_2$; although at higher pHs, 100 mM became very inhibitory. At pH 6.2, even a low concentration, *i.e.* 10 mM $CaCl_2$ was inhibitory.

In protoplast culture of *P. alba*, the effects of pHs 5.7, 4.5 and 3.5 were investigated (**Fig. 3**). The number of colonies obtained without the addition of salts was 30 ± 4 (standard error) at pH 4.5 and 42 ± 4 at pH 5.7, respectively. At pH 4.5, similar inhibitory patterns of all ions were observed as at pH 5.7 as shown in **Fig. 1**. Fifty mM KCl and NaCl, 25 mM of $MgCl_2$ and $CaCl_2$, totally inhibited colony formation at all pHs tested. At pH 3.5, only occasional (once per eight repeats) one colony formation was observed without the addition of salts. Effects of salts at pH 3.5 were not clearly observed, though small numbers (2-4) of colonies were obtained at low concentrations (10 mM and 25 mM) of KCl and NaCl, while no colony was at all concentrations of $MgCl_2$ and $CaCl_2$ tested.

Discussion

The mechanisms for tolerance of cells to Na^+ ions and Cl^- ions were investigated in herbaceous as well as mangrove plants (Ashihara *et al.*, 2003; Mimura *et al.*, 2003). High concentrations of these two ions are prominent in the environment surrounding the mangroves. Sea water contains approximately 300 mM of Na^+ ions and 390 mM of Cl^- ions. It is also known that constituents of magnesium and calcium ions are also high, *e.g.* about 2000 ppm, in the mangrove growing soil, while that of sodium ions is around 5000 ppm (Dagar *et al.*, 1993). Although low concentrations, *e.g.* less than 10 mM of Ca^{2+} ions were investigated in a cell culture system (Anil and Rao, 2000), the effects of high concentrations of these ions were not well studied in cell culture except for a few reports. High concentrations of Mg^{2+} and Ca^{2+} ions are inhibitory for cell divisions in a protoplast culture of a broad-leaved tree species, *Betula platyphylla*, though Ca^{2+} ions promote fiber formation of cell wall component (Sasamoto *et al.*, 2003).

In this report, we found the stimulatory effects of Mg^{2+} ions and Ca^{2+} ions on the growth of the mangrove, *B. sexangula* protoplasts at the usual medium pH 5.7. While these salts were inhibitory to tobacco and *P. alba* in all concentrations tested. 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 *P. alba* and tobacco protoplasts, though the latter was more tolerant.

As original suspension cells of *B. sexangula* were subcultured at pH 6.2 (Mimura *et al.*, 2003), which is higher than the usual medium pH 5.7, effects of higher and lower pHs were investigated. Though the highest number of colony formation was obtained at pH 5.7 without additional salts, patterns of stimulatory and inhibitory effects of four salts differed when the pH of the culture medium was varied. A low concentration of NaCl stimulated growth at pH 6.2. And at lower pHs, we found the stimulatory effects of low concentration of NaCl and a wide range of concentrations of $MgCl_2$ and $CaCl_2$ in *B. sexangula*. In a protoplast culture of *Betula platyphylla*, acidic pH condition *e.g.* 4.5 and 3.5 was better for cell divisions than the usual pH 5.8 (Wakita *et al.*, 1996a; Sasamoto *et al.*, 2003). However, stimulatory effect of salts at lower pHs was not found in *P. alba* protoplasts. Drastic change of tolerance in *B. sexangula* protoplasts to the four salts at different pHs might be related to the facts that soil conditions and sea water and river water where the mangrove trees are growing give different salinity and pHs, *e. g.* 3.3 to 8.2 (Dagar *et al.*, 1993; Wakushima *et al.*, 1994). Mangrove plants may have developed a higher tolerance in changes in ion concentrations. This adaptability may prove to be a key survival strategy for these plants in the severe environment.

The 96-well culture plate method developed in leaf protoplast culture of *B. platyphylla* (Wakita *et al.*, 1996b) was found to be effective for surveying many concentrations of salts and pHs. Only 250 to 2500 protoplasts in 55 μl medium per one treatment were sufficient. Such a method could be usable for selection of salt-tolerant cells at early cell culture stage after cell fusion (Sasamoto *et al.*, 2000a, 2000b). Also the purification method of the protoplasts using only mannitol solution without addition of salt ions was effective for clarifying the precise effects of salts in culture.

Many mangroves remain very recalcitrant for tissue culture manipulation (Mimura *et al.*, 1997a, 1997b). The generation and maintenance of a callus

culture remain difficult. The stimulatory effects of $MgCl_2$ and $CaCl_2$ found in *B. sexangula* protoplasts indicate that the incorporation of a high concentration of these salts in a culture medium could be beneficial in the generation of viable cell lines of mangrove for micropropagation and theoretical studies on salt tolerance.

Acknowledgements

Author (H.S.) thanks to Dr. E. C. Yeung of the University of Calgary for his critical reading of this ms. This paper was partly supported by the Grant-in-Aid for Scientific Research on Priority Areas No. 15657010 from the Ministry of Education, Sports, Science ,Technology, Japan and by the Salt Science Research Fund to H.S. .

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