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Strong Allelopathic Activity in Purple Leaves of Transgenic *Spiraea cantoniensis* Containing Cyanidin 3-Glucoside Assayed by the Protoplast Co-culture Method with Digital Image Analysis

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Abstract

The protoplast co-culture method with digital image analysis (DIA-PP method), was used for *in vitro* bioassay of allelopathic activity in the leaves of *in vitro* cultured plants of *Spiraea cantoniensis*. Purple leaves of transgenic *S. cantoniensis*, containing an anthocyanin, cyanidin 3-glucoside showed strong inhibitory allelopathic activity, 100% inhibition of recipient lettuce protoplast division by 80×10^3 mL^{-1} of co-cultured protoplasts, while green leaves of non-transgenic *S. cantoniensis* showed only 20% inhibition. Cyanidin 3-glucoside which inhibited lettuce protoplast division 80% at 100 μM was evaluated as an allelochemical of transgenic *S. cantoniensis*. Inhibition was stronger at the cell division stage of lettuce protoplast growth than that at the other stages, *i.e.*, cell wall formation stage and yellow pigment accumulation stage. The results were discussed comparatively with the strong inhibitory activity of non-transgenic *S. thunbergii* and other allelopathic plant species containing different anthocyanin or carotenoid pigments as putative allelochemicals.

Keywords: allelopathy, anthocyanin, bioassay, image analysis, protoplast culture, *Spiraea*

1. Introduction

Spiraea cantoniensis, Lour. (Rosaceae) is a white flowering shrub called kodemari in Japanese meaning small handballs. Breeding of flowers with different colors has been a theme in horticulture. The *Agrobacterium*-mediated transformation method has been used to introduce the anthocyanin-synthesizing gene, *Atpap 1* into the plant cells, and succeeded in constitutive expression of cyanidin-glucoside in the leaf extract of regenerated transgenic plants (Nakagawa & Suzuki, 2015).

Spiraea thunbergii, Sieb. ex. Blume is another white flowering shrub called yukiyanagi in Japanese meaning snow willow known to have strong allelopathic activity due to allelochemicals identified as *cis*-cinnamoyl derivative and *cis*-cinnamic acid (Hiradate *et al*., 2005; Wasano *et al*., 2013). By contrast, weak allelopathic activity of *S. cantoniensis* was found in a lettuce seedling growth test (Takemura *et al*., 2013).

The protoplast co-culture method for bioassay of allelopathy was developed to elucidate the underlying cellular mechanisms of allelopathy, at the cell wall formation and cell division stages of recipient lettuce protoplast growth, as well as to simulate future environmental risks of genetically modified plants (Sasamoto *et al*., 2013). Digital image analysis with the protoplast co-culture method (DIA-PP method), was further developed to examine the effects of allelochemicals on the yellow pigment accumulation, which is specific to recipient lettuce protoplast growth at the late stage of co-culture (Sasamoto *et al*., 2017a, b). Many plant species and allelochemicals have been examined using the DIA-PP method (Sasamoto *et al.*, 2022).

Leaf protoplasts of *in vitro* cultured plant of *Spiraea thunbergii* and *cis*- and *trans*- cinnamic acid were found to have high allelopathic activities by the DIA-PP method (Suzuki *et al*., 2021).

In this paper, using the DIA-PP method, we examined the allelopathic activities in green leaves of *in vitro* cultured plant of *Spiraea cantoniensis* and purple leaves of transgenic plant of *S. cantoniensis*, and identified cyanidin 3-glucoside as an allelochemical in the transgenic *S. cantoniensis*.

The difference in inhibition with the growth stages of recipient lettuce protoplasts was compared with the results obtained by the DIA-PP method in non-transgenic *Spiraea thunbergii* and *cis*- and *trans*- cinnamic acids (Suzuki *et al*., 2021), and another anthocyanin, cyanidin 3,5-di-glucoside (cyanin), which was evaluated as an allelochemical of red callus of a mangrove *Sonneratia ovata*, (Sasamoto *et al*., 2018), in comparison with that of carotenoid pigments, which showed strong inhibition at the yellow pigment accumulation stage (Sasamoto *et al*., 2021).

