Scientific Research Publishing

American Journal of Plant Sciences, 2021, 12, 376-393
https://www.scirp.org/journal/ajps
ISSN Online: 2158-2750
ISSN Print: 2158-2742

Evaluation of Isoflavones as Allelochemicals with Strong Allelopathic Activities of Kudzu Using Protoplast Co-Culture Method with Digital Image Analysis

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How to cite this paper: Kobayashi, K., Sasamoto, H., Sasamoto, Y., Sugiyama, A. and Fujii, Y. (2021) Evaluation of Isoflavones as Allelochemicals with Strong Allelopathic Activities of Kudzu Using Protoplast Co-Culture Method with Digital Image Analysis. *American Journal of Plant Sciences*, **12**, 376-393.
https://doi.org/10.4236/ajps.2021.123024

Received: February 8, 2021
Accepted: March 27, 2021
Published: March 30, 2021

Abstract

The inhibitory allelopathic activity of *Pueraria montana* (Kudzu), and activities of two putative allelochemical isoflavones, puerarin and daidzein, were evaluated using the protoplast co-culture method with digital image analysis using lettuce as a recipient (DIA-PP method). Cotyledon protoplasts of Kudzu were isolated using Cellulase R10 and Driselase 20 in 0.6 M mannitol solution. Optimal hormonal condition and density for growth of Kudzu protoplasts were surveyed. Medium for co-culture of Kudzu or isoflavones with lettuce protoplasts was 50 µl liquid MS basal medium containing 1 µM 2,4-dichlorophenoxyacetic acid, 0.1 µM benzyladenine, 3% sucrose, and 0.4 M or 0.6 M mannitol. Protoplast division of lettuce was strongly inhibited by Kudzu at a low density (10^4/ml). Slightly less inhibition by Kudzu on cell wall formation and yellow pigment accumulation stages of lettuce growth was also observed. Puerarin did not inhibit the growth of lettuce protoplasts at three growth stages but slightly stimulated growth at high concentrations. By contrast, daidzein, aglycon of puerarin, inhibited growth at three stages of lettuce protoplast growth and strongly inhibited cell division at 100 µM. Daidzein might be one cause of the strong inhibitory allelopathic activity of Kudzu. Grade of inhibitory activities was compared with that of other allelopathic plants including an invader plant and their allelochemicals studied using the DIA-PP method.

Keywords

Allelopathy, Bioassay, Daidzein, Isoflavone, Protoplast Culture, Puerarin

1. Introduction

Pueraria montana (Lour.) Merr. var. *lobata* (Willd.) (Kudzu), also known as *P. lobata* Ohwi, and *P. thunbergiana,* is a leguminous plant, naturally grown in Japan and East Asia and introduced to other countries for its rapid growth in the field to stop soil erosion. However, problems of invasive weeds occurred [1]. On the other hand, Kudzu root and Kudzu starch and flowers, and their extracts are also used as traditional medicine for human health and food. High content of an isoflavone, puerarin, was reported in the root [2] [3] [4] and stalk [5] of Kudzu, which is a glycoside of an aglycon, daidzein, though the content of daidzein was low. Different types of aglycons and their glycosides of isoflavones are also known in different organs at different growth stages of Kudzu plants and tissue cultured cells [2]-[8]. In the green cotyledons-derived suspension-cultured cells of Kudzu, content of puerarin was the highest in isoflavones [2].

Allelopathy is a strategy of plants to survive by inhibiting the growth of neighboring plants of different species sharing the same habitat through formation of allelochemical(s). Surveying of the many plant species of strong allelopathic activities had been studied using bioassay methods of allelopathy of lettuce seedlings growth test, for their use in agriculture through weed management [9]. Though the allelopathic activities of test plants vary depending on neighboring plant species, high sensitivity of recipient lettuce seedlings was reported compared with other recipient plants [10]. In the sandwich method, which measures the effects of dried leaves [11] [12], the values of 48% and 65% growth of control were reported in Kudzu [10] [13]. In the plant box method, which measures the effects of intact root of small plant [14], the stronger inhibitory values of 20% and 28% growth of control were reported in Kudzu. Such values of roots of kudzu were stronger compared with 76% and 84% of a leguminous agricultural crop, soybean, similar to 30% and 20% of a cover crop, *Vicia villosa*, but less than 10% of strong allelopathic plant, *Mucuna pruriens* [13] [15].

Protoplast co-culture bioassay method of allelopathy was recently developed to know cellular mechanisms underlying allelopathy and to contribute to predicting future environmental risk [16]. Inhibitory effects of protoplasts of *M. pruriens* [16] and *V. villosa* [17] and their allelochemicals, L-DOPA and canavanine, on the growth of recipient plant protoplasts were determined in 50 µl liquid medium in a 96-well culture plate using an inverted microscope. Numbers of plants (and their putative allelochemicals) tested are increasing using the protoplast co-culture method with recipient lettuce, e.g., an invader plant, *Leucaena leucocephala* (mimosine) [18]; *M. gigantea* (L-DOPA) [18]; *Derris indica* (isoflavonoid, rotenone) [19]; *Prunus yedoensis* (coumarin and abscisic acid) [20]; three *Sonneratia* mangrove species of different salt tolerance [21]. The digital image analysis with protoplast co-culture method (DIA-PP method), which is the analysis of scanned image of yellow pigment accumulation in co-cultured (with lettuce protoplast) 96-well plate, was further developed. Numbers of stu-

died plants and their putative allelochemicals are increasing as follows: *Arabidopsis thaliana* [22] [23]; four bamboo species [24]; *Sonneratia ovata* (anthocyanin [25]); *Spiraea thunbergii* (cinnamic acid) and *S. cantoniensis* (anthocyanin) [26]; halophilic mangrove plant, *Avicennia alba* (carotenoid) [27]; *Coffea canephora* (caffeine and metabolites) [28] [29] [30]; *V. villosa* (canavanine and cyanamide) [17]. Very recently, volatile compounds of Saffron (safranal) and *Spiraea thunbergii* (tulipalin A) were reported [31].

