

Evaluation of an Anthocyanin, Cyanidin 3,5-di-*O*-glucoside, as an Allelochemical in Red Callus of a Mangrove *Sonneratia ovata*, Using Protoplast Co-Culture Bioassay Method with Digital Image Analysis

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Abstract

Protoplasts isolated from red callus of a mangrove, *Sonneratia ovata* were co-cultured with recipient lettuce protoplasts to examine the allelopathic activities. Protoplasts were isolated with Cellulase R10 and Driselase 20 in 0.6 M mannitol solution and purified by density gradient centrifugation on 0.6 M sucrose. Protoplasts were co-cultured in 50 μ L of liquid Murashige and Skoog's (MS) basal medium containing 1 μ M 2,4-dichlorophenoxyacetic acid and 0.1 μ M benzyladenine and 0.6 M mannitol solution in a 96-well culture plate. Protoplast density ranged from 5×10^3 /mL to 10^5 /mL. Cell division of lettuce protoplasts was strongly inhibited by addition of *S. ovata* protoplasts, and non-spherical cell enlargement was slightly inhibited. By contrast, digital image analysis of scanned 96-well culture plates revealed no inhibition in accumulation of yellow color in lettuce protoplasts. An anthocyanin, cyanidin 3,5-di-*O*-glucoside (cyanin), was identified and its content in the red callus was ca. 1 mM of fresh weight. The effects of cyanin on the growth of lettuce protoplasts at three stages were similar to those of red *S. ovata* protoplasts. From these results, cyanin was most likely the allelochemical contained in red callus of *S. ovata*. The allelopathic activity of cyanin was compared with that of other putative allelochemicals in several plant materials, using the protoplast co-culture method with digital image analysis.

Keywords: allelopathy, anthocyanin, mangrove plant, protoplast culture, image analysis

1. Introduction

1.1 Salt Tolerance and Allelopathy of Mangrove Cultured Cells

Mangrove plants are mainly tree species growing in brackish water of different salinity in tropical and subtropical areas (Tomlinson, 1986; Spalding et al., 2010). Strong salt tolerance at the cellular level of several mangrove plants, growing in the coastal region with a high salinity, has been found using tissue cultured cells and protoplasts (Kawana & Sasamoto, 2008; Hayashi et al., 2009; Yamamoto et al., 2011; Hasegawa et al., 2013).

A survival strategy of the upstream-grown mangrove plants could be the inhibition of the growth of neighboring plants through production of allelochemicals (Hasegawa, 2014; Hasegawa et al., 2014). Allelopathic activities of many non-mangrove test plants using lettuce seedlings as recipients have been studied by the *in vitro* bioassay sandwich method (Fujii, 2000; Takemura et al., 2013). However, studies on allelopathy of mangrove plant cells were started only recently, after the *in vitro* bioassay method of allelopathy using tissue cultured cells and protoplasts was developed.

1.2 Protoplast Co-culture Method for Bioassay of Allelopathy

The protoplast co-culture method for *in vitro* bioassay of allelopathy using lettuce protoplasts as the recipient was recently developed for examining not only herbaceous plant species, *e.g.*, leguminous *Mucuna pruriens* (Sasamoto et al., 2013) and *Vicia villosa* (Sasamoto et al., 2015b), and *Arabidopsis thaliana* (Sasamoto et al., 2017b), but also woody and tree plant species (Hasegawa et al., 2014; Inoue et al., 2015; Mori et al., 2015; Ogita & Sasamoto, 2017; Fujise et al., 2018) including a tropical invader leguminous tree plant, *Leucaena leucocephala* (Mori et al., 2015). Using the same lettuce protoplast co-culture method, the inhibitory effects of putative allelochemicals in plants, *e.g.*, L-DOPA (Sasamoto et al., 2013); purine alkaloids, caffeine metabolites (Sasamoto et al., 2015a); pyridine alkaloids, mimosine (Mori et al., 2015); nicotinic acid, nicotinamide and trigonelline (Sasamoto & Ashihara, 2014); isoflavonoid, rotenone (Inoue et al., 2015); abscisic acid and coumarin (Fujise et al., 2018), were determined.

