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Mg^{2+} Ions Stimulate both Helical Protoplast-Callose-Fiber Formation and Protoplast Division in a Mangrove Tree, *Sonneratia caseolaris*: Analysis of Sub-fibril Structures of PCF by Atomic Force Microscopy

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Abstract

The effects of four salts, NaCl, KCl, $MgCl_2$ and $CaCl_2$, on protoplast division and protoplast-callose-fiber (PCF) formation were examined using suspension cultured cells of a mangrove tree, *Sonneratia caseolaris.* Basal medium was hormone-free Murashige & Skoog's medium containing 0.8 M sorbitol and 3% sucrose. Addition of 50-100 mM of Mg^{2+} ions highly stimulated both cell division and PCF formation, while addition of Ca^{2+} ions was inhibitory. Addition of Na^+ ions at 10-25 mM and K^+ ions at 50-100 mM stimulated protoplast division but not PCF formation. Helical PCF rapidly elongated from a specific site of cell division plate after 3 days of culture. The β-1,3-glucan (callose) component of the protoplast-fibers was stained with Aniline Blue fluorescent dye and was re-certified enzymatically using laminarinase. After selecting the PCF using a micromanipulator, we analyzed the fibril- and sub-fibril- structures using laser confocal scanning microscopy (LCSM), and atomic force microscopy (AFM). We discuss the uniqueness of the PCF of *S. caseolaris* as compared to PCFs of several plants.

Keywords: AFM, *β*-1,3-glucan, LCSM, protoplast culture, protoplast-fiber, salt tolerance

1. Introduction

1.1 Protoplast-Callose-Fiber (PCF) Formation in Larix and Betula

Novel protoplast-callose-fiber (PCF) formation in high concentrations of divalent cations, was discovered in protoplasts of suspension cultured cells of *Larix leptolepis* and those of the leaf of *Betula platyphylla.* Culture with additional 50 mM $MgCl_2$ for two weeks or 100 mM $CaCl_2$ for one month, was needed for efficient PCF formation. Helical PCF (~2 mm long) elongated from a specific site on the surface of spherically enlarged protoplasts without division. β-1,3-glucan (callose) component of PCF was stained with a fluorescent dye Aniline Blue (Sasamoto *et al*., 2003).

The callose component of *Larix* PCF was confirmed using two enzymes specific for callose degradation and specific for cellulose degradation (Fukumoto *et al*., 2005).

The site of PCF elongation on the surface of spherically enlarged single protoplasts of *Larix* was investigated by laser confocal scanning microscopy (LCSM) using Alexafluor 488 phalloidin, and by transmission electron microscopy (TEM) (Oyanagi *et al*., 2014).

Fibril and sub-fibril structures of *Larix* PCF were analyzed by LCSM and atomic force microscopy (AFM) using Image J analysis (Fukumoto *et al*., 2005).

Mechanical properties (Young's modulus) of elongated PCFs of *Larix* (*ca*.10 μm diameter) and *Betula* (*ca*. 25-30 μm diameter) were investigated using a unique tensile test under an inverted microscope. Young's modulus values (the area base) were similar, which suggested that the sub-structures of their PCFs were related, and application of alkali-labile PCFs in medicinal use was discussed (Oyanagi *et al*., 2014, 2017).

1.2 Different Factors for PCF Formation and Protoplast Growth in Different Plants

Effects of not only divalent cations, but also monovalent cations on protoplast growth and PCF formation have been studied in several mangrove tree species and herbaceous plant species. For example, PCF formation by protoplasts of suspension cultured cells of a halophilic mangrove, *Avicennia alba* (Sasamoto *et al*., 2020a) was stimulated by Na^+ ions only at low concentrations (Kurita-Tashiro *et al*., 2020). Another factor for PCF formation, electric treatment, was found in suspension cultured cells of a salt-tolerant *Bruguiera sexangula* (Kurita-Tashiro *et al*., 2020). Ca^{2+} ions stimulated PCF formation in suspension cultured cells of herbaceous *Vinca rosea*, but not in those of tobacco (Fukumoto, 2006). K^+ and Ca^{2+} ions stimulated PCF formation in leaf protoplast cultures of herbaceous *Arabidopsis thaliana* (Sasamoto *et al*., 2020b; Hayatsu *et al*., in preparation).

