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Strong Allelopathic Activities of Leaves and Cultured Cells of *Spiraea thunbergii* Assayed by the Protoplast Co-Culture Method with Digital Image Analysis: Evaluation of *Cis*- and *Trans*-Cinnamic Acid as Allelochemicals

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Abstract

The inhibitory allelopathic activities of leaves and leaf-origin suspension cultured cells of *Spiraea thunbergii*, and putative allelochemicals, *cis*- and *trans*-cinnamic acid, were investigated by the protoplast co-culture method with digital image analysis (DIA-PP method) using lettuce as a recipient. The optimal conditions of 2,4-dichlorophenoxyacetic acid (2,4-D) and benzyladenine (BA) for the cell division of *S. thunbergii* protoplasts were first examined using 50 μL liquid MS basal medium containing 3% sucrose, and 0.8 M mannitol in a 96-well culture plate. The hormonal condition for co-culture, 1 μM 2,4-D plus 0.1 μM BA, which was optimal for lettuce protoplast growth, was sub-optimal for *S. thunbergii* protoplasts. Effects of co-culture on the three stages of lettuce protoplast growth, *i.e.*, cell wall formation, cell division, and yellow pigment accumulation, were examined. Protoplasts of leaf and suspension cells of *S. thunbergii* strongly inhibited lettuce protoplast growth at the cell division stage (100% inhibition at 80 - 100 × 10^3 mL^{-1}), but not so much at the other two stages. Both *cis*- and *trans*-cinnamic acid, showed the strongest inhibition at the cell wall formation stage, and 100% inhibition at the cell division stage at 100 μM. These results were compared with those obtained in a lettuce seedling growth test, using different allelopathic plants, and their allelochemicals were studied by the DIA-PP method.

Keywords

Allelopathy, Cinnamic Acid, Protoplast Co-Culture, *Spiraea thunbergii*

1. Introduction

As a strategy for survival, plants release allelochemicals into the surrounding environment thereby inhibiting the growth of neighboring plants that share the same habitat. This is called allelopathy. The broad definition of allelopathy includes effects of stimulation, insects, microorganisms and animals [1] [2]. Allelopathic plants could become a natural herbicide for weed control in agriculture [3].

Spiraea thunbergii, Sieb. ex. Blume (Rosaceae) is a white flowering shrub, that grows on the riverside. In horticulture, the inter-specific hybrid between *S. japonica* [4] has been made and anthocyanin-synthesizing genes introduced by *in vitro* culture to change the flower-color in *S. thunbergii* and *S. cantoniensis* [5] [6]. On the other hand, the second strongest inhibitory allelopathic activity of *S. thunbergii* and less inhibitory activity of *S. cantoniensis* have been observed in 168 plant species from 68 families tested using a lettuce seedling growth test [3] [7]. Leaf litters of *S. thunbergii* showed strong inhibitory allelopathic activity on cyanobacteria *Microcystis*, but not on green algae, in an algal growth test [8] [9].

The protoplast co-culture method for bioassay of allelopathy was developed to elucidate the underlying cellular mechanisms, and to simulate future environmental risks. Effects on cell division were examined during co-culture of test plant protoplasts with protoplasts of different recipient plant species, such as lettuce and rice [10]. Similar inhibitory allelopathic activities have been obtained by the protoplast co-culture method and the seedling growth test (the sandwich method [11]), using lettuce as a recipient, *e.g.*, *Leucaena leucocephala*, and *Mucuna gigantea* [12]; *Sonneratia* mangrove trees [13] and *Derris indica* [14]; *Prunus* flower trees [15].

Recently, digital image analysis with the protoplast co-culture method (DIA-PP method), was used to examine the effects on the yellow pigment accumulation, specific to recipient lettuce protoplast at the late stage of co-culture [16] [17]. 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, may reflect the difference in the action site of each allelochemical. The numbers of tested plants and putative allelochemicals found by the DIA-PP method have increased [17]-[25].

The water-soluble volatile compound tulipalin A, was found in leaves of *Spiraea thunbergii*, and the strong inhibitory allelopathic activity of tulipalin A was assayed against lettuce seedling growth test [26], and compared with the DIA-PP method [19].

Hiradate *et al.* [27] [28] also found *cis*-cinnamoyl glucosides as allelochemicals in leaves of *S. thunbergii*, and found that the essential chemical structure, *cis*-cinnamic acid, had more than 100 times stronger inhibitory allelopathic activity than *trans*-cinnamic acid against seedling growth test of lettuce and four other recipient plant species. While a synthetic auxin, 2,4-dichlorophenoxyacetic acid (2,4-D) had strong inhibitory activity and the growth retardant phytohor-

mone, *cis*-abscisic acid had weaker inhibitory activity than *cis*-cinnamic acid. Inhibitory effects of *cis*-cinnamic acid on the root growth and auxin-related genes have been investigated in *Arabidopsis thaliana* [29].

We examined the effects of plant hormones, 2,4-D and benzyladenine (BA) on the protoplast divisions in the leaves of aseptic cultured seedlings, and leaf-origin suspension culture of *S. thunbergii*, using 50 µl liquid medium in a 96-well culture plate. Optimal hormonal conditions for the protoplast culture of test plants were compared with those for protoplast co-culture, *i.e.*, 2,4-D 1 µM and BA 0.1 µM, which was optimal for lettuce protoplast growth [10].