1.3 Allelopathic Activities of *Sonneratia Mangrove Cells*

After development of the protoplast co-culture method, an inverse relationship was found between allelopathic activity and salt tolerance using dark-grown suspension cultured cells of three *Sonneratia* mangrove species, *S. alba*, *S. ovata*, and *S. caseolaris* (Hasegawa et al., 2014). Salt tolerance to NaCl and allelopathic activity of suspension cells of *S. ovata* were in-between those of *S. alba* and *S. caseolaris*. However, mangrove cultured cells have not been examined for putative allelochemicals, except for a leguminous tree mangrove, *Derris indica*, which contained rotenone an isoflavonoid, in roots, but its content in cultured cells remains unknown (Inoue et al., 2015).

1.4 Allelopathic Activities of Red Callus Culture of *S. ovata* and Anthocyanins

Red calluses were induced from hypocotyls, cotyledons and roots of *S. ovata*, and could be sub-cultured in a light condition (Hasegawa, 2014). In contrast, from *S. alba* and *S. caseolaris*, no red callus strain was obtained (Sasamoto et al., 2017c). Anthocyanins are natural plant pigments (Takeda et al., 2013), but allelopathic activities of anthocyanins have not been reported.

Here, we identified an anthocyanin in red *S. ovata* callus cultured in the light condition, as cyanidin 3,5-di-*O*-glucoside (cyanin). Then, using the protoplast co-culture bioassay method, allelopathic activities of protoplasts of red callus of *S. ovata* and the putative allelochemical, cyanin, were investigated. In addition, we used digital image analysis developed recently for protoplast co-culture method of allelopathy, to quantify the effects on the yellow color accumulation during lettuce protoplasts growth (Ogita & Sasamoto, 2017; Sasamoto et al., 2017a-c). Other allelopathic plants and their putative allelochemicals were also investigated by the protoplast-co-culture bioassay method for comparison.

2. Method

2.1 Materials

Red callus culture of *S. ovata* was induced from cotyledons of *Sonneratia ovata* in the MS (Murashige & Skoog, 1962) basal medium containing 0.1 μM of 2,4-dichlorophenoxyacetic acid (2,4-D), 3% sucrose (Hasegawa, 2014), and sub-cultured on agar medium (0.8% agar) of the same composition for induction, in a light condition (16 hrs photoperiod, 90-140 $\mu\text{mol m}^{-2}\text{s}^{-1}$) at 25°C.

Lettuce seedlings were cultured as described previously (Sasamoto et al., 2013). Briefly, lettuce seeds (Great Lakes) were sterilized with 1.5% NaClO solution for 15 min and washed three times with autoclaved water. They were aseptically cultured on 0.8% agar medium in the light condition for 6-8 days at 25°C.

2.2 Identification and Quantification of Anthocyanin in Red Callus

2.2.1 Isolation and Identification of Anthocyanin in Red Callus

Red callus (fresh weight, 15.8 g) was extracted with methanol/formic acid (92:8). After concentration, the extract was applied to preparative paper chromatography (PC) using a solvent system, BAW (*n*-butanol/acetic acid/water = 4:1:5, upper phase). The isolated anthocyanin was purified by Sephadex-LH-20 column chromatography using solvent system, methanol/water/formic acid (70:25:5). Analytical high performance liquid chromatography (HPLC) of the isolated anthocyanin was performed with a Shimadzu HPLC systems using L-column2 ODS column (I.D. 6.0 \times 150 mm, Chemicals Evaluation and Research Institute) at a flow-rate of 1.0 mL/min, detection wave-length of 530 nm, and eluent was acetonitrile/phosphoric acid/acetic acid/water (8:3:8:81). Liquid chromatograph-mass spectra (LC-MS) was measured with Shimadzu LCMS-2010 EV systems using L-column2 ODS column (I.D. 2.1 \times 100 mm) at a flow-rate of 0.2 mL/min, detection wavelength of 530 nm, ionizing voltage, 4.5 kV for ESI⁺, and eluent was acetonitrile/water/formic acid (5:90:5). Acid hydrolysis