2. Methods

2.1 Materials

Small aseptic seedlings of *Spiraea cantoniensis* were cultured on 1/2 MS medium (Murashige & Skoog, 1962) containing 2% sucrose, 0.2% Gellan Gum, pH 5.8, and allowed to grow under a continuous light condition (60 μmoles $m^{-2}s^{-1}$) at 25°C before use. Over-expressed *Atpap 1* gene, which was a transcription factor of the anthocyanin biosynthetic pathway in *Arabidopsis*, was introduced by *Agrobacterium*-mediated transformation (Nakagawa & Suzuki, 2015).

Lactuca sativa (lettuce) seedlings were prepared as described previously (Sasamoto *et al*., 2013). Briefly, lettuce seeds (Great Lakes 366) in a small bag of Miracloth were washed with a neutral detergent and tap water, and then sterilized with 1.5% NaClO solution for 15 min and washed three times with autoclaved water. They were aseptically cultured on 5 ml of 0.8% agar medium in a 15 mL tube in a light condition (60 μ*E*) at 24°C for 6-8 days.

2.2 Protoplast Isolation and Purification

Optimal combination of cell wall degrading enzymes and osmotic conditions for protoplast isolation of leaves of *Spiraea cantoniensis* were determined from preliminary experiments, using 24 combinations of six kinds of enzymes, *i.e.*, Cellulase R10, Cellulase RS, Hemicellulase, Driselase 20, and Macerozyme R10, and Pectolyase Y-23, in 0.6 M to 0.8 M mannitol solution in a 24-well plastic culture plate (Sasamoto *et al*., 2017c, Sasamoto *et al*., 2019). Protoplasts of green leaves were isolated by the enzyme combination, 1% each of Cellulase R10, Hemicellulase, and Driselase 20 in 0.8 M mannitol. Protoplasts of purple leaves of transgenic *S. cantoniensis* were isolated by 1.8% each of the enzyme combination, Cellulase R10, Hemicellulase, Driselase 20 and Macerozyme R10 in 0.8 M mannitol solution. Protoplasts were passed through a 42 μm pore size nylon mesh and washed with 0.8 M mannitol solution three times by centrifugation at 1000 rpm (green leaves) or at 1500 rpm (purple leaves) for 5 min.

Protoplasts of lettuce cotyledons were isolated for 20-24 hrs in 1% each of Cellulase RS and Macerozyme R10 in 0.8 M mannitol solution in a 100 mL flask (Sasamoto *et al*., 2013). Protoplasts were passed through a 63 μm pore size mesh, and washed three times with 0.8 M mannitol solution by centrifugation at 900 rpm (100*g*) for 5 min.

2.3 Protoplast Culture of Lettuce with Spiraea cantoniensis or Cyanidin 3-glucoside

Fifty μL of liquid MS basal medium containing 1 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.1 μM benzyladenine (BA), 3% sucrose and 0.8 M mannitol were put in each well of a 96-well culture plate (Falcon No.3075). The medium pH was adjusted at pH5.8 before autoclaving at 120°C for 20 min. After addition of protoplasts, 100 μL of autoclaved pure water was added to the space between the wells and the plate was sealed with two layers of Parafilm^R. The protoplasts were cultured at 28°C in the dark in a humid incubator (CO_2-incubator without the supply of CO_2, APC-30DR, ASTEC Co. LTD.). All the experiments were repeated once.

2.3.1 Protoplast Co-culture of Lettuce with *Spiraea cantoniensis*

Five μL each of the suspension of *S. cantoniensis* and lettuce protoplasts at 10 times the final protoplast density in 0.8 M mannitol solution was added to each well. The final protoplast density was 6-100 $\times 10^3$ mL^{-1}.

After 5 days of co-culture, the non-spherically enlarged protoplasts of lettuce (which shows cell wall formation stage) were counted under an inverted microscope (Olympus CK40 or IX71). After 12 days of co-culture, the divided protoplasts and colonies were counted. The percentage of control values without *S. cantoniensis* protoplasts was calculated, and the average percentage of control at different densities of lettuce protoplasts (6-50 $\times 10^3$ mL^{-1}) with standard error (SE) was obtained at different densities of *S. cantoniensis* protoplasts. Yellow pigment accumulation of lettuce protoplasts was analyzed using digital image analysis described in *2.4*.