The DIA-PP method with leaf and suspension cells of *S. thunbergii* was used to determine the effective protoplast densities at the three growth stages of recipient lettuce protoplasts. Effects of putative allelochemicals, *cis*- and *trans*-cinnamic acid, were also examined. The results obtained by the seedling growth test and the DIA-PP method using different allelopathic plants containing different putative allelochemicals were analyzed.

2. Materials and Methods

2.1. Materials

Small aseptic seedlings of *Spiraea thunbergii* were obtained as described previously [30]. They were cultured on 1/2 MS (Murashige and Skoog [31]) medium containing 2% sucrose, 0.2% Gellan Gum, pH 5.8, and allowed to grow under a continuous light condition (60 $\mu moles \cdot m^{-2} \cdot s^{-1}$) at 25˚C before use. Leaves were used directly for protoplast isolation or for the induction of suspension cultured cells. Suspension cultured cells were induced in 1 mL of MS basal medium containing 3% sucrose, 10 µM each of 2,4-dichlorophenoxyacetic acid (2,4-D) and thidiazuron in a 10-mL flat-bottomed culture tube covered with translucent film. The hormonal condition was selected from 20 combinations of concentrations of 2,4-D (0, 0.1, 1, 10, and 100 µM) and thidiazuron (0, 0.1, 1, and 10 µM) [32] [33]. Proliferated cells were observed under an inverted microscope (Olympus CK40) and sub-cultured in 10 mL medium in a 100 mL flask at 100 rpm speed, at 27˚C in the dark for 9 months. Three-week-old cells were used for protoplast isolation.

Lactuca sativa (lettuce) seeds "Great Lakes 366" were wrapped in a Miracloth bag (CALBIOCHEM, Cat; 475855), washed with a neutral detergent and tap water, and sterilized with 1.5% NaClO solution for 15 min and then washed with autoclaved water three times. Seeds were cultured on 0.8% agar medium and allowed to grow under a continuous light condition at 25˚C for 6 - 7 days.

2.2. Protoplast Isolation

The best combination of 6 cell wall degrading enzymes was selected using Cellulase R10, RS, Hemicellulase, Driselase 20, Macerozyme R10 at 1%, and Pectolyase Y-23 at 0.25% in a 24-well culture plate [23]; and the optimal osmotic condition was surveyed using mannitol at 0.4, 0.6 and 0.8 M.

Leaf protoplasts of *S. thunbergii* were isolated using 1% each of Cellulase R10, Driselase 20, and Macerozyme R10 in 0.8 M mannitol for 25 hrs. Separate wells method in a 24-well culture plate was used to reduce the formation of inhibitory substances [10] [23]. After filtration through a 42 μm pore size nylon mesh, washed three times with mannitol solution by centrifugation at 1200 rpm for 5 min.

Protoplasts of suspension cultured cells of *S. thunbergii* were isolated using 1% each of Cellulase RS and Driselase 20 in 0.8 M mannitol, at 70 rpm for 21 hrs. After filtration through 63 μm mesh, washed with mannitol solution three times by centrifugation at 1000 rpm for 5 min.

Protoplasts of lettuce cotyledons were isolated with 1% each of Cellulase RS and Macerozyme R10 in 0.8 M mannitol solution in the dark for 20 - 24 hrs. After filtration through 63 or 80 μm mesh, they were washed three times with osmotic solution by centrifugation at 800 rpm (100 g) for 5 min [10].

2.3. Effects of Plant Hormones in Protoplast Culture of *Spiraea thunbergii*

The combinations of 2,4-D (0, 0.1, 1, 10, 100 μM) and benzyladenine (BA) (0, 0.1, 1, 10 μM) were tested using the MS basal medium containing 3% sucrose and 0.8 M mannitol in 50 μl of liquid medium. Protoplast densities tested were 10 - 80 × 10^3 mL^{-1} (leaf) and 12 - 150 × 10^3 mL^{-1} (suspension cells), using the inside 60 wells of a 96-well plastic culture plate (Falcon No. 3072). 100 μL of autoclaved pure water was added in between the wells and the plate was tightly sealed with two layers of Parafilm^R. Cultures were kept in the dark at 28˚C in a humid incubator (CO_2-incubator without supply of CO_2 gas, APC-30DR, ASTEC Co. Ltd.). Numbers of non-spherically enlarged and divided protoplasts were counted under an inverted microscope. Average plating efficiency (% of reacted protoplasts per total protoplasts plated in a well, *e.g.*, 500 at 10 × 10^3 mL^{-1}, 7500 at 150 × 10^3 mL^{-1}) was calculated.

2.4. Protoplast Co-Culture with Lettuce

As described previously [10], 5 μL of each protoplast suspension in mannitol solution in ten times concentration of 6 to 150 × 10^3 mL^{-1} was put into 50 μl of liquid medium. The medium was MS basal medium containing 1 μM of 2,4-D, 0.1 μM of BA, 3% sucrose and 0.8 M mannitol. The pH was adjusted to 5.8 - 5.9 before autoclaving at 121˚C for 20 min. *cis*-Cinnamic acid, which was artificially synthesized according to the method of Abe *et al.* [34], and *trans*-cinnamic acid (Fujifilm Wako Pure Chemical) was dissolved in ethanol and diluted in the co-culture medium with or without pH adjustment at ca. 6 before filter sterilization (Milipore GV4). The final ethanol concentration was less than 5% in the test medium.