was performed in 12% HCl at 100 °C for 30 min. After cooling, iso-amyl alcohol was added in mother liquor, and anthocyanidin and sugar were obtained from the organic layer and aqueous layer, respectively. Anthocyanidin was characterized by HPLC using the authentic sample for comparison. Sugar was identified by PC with the authentic sample for comparison using solvent systems, BBPW (*n*-butanol/benzene/pyridine/water = 5:1:3:3) and BTPW (*n*-butanol/toluene/pyridine/water = 5:1:3:3). Sugar spot was visualized by spraying to the chromatogram with 1% methanolic aniline hydrochloride and then heating.

2.2.2 Quantification of Anthocyanin in Red Callus

Red callus (100 mg fresh weight) was extracted in 1 mL of 2% trifluoroacetic acid (TFA) / methanol for more than 4 hrs in a dark condition at 4 °C. The extracts were centrifuged at 13,000 rpm for 10 min at 4 °C, and the supernatant were filtered through a 13 mm syringe filter with a PVDV membrane (polyvinylidene difluoride, 0.45 µm, Whatman, Japan) and used for HPLC analysis. Anthocyanin was identified using a Shimadzu HPLC system (Shimadzu Co., Kyoto, Japan) equipped with a Prominence HPLC system (LC-20AD pumping system, a SIL-20AHT autosampler, SPD-M20A diode array detector (200~600 nm detection range). A Shim-pack VP-ODS column (4.6 mm Φ x 150 nm, Shimadzu, Co., Kyoto, Japan) at 40°C was used for chromatography to separate anthocyanin species using 0.5% TFA in ultrapure water as solvent A and 0.1% TFA in acetonitrile as solvent B. The solvent gradient was %B, initial 8%; 50 min, 15%; 60 min, 30%; 65 min, 60% and 75 min, 65% (run time 75 min); at flow rate of 1 mL/min and injection volume of 10 µL. Cyanidin 3-*O*-glucoside (Cy-3-glu, Wako Chemical Co. Ltd.) was used to form a standard curve and the value of each anthocyanin present was calculated in µg Cy-3-glu equivalents/g fresh weight. Peaks were identified by comparison with a standard of Cy-3-glu and retention times of blueberry-fruit extracts (Ogawa et al., 2008).

2.3 Protoplasts Isolation and Purification

Red purple callus of *S. ovata* at 1.5-2 months of culture were incubated in 1% each of Cellulase R10 (Yakult) and Driselase 20 (Kyowa Hakko Kogyo Co. Ltd.) in 0.6 M mannitol solution, shaking at 80 rpm speed for 24 hrs. This combination of enzymes was selected from 24 combinations in a preliminary experiment using six kinds of cell wall degrading enzymes as described previously (Sasamoto et al., 1997; Azumi & Sasamoto, 2017). After filtration through 42 µm mesh, protoplasts were purified by floatation in density gradient on 0.6 M sucrose solution and washed with mannitol solution by centrifugation at 100 g 5min three times.

Lettuce cotyledon protoplasts were isolated as described previously (Sasamoto et al., 2013). Briefly, protoplasts of lettuce were isolated in 1% each of Cellulase RS (Yakult) and Macerozyme R10 (Yakult) in 0.6 M mannitol. Protoplasts were passed through a 63 µm nylon mesh and purified with mannitol solution by centrifugation at 100 g 5 min three times.