In this study, we isolated cotyledon protoplasts of Kudzu and investigated allelopathic activity of Kudzu protoplasts at three growth stages of lettuce protoplast, *i.e.,* cell wall formation, cell division, and yellow pigment accumulation, using the DIA-PP method. We also examined the effects of isoflavones, puerarin and daidzein using the same lettuce protoplast method to evaluate the putative allelochemicals of Kudzu, in comparison with the protoplasts of different plant species and different allelochemicals reported.

2. Materials and Methods

2.1. Seedlings Growth of Kudzu and Lettuce

Seeds of *Pueraria montana* var. *lobata* (Kudzu) were collected at Nagoya city, Japan and stored at room temperature for 3 years. Seeds were washed with a neutral detergent and tap water, and sterilized with 2% NaClO solution for 5 min. The hard seed coat was cut with a knife for imbibition. After 10 min further sterilization, they were washed three times with autoclaved water in a clean bench, and were aseptically cultured on 0.8% agar medium in a 50 ml tube in the dark at 24˚C for 6 to 14 days. *Lactuca sativa* (lettuce) seedlings were prepared as described previously [16]. Briefly, lettuce seeds (Great Lakes) in a small bag of Miracloth were washed with a neutral detergent and tap water, and 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 a 15 ml tube in the light condition (60 $\mu E\,s^{-1}$) at 24˚C for 5 - 7 days.

2.2. Protoplast Isolation

Optimal combination of cell wall degrading enzymes and osmotic condition for Kudzu protoplast isolation was determined from preliminary experiments using 14 day-old seedlings, using 24 combinations of six kinds of enzymes, *i.e.,* Cellulase R10, Cellulase RS, Hemicellulase, Driselase 20, Macerozyme R10, Pectolyase Y-23 [17], in 0.4 M to 0.8 M mannitol solution. Cotyledons of 6 day-old Kudzu seedlings aseptically grown, were cut and treated 20 - 24 hrs in the enzyme combination, 1% each of Cellulase R10 and Driselase 20, in 0.6 M mannitol solution in a 24-well plastic culture plate at static condition at 27˚C in the dark. Protoplasts of lettuce cotyledons were isolated in 1% each of Cellulase RS and Macerozyme R10 [16] in 0.6 M mannitol solution in a 100 ml flask. After filtration with a mesh size of 80 μm (Kudzu) or 63 μm (lettuce), the protoplasts were washed three times with 0.6 M mannitol solution by centrifugation at 900 rpm (100 *g*)

for 5 min.

2.3. Protoplast Culture of Kudzu

Effects of different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D), 0, 0.1, 1, 10, 100 μM, and benzyladenine (BA), 0, 0.1, 1, 10 μM, were investigated using 50 μl each liquid Murashige and Skoog's (MS) [32] basal medium containing 3% sucrose and 0.6 M mannitol in each well of a 96-well culture plate (Falcon No. 3072). Five μl of Kudzu protoplasts in 0.6 M mannitol solution (0.6 - 70 × 10^4/ml) were put into the 50 μl medium. Initial protoplasts densities of culture were described to be 0.6 - 70 × 10^3/ml. Numbers of non-spherically enlarged protoplasts and divided protoplasts of Kudzu were counted under an inverted microscope periodically (2 to 12 days of culture). Protoplast growth was described as the percentage of initial plated numbers of protoplasts in each well.

2.4. Protoplast Co-Culture of Kudzu and Lettuce

Fifty μl of liquid MS basal medium containing 1 μM 2,4-D and 0.1 μM BA, 3% sucrose and 0.6 M mannitol was put in a well of 96-well culture plate. Five μl each of protoplast suspensions of Kudzu and lettuce at 10 times of final protoplast densities in 0.6 M mannitol solution were added to each well. Final protoplast densities were 0.6 - 30 × 10^3/ml for Kudzu and 6 - 100 × 10^3/ml for lettuce, respectively. Numbers of non-spherically enlarged and divided protoplasts were counted under an inverted microscope. After 6 days of co-culture, lettuce protoplast growth was calculated by deduction of the numbers of dark-yellow non-spherically enlarged Kudzu protoplasts from the total numbers of enlarged protoplasts of lettuce and Kudzu. After 10 days of co-culture, the numbers of divided lettuce protoplasts were counted. Percentages of control values without Kudzu protoplasts were calculated and then, the percentages of control at different densities of lettuce protoplasts (6, 12, 25, 50, 100 × 10^3/ml) were averaged with standard error (SE). Yellow pigment accumulation of lettuce protoplasts after 28 days of co-culture was analyzed using digital image analysis described in 2.6.

2.5. Protoplast Culture of Lettuce with Isoflavones, Puerarin and Daidzein

Isoflavones, puerarin and daidzein, were dissolved at 10 mg/ml and diluted in filter-sterilized DMSO (Milipore PTFE membrane). And one μL of each solution was put into 50 μl of the same medium described in 2.4. except for 0.4 M mannitol. Final concentration of DMSO was 2% as previously described [19]. Final protoplast densities of lettuce were the same as in 2.4 (6 - 100 × 10^3/ml). Numbers of non-spherically enlarged and divided protoplasts of lettuce were counted after 4 and 8 days of culture. Percentages of control values without isoflavone were calculated and then, the percentages of control at different densities of lettuce protoplasts (6, 12, 25, 50, 100 × 10^3/ml) were averaged with SE. Yellow pig-

ment accumulation of lettuce protoplasts after 27 days of co-culture was analyzed as in 2.6.