As described previously [35], under an inverted microscope, numbers of non-spherically enlarged protoplasts (cell wall formation) (E) and numbers of divided protoplasts (D) of lettuce were counted after 4 - 5 days of culture: 93% -

98% were (E) at the zero control. The numbers of (D) and colonies composed of more than four cells (C) of lettuce were counted after 10 to 12 days of culture. The percentage of control with neither test plant protoplasts nor cinnamic acid was calculated at each lettuce protoplast density, and the percentages for the control were averaged with standard error (SE) at different protoplast densities of lettuce (6×10^3 mL^{-1} to 10^5 mL^{-1}).

2.5. Digital Image Analysis

Image analysis of yellow pigment accumulation of lettuce protoplasts was performed as described previously [16] [17]. Digital image of a 96-well culture plate was captured using a scanner (Epson GTX-970) after about one month of co-culture. The image was analyzed using Image J software [36]. An image of the 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 then analyzed. The data of the blue plot values were recorded using Excel software, and the average of "blue plot values" was determined for each well. The yellow value was converted by deduction of each averaged blue value from the highest blue value of the control well with neither lettuce nor cinnamic acid. The yellow values at each density of *Spiraea* protoplasts or at each concentration of cinnamic acid were subtracted from yellow values of lettuce-containing wells. The percentage of the yellow value to the control value with neither *Spiraea* protoplasts nor cinnamic acid was calculated at each lettuce protoplast density. Finally, the percentages of control with neither test plant protoplasts nor cinnamic acid were averaged with SE at different densities of lettuce (6×10^3 mL^{-1} to 10^5 mL^{-1}).

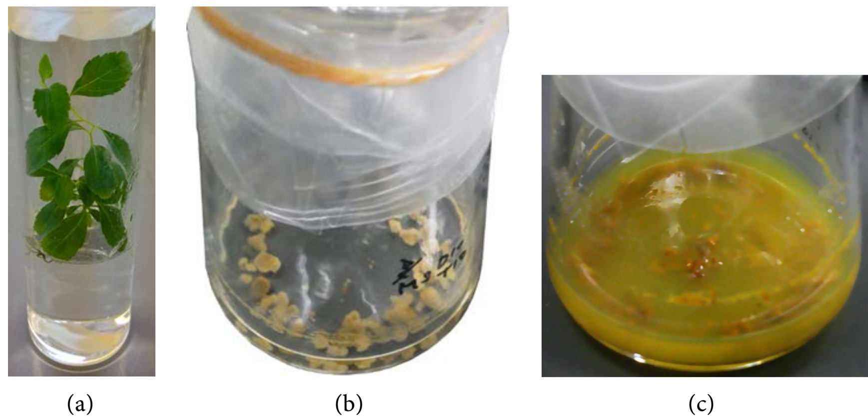
2.6. HPLC Analysis of *Cis*- and *Trans*-Cinnamic Acid

Cis- and *trans*-Cinnamic acid were determined by Shimadzu HPLC system (Kyoto, Japan); pump (LC-20AD) and photodiode array detector (SPD-M20A). Reversed-phase column; CLC-ODS (M), 4.6 mm i.d., 250 mm length. Oven temperature; 40˚C. Flow rate; 0.6 ml/min, isocratic mode, MeOH/2% acetic acid; 6:4 elution system for 20 min. UV spectra; 254 nm. RT were 9 min (*cis*) and 11 min (*trans*).

3. Results

3.1. Protoplast Cultures of *Spiraea thunbergii*

Figure 1 shows the results for the aseptic culture of *Spiraea thunbergii*. Fresh green leaves (**Figure 1(a)**) were used for protoplast isolation. Leaf-derived suspension culture of *S. thunbergii* was induced and sub-cultured in the MS basal medium containing 10 μM each of 2,4-D and thidiazuron and 3% sucrose in a 100 mL flask. Three-week-old, dim yellow suspension cultured cells (**Figure 1(b)**) were used for protoplast isolation. After 1 - 2 months, the sub-cultured suspension culture was dark yellow to orange and precipitates were observed in the culture medium (**Figure 1(c)**).



(a) (b) (c)

Figure 1. Aseptic seedling (a) and leaf-derived suspension culture ((b), (c)) of *Spiraea thunbergii*.

3.1.1. Leaf Protoplast Culture of *S. thunbergii*

Diameter of the isolated leaf protoplasts of *S. thunbergii* was about 20 μm (**Figure 2(a)**). A high mannitol concentration (0.8 M) was selected as the optimal osmoticum. Effects of the concentrations of 2,4-D and BA on the growth of leaf protoplasts of *S. thunbergii* were examined (**Figure 3**). The numbers of non-spherically enlarged protoplasts and divided protoplasts (**Figure 2(b)**) were counted after 13 days of culture. Reaction was observed at a wide range of concentrations of 2,4-D (0 - 10 μM) and BA (0 - 100 μM). The optimal condition was 1 μM 2,4-D without BA. At the optimal condition of 2,4-D (1 μM), 100 μM BA was still effective. The presence of 2,4-D 1 μM and BA 0.1 μM, which was optimal for lettuce protoplast growth [10], was sub-optimal condition for leaf protoplast culture of *S. thunbergii*.

3.1.2. Protoplast Culture of Suspension Cultured Cells of *S. thunbergii*

Diameter of the isolated protoplasts of *S. thunbergii* suspension cells was about 20 μm (**Figure 4(a)**). The high concentration of mannitol (0.8 M) was the same optimal osmoticum as that in leaf protoplasts.