The growth of protoplasts of *Betula* leaf was stimulated by an acidic pH (pH 3.5), while addition of high concentrations of Ca^{2+} ions (200 mM) inhibited the growth and stimulated PCF formation (Sasamoto *et al*., 2003; Seyama *et al*., 2008, though culture condition was wrongly described). Therefore, a high concentration of Ca^{2+} ions was not the sole factor for PCF formation in all plant species.

1.3 Effects of Nutrient Sugars, Sucrose and Galactose, in Suspension Cultured Cells and their Protoplasts of Salt-Tolerant and Halophilic Sonneratia caseolaris

Sucrose is a common nutrient sugar in plant tissue and cell cultures and has been used in the plant species described above (*1.1, 1.2*). However, suspension culture of another salt-tolerant mangrove, *Sonneratia caseolaris* was originally induced and sub-cultured in medium containing galactose (Yamamoto *et al*., 2009; Hasegawa, 2014). In galactose medium, salt tolerance of suspension cultured cells of *Sonneratia caseolaris* was weaker than that of other *Sonneratia* species, *i.e.*, Na^+ halophilic *S. alba* (Kawana and Sasamoto, 2008) and salt-tolerant *S. ovata* cultured in sucrose medium (Hasegawa *et al*., 2014).

Recently, Sasamoto & Hasegawa (2021) reported the halophilic nature (not only to Na^+, but also to low concentrations of K^+, Mg^{2+}, and Ca^{2+} ions), of suspension cultured cells of *Sonneratia caseolaris*, cultured in sucrose-containing medium instead of original galactose medium. On the other hand, their protoplast growth was better in sucrose medium than in galactose medium, which suggested that the cell wall is needed for efficient use of galactose as a nutrient sugar (Hasegawa *et al*., 2012; Oyanagi *et al*., 2012a, b).

1.4 PCF Formation in Sonneratia caseolaris

In this report, the effects of four salts, NaCl. KCl, $MgCl_2$ and $CaCl_2$ on protoplast growth and PCF formation were investigated using protoplast cultures of *Sonneratia caseolaris* in sucrose medium as a nutrient sugar. The results were compared with those of other plant species cultured in sucrose medium. Enzymatic certification of callose component of PCF was performed. Sub-structures of PCF of *Sonneratia caseolaris* were investigated using LCSM and AFM with Image J analysis. They were compared with sub-structures of PCFs of other plant species, *e.g*., *Larix* (Fukumoto *et a*l., 2005), *Avicennia alba* and *Bruguiera sexangula* (Kurita-Tashiro *et al*., 2020).

2. Method

2.1 Suspension Culture of Sonneratia caseolaris

Suspension cultures of *S. caseolaris* were induced from cotyledons or hypocotyls and sub-cultured (Yamamoto *et al*., 2009; Hasegawa *et al*. 2014) in Murashige and Skoog's (MS; Murashige & Skoog, 1962) basal medium containing 0.1 μM of 2,4-dichlorophenoxyacetic acid (2,4-D) and 3% galactose. Basal medium contained 0.2 mM Na, 21.25 mM K, 1.5 mM Mg, 3 mM Ca. Medium pH was adjusted at 5.8 with KOH before autoclaving at 121^{o}C, 20 min. Cultures (10 ml medium in a 100 ml culture flask) were incubated at 30^{o}C on a rotary shaker at 100 rpm speed, and sub-cultured at 10-day intervals (Oyanagi *et al*., 2012a, b; Sasamoto & Hasegawa, 2021).

2.2 Protoplast Isolation

Protoplasts of suspension cells (one-week-old culture) of *S. caseolaris* were isolated in 1% Cellulase R10 and 1% Driselase 20 in 0.8 M sorbitol solution for 3 hrs. Protoplasts were purified by passing through a mesh with a pore size of 42 μm and washed three times with sorbitol solution by centrifugation at 100*g* 3 min.

2.3 Protoplast Culture

Protoplasts of *S. caseolaris* were cultured in hormone-free MS basal medium containing 3% sucrose and 0.8 M sorbitol. NaCl, KCl, $MgCl_2$, and $CaCl_2$ were added to the medium at final concentrations of 0, 10, 25, 50, 100 and 200 mM. The medium pH was adjusted to 5.8 with KOH before autoclaving at 121^{o}C, 20 min. When cited in the text, 0.1 μM 2,4-D or 1 μM abscisic acid (ABA) were added.