2.3.2 Protoplast Culture of Lettuce with Cyanidin 3-glucoside

Cyanidin 3-glucoside chloride (Tokiwa Phytochemical Co. Ltd.) was dissolved in filter-sterilized H_2O, and

diluted with the autoclaved medium. The media pH was measured using a micro-pH-electrode (pHBOY-P2, SHINDENGEN) before and after culture with lettuce protoplasts. Growth of lettuce protoplasts was determined at different concentrations (0.1 μM up to 1 mM) of cyanidin 3-glucoside as described in 2.3.1 and *2.4.*

2.4 Digital Image Analysis of 96 Well Culture Plate

After about one month of culture, yellow pigment accumulation in lettuce protoplasts (Figure A1) were subjected to image analysis as described previously (Sasamoto *et al*., 2017a, b). Digital image (jpg file) of the 96-well culture plate (Figure A2) was obtained using a scanner (Epson GTX-970). Image analysis was performed using software Image J (Rasband, 1997-2016). An image was selected from the blue channel. A horizontal straight line was drawn at the center of the wells. The plot profile of the line was analyzed. Using Excel software, we determined the averages of blue plot values for each well. The yellow value was converted by deduction of each averaged blue value from the highest blue value (control). The yellow value at each density of *Spiraea cantoniesis* protoplast or at each concentration of cyanidin 3-glucoside was subtracted as a control. The % yellow value of control without cyanidin-glucoside or *S. cantoniensis* protoplasts was calculated at each lettuce protoplast density. Finally, the percentages were averaged with standard errors at different protoplast densities of lettuce (6-100 $\times 10^3$ mL^{-1}).

3. Results and Discussion

3.1 Protoplast Isolation and Purification

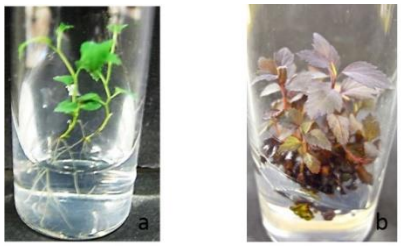

Figure 1. *In vitro* cultured plants of non-transgenic (a), and transgenic (b) plants of *Spiraea cantoniensis*

A high 0.8 M mannitol solution was needed for protoplast isolation from green leaves of non-transgenic *Spiraea cantoniensis* (Figure 1a), with three cell wall degrading enzymes, Cellulase R10, Hemicellulase and Driselase 20. Higher concentrations of three enzymes, and addition of Macerozyme R10 in 0.8 M mannitol was needed for protoplast isolation from purple leaves of transgenic *Spiraea cantoniensis* (Figure 1b).

Protoplasts of *Spiraea cantoniensis* (20-25 μm diameter) (Figure 2b, c) were smaller than those of lettuce (Figure 2a). A higher centrifugation speed than that used for lettuce was needed to purify protoplasts of green or purple leaves of *S. cantoniensis*. The protoplasts of purple leaves of *S. cantoniensis* showed a prominent red color (Figure 2c), but green protoplasts were also observed. The intensity of red color of protoplasts of purple leaves decreased during culture.

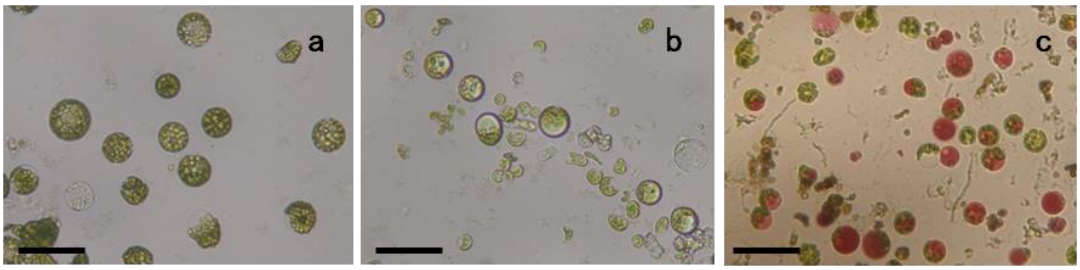

Figure 2. Photographs of protoplasts of lettuce and *in vitro* cultured plants of *Spiraea cantoniensis*

a): Green protoplasts of lettuce in the co-culture medium. b): Protoplasts of green leaves of non-transgenic *S. cantoniensis* in the co-culture medium. c): Protoplasts of purple leaves of transgenic *S. cantoniensis* in enzymatic solution. Bar= 50 μm.