Effects of concentrations of 2,4-D and BA on protoplast growth of *S. thunbergii* were examined (**Figure 5**). The numbers of non-spherically enlarged protoplasts and divided protoplasts (**Figure 4(b)** and **Figure 4(c)**) were counted after 10 days of culture. Averages of plating efficiency (%) at densities of 50, 100, 150 $\times 10^3$ mL^{-1} were calculated. The optimal condition was 10 μM 2,4-D and BA 0.1 μM. Although the original suspension culture was obtained and sub-cultured with 10 μM each of 2,4-D and thidiazuron, a strong cytokinin, their protoplast divisions occurred at lower concentrations of a weak cytokinin, BA. The presence of 2,4-D 1 μM and BA 0.1 μM, which was optimal for lettuce protoplast growth [10], was sub-optimal for protoplasts of suspension cultured cells of *S. thunbergii*.

A higher 2,4-D (10 μM) condition with BA at 0.1 μM was optimal for suspension cell protoplasts of *S. thunbergii* (**Figure 5**) while the optimal hormonal condition for leaf protoplasts was 0.1-1 μM 2,4-D and without BA (**Figure 3**).

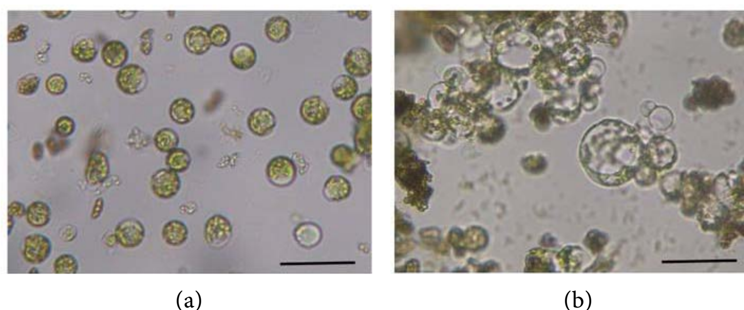


Figure 2. Isolated leaf protoplasts of *Spiraea thunbergii* (a), and after 11 days of culture (b). Medium in (b) was MS basal medium containing 1 μM 2,4-D and 0.1 μM BA, 3% sucrose and 0.8 M mannitol. Protoplast density was 80×10^3 mL^{-1}. Bar = 50 μm

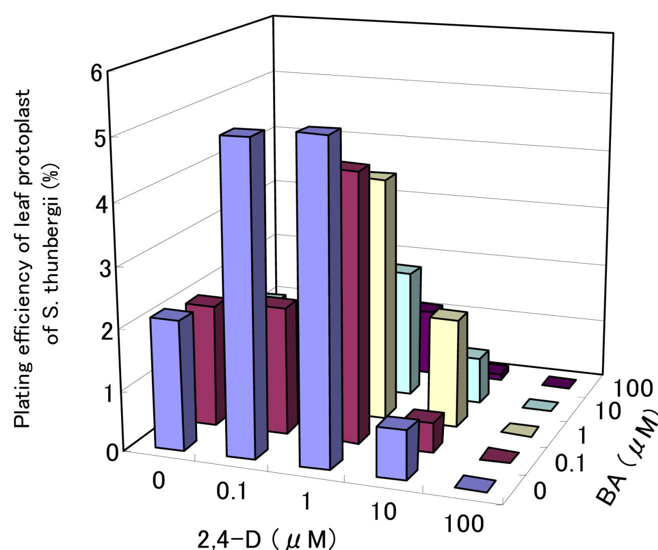


Figure 3. Effects of 2,4-D and BA on the growth (the plating efficiency) of leaf protoplasts of *Spiraea thunbergii* after 13 days of culture. Basal medium was MS medium containing 3% sucrose and 0.8 M mannitol. Values at densities of 10 - 80×10^3 mL^{-1} were averaged.

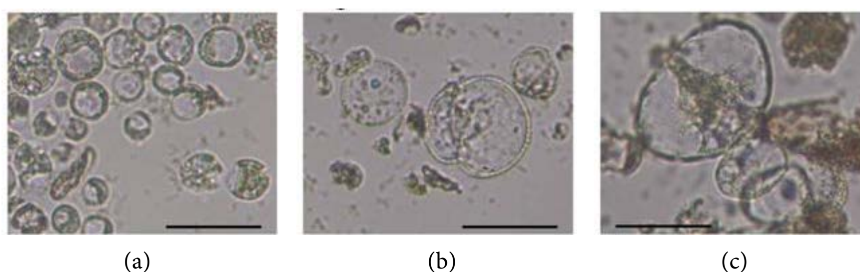


Figure 4. Protoplast culture of suspension cultured cells of *Spiraea thunbergii* at day 0 (a) and at day 5 ((b), (c)). Medium was MS basal medium containing 1 μM 2,4-D, 3% sucrose, 0.8 M mannitol and 0.1 μM BA ((a), (b)) or 1 μM BA (c). Protoplast density was 100 - 150×10^3 mL^{-1}. Bar = 50 μm.