Five μL of isolated protoplast suspension in osmoticum solution was put in 50 μL liquid medium in each well of a 96-well culture plate (Falcon No.3075). Ultra-pure water (100 μL) was supplied between wells. The culture plate was sealed with two layers of Parafilm^{R}, and incubated at 30^{o}C in a humid incubator (CO_2 incubator without the supply of CO_2 gas, APC-30D/CL-30, ASTEC Co. Ltd.). Protoplast density was adjusted to 1-100 x 10^4 mL^{-1}.

Cultures were observed periodically under an inverted microscope (Olympus CK40 or IX71). Numbers of enlarged and divided protoplasts were counted from photographs of three different areas in each well after Image J analysis (2.4.5). Protoplast growth was described as the % of control without addition of salts.

2.4 Observation of Fibers

All procedures were performed as described previously for protoplast-fibers of a conifer, *Larix* (Fukumoto et al., 2005; Oyanagi *et al*., 2014) and those of a mangrove, *Bruguiera sexangula* (Kurita-Tashiro *et al*., 2020).

2.4.1 Observation of Fibers after Aniline Blue Staining and LCSM

Fibers were first observed under an inverted microscope. Blue-green fluoresced fibers were observed and numbers were counted using a fluorescence inverted microscope (Olympus IX71, UV filter U-MWU2) after staining with Aniline Blue solution (0.5%, pH8.5), which was added to the culture medium at a final concentration of 0.05%. Numbers of PCF were counted in 2-4 wells and averaged. When cited in the text, the average was described as % of initial plated protoplast numbers. Fibers were also observed using a laser confocal scanning microscope (Nikon C1, Differential interference contrast (DIC) and fluorescence inverted microscope, Nikon). Diameters of fibers and fibrils were determined after Image J analysis (2.4.5).

2.4.2 Micromanipulation

Fibers and fiber-forming protoplasts were selected using micromanipulators (Narishige MO-202, MM-89 or Narishige-Nikon MM-188) with a microinjector (IM-188) under an inverted microscope (Olympus IX-71, CK-40). Micropipettes were made from a glass capillary (10 μL, Drummond) using a micropipette puller (PB-7, Narishige), and were bent with a micro-burner (Pen burner, Prince) (Ogita *et al.*, 1999).

2.4.3 Enzymatic Treatment of Protoplast-Fibers

Fibers selected with a micromanipulator (2.4.2), were treated with laminarinase, specific to β-1,3-glucan, or with β-1,4-glucan cellobiohydrolase I (CBH1, Hayashi *et al*., 1997a,b), specific to cellulose. After 24 hr of incubation in a 96-well culture plate, degradation by laminarinase was observed under an inverted microscope. Cotton cellulose was used as a control. Finally, the absence of remaining callose fibers was checked by staining with Aniline Blue (2.4.1).

2.4.4 AFM of a Protoplast-Callose-Fiber

Using a micromanipulator, each fiber, after 30 days of culture, was put on a piece of Isopore^{TM} Membrane filter (Milipore) taped onto a cover glass with cut double-sided tape and dried. The fiber-structures were observed under an atomic force microscope (Nanoscope III; Digital Instruments, Bruker) equipped with a scanner with a 10 μm range (E-scanner).

2.4.5 Image J Analysis

The sub-structures of the protoplast-fibers were predicted from the lines perpendicular to the fiber axis or (sub)-fibril-structures in AFM images, and analyzed by software Image J (NIH, Rasband, 1997-2016). Plot profile data of gray values were obtained from the lines. Peak distances were calculated, and averaged to determine the diameter of the fibrils or sub-fibrils.

3. Results & Discussion

3.1 Effects of Salts on Protoplast Growth of Sonneratia caseolaris

3.1.1 Protoplast Isolation and Culture

Conditions for subculture of suspension cultured cells and enzymatic condition for protoplast isolation of *Sonneratia caseolaris* in this report (Cellulase R10 and Driselase 20) were a modification of previously reported protoplast co-culture method (Cellulase RS, Driselase 20 and Pectolyase Y-23) (Hasegawa *et al*., 2014).