2.6. Digital Image Analysis of 96 Well Culture Plate

Image analysis of yellow pigment accumulation of lettuce protoplasts was performed after 3 weeks to 1.5 months of culture as described previously [17] [22] [23] [24] [25] [27] [29] [31]. Digital image of a 96-well culture plate was scanned using a scanner (Epson GTX-970). Image analysis by software Image J [33] was performed. 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 average 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 concentration of isoflavones was deduced as a control. The yellow values of Kudzu protoplasts at each density were not deduced or deduced as noted in the text. The % yellow value of control without protoplasts of Kudzu or isoflavones was calculated at each lettuce protoplast density. Finally, the percentages of control were averaged with SE at different cell densities of lettuce (6, 12, 25, 50, 100 × 10^3/ml).

3. Results

3.1. Effects of Plant Hormones on the Growth of Kudzu Protoplast

Isolated protoplasts of Kudzu cotyledons were non-green and around 40 μm diameter (similar to **Figure 1(a)**). No difference was observed during 1 day of culture. In the optimal hormonal conditions, non-spherical enlargement (**Figure 1(f)**) after 2 days of culture and cell division (**Figure 1(g)**) after 3 days of culture were observed.

Figure 2(a) shows the effects of 2,4-D and BA on non-spherical enlargement of Kudzu protoplasts after 2 days of culture at 17 × 10^3/ml. All hormonal conditions tested gave cell enlargement, while 0.1 - 10 μM of 2,4-D were better than without 2,4-D. Enlargement occurred without BA. Combination of 10 μM 2,4-D and 0.1 μM BA was the best condition. **Figure 2(b)** shows the effects of 2,4-D and BA on the cytoplasm-rich cell division of Kudzu protoplasts after 5 days of culture at 17 × 10^3/ml. Although enlarged protoplasts (including vacuolated cells) can be observed in all hormonal conditions, cytoplasm-rich division can be observed in both 2,4-D and BA containing media. High division efficiency was observed at 2,4-D 10 μM and BA 0.1 - 10 μM (8.9%, 5.9%, 7.7%). After 12 days of culture, large brown colored colony development was observed at the similar hormonal conditions as of **Figure 2(b)**. At higher initial protoplast densities (35, 70 × 10^3/ml) (**Figure 2(c)** and **Figure 2(d)**), division occurred after 5 days of culture at 1 - 10 μM 2,4-D and BA 0.1 - 10 μM. Although 10 μM each of 2,4-D and BA was the best condition, the percentages of division were lower (5.0, 2.9%) than those at 17 × 10^3/ml (**Figure 2(b)**).

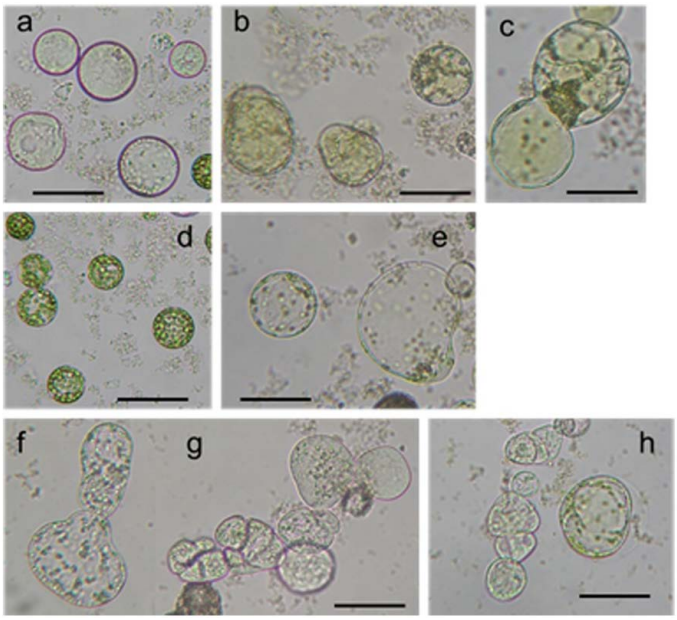

Figure 1. Photographs of cultured protoplasts of Kudzu or lettuce. a: non-green Kudzu protoplasts. b, c: dark yellow enlargement of Kudzu protoplasts. d: green lettuce protoplasts. e: spherical or non-spherical enlargement of lettuce. f: non-spherical enlargement of Kudzu. g: division of Kudzu. h: division or non-spherical enlargement of lettuce. a-e: co-cultured. f-h: not co-cultured. a, d: at 1st day. b, e, f, g: at 6th day. c, h: at 10th day of culture. Medium was MS basal medium containing 1 μM 2,4-D, 0.1 μM BA, 3% sucrose and 0.6 M mannitol. Bar = 50 μm.