3.2 Protoplast Co-culture of Spiraea cantoniensis with Lettuce

3.2.1 Effects of Protoplasts of Green Leaves of *Spiraea cantoniensis* on the Growth of Lettuce Protoplasts

As shown in Figure 3, non-spherical enlargement was slightly increased after 5 days of co-culture. After 12 days of co-culture, cell division of lettuce protoplasts was inhibited only 20% by green protoplasts of *S. cantoniensis* at up to 100 $\times 10^3$ mL^{-1}. Yellow pigment accumulation was not inhibited.

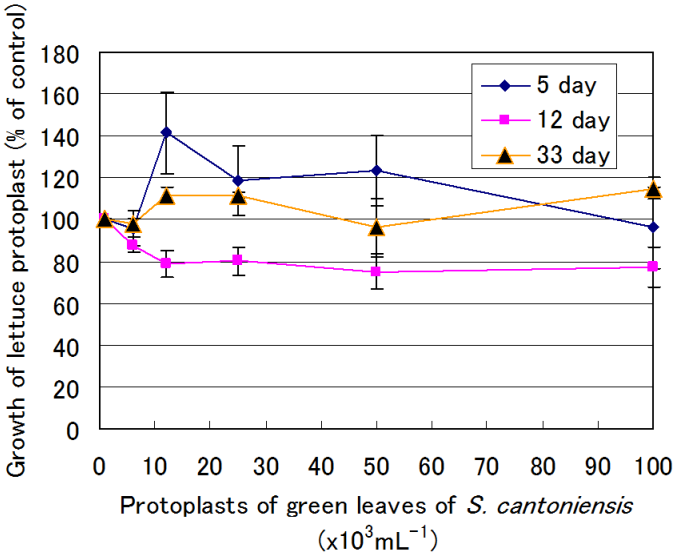

Figure 3. Effects of co-culture of green protoplasts of *Spiraea cantoniensis* at three stages of lettuce protoplast growth examined by the DIA-PP method. The medium used was MS basal medium containing 1 μM 2,4-D and 0.1 μM BA, 3% sucrose and 0.8 M mannitol. Growth % of control (non-spherically enlarged after 5 days, and divided lettuce protoplasts after 12 days and yellow pigment accumulation after 33 days of culture) was averaged at 6-100 $\times 10^3$ mL^{-1} of lettuce protoplasts with SE