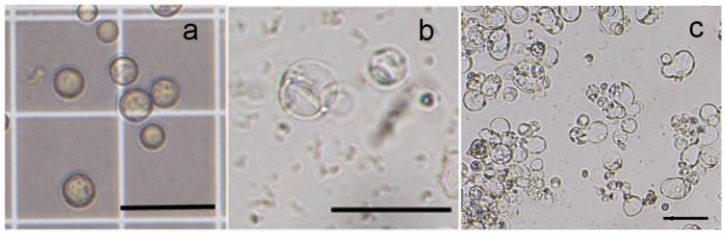

Figure 1. Isolated protoplasts of *Sonneratia caseolaris* on a hemocytometer (a). Enlarged and divided protoplasts after 3 days of culture at 2.5 $\times 10^4$ mL^{-1} (b), and after 7 days of culture at 10^6 mL^{-1} (c). Bar=50 μm

Figure 1a shows the isolated protoplasts of *S. caseolaris* on a hemocytometer. Diameters were 20 μm or less. Figure 1b shows the enlarged and divided protoplasts after 3 days of culture at 5 x 10^4 mL^{-1}. Figure 1c shows non-spherically enlarged and divided protoplasts at 10^6 mL^{-1}. Higher protoplast density (10^6 mL^{-1}) gave better protoplast division, and large colony development after 2 weeks of culture (data not shown).

3.1.2 Effects of Four Salts on Protoplast Growth

Figure 2 shows the effect of addition of four salts on protoplast growth (enlargement and cell division as shown in Figure 1b,c). Growth of protoplasts was stimulated by $MgCl_2$ at 10-100 mM, especially by Mg^{2+} ions at 50-100 mM, but strongly inhibited by $CaCl_2$ at 50 mM or higher concentration. Na^+ ions at 10-25 mM and K^+ ions at 50-100 mM stimulated protoplast growth.

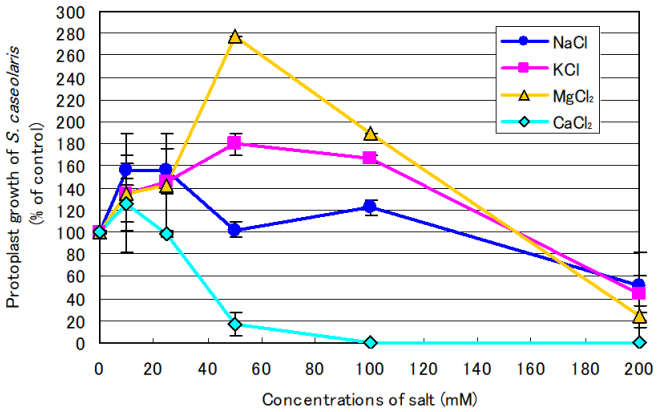

Figure 2. Effects of additional salts on the growth (enlargement and division) of protoplasts of *Sonneratia caseolaris* after 3 days of culture. Medium was hormone-free MS basal medium containing 3% sucrose and 0.8 M sorbitol. Protoplast density was 5 $\times 10^4$ mL^{-1}

Similar patterns of stimulation and inhibition of protoplast growth by the four salts shown in Figure 2, have been reported using the medium containing 3% sucrose, in combination with 0.1 μM 2,4-D or 1 μM ABA, though stimulation by Na^+ ions was not observed in the presence of ABA. In *Sonneratia caseolaris*, $MgCl_2$ at 50 mM showed the strongest stimulation of protoplast growth in the three hormonal conditions (Oyanagi *et al*., 2012a,b; Sasamoto & Hasegawa, 2021).

High concentrations of Mg^{2+} ions strongly stimulated protoplast growth while high concentrations of Ca^{2+} ions strongly inhibited protoplast growth in protoplast cultures of the Na+ halophilic mangrove plants, *Sonneratia alba* (Hasegawa *et al*., 2013) and *Avicennia alba* (Sasamoto *et al*., 2020a), and salt-tolerant *Sonneratia ovata* (Hasegawa *et al*., 2012), when cultured in sucrose medium (Sasamoto & Hasegawa, 2021).

High concentrations of Mg^{2+} ions were reported to stimulate protoplast growth in the leaf protoplast culture of another mangrove plant, *Kandelia obovata* (Kaai *et al*., 2006; Sasamoto & Yokota, 2021).