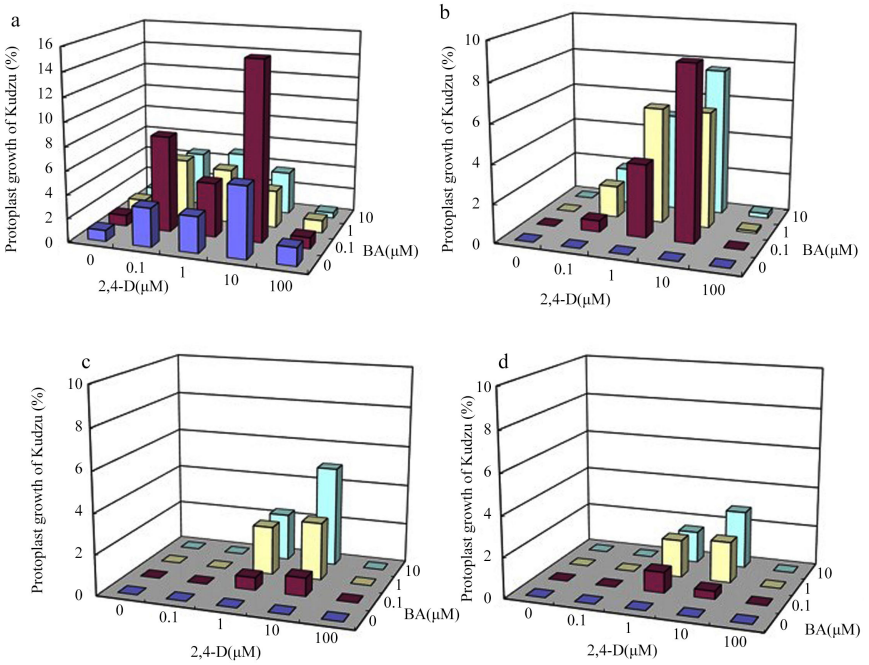

Figure 2. Effects of 2,4-D and BA on protoplast growth of Kudzu after 2 days (a) and 5 days (b, c, d) of culture. a: non-spherical enlargement. b, c, d: cytoplasm-rich division. Medium was MS basal medium containing 3% sucrose, and 0.6 M mannitol. Initial protoplast densities were 17 × 10^3/ml (a, b), 35 × 10^3/ml (c) and 70 × 10^3/ml (d), respectively.

Figure 3 shows the effects of different protoplast densities of Kudzu at 1 μM 2,4-D and 0.1 μM BA condition. Good growth of Kudzu protoplasts was obtained at 4.2 × 10^3/ml up to 17 × 10^3/ml of protoplast densities. After 2 days of culture, efficiency of highest density (70 × 10^3/ml) was inhibitory for non-spherical enlargement (E). After 5 days of culture, non-spherical enlargement (E) occurred at this highest density, however, much less division (D) was observed. In another independent experiments for lower protoplast densities (0.6, 1.2, 2.5, 5, 10 × 10^3/ml), after 6 days of culture, growth (E + D) of 10%, 7%, 18%, 13%, 16% were obtained, respectively. Average % was 13 ± 2 (SE).

3.2. Protoplast Co-Culture of Kudzu and Lettuce

The lettuce cotyledon protoplasts were green and ca.30 μm diameter (Figure 1(d)) and a little smaller than of Kudzu protoplasts (Figure 1(a)) at the 1st day of co-culture. Lettuce protoplasts did not enlarge before 3 days of culture, when the Kudzu protoplast started to grow without lettuce. Non-spherically enlarged or divided Kudzu protoplasts without lettuce had no yellow color (Figure 1(f), Figure 1(g).

Figure 4 shows the inhibition of lettuce protoplast growth by co-cultured Kudzu protoplasts at very low densities (0.6 - 10 × 10^3/ml) in the hormonal condition of 1 μM 2,4-D and 0.1 μM BA.

After 6 days of co-culture, dark yellow Kudzu protoplasts can be distinguished under an inverted microscope (Figure 1(b), Figure 1(c)). From the total numbers of non-spherically enlarged protoplasts, such Kudzu-specific numbers were deducted and described as lettuce-specific non-spherically enlarged lettuce protoplasts. After 10 days of co-culture, only the numbers of divided cells including developed colonies of lettuce protoplasts were counted. Cell division of lettuce protoplasts and of Kudzu was distinguished easily under an inverted microscope. Almost total inhibition of lettuce division was observed at 10 × 10^3/ml of Kudzu protoplasts, which was similar density to the lowest density of lettuce tested in co-culture (6 × 10^3/ml). Kudzu protoplasts clearly inhibited growth of lettuce protoplasts at two stages, *i.e.*, cell wall formation stage after 6 days and cell division stage after 10 days of co-culture.

At the lowest lettuce protoplast density tested (6 × 10^3/ml), Kudzu specific growth was prominent at high protoplast densities of Kudzu (10, 30 × 10^3/ml). Averaged counted numbers of lettuce protoplast growth at control without Kudzu were 85 ± 21 (SE) at 6 days of culture (91% was non-spherically enlargement) and 48 ± 8 (SE) at 10th day of culture, respectively.

As shown in the scanned image of 96-well plate co-cultured for 28 days (Figure 5(a)), at the highest Kudzu density, 30 × 10^3/ml, brown colored Kudzu growth was prominent. Though, lettuce protoplasts inhibited the brown colored Kudzu growth at density dependent manner. At high Kudzu density (30 × 10^3/ml), the calculated yellow values increased at lowest lettuce density (6 × 10^3/ml). But decrease of yellow color at the high Kudzu density (10 × 10^3/ml)

and low lettuce density (6 × 10^3/ml) was clearly shown in Figure 5(a). On line b1 of yellow pigment accumulation (Figure 5(b)), when the yellow values of Kudzu protoplasts at each density were not deducted, less inhibition of yellow pigment accumulation by Kudzu was observed compared with inhibition on cell wall formation and cell division stages (Figure 5). In line b2 of yellow pigment accumulation (Figure 5(b)), where each zero control value without lettuce protoplasts was deducted from each yellow value of co-cultured well, inhibition by Kudzu was similar to that at 6 days of co-culture (Figure 4).