2.4 Protoplasts Co-Culture of *S. ovata* and Lettuce

Protoplasts were counted using a hemocytometer. Five µL each of protoplasts in 0.6 M mannitol solution was put in 50 µL of liquid MS medium containing 1 µM of 2,4-D and 0.1 µM of benzyladenine (BA), and 3% sucrose and 0.6 M mannitol in a well of 96-well culture plate (Falcon 3075). Final protoplast densities of *S. ovata* were 1.5×10^3 /mL to 10^5 /mL, and of lettuce were 6×10^3 /mL to 10^5 /mL. The protoplasts were cultured at 28°C in a humid incubator (CO₂-incubator without the supply of CO₂, APC-30DR, ASTEC Co. Ltd.). The non-spherically enlarged lettuce protoplasts and divided cells were counted under an inverted microscope (Olympus IX-71) after 5 days of culture. The numbers of divided protoplasts including colonies composed of more than 4 cells were counted after 12 days of culture. Percentages of control without addition of *S. ovata* protoplasts were calculated and averaged with standard error (SE) at different densities of lettuce protoplasts.

2.5 Effects of Cyanin on Lettuce Protoplasts

Cyanin (cyanidin 3,5-di-*O*-glucoside chloride, PhytoLab, Kanto Chemical Co. Ltd.) was dissolved in water (1 mM) and filter sterilized (Millex-GV, 0.22 µm, Millipore): 25 µL was added to 25 µL of double concentrations of each medium composition described in the co-culture method except for mannitol. The final mannitol concentration was 0.4 M instead of 0.6 M. The numbers of non-spherically enlarged lettuce protoplasts and divided cells were counted after 4 days of culture. The numbers of divided cells and colonies were counted after 8 days of culture. Percentages of control without cyanin were calculated and averaged with SE at different lettuce protoplast densities.

2.6 Digital Image Analysis

Image analysis of yellow color accumulation of lettuce protoplasts after protoplast co-culture was performed as described previously (Ogita & Sasamoto, 2017; Sasamoto et al., 2017a-c). Digital image of a 96-well culture plate was scanned using a scanner (Epson GTX-970) after 27-35 days of culture. Image analysis by software

Image J (NIH, Rasband, 1997-2016) was performed. An image of blue channel (jpg file) was selected. A horizontal straight line was drawn at the center of the wells. The plot profile of the line was analyzed. The data of the blue plot values were saved as an excel file. In excel software, the average of blue plot values were determined for each well. The yellow value was converted by deduction of each averaged blue value from the highest blue value (control). The yellow values were deduced at each density of *S. ovata* protoplasts or each concentration of cyanin themselves. The % yellow value of control without *S. ovata* protoplasts or cyanin was calculated at each lettuce protoplast density. Finally, the percentages of control were averaged with SE at different cell densities of lettuce ($6 \times 10^3/\text{mL}$ to $10^5/\text{mL}$).

3. Results and Discussion

3.1 Red Callus of *S. ovata*

Red calluses were induced from all tissues of germinated seedlings, i.e., hypocotyls, cotyledons and roots, of *S. ovata* (Hasegawa, 2014), and red calluses originating from cotyledons could be sub-cultured for more than four years in the light condition. Figure 1 shows red to red-purple *S. ovata* callus originating from cotyledons. After sub-culture at two-week intervals, the red-purple color was maintained during growth. However, when the sub-culture interval was prolonged, the red color diminished after transfer to fresh medium, and red-purple color increased after one month of culture, before browning proceeded.

Several tissue parts of *Sonneratia* plants were red, e.g., small parts of flowers (Minagawa et al., 2016), and transiently pink seeds of *S. alba* when imbibed in water, but not the seeds of *S. ovata* (Sasamoto et al., 2017c). However, no red callus strain was obtained from young seedlings of *S. alba* and *S. caseolaris* even in the light condition. On the other hand, green callus cultures were obtained from *S. alba* in the light condition (Kawana et al., 2007), and blue-green suspension cultured cells were obtained from *S. caseolaris* in the dark (Hasegawa et al., 2014; Sasamoto et al., 2017c). Red callus might be unique to *S. ovata*.