3.2. Protoplast Co-Culture of *Spiraea thunbergii* with Lettuce Protoplasts

Diameter of green cotyledon protoplasts of lettuce is ca.30 μm, and under an

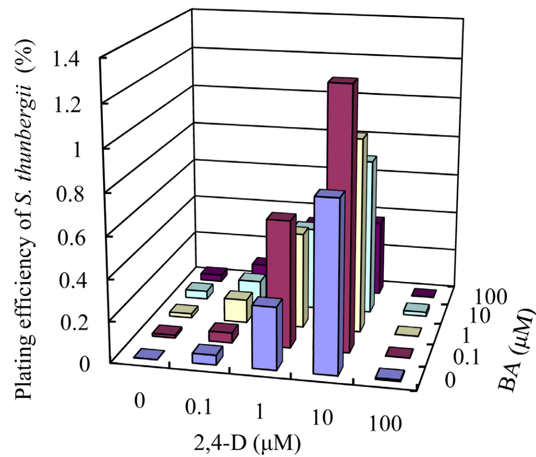


Figure 5. Effects of 2,4-D and BA on the protoplast growth (the plating efficiency) of suspension cultured cells of *Spiraea thunbergii* after 10 days of culture. Basal medium was MS medium containing 3% sucrose and 0.8 M mannitol. Values at densities of 50 - 150 × 10^3 mL^{-1} were averaged.

inverted microscope, lettuce protoplast growth, *i.e.* non-spherical enlargement (cell wall formation) and divisions [35] [37], can be distinguished from the protoplast growth of leaf (**Figure 2**) and that of suspension cultured cells (**Figure 4**) of *S. thunbergii*. Yellow pigment accumulation is specific to lettuce protoplast growth [16] [17].

3.2.1. Protoplast Co-Culture of Leaf Protoplasts of *S. thunbergii* with Lettuce

As shown in **Figure 6**, protoplast division of lettuce protoplasts was inhibited by leaf protoplasts of *S. thunbergii* after 11 days of co-culture with 100% inhibition observed at 80 × 10^3 mL^{-1} of *S. thunbergii* and *ca.*50% inhibition at 15 × 10^3 mL^{-1}.

Less inhibition than at cell division stage was observed at cell wall formation stage after 5 days of co-culture. No stimulation at the cell wall formation stage was observed, even at low protoplast densities of *S. thunbergii*. Inhibition rate of yellow pigment accumulation of lettuce protoplasts after one month of co-culture was in between of those at the cell wall formation stage and at the cell division stage.

3.2.2. Protoplast Co-Culture of Suspension Cell Protoplasts of *S. thunbergii* with Lettuce

As shown in **Figure 7**, protoplast division of lettuce protoplasts was inhibited by suspension cell-protoplasts of *S. thunbergii* after 10 days of co-culture with 100% inhibition observed at 100 × 10^3 mL^{-1} of *S. thunbergii* and ca. 50% inhibition at 25 × 10^3 mL^{-1}.

At the cell wall formation stage, stimulation (40% - 140%) was observed at low protoplast densities (5 - 25 × 10^3 mL^{-1}) of *S. thunbergii*. Less inhibition than on cell division was observed at higher densities of *S. thunbergii* (50 - 100 × 10^3 mL^{-1}).

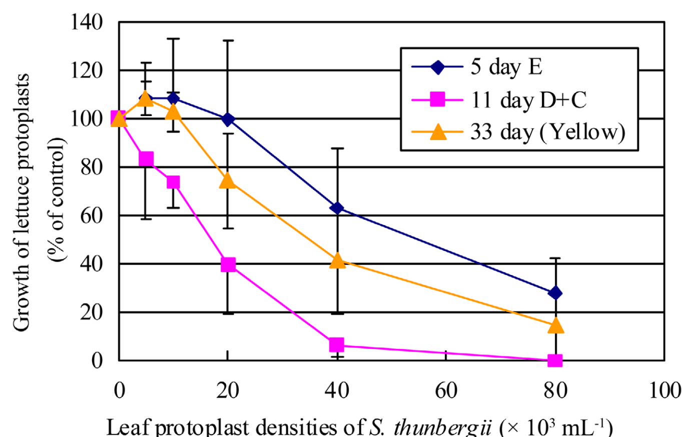


Figure 6. Effects of leaf protoplasts of *Spiraea thunbergii* on lettuce protoplast growth (cell wall formation stage at day 5, cell division stage at day 11, and yellow pigment accumulation stage at day 33). Medium was MS basal medium containing 1 μM 2,4-D, 0.1 μM BA, 3% sucrose and 0.8 M mannitol. At day 11, 87% was division (D) at the zero control.

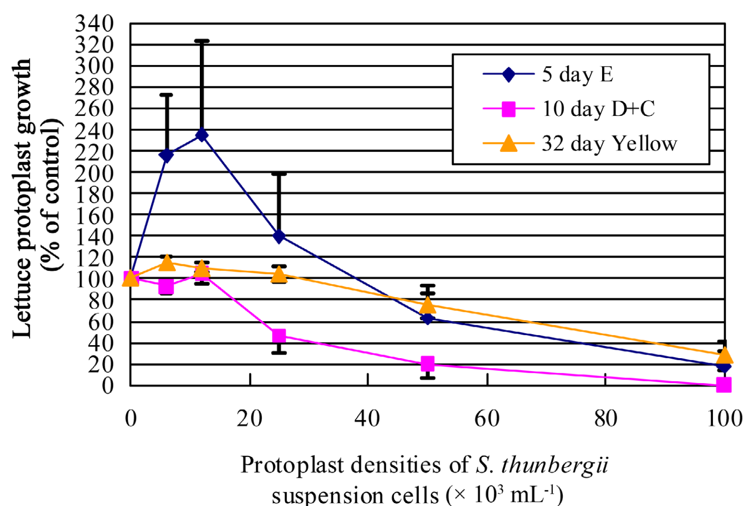


Figure 7. Effects of suspension cell protoplasts of *Spiraea thunbergii* on lettuce protoplast growth (cell wall formation stage at day 5, cell division stage at day 10, and yellow pigment accumulation stage at day 32). Medium was MS basal medium containing 1 μM 2,4-D, 0.1 μM BA, 3% sucrose and 0.8 M mannitol. Medium was the same as of Figure 6. At day 10, 70% was division (D) at the zero control.