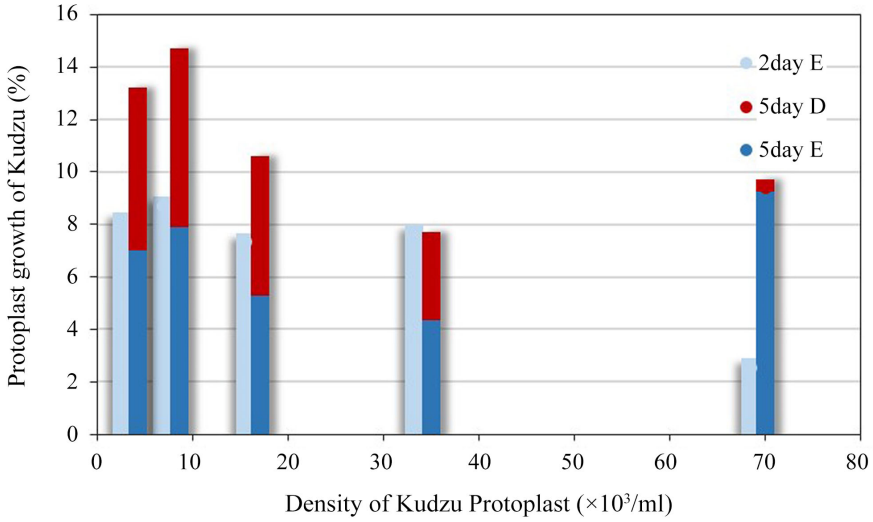

Figure 3. Effects of protoplast densities on protoplast growth of Kudzu after 2 days and 5 days of culture. E: non-spherical enlargement. D: division. The medium was MS basal medium containing 1 μM 2,4-D, 0.1 μM BA, 3% sucrose and 0.6 M mannitol.

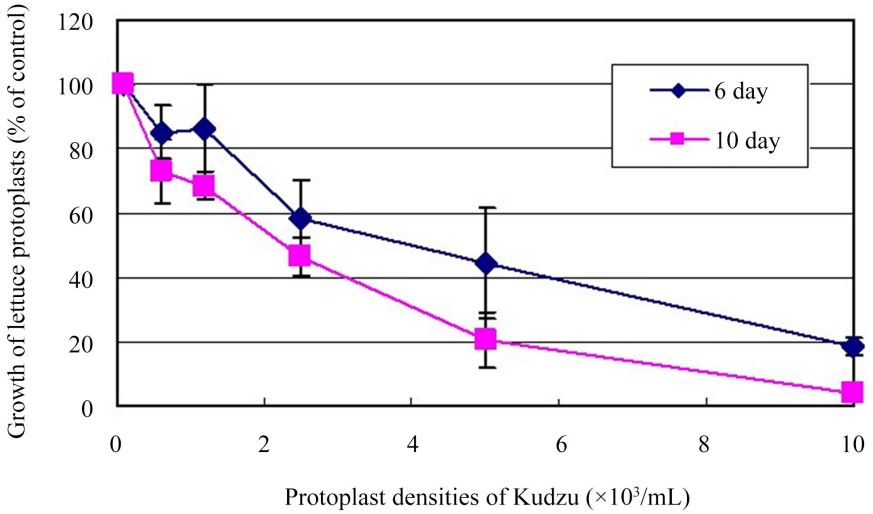

Figure 4. Effects of Kudzu protoplasts on the growth of lettuce protoplasts after 6 and 10 days of co-culture. Medium was MS basal medium containing 1 μM 2,4-D and 0.1 μM BA, 3% sucrose, 0.6 M mannitol. 6 day: cell wall formation stage, average % of 6 - 50 × 10^3/ml of lettuce with SE. 91% was non-spherical enlargement at control without Kudzu. 10 day: cell division stage, average % of 12 - 100 × 10^3/ml of lettuce with SE.

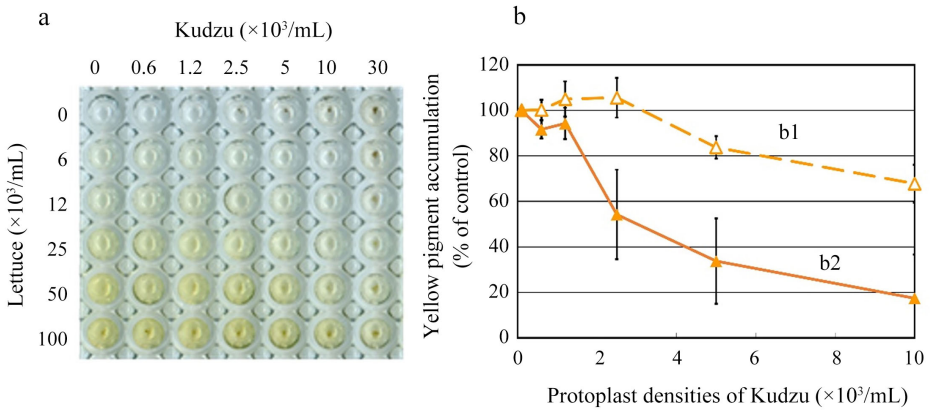

Figure 5. Scanned image (a) and Image J analysis (b) of yellow pigment accumulation after 28 days of co-culture of Kudzu and lettuce protoplasts. Line b1: averages with SE of 12 - 100 × 10^3/ml of lettuce without automatic deduction of yellow values of Kudzu. Line b2: averages with SE of 6 - 100 × 10^3/ml of lettuce after automatic deduction of yellow values of Kudzu. Medium was the same as of **Figure 4**.

3.3. Lettuce Protoplast Culture with Isoflavones, Puerarin and Daidzein

Two putative allelochemicals, puerarin and daidzein of Kudzu were tested using protoplast co-culture method with digital image analysis. As shown in **Figure 6**, big differences of effects on three stages of lettuce protoplast growth were found between puerarin and daidzein. **Figure 7** shows the scanned image of 96-well plate in **Figure 6(c)**. In the daidzein co-culture with lettuce protoplasts, many crystals were found in spherical and non-spherical protoplasts at 100 μM (**Figure 8**), which almost totally inhibited cell division after 8 days of co-culture (**Figure 6(b)**). At higher concentrations of daidzein, many crystals were prominent in the culture medium. No such crystals of puerarin were observed. In contrast, stimulation of cell division was observed at high concentrations of puerarin (**Figure 6(b)**). At up to 10 μM, the 20% inhibition rate of daidzein was not different among the three stages of lettuce growth (**Figures 6(a)-(c)**). At 100 μM, inhibition was the strongest at the cell division stage (**Figure 6(b)**). Less inhibition was observed on early cell wall formation stage (**Figure 7(a)**), or yellow pigment accumulation stage (**Figure 6(c)**).