3.2.2 Effects of Protoplasts of Purple Leaves of Transgenic *Spiraea cantoniensis* on the Growth of Lettuce Protoplast

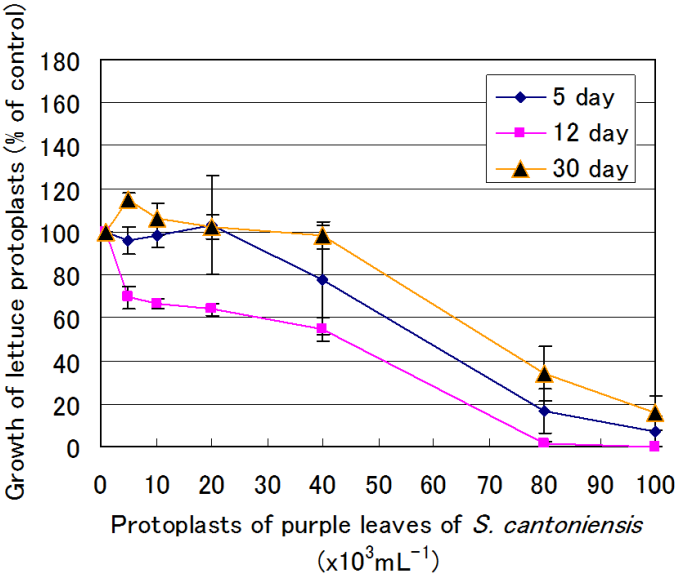

Figure 4. Effects of co-culture of protoplasts of purple leaves of transgenic *Spiraea cantoniensis* examined by the DIA-PP method. Growth % of control (non-spherically enlarged after 5 days, and divided lettuce protoplasts after 12 days and yellow pigment accumulation after 30 days of culture) was averaged at 6-100 $\times 10^3$ mL^{-1} of lettuce protoplasts with SE. Medium was MS basal medium containing 1 μM 2,4-D and 0.1 μM BA, 3% sucrose and 0.8 M mannitol

As shown in Figure 4, protoplasts of purple leaves of transgenic *Spiraea cantoniensis* at high protoplast densities inhibited the cell division of lettuce (100% by 80 $\times 10^3$ mL^{-1}) while those of green leaves of non-transgenic *S. cantoniensis* showed only 20% inhibitory activity (Figure 3). The purple leaf protoplasts showed strong inhibition like the green leaf protoplasts of *Spiraea thunbergii*, though the latter showed 90% inhibition at 40 $\times 10^3$ mL^{-1} (Suzuki et al., 2021). *Spiraea thunbergii* showed strong inhibitory allelopathic activity and *S. cantoniensis* showed weak activity in the lettuce seedlings growth test for *in vitro* bioassay of allelopathic activities (Takemura *et al*., 2013).

Inhibition was stronger at the cell division stage than at the cell wall formation stage, similar to that obtained with green leaf protoplasts of non-transgenic *Spiraea thunbergii*, but not to that obtained with its allelochemical, cinnamic acid (Suzuki et al., 2021).

At the yellow pigment accumulation stage, protoplast of transgenic *S. cantoniensis* at high densities (80 $\times 10^3$ mL^{-1}) clearly showed inhibition (70%) which was weaker than that of protoplasts of green leaves of non-transgenic *Spiraea thunbergii* which showed 90% inhibition at 80 $\times 10^3$ mL^{-1} (Suzuki et al., 2021).

3.3 Evaluation of Cyanidin glucoside as an Allelochemical of Transgenic Spiraea cantoniensis

3.3.1 Effects of Cyanidin 3-glucoside on the Three Growth Stages of Lettuce Protoplasts

As shown in Figure 5, the pattern of inhibition at 10 μM up to 1000 μM of cyanidin 3-glucoside was similar to that of purple leaf protoplasts of transgenic *S. cantoniensis* (Figure 4). At high concentrations, inhibition was stronger at the cell division stage than at the other growth stages of lettuce.

The inhibitory pattern of green leaf protoplasts of non-transgenic *Spiraea thunbergii* (Suzuki et al., 2021) was similar to that of transgenic *S. cantoniensis* as shown in Figure 3. Inhibition was strongest at the cell division stage. In contrast, putative allelochemical of *Spiraea thunbergii*, cinnamic acid, was not so, as discussed in *3.4* (Sasamoto *et al*., 2021).

The content of cyanidin-glucoside in the leaf extract of transgenic *Spiraea cantoniensis* (110 μg/mL from 50 mg fresh weight (Nakagawa & Suzuki, 2015) was calculated to be *ca*. 4 mM.