At the yellow pigment accumulation stage, inhibition was observed at higher densities of *S. thunbergii* (50 - 100 × 10^3 mL^{-1}). Neither stimulation nor inhibition was observed at low protoplast densities of *S. thunbergii* up to 25 × 10^3 mL^{-1}.

3.3. Effects of *Cis*- and *Trans*-Cinnamic Acid on Lettuce Protoplast Culture

As shown in Figure 8 and Figure 9, no significant difference was found between *cis*- and *trans*-cinnamic acid. At 100 μM, both *cis*- and *trans*-cinnamic acid

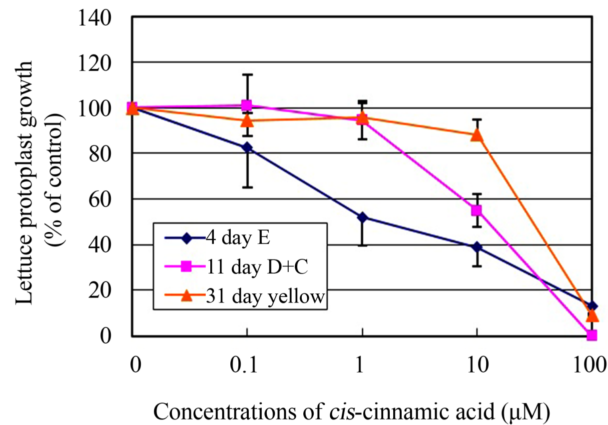


Figure 8. Effects of *cis*-cinnamic acid on lettuce protoplast growth (cell wall formation stage at day 4, cell division stage at day 11, and yellow pigment accumulation stage at day 31). Medium was MS basal medium containing 1 μM 2,4-D, 0.1 μM BA, 3% sucrose and 0.8 M mannitol. At day 11, 66% was division (D) at the zero control.

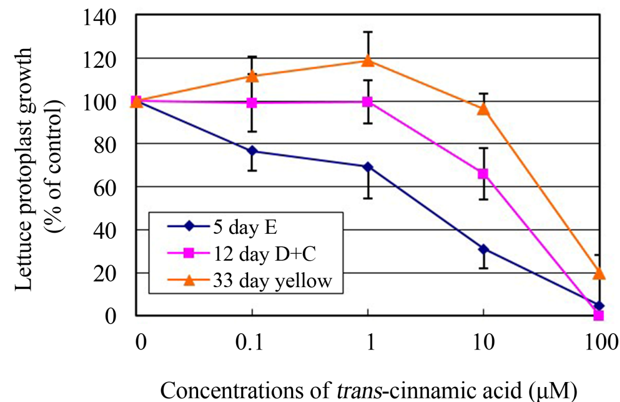


Figure 9. Effects of *trans*-cinnamic acid on lettuce protoplast growth (cell wall formation stage at day 5, cell division stage at day 12, and yellow pigment accumulation stage at day 33). Medium was MS basal medium containing 1 μM 2,4-D, 0.1 μM BA, 3% sucrose and 0.8 M mannitol. At day 12, 82% was division (D) at the zero control.

showed 100% inhibition at the cell division stage of lettuce protoplast growth, but no inhibition was observed at up to 1 μM.

At the cell wall formation stage, *cis*- and *trans*-cinnamic acid both showed inhibitory activity depending on the concentration (0.1 - 100 μM): 50% inhibition was observed at 1 - 3 μM.

At the yellow pigment accumulation stage, no inhibition was observed at up to 10 μM. Both *cis*- and *trans*-cinnamic acid showed strong inhibitory activity at 100 μM.

In Figure 8 and Figure 9, medium pH was adjusted before filtration sterilization. Without pH adjustment before culture, pH values were 0.3 - 0.5 lower in 100 μM medium and 1.2 - 1.6 lower in 1 mM medium. However, in the co-culture experiments, no significant difference was found at up to 100 μM between the pH adjusted medium and non-adjusted medium (data not shown).

4. Discussion

4.1. Effects of *Spiraea thunbergii* by the Protoplast Co-Culture Method (At Cell Division Stage) and Seedling Growth Test

4.1.1. Effects of Co-Cultured Protoplasts of *S. thunbergii*

At the cell division stage of recipient lettuce protoplasts, both leaf protoplasts (**Figure 6**) and suspension cell protoplasts (**Figure 7**) of *S. thunbergii* showed inhibitory allelopathic activities (**3.2**). In the plant group with strong allelopathic activity, *e.g. Arabidopsis thaliana* leaf [17], calluses of a mangrove tree, *Sonneratia ovata* [22], and *Coffea canephora* [21], 100% inhibition was obtained at 80 - 100 × 10^3 mL^{-1}.