Averaged counted numbers of lettuce protoplast growth at zero control without isoflavone were 50 ± 12 (SE) at 4 days of culture (**Figure 6(a)**) and 24 ± 2.7 (SE) at 8 days of culture (**Figure 6(b)**), respectively.

4. Discussion

4.1. Hormonal Conditions and Densities for Protoplast Growth of Kudzu

In the protoplast cultures of Kudzu, optimal hormonal condition for cell division and colony development was a combination of 10 μM 2,4-D and 0.1 - 10 μM BA in MS basal medium containing 0.6 M mannitol. 2,4-D only medium

was good for early cell wall formation, but both 2,4-D and BA were needed for further growth. Such a result was repeatedly obtained in experiments using different protoplast densities (17 - 70 × 10^3/ml) (Figure 2).

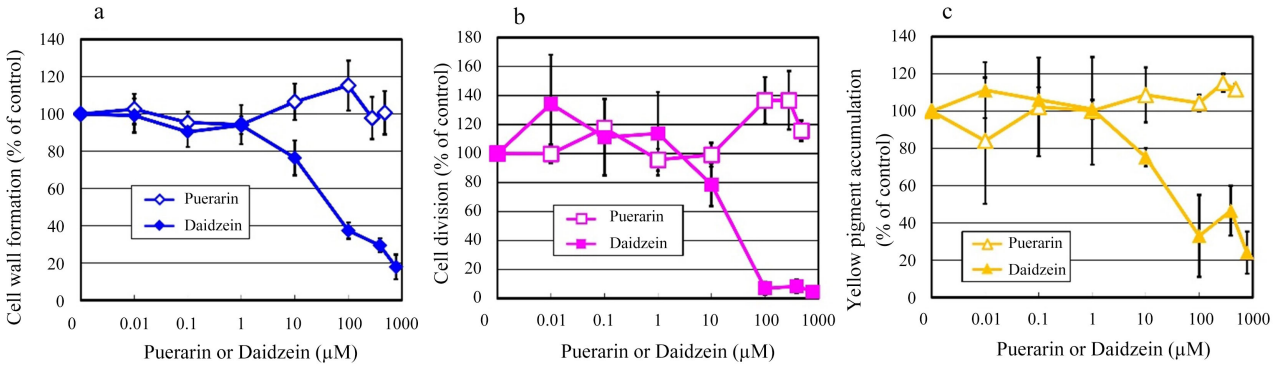

Figure 6. Effects of puerarin and daidzein on three growth stages of lettuce protoplasts after 4 (a), 8 (b), and 27 (c) days of culture. Medium was MS basal medium containing 1 μM 2,4-D and 0.1 μM BA, 3% sucrose, 0.4 M mannitol. a: cell wall formation stage, average % with SE of 6 - 50 × 10^3/ml of lettuce. 84% was non-spherical enlargement at zero control. b: cell division stage, average % with SE of 6 - 50 × 10^3/ml of lettuce. c: yellow pigment accumulation stage, average % with SE of 12 - 100 × 10^3/ml of lettuce.

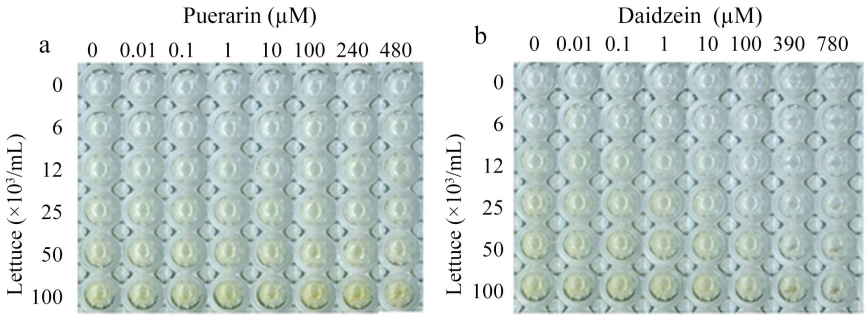

Figure 7. Scanned images of 96-well plate of puerarin (a) and daidzein (b) after 27 days of co-culture. Correspond to Figure 6(c).

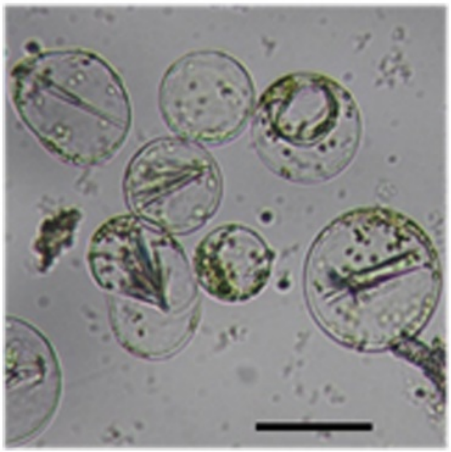

Figure 8. Crystals incorporated into lettuce protoplasts co-cultured with 100 μM daidzein for 5 days. Protoplast density was 50 × 10^3/ml. Bar = 50 μm.