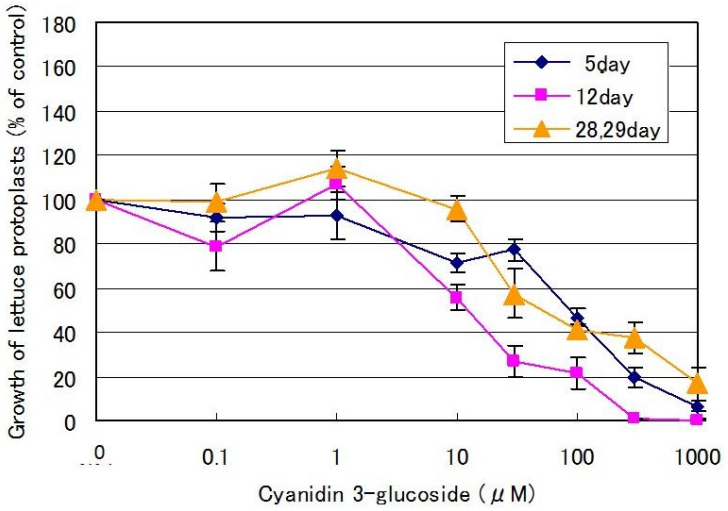

Figure 5. Effect of cyanidin 3-glucoside at three stages of lettuce protoplast growth examined by DIA-PP method. Medium was MS basal medium containing 1 μM 2,4-D and 0.1 μM BA, 3% sucrose and 0.8 M mannitol. Cell wall formation stage (day 5), cell division stage (day 12), and yellow pigment accumulation stage (day 28, 29)

3.3.2 Effect of Medium pH

After autoclaving, pH was 5.2, before co-culture. The pH was not lowered by the addition of 10 μM of cyanidin 3-glucoside chloride but pH 0.3 and 1.8 was lowered at 100 μM and 1 mM of cyanidin 3-glucoside chloride, respectively. The pH was increased to 4 after co-culture with lettuce protoplasts, in 1 mM cyanidin 3-glucoside. Though a lower pH was observed at high lettuce protoplast densities, pH was not lowered by changing the concentration of cyanidin-glucoside.

When the pH of the medium containing cyanidin 3-glucoside chloride was aseptically adjusted to 5.2-5.3 before

culture, a different pattern of inhibition was observed (Sasamoto *et al*., 2017c). In the pH-adjusted medium, protoplast division was stimulated at concentrations of up to 10 μM, but was inhibited at higher concentrations as it was in non-pH adjusted media (Figure 4).

Similar reduction of pH in the medium containing *cis*- and *trans*-cinnamic acids at high concentrations (0.3-0.5 at 100 μM, and 1.2-1.6 at 1 mM), was observed which were identified as allelochemicals of *Spiraea thunbergii* by the DIA-PP method. However, inhibitory patterns did not differ between pH-adjusted and non-pH adjusted medium experiments (Suzuki *et al*., 2021).

Such pH-adjusted medium is the same condition as that in the protoplast co-culture method using coumarin with *Prunus yedoensis* protoplasts, in which gradual inhibition of cell division of lettuce protoplasts depended on the concentration of coumarin (Fujise *et al*., 2018).

Reduction of the medium pH before culture is not necessarily the only cause of the inhibition of lettuce protoplast growth in the DIA-PP method.

3.4 Different Patterns of Inhibition among Three Growth Stages of Lettuce Protoplasts: Cell Wall Formation, Cell Division, and Yellow Pigment Accumulation

As shown in Figures 3 (non-transgenic), Figure 4 (transgenic), and Figure 5 (cyanidin 3-glucoside), inhibition % was highest at the cell division stage than at the cell wall formation and yellow pigment accumulation stages using the DIA-PP method of protoplasts of *Spiraea cantoniensis* and their putative allelochemical, cyanidin 3-glucoside.

Such inhibitory pattern, *i.e*., strongest inhibition at the cell division stage was observed repeatedly. For example, another anthocyanin, cyanidin 3,5-di-glucoside (cyanin), which was evaluated as an alleochemical in red purple callus of a mangrove plant, *Sonneratia ovata* was examined by the DIA-PP method. Inhibition at three growth stages by plant protoplasts was similar to the inhibition by putative allelochemicals, and inhibition at the cell division stage (60% by 100 μM cyanin) was the strongest, while yellow pigment accumulation was not inhibited (Sasamoto et al., 2018).

Allelopathic activity of a carotenoid (neoxanthin)-accumulating yellow callus of a mangrove plant, *Avicennia alba* was examined using the DIA-PP method (Sasamoto *et al*., 2020). Strong inhibition was observed at the cell division stage (100% inhibition by 100×10^3 mL^{-1}), slight stimulation was observed at the cell wall formation stage, and intermediate inhibition was observed at the yellow pigment stage. On the other hand, the inhibitory allelopathic activity of neoxanthin was very high at the yellow pigment accumulation stage (Sasamoto *et al*., 2021), which can be partly explained by the yellow pigment, a carotenoid, accumulating in lettuce protoplasts (Sasamoto et al., 2017a), which might be inhibited by the exogenously supplied carotenoid.