3.2 Formation of Protoplast-Callose-Fiber (PCF) in protoplast cultures of Sonneratia caseolaris

3.2.1 Observation of PCF

Figure 3a was photographed under an inverted microscope. Non-spherical enlargement of protoplast and cell

division plate was observed. A helical fiber elongated from the black spot at the division plate. Figures 3b and 3c show helical blue-green protoplast-fibers detected under a fluorescence inverted microscope after Aniline blue staining.

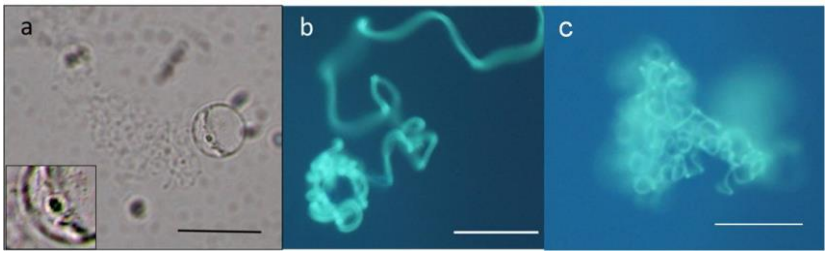

Figure 3. Protoplast-callose- fibers of *Sonneratia caseolaris* cultured for 3-7 days. Protoplast density was 5×10^4 mL^{-1}. a: under an inverted microscope. b,c: under a fluorescence microscope after Aniline Blue staining. Bar=50 μm

PCF elongation from a divided protoplast *of Sonneratia caseolaris* is a unique phenomenon. PCF of other plant species (*Betula*, *Larix*, *tobacco*, *Vinca rosea* and *Arabidopsis thaliana*) elongated from a large spherically enlarged protoplast without cell division (Fukumoto, 2006; Fukumoto *et al*., 2005; Oyanagi *et al*., 2014; Sasamoto *et al*., 2003, 2020b; Hayatsu *et al*., in preparation).

3.2.2 Enzymatic Certification of Callose Component of a PCF

A protoplast fiber of *Sonneratia caseolaris* was selected using a micromanipulator, and treated with laminarinase or CBH1 in a well of 96-well plate. No fiber structures could be observed under an inverted microscope after a 24-hr incubation with laminarinase, which is specific to degrade *β*-1,3-glucans, and the absence was confirmed under a fluorescence microscope after incubation with Aniline Blue.

After CBH-1 treatment, *Sonneratia caseolaris* protoplast-fibers remained as was reported for *Larix* PCF (Fukumoto *et al*., 2005) and PCF of *Bruguiera sexangula* (Kurita-Tashiro *et al*., 2020), while the cotton cellulose fibers were degraded.

Blue-green fluorescence with Aniline Blue staining can be used to distinguish the protoplast-callose-fiber (PCF).

3.2.3 Effects of Four Salts on the PCF Formation

Figure 4 shows the effects of four salts on the number of fibers per well after culture of protoplasts of *Sonneratia caseolaris* for 4 days. Fibers were counted after Aniline Blue staining. The number was slightly increased by 25-50 mM KCl, 50 mM NaCl and 10 mM $CaCl_2$. However, 10-100 mM $MgCl_2$ strongly stimulated PCF formation.

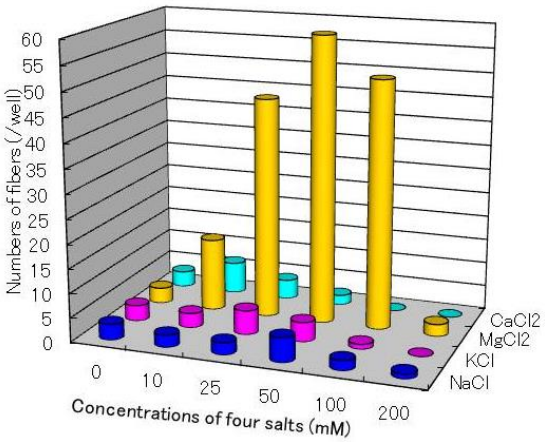

Figure 4. Effects of four salts on PCF formation of *Sonneratia caseolaris* after 4 days of culture. Protoplast density was 5×10^4 mL^{-1}. Medium was hormone-free MS basal medium containing 3% sucrose and 0.8 M sorbitol

50 mM Mg^{2+} ions highly stimulated PCF formation in *Sonneratia caseolaris.* PCF formation was more efficient

in hormone-free medium (Figure 4) than those in plus 2,4-D 0.1 μM or 1 μM ABA medium (data not shown, Oyanagi *et al*., 2012a,b).