As for the lettuce cotyledon protoplast growth, lower concentrations of 2,4-D (0.1 - 1 μM) and BA (0.1 - 1 μM) were optimal [16]. The combination of 1 μM 2,4-D and 0.1 μM BA in MS basal medium, was used for co-culture with recipient lettuce cotyledons protoplasts in bioassay of allelopathy of many test plants [16] [18] [19] [24] and of Kudzu in this paper. It was a suboptimal condition for Kudzu protoplast growth. MS basal medium was not optimal for protoplast growth of lettuce instead ammonium nitrate-free MS basal medium was optimal [16]. However, the hormonal condition and basal medium for co-culture bioassay of allelopathy were not necessary the optimal condition for each test plant protoplasts as studied with *Mucuna pruriens* [16], *Derris indica* [19], *Leucaena leucocephala and M. gigantea* [18], and bamboo species [24]. At sub-optimal hormonal condition for cell division of test plants protoplasts, a similar strong allelopathic activity was observed.

Kudzu protoplasts could grow in a wide density range (0.6 - 70 × 10^3/ml) at sub-optimal hormonal conditions, though quantitative evaluation was more difficult at high densities under an inverted microscope, which was much low density compared with the optimal high density of *Mucuna pruriens*. Inhibition of lettuce protoplast growth was observed at lower densities than optimal high densities (300 × 10^3/ml or more) for *M. pruriens* [16].

4.2. Protoplast Co-Culture of Kudzu with Lettuce

About 50% inhibition of lettuce protoplast division by 2.5 × 10^3/ml Kudzu and almost total inhibition by 10 × 10^3/ml (10^4/ml) Kudzu indicates very strong inhibition (**Figure 4**). Compared with the effective protoplast density of other plant species reported, the grade of inhibition of Kudzu was as high as the protoplasts of an invader plant, *L. leucocephala* (1.5 × 10^3/ml, 20 × 10^3/ml) and *M. gigantea* (1.5 × 10^3/ml, 20 × 10^3/ml) [18]. Inhibition by epicotyl protoplasts of *V. villosa* (1 × 10^3/ml, 3 × 10^3/ml) [17], which was three times stronger inhibition than of Kudzu, was the strongest among those tested using protoplast co-culture method.

Compared with *Arabidopsis* leaves (20 × 10^3/ml, 100 × 10^3/ml) [23], one tenth lower density was effective in Kudzu, while a similar pattern of inhibition among three growth stages (less inhibition on yellow pigment accumulation compared to that on cell wall formation or cell division) was observed. An anthocyanin accumulating *Sonneratia ovata* (10 × 10^3/ml, 100 × 10^3/ml<) showed much lower activity, and different patterns (no inhibition on the yellow pigment accumulation stage) were observed among three growth stages [25].

Mutual inhibition was observed by lettuce protoplasts on Kudzu protoplast growth. In early cell wall formation stage in co-culture, the number of the dark-yellow non-spherically enlarged protoplasts of Kudzu was deducted from the total number of non-spherically enlarged protoplasts, which included both lettuce and Kudzu reacted. Division of lettuce protoplasts was detected under an inverted microscope. However, yellow pigment accumulation of lettuce protop-

lasts at low densities (6, 12 × 10^3/ml) is difficult to separate from the brown colored colony development of Kudzu at high densities (10, 30 × 10^3/ml). Similar mutual inhibition by bamboo protoplasts and by lettuce protoplasts has been reported [24]. Strong inhibition of yellow pigment accumulation by *Sasa kurilensis* was calculated as in line b2 in **Figure 5(b)**, which shows strong inhibition at the cell wall formation stage similar to Kudzu. Such a calculation method (Subtraction of the control yellow values without lettuce protoplasts, at each density of test plant protoplasts or at each concentration of allelochemicals) had been employed in the previously reported papers using the DIA-PP method [17] [22]-[27] [29] [31].

Yellow pigment accumulation at late growth stage (after 3 weeks of culture) of lettuce protoplast might be related to the synthesis and degradation of secondary metabolites. A carotenoid was extracted to hexane fraction from lettuce protoplasts that had accumulated the yellow pigment [22]. Recently, a very different pattern of inhibition at three growth stages of lettuce by a carotenoid-accumulating callus of *Avicennia alba* has been reported [27]. Very recently, unique allelopathic activities of three carotenoids were investigated at three growth stages of lettuce protoplast using the DIA-PP method [34].

4.3. Lettuce Protoplast Culture with Isoflavones

Though, lettuce protoplast has a broad range of optimal osmotic condition, e.g., 0.4 M to 0.8 M mannitol, better and rapid growth is observed in low 0.4 M mannitol condition. We selected 0.6 M in co-culture with Kudzu as it was the optimal osmotic condition for Kudzu protoplasts. However, we used 0.4 M for testing the allelochemicals, as dissolving solvent DMSO inhibits lettuce protoplast growth at a concentration higher than 2% [19]. We found no differences in the inhibition patterns among different osmotic conditions. Though, growth stages of lettuce proceed earlier under lower osmotic conditions.

Complete inhibition of lettuce protoplast division by daidzein at 100 μM (**Figure 6(b)**) is in the group of chemicals with strong allelopathic activity studied using protoplast co-culture method. Similar strong inhibition has been observed by, e.g., canavanine in *Vicia villosa* [18]; L-DOPA in *Mucuna pruriens* [16]; isoflavonoid, rotenone in *Derris indica* [19]; cinnamic acid [26] and tulipalin A [31] in *Spiraea thunbergii*. In contrast, coumarin in *Prunus yedoensis* was 25% of control at 100 μM [20]. An anthocyanin, cyanin in *Sonneratia ovata* inhibited growth to 40% of the control [25], respectively. Although, purine alkaloid, caffeine was the strongest in the group of related metabolites [28] [29], no inhibition was observed at 100 μM. Stimulation effect of trigonelline was reported [35].