Inhibitory patterns examined using the DIA-PP method were different in green leaves of non-transgenic *Spiraea thunbergii*, and the putative allelochemical, *cis*- and *trans*- cinnamic acid. Inhibition by cinnamic acid was the strongest at the cell wall formation stage, though inhibition by protoplasts of leaf and yellow suspension cultured cells of *Spiraea thunbergii* was strongest at the cell division stage. The stimulation by the suspension cell protoplasts was strong at the cell wall formation stage, but was not observed with green leaves. (Suzuki et al., 2021).

Yellow suspension cells of *Spiraea thunbergii* were obtained in the dark from leaves with high concentrations of 2,4-D and a strong cytokinin, thidiazuron (Suzuki et al., 2021). Similarly, in *S. cantoniensis*, high concentrations of a cytokinin, BA promoted the proliferation of callus and yellow suspension culture in the dark (Kubota *et al*., 2009; Sasamoto *et al*., in preparation). Identification of the yellow substance(s) in suspension cultured cells of two *Spiraea* species and application of DIA-PP method might clarify different factor(s) for different inhibitory or stimulatory patterns of allelopathic activities. A single allelochemical might not be the only cause of strong allelopathic activities of plant cells and of tissues and whole plants regenerated.

Different patterns of inhibition or stimulation at three growth stages of lettuce protoplasts, *i.e.*, cell wall formation, cell division, and yellow pigment accumulation stages, might reflect not only different allelochemicals but also the difference in the metabolic action site of each allelochemical (Sasamoto & Ashihara, 2014; Sasamoto *et al*., 2015; Ogita & Sasamoto, 2018; Ogita *et al*., 2020).

4. Conclusion

Protoplasts obtained from purple leaves of *in vitro* cultured plants of transgenic *Spiraea cantoniensis*, showed strong inhibitory allelopathic activity (100% inhibition of recipient lettuce protoplast division by 80×10^3 mL^{-1}) when examined with protoplasts by the DIA-PP method, while only 20% inhibition was obtained with green

leaves of non-transgenic *S. cantoniensis.* Inhibition was the strongest at the cell division stage among the three growth stages of lettuce protoplasts, *i.e.*, cell wall formation stage, cell division stage, and yellow pigment accumulation stage.

An anthocyanin, cyanidin 3-glucoside at 100 μM, inhibited lettuce protoplast division 80% and was evaluated as an allelochemical of transgenic *Spiraea cantoniensis*, which has a high cyanidin-glucoside content. Inhibition at the cell division stage was stronger than that at the cell wall formation and yellow pigment accumulation stages.

These results were comparable to those obtained with green leaves of non-transgenic *Spiraea thunbergii*, and also with different allelopathic plants containing different pigment-allelochemicals, *e.g.*, anthocyanin and carotenoid, examined using the DIA-PP method.

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Appendix

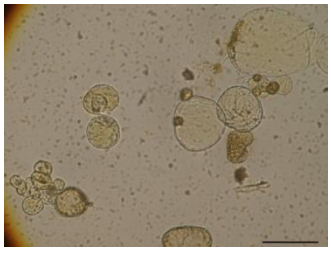

Figure A1. Lettuce protoplasts cultured for 26 days at density of 12 $\times 10^3$ mL^{-1}. Yellow spherically-enlarged, yellow non-spherically-enlarged, and yellow divided cells, and dead cells were observed. Bar=100 μm

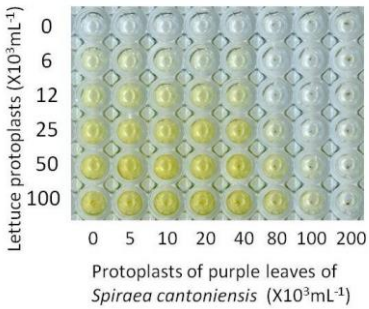

Figure A2. A scanned image of yellow pigment accumulation in protoplast co-culture of lettuce with purple leaves of *Spiraea cantoniensis* after 30 days of culture