Figure 1. Red-purple *Sonneratia ovata* callus at 1.5 months of culture in a 100 ml flask

3.2 Identification and Quantification of Anthocyanin in Red Callus of *S. ovata*

The anthocyanin was obtained as a pure red solution (see the subsection 2.2.1). Cyanidin and glucose were liberated by acid hydrolysis of this anthocyanin. Since the molecular ion peak, m/z 611 $[\text{M}]^+$ appeared by LC-MS, 2 mol glucose was shown to be attached to cyanidin. Finally, anthocyanin was identified as cyanidin 3,5-di-*O*-glucoside (cyanin) (Figure 2), which was not found in the fruit of *S. ovata* (Wu et al., 2009), by HPLC using the authentic sample for comparison from the flowers of *Dahlia variabilis* (Hayashi, 1933).

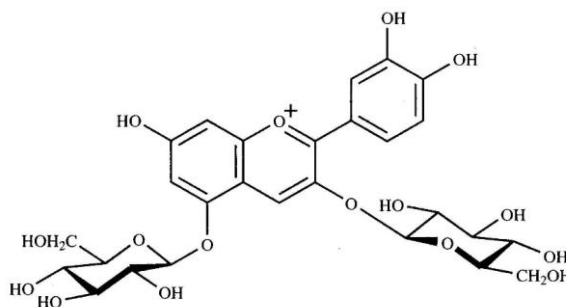


Figure 2. Cyanidin 3,5-di-*O*-glucoside (cyanin)

Figure 3 shows the HPLC profile of anthocyanin in red callus of *S. ovata* (see the subsection 2.2.2). The peak at 7 min was the main peak of cyanin, which was very rapidly eluted than those of the peaks of blueberry extracts (Ogawa et al., 2008), which contained delphinidin 3-*O*-galactoside (9 min). The calculated content of the main peak was 573 $\mu\text{g eq./g}$ fresh weight. The cyanin content in the red callus of *S. ovata* was ca. 1 mmole/kg fresh weight.

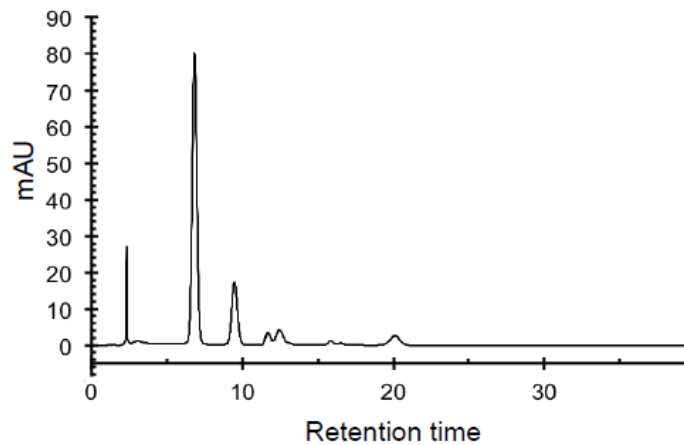


Figure 3. HPLC profile of an extract of *S. ovata* red callus

3.3 Isolation and Purification of Protoplasts of *S. ovata* Callus

The cell-wall-degrading enzyme combination of Cellulase R10 and Driselase 20 was effective for protoplast isolation from red-purple-colored callus of *S. ovata*. The strong enzyme combination consisting of Cellulase RS, Hemicellulase and Driselase 20, which was used for dark-grown suspension cells of *S. ovata* (Hasegawa et al., 2014), was not used for the red callus in the 24-well method (Azumi & Sasamoto, 2017; Sasamoto et al., 1997). The density gradient centrifugation method for purification of intact protoplasts was effective for separation from broken protoplasts. Such a method was also effective for protoplast purification of callus culture of mangrove plants, *Avicennia alba* (Tsuchiya et al., 2013), and suspension cells of *S. alba* (Hasegawa et al., 2014). As dark-grown *S. ovata* suspension cells could be purified by centrifugation (Hasegawa et al., 2014), cell wall components of red callus might be different from dark-grown suspension cells. The protoplast precipitate of *S. ovata* red callus was pink.