High concentrations of Mg^{2+} ions stimulated both protoplast division (3.1.2) and PCF formation. In the present study, PCF elongated from the center of the division plate with non-spherical enlargement of protoplasts of *Sonneratia caseolaris* (Figure 3a, 3.2.1). These results were different from those previously reported for PCFs of *Larix*, in which PCF elongated from the surface of large spherically enlarged protoplasts without cell division, though PCF formation was stimulated by 50 mM Mg^{2+} ions (Sasamoto *et al*., 2003; Fukumoto *et al*., 2005; Oyanagi *et al*., 2014).

In *Larix* and *Bruguiera sexangula*, the single cell TEM was difficult for clarifying the specific cell surface site of PCF elongation because their PCFs easily separate from the elongation site after fixation (Kurita-Tashiro *et al*., 2020; Oyanagi *et al*., 2014). However, in *Sonneratia caseolaris*, single cell TEM might be useful after manipulation of PCF-elongating and -dividing protoplasts.

3.2.4 Effect of Protoplast Density on PCF Formation

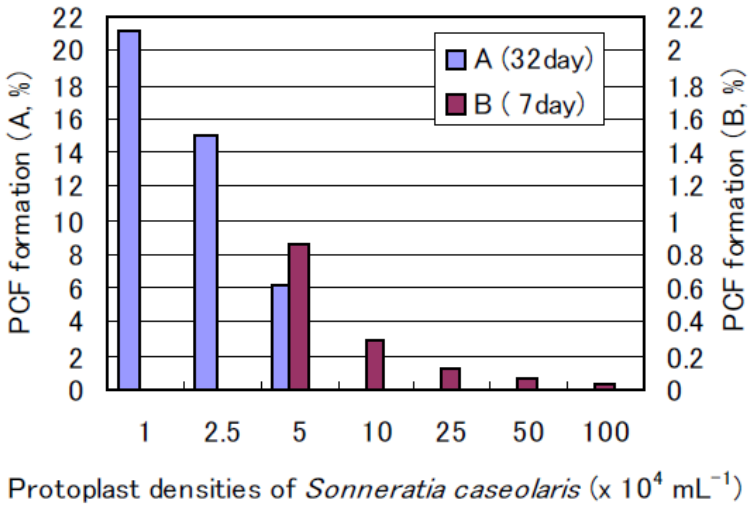

Figure 5. Effects of protoplast densities on formation of PCF of *Sonneratia caseolaris* after 32 days (A) and 7 days (B) of culture in MS basal medium containing 50 mM $MgCl_2$, 3% sucrose, and 0.8M sorbitol

As shown in Figure 5, at a high protoplast density of up to 10^6 mL^{-1}, the % of PCF formation was decreased. Non-spherical enlargement and protoplast division were prominent as shown in Figure 1c (3.1.1). At lower densities, the % of PCF formation increased, and spherical enlargement was prominent.

At 5 $\times 10^4$ mL^{-1}, average % of PCF formation in each well was 3.2%±1.1% in independent experiments (0.86% after 7 days, 2.3% after 4 days, 3.3% after 6 days, 6.1% after 32 days of culture).

3.2.5 Mg^{2+} Stimulation of Formation of PCF of *Sonneratia caseolaris* in Sucrose-free Medium

In this study, we used hormone-free medium containing 3% sucrose for examination of both protoplast growth and PCF formation using *Sonneratia caseolaris* (Figures 1-5). Protoplast growth was suppressed in the sucrose-free medium (data not shown), but PCF formation was stimulated by Mg^{2+} ions at 50 mM to the same level as observed in 3% sucrose medium within a short culture period (Table S1).

Such simple medium condition (sucrose-free medium) for PCF formation in *Sonneratia caseolaris* might serve a good material for large scale production of callose fibers. (Non-fiber) callose accumulation is well known as stress response of plants at pathogen attack (Fukumoto, 2006; Wang *et al*., 2021).

3.3 Analysis of Sub-structures of PCF of Sonneratia caseolaris with LCSM and AFM

3.3.1 Laser Confocal Scanning Microscopy (LCSM)

After Aniline Blue staining, helical protoplast-callose-fibers were observed under LCSM (Figure 6). Average diameters of PCF at 20 points in Figures 6a, 6b, and 6c, were calculated to be 3.3±0.34 μm using Image J software. In Figure 6c, at 2 points, PCF was composed of two fibrils, which had an average diameter of 1.4±0.3 μm.