Calluses or suspension cultured cells of *Leucaena leucocephala* and *Mucuna gigantea* [12], a mangrove tree *Derris indica* [14], *Prunus yedoensis* flower tree [15], *Vicia villosa* epicotyl [23] and Kudzu cotyledon [18] showed stronger inhibitory activity with 100% inhibition obtained at 10 - 50 × 10^3 mL^{-1}. *L. leucocephala* and Kudzu are well known invader plants.

4.1.2. Protoplast Co-Culture Method *vs.* Seedling Growth Test

Strong inhibitory allelopathic activity of *Spiraea thunbergii* has also been obtained using the lettuce seedling growth test [3]. In all plant species tested using the protoplast co-culture method described in (**4.1.1**), inhibitory allelopathic activities have been obtained using the lettuce seedlings growth test, *e.g.*, the sandwich method or the plant box method [38]. The extent of inhibitory activity obtained by the protoplast co-culture method was not always the same as that obtained in the seedling growth test. For example, in Kudzu, only moderate inhibitory activity was obtained in the seedling growth test, but strong inhibitory allelopathic activity was obtained by the protoplast co-culture method [18].

By contrast, leaf protoplasts of poplar (200%) and birch (60%) strongly stimulated growth of co-cultured lettuce protoplasts at the cell division stage, but only moderately inhibited growth in the seedling growth test (the sandwich method). These phenomena were explained by the effects of an allelochemical, abscisic acid (ABA) [39] (**4.2.2**).

4.2. Activity of Putative Allelochemicals by Protoplast Co-Culture Method (At Cell Division Stage) *vs.* Seedling Growth Test

4.2.1. Inhibition by Cinnamic Acid and Tulipalin A

Using the lettuce seedling growth test, *cis*-cinnamic acid was evaluated as an allelochemical of *S. thunbergii* which has strong inhibitory allelopathic activity [28]. Strong allelopathic activity, *i.e.*, 100% inhibition by *cis*-cinnamic acid at 100 μM was also observed at the cell division stage by the protoplast co-culture method (**Figure 8**). However, *cis*-cinnamic acid showed more than 100 times stronger inhibitory activity than *trans*-cinnamic acid in the lettuce seedling growth test [28], which was not observed by the protoplast co-culture method in the present study. No significant difference in effect was observed between *cis*- and *trans*-cinnamic acid at the cell division stage (**Figure 8** and **Figure 9**).

Reported effects of *cis*- and *trans*-cinnamic acid varied with the organ in different recipient plant species, *e.g.*, *cis*-cinnamic acid inhibited root growth [29] but stimulated leaf growth [40] in *Arabidopsis*; *trans*-cinnamic acid stimulated leaf expansion [41] but inhibited root growth [42] in cucumber and inhibited leaf growth in maize [43]. Such variation might partly be caused by the light conditions (by UV [40]) used in the experiments. In the present study, we cultured protoplasts in the dark under which condition, efficient conversion of *trans*- to *cis*-cinnamic acid by UV was not confirmed by HPLC analysis (data not shown).

In the study using the protoplast co-culture method, tulipalin A (100% inhibition at 100 μM), which is another allelochemical of *S. thunbergii* leaves, showed stronger inhibitory activity at the cell division stage than another volatile allelochemical, safranal, of *Crocus sativus*. However, tulipalin A had weaker inhibitory activity than safranal on the growth of hypocotyls and roots in the lettuce seedling growth test [19].

4.2.2. Effects of ABA Obtained by Seedling Growth Test and Protoplast Co-Culture Method

A plant hormone, ABA was inhibitory in the lettuce seedling growth test [28]. However, ABA was stimulatory at 0.1 - 10 μM while the antagonistic plant hormone, gibberellic acid, was inhibitory on lettuce protoplast growth [10]. ABA and its contents were discussed as an allelochemical with stimulatory and less inhibitory activities in leaf protoplasts of poplar and birch in studies using the protoplast co-culture method (**4.1.2**) [39].

Therefore, inhibitory and stimulatory effects of allelochemicals obtained by the protoplast co-culture method (at cell division stage) are not always the same as those obtained by the seedling growth test using the same recipient plant, lettuce.

4.3. DIA-PP Method of *Spiraea thunbergii*

4.3.1. Effects at the Cell Wall Formation Stage of Lettuce Protoplast Growth

At the cell wall formation stage of lettuce protoplasts, inhibitory activity of both co-cultured protoplasts of leaf (**Figure 6**) and suspension cells (**Figure 7**) of *S. thunbergii* was weaker than that at the cell division stage. Stimulation at a low protoplast density (140% at 10×10^3 mL^{-1}) was observed in suspension cell protoplasts (**Figure 7**).

Such strong stimulation at the cell wall formation stage was also observed in the co-culture with coffee callus protoplasts at a low protoplast density (150% - 300% at 3 - 26×10^3 mL^{-1}) [21]. Weak or no inhibition at early cell wall formation, but strong inhibition at the cell division stage might cause such transient stimulation of cell wall formation at low protoplast densities. These phenomena may be due to differences in the concentration of chemicals studied, or the site, each allelochemical is effective, *e.g.* caffeine, in the cell cycle of each plant cell [44].