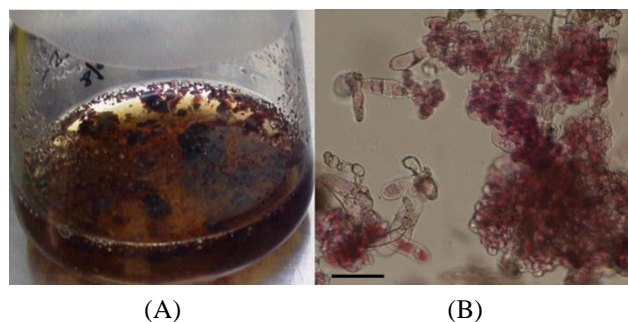


Figure 4. Red cell clumps of *S. ovata* callus, after 10 min in enzyme solution in a 100 ml flask (A) and the cell clumps under an inverted microscope (B). Bar=100 μm

3.4 Protoplasts Co-Culture of *S. ovata* Red Callus and Lettuce

Figure 5 shows green lettuce protoplasts 30-50 μm in diameter and red to light red *S. ovata* protoplasts 15-25 μm in diameter. The size of *S. ovata* was similar to those of *S. caseolaris* suspension cells, but smaller than protoplasts of dark-grown *S. ovata* suspension cells (30-50 μm diameter, Hasegawa et al., 2014). The vacuole of protoplasts was red.

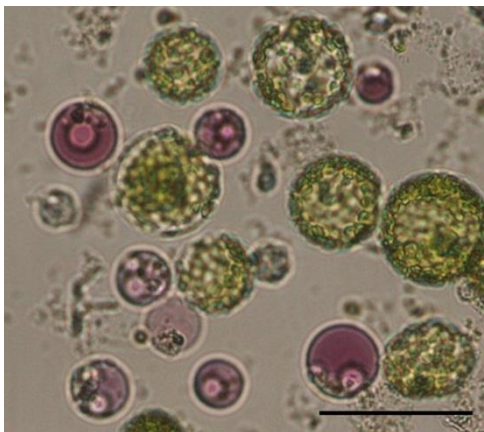


Figure 5. Protoplasts of *S. ovata* at 5×10^4 /mL and of lettuce at 10^5 /mL in MS basal medium containing 1 μ M of 2,4-D and 0.1 μ M of BA, 3% sucrose and 0.6 M mannitol on the first day of culture. Bar=50 μ m

Though protoplasts of *S. ovata* changed (non-spherical shape change, cell wall formation) after several days of culture, they did not enlarge or divide well in the medium of co-culture and their red color decreased. The change in lettuce protoplasts was well distinguished from their size and divisions under an inverted microscope (Sasamoto & Ashihara, 2014; Sasamoto et al., 2015a).

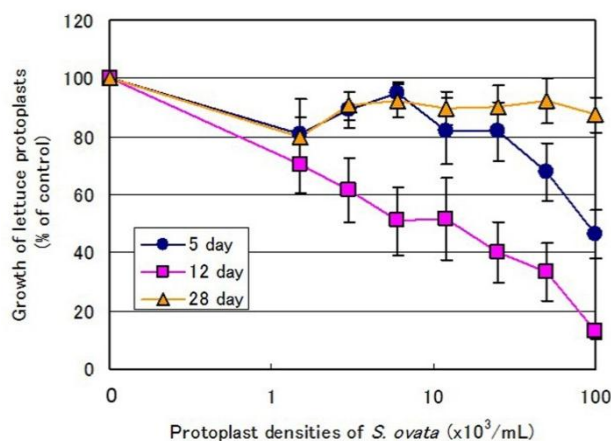


Figure 6. Effects of *S. ovata* protoplasts on growth of lettuce protoplasts by the protoplast co-culture method. Basal medium is shown in Figure 5. Numbers of non-spherically enlarged and divided lettuce protoplasts were counted after 5 or 12 days of co-culture. Digital image analysis of yellow accumulation was performed after 28 days of co-culture. Data were described as % of control without *S. ovata* protoplasts and averaged with SE at lettuce protoplast densities of $6-100 \times 10^3$ /mL