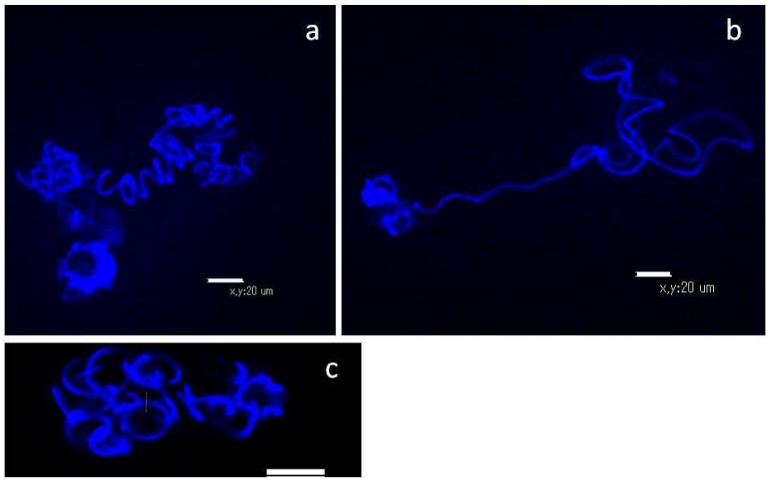

Figure 6. LCSM of PCF of *Sonneratia caseolaris* after 4 days of culture.Bar=20μm

The diameter of *Larix* PCF determined by LCSM was 10 μm (with fibrils of 0.7 μm diameter) (Fukumoto *et al*., 2005). The diameters of PCF of two mangrove species, *i.e*., *Avicennia alba* and *Bruguiera sexangula*, were calculated using LCSM (Kurita-Tashiro *et al*., 2020). PCF of *Avicennia alba* elongated after more than one month of culture, was 15 μm (2-3 fibrils of 6 μm diameter). After 1-2 days of culture after electric treatment, PCF of *Bruguiera sexangula* elongated rapidly (2 μm diameter with 2 fibrils of 1.1 μm diameter).

The calculated diameter of PCF of *Sonneratia caseolaris* after 4 days of culture was in between that of PCFs of *Bruguiera sexangula* and *Avicennia alba*.

3.3.2 Atomic Force Microscopy (AFM)

Fibrils and sub-fibrils were observed in parallel to the longitudinal direction of the fibers by AFM (Figure 7). Sub-fibrils were also in an oblique direction to the fibers. Lines perpendicular to the fibril and sub-fibril structures were drawn and the plot profiles of grey values (a1, a2, b1, c1) were analyzed using image J software to calculate the average diameters (peak distance) (Table 1).

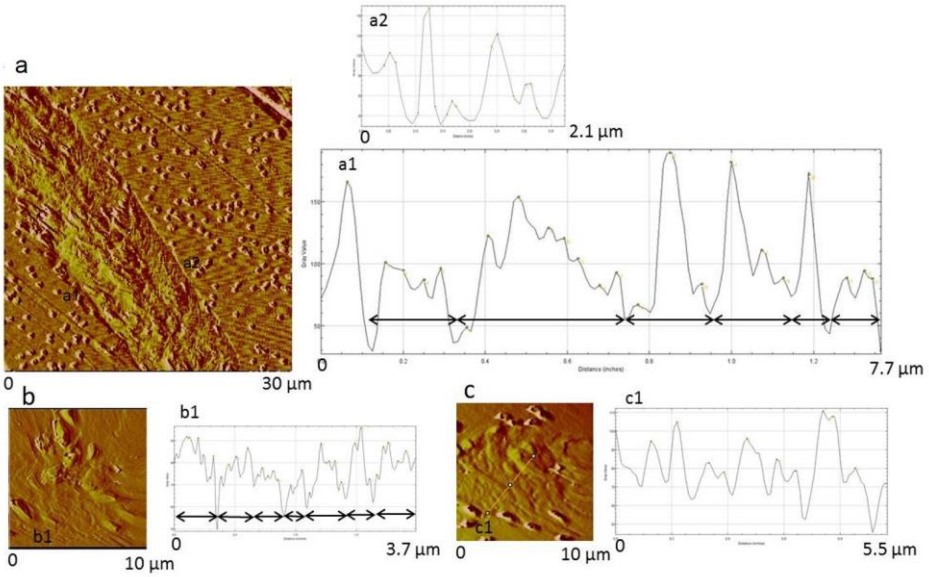

Figure 7. Image J analysis of AFM images of PCF of *Sonneratia caseolaris* (a,b,c) selected after 30 days of culture. Gray values of the lines in images a,b,c are a1, a2, b1 and c1, respectively