4.3.2. Effects at the Yellow Pigment Accumulation Stage of Lettuce Protoplast Growth

Inhibition rate of co-cultured *S. thunbergii* protoplasts at the yellow pigment accumulation stage of lettuce was in between that of the cell division stage and cell wall formation stage (**Figure 6**). Such a pattern was similar to that seen in coffee callus [21], and *Avicennia alba* callus [24]. By contrast, less inhibition was seen at the yellow pigment accumulation stage than at the other two stages in the leaf of *Arabidopsis thaliana* [17]. No inhibition was observed in callus of *Sonneratia ovata* [22]. Such different effects on the yellow pigment accumulation must reflect the differences of the cellular action site of each allelochemical.

4.4. DIA-PP Method of Putative Allelochemicals of *Spiraea thunbergii*

4.4.1. Effects at the Cell Wall Formation Stage

In the DIA-PP method, both *cis*- (**Figure 8**) and *tran*s- (**Figure 9**) cinnamic acid showed the strongest inhibitory activity at the cell wall formation stage among three growth stages of lettuce. Tulipalin A, another volatile allelochemical of *S. thunbergii*, showed a pattern of inhibition at the three growth stages similar to that of *cis*- and *trans*-cinnamic acid using the DIA-PP method [19]. The stronger inhibition at the cell wall formation stage than at the cell division stage was in contrast to that obtained in the co-culture with *S. thunbergii* protoplasts (**4.3.1**). In addition to these putative allelochemicals, different allelochemical(s) might be present in the co-cultured protoplasts of *S. thunbergii*.

Among the other putative allelochemicals evaluated using the DIA-PP method, the inhibition rate at the cell division stage was stronger than that at the cell wall formation stage. Such a pattern is common in many test plants protoplasts and allelochemicals, *e.g.*, caffeine in coffee [21] [37] [45], a carotenoid, neoxanthin in *Avicennia alba* [24], an anthocyanin in *Sonneratia ovata*. [22], mimosine in *Leucaena leucocephala* [12], and an isoflavone, daidzein in Kudzu [18].

Inhibition rate at the cell wall formation stage was the same as that at the cell division stage for the isoflavonoid, rotenone in co-culture with *Derris indica* protoplasts [14].

4.4.2. Effects at the Yellow Pigment Accumulation Stage

The yellow pigment accumulated in lettuce protoplast was found to be a carotenoid at least partly [16]. Exogenous carotenoids, neoxanthin and crocin, have been reported to show strong inhibitory activity at the yellow pigment accumulation stage when examined by the DIA-PP method [25].

The DIA-PP method did not show inhibition at the yellow pigment accumulation stage by *cis*-cinnamic acid (**Figure 8**) and *trans*-cinnamic acid (**Figure 9**) and tulipalin A [19], at up to 10 μM, and showed weaker inhibitory activity than at the cell wall formation stage and cell division stage. These patterns are different from the effects of co-culture with protoplasts of *S. thunbergii* (**Figure 6** and **Figure 7**). Therefore, these putative allelochemicals of *S. thunbergii* are not like-

ly to be directly related to the synthesis and degradation of yellow carotenoid(s) in lettuce protoplasts.

On the other hand, yellow pigment accumulation in lettuce protoplasts was not inhibited by an anthocyanin pigment, cyanidin-di-glucoside, which is the allelochemical of red callus of *Sonneratia ovata* [22].

4.5. Finding New Allelochemicals Using the DIA-PP Method

Chemicals found in high concentrations in plants are not always the allelochemicals responsible for the strong allelopathic activity. For example, in Kudzu, different isoflavones found in low concentrations were suggested as candidates for the strongest inhibitory activity of Kudzu protoplasts found by the DIA-PP method [18]. Purine alkaloids, and phenolic acids including *p*-coumaric acid, were also discussed as candidates of allelochemicals in coffee cells [21]. Several purine alkaloids related to biosynthesis and catabolism of caffeine, have been investigated by the DIA-PP method [37] [45].

Spiraea cantoniensis was found to have weaker inhibitory allelopathic activity than *S. thunbergii* in a seedling growth test [3]. Very recently, the protoplasts of purple leaves of *S. cantoniensis* with anthocyanin-synthesizing genes introduced showed strong inhibitory activity, while the protoplasts of green leaves of *S. cantoniensis* showed no inhibitory activity at three stages of growth [6]. Unidentified yellow substance(s) were observed in suspension cultures of both *S. thunbergii* (**3.1**) and *S. cantoniensis* (in preparation). New allelochemicals may be found by comparative studies using *S. cantoniensis* and *S. thunbergii* and their allelochemicals.

Further studies using the DIA-PP method are needed to elucidate the cellular mechanism(s) of allelopathy, in relation to the synthesis and degradation pathways of putative allelochemicals, and to further clarify the functions of allelochemicals in protoplasts to help predict environmental risks in the field [10].

5. Conclusion

Using *in vitro* bioassay method of allelopathy, the protoplast co-culture method with digital image analysis (DIA-PP method), strong inhibitory activities of protoplasts of leaves and leaf-origin suspension cultured cells of *Spiraea thunbergii*, and putative allelochemicals, *cis*- and *trans*-cinnamic acid, were found. Different patterns of inhibition were observed at the three growth stages of recipient lettuce protoplasts, *i.e.*, cell wall formation, cell division and yellow pigment accumulation, which must reflect the different cellular action site of each allelochemical. Results were compared with reports on another putative allelochemical of *S. thunbergii*, tulipalin A. Reports of DIA-PP method on different allelopathic plants containing different putative allelochemicals were also compared with another bioassay method of allelopathy, the lettuce seedling growth test. Several allelochemicals, including *trans*-cinnamic acid, showed different strength of allelopathic activities between the two methods.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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