Figure 6 shows the effects of *S. ovata* protoplasts on the growth of lettuce protoplasts. At 5 days of co-culture, non-spherical enlargement of lettuce protoplasts was clearly inhibited by the addition of 5×10^4 /mL or more of *S. ovata* protoplasts. After 12 days of co-culture, cell division of lettuce protoplasts was inhibited by the addition of *S. ovata* protoplasts at low (3×10^3 /mL) to high protoplast densities (up to 10^5 /mL). Formation of colonies composed of more than 4 cells was more strongly inhibited than early cell division (data not shown). Such inhibition pattern was different from that of *Derris indica* which showed the same inhibitory patterns at two stages of growth (cell enlargement stage and division stage).

Allelopathic activity of protoplasts at low densities of red callus of *S. ovata* sub-cultured in the light condition was stronger than that of dark-grown suspension cells of *S. ovata* (Hasegawa et al., 2014), which might be related to the differences of allelochemical(s) between the two cell lines of *S. ovata*.

By contrast, no inhibition was observed on the yellow color accumulation in lettuce protoplasts after 28 days of co-culture (Figure 6). Data were similar to those at 35 days of co-culture (data not shown). The yellow substance

formed during the growth of lettuce protoplasts was identified as a carotenoid (Sasamoto et al., 2017a). Effects on the yellow color accumulation in protoplasts by the co-culture method were very recently investigated using protoplasts of several plant species, e.g., *Vicia villosa* (Sasamoto et al., 2015b); bamboo species (Ogita & Sasamoto, 2017); *Arabidopsis thaliana* (Sasamoto et al., 2017b). In these plants, the yellow color accumulation was inhibited, though the inhibition was weaker than that at other growth stages (cell wall formation and cell divisions). These results are different from the results we obtained using red *S. ovata* protoplasts, which did not inhibit the yellow color accumulation at a protoplast density of up to 10^5 /mL. Specific allelochemical(s) in red callus of *S. ovata* might be considered for this phenomenon.

3.5 Allelopathic Activities of Cyanin

Figure 7 shows the effects of an anthocyanin, cyanin on the growth of lettuce protoplasts. The inhibitory effect at the cell division stage was stronger than that at the non-spherically cell enlargement stage (cell wall formation). By contrast, yellow color accumulation was not inhibited after 27 days of co-culture. The same pattern of inhibition as shown in Figure 6 was obtained at a high concentration of cyanin.

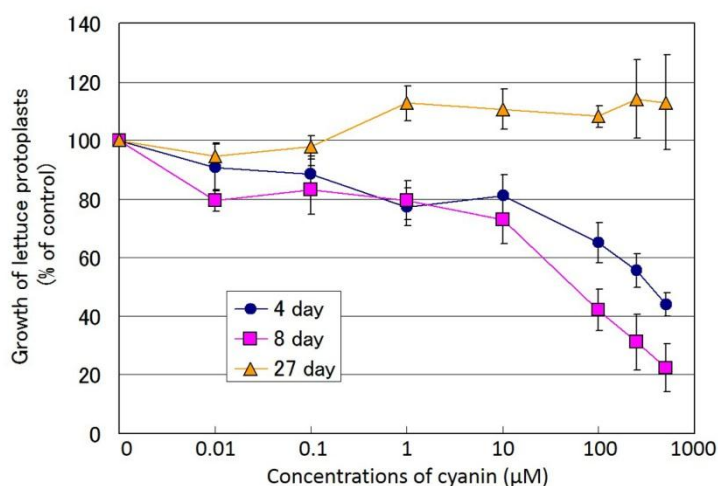


Figure 7. Effects of cyanin on growth of lettuce protoplasts by the protoplast co-culture method. Basal medium was the same as in Figure 5 except for 0.4 M mannitol. Numbers of non-spherically enlarged and divided lettuce protoplasts were counted after 4 or 8 days of co-culture. Digital image analysis of yellow accumulation was performed after 27 days of co-culture. Data were described as % of control without cyanin and averaged with SE at lettuce protoplast densities of $6-90 \times 10^3$ /mL