Table 1. Sub-structures of protoplast-callose-fibers of *Sonneratia caseolaris*.

Fibers	a	b	c
Diameters (μm)	9	7	8
Fibrils	1.17±0.26 (a1) *	0.52±0.04 (b1)	0.46±0.05 (c1)
Sub-fibrils	0.33±0.02 (a1)	0.11±0.01 (b1)	
Sub-fibrils	0.15±0.025 (a2)		

*Average diameters of fibrils, and sub-fibrils after Image J analysis (a1, a2, b1, c1) of AFM images in Figure 7.

PCF was manipulated after 30 days of culture for observation by AFM (Figure 7). Diameters of PCF of *Sonneratia caseolaris* (7-9 μm in Table 1) were similar to those of previously reported *Larix* PCF (10 μm), which took about 2 weeks of culture. PCF of *Larix* had similar fibril diameters (0.7 μm, sub-fibril 0.17μm) on both LCSM and AFM (Fukumoto *et al*., 2005). PCF of *Sonneratia caseolaris* had a smaller diameter on LCSM (3.3 μm, in 3.3.1) than AFM (Table 1) might depend on the culture period. More than one month of culture was needed to observe the large diameters of PCF of *Betula* leaf (25-30 μm, Sasamoto *et al*., 2003; Oyanagi *et al*., 2017) and of *Avicennia alba* (22 μm, Kurita-Tashiro *et. al*., 2020).

Fibril-structures were parallel to the longitudinal fiber in the previous reports of PCF of *Larix* (Fukumoto et al., 2005) and *Bruguiera sexangula* (Kurita-Tashiro *et al*., 2020).

In PCF of *Sonneratia caseolaris*, part of the fiber showed different sub-structures, *e.g*., smaller diameters of sub-fibrils of oblique direction (a2 in Figure 7). Similarly, the fibril structure of the PCF of *Avicennia alba* was not parallel to the longitudinal fiber (Kurita-Tashiro *et al*., 2020).

Such sub-structures of PCF might affect the mechanical properties of PCF, though PCF of *Larix* and *Betula* with different diameters showed similar Young's modulus (7-9 KPa) and tensile strain at break (0.12-0.26) using a unique tensile test under an inverted microscope (Oyanagi *et al*., 2017). However, application of the tensile test for the PCF of *Sonneratia caseolaris* might need further improvement, because of the short length of PCF (~0.5 mm) compared with ~2 mm length of PCFs of *Larix* and *Betula*.

4. Conclusion

In a mangrove plant *Sonneratia caseolaris*, Mg^{2+} ions stimulated both the growth of protoplasts and rapid protoplast-callose-fiber (PCF) formation within a few days of culture. PCF elongated from a specific site (at the center of a division plate) of small divided protoplasts. Using micromanipulation and AFM with Image J analysis, we analyzed the sub-fibril structures of PCF of *Sonneratia caseolaris*. The direction of sub-fibril elongation was not always longitudinal to the fiber elongation, which is different from the PCF of *Larix* and *Bruguiera sexangula*, but similar to the PCF of *Avicennia alba.*

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Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Informed consent

Obtained.

Ethics approval

The Publication Ethics Committee of the Canadian Center of Science and Education.

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Data availability statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Data sharing statement

No additional data are available.

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Appendix

Table S1. Effects of Mg^{2+} ions and sugar on PCF formation in *Sonneratia caseolaris*

	Sugar free	3% sucrose
Additional Salts		
No	0	3.5
50 mM $MgCl_2$	78	81.5

Protoplasts were cultured in the MS basal medium containing 0.8 M sorbitol without sugar or with 3% sucrose. Numbers of PCF were counted after 9 days of culture and averaged in two wells. Protoplast density was 5×10^4 mL^{-1}.