As the cyanidin 3,5-di-*O*-glucoside chloride could not be dissolved directly in the co-culture medium, the 1 mM solution was dissolved in water and filter-sterilized before mixing with medium of double concentrated composition. By the addition of cyanidin 3,5-di-*O*-glucoside chloride, the initial medium pH 5.2-5.4 was slightly reduced (4.9, 4) at high concentrations (250 and 500 μM). However, the difference was not as large as the reduction in pH (4.5-4.0) after culture of protoplasts of lettuce. This indicated that the inhibition of cell division and absence of inhibition of yellow accumulation at 100 μM was not the effect of a low pH.

Though lettuce protoplasts can grow in a wide range of osmotic conditions, i.e., 0.4 to 0.8 M mannitol, lettuce protoplasts grow better in 0.4 M mannitol solution than in 0.6 M mannitol solution. Four days and 8 days were selected for counting the non-spherical enlargement and cell divisions, respectively in 0.4 M mannitol solution, as in the protoplast co-culture with *Derris indica* and rotenone (Inoue et al., 2015).

3.6 Anthocyanin is the Allelochemical of *S. ovata* Red Callus Evaluated Using Protoplast Co-Culture Method with Digital Image Analysis

This is the first report on allelopathic activity of red colored callus of a mangrove *S. ovata* containing an anthocyanin, cyanidin 3,5-di-*O*-glucoside (cyanin). Patterns of inhibition at three different stages of growth of lettuce protoplasts, i.e., cell wall formation, cell division and yellow color accumulation, were similar in both *S. ovata* protoplasts and cyanin. Content of cyanin in red callus of *S. ovata* (ca. 1 mM) was sufficient for inhibition of lettuce protoplast growth (Figure 7). Though the content of cyanin in *S. ovata* protoplasts was not investigated, a higher concentration in protoplasts is expected without cell walls. Cyanin is most likely the allelochemical in

red callus of *S. ovata*, and it is the only allelochemical evaluated in *Sonneratia* mangrove species using the protoplast co-culture method.

Inhibitory effect of cyanin on cell divisions was weak compared with another putative allelochemical of a mangrove *Derris indica*, rotenone, which is known in the plant roots. However, no direct investigation was made on its content in the suspension cells of *D. indica* (Inoue et al., 2015). The inhibition by cyanin was stronger than that by caffeine (Sasamoto et al., 2015a) or mimosine (Mori et al., 2015) at 100 μ M. Not only the endogenous contents of allelochemicals in the protoplasts themselves, but also the uptake efficiency of these chemicals from culture media and the cellular mechanisms of inhibition on lettuce protoplasts growth might be considered.

This is also the first report of an anthocyanin with strong allelopathic activity identified as cyanin. The antioxidant and antimicrobial activities of anthocyanins have been studied (Kähkönen & Heinonen, 2003; Cisowska et al., 2011; Takeda et al., 2013), but not the allelopathic activities of anthocyanins. We have been studying the allelopathic activities of chemicals containing the cyanidin-structure using the protoplast co-culture method (Sasamoto et al., 2017d).

We found the protoplast co-culture method with digital image analysis useful to study the allelopathic activities of chemicals in lettuce protoplasts at three different growth stages. Though the yellow color accumulation is observed only during the growth of lettuce protoplasts, the protoplast co-culture method can be applied not only using lettuce as a recipient plant, but also neighboring plants in the field (Sasamoto et al., 2013).

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