This is the first study showing the strong inhibitory allelopathic activity of isoflavone, daidzein, at the cellular level, using the protoplast co-culture method with digital image analysis. Among the three growth stages of lettuce, inhibition at the cell division stage was the strongest. Such inhibitory patterns are similar to

those of the Kudzu protoplast in co-culture. By contrast, puerarin, which is a glucoside of daidzein, was not inhibitory, and showed a rather stimulatory effect at high concentrations at all three growth stages of lettuce protoplasts (**Figure 6**).

Differences in the stimulation and inhibition patterns among three growth stages were observed among plant species and putative allelochemicals studied [17] [23] [24] [25] [26] [27] [30] [31], which must reflect the cellular action site of each chemical.

4.4. Evaluation of Isoflavones as Allelochemicals in Kudzu Protoplasts

In the present study, strong allelopathic activity of cotyledon protoplasts of Kudzu was found in co-culture with lettuce protoplasts (**Figure 4**). Such strong inhibitory activity is consistent with the data of the plant box method, *i.e.,* 20% to 28% growth of control [13] [15], which measured the effect of the intact root of Kudzu on the growth of lettuce seedlings. These were stronger compared with leaves of kudzu, 48% to 65% growth of control, using the sandwich method [10] [13].

High content of isoflavone, puerarin, in the roots (e.g., 13 mg/g dry weight, 5 mg/g dry weight) and immature cotyledon-derived callus or suspension cultured cells (1 - 2 mg/g dry weight), but not in the leaves of Kudzu had been reported [2] [3] [4]. These phenomena suggested the possibility of puerarin as an allelochemical in Kudzu protoplasts.

However, in this report, puerarin itself was not likely the allelochemical of Kudzu protoplasts since no inhibitory activity of puerarin was observed up to 780 μM in the protoplast co-culture method (**Figure 6**). By contrast, crystal of daidzein was incorporated into the lettuce protoplasts (**Figure 8**), and showed strong inhibitory activity on lettuce protoplast growth at 100 μM (**Figure 6**). In Kudzu plant tissues, content of daidzein is not high (e.g., 0.07 mg/g dry weight) compared with that of puerarin, though the extraction method affects their contents [2] [3] [4] [5] [6]. In Kudzu root prepared after water extraction, the glycoside, puerarin content decreased and aglycon, daidzein content increased [6]. When the water content of Kudzu tissue is predicted to be 90%, concentration of daidzein is calculated to be ca. 30 μM, which showed inhibition of division of lettuce protoplasts (**Figure 6(b)**). Even 100 times lower content of daidzein than puerarin might still be effective as an allelochemical in Kudzu plant. These findings suggest the need of further study on the allelopathic activities of other isoflavones of different aglycon types, e.g., daidzein, genistein, glycitein and their glucosides, e.g., daidzin, genistin, glycitin, and glycosides, whose low content had been found in different organs and at different growth stages of Kudzu plants [2] [3] [4] [5] [6] and in the tissue culture of Kudzu [7] [8]. Several isoflavones are under investigation using the DIA-PP method. Crystal found in co-cultured protoplasts is also an interesting theme.

On the other hand, in another isoflavone-containing plant, *Trifolium pratense* (red clover) [36] [37], activities of isoflavones themselves were not so high when red clover was used as a recipient, and isoflavonoid-origin phenolic acids in the soil environment were discussed as germination retardant [36]. Soluble phenolics in leaves, roots and soil of Kudzu were reported as allelochemicals using lettuce and radish as recipient plants [38]. In soybean, different amounts of isoflavones and glycosides were reported in sprouts [39], leaves and roots [40], of low allelopathic activities [15]. Recently, a high content of daidzein, which was not found in leaves, was found in the rhizosphere soil, which affects soybean growth through rhizosphere bacterial community [40] [41]. Xanthoxin, which is known as an intermediate of a growth retardant hormone, abscisic acid (ABA) synthesis, was reported as an allelochemical of leaves of Kudzu using cress as a recipient plant [42]. Using the protoplast co-culture method, differences in activities among different recipient plants are known, e.g., inhibition activity of L-DOPA, which is the allelochemical in *Mucuna pruriens*, was weak on rice protoplasts [16]; Exogenously supplied ABA was strongly inhibitory on *Prunus yedoensis* protoplasts, in which coumarin was an allelochemical [20], but ABA was stimulatory on lettuce protoplasts [16]. As we found that Kudzu protoplasts can grow at low densities, effects of chemicals, such as coumarin and hinokitiol, were investigated using only 500 Kudzu protoplasts in a well, at optimal hormonal condition of Kudzu (2,4-D 10 μM and BA 1 μM) [43].

Therefore, a single allelochemical in an allelopathic plant might not be the only cause of the allelopathy of the whole plant. The volatile compounds [13] [31], and metabolites in rhizosphere [40] [41] might also be considered. The protoplast co-culture method with digital image analysis using lettuce as a recipient for bioassay of allelopathy will contribute to identification of the underlying mechanism(s) of allelopathy at a cellular level.

5. Conclusions

Strong inhibition by Kudzu cotyledon protoplast on lettuce protoplast growth was found using the protoplast co-culture method with digital image analysis. Kudzu protoplasts at 10^4/ml totally inhibited cell division of lettuce protoplasts, which is inhibitory allelopathic activity similar to that of invader plants with high allelopathic activities. Less inhibition on cell wall formation and yellow pigment accumulation stages of lettuce growth was also found.

Two putative allelochemicals in Kudzu plants, the isoflavones, daidzein and puerarin were investigated using the protoplast co-culture method with digital image analysis. Puerarin content is known to be high in Kudzu plant and tissue cultured cells, was not inhibitory or some stimulation was found at high concentrations. By contrast, aglycon of puerarin, daidzein, whose content in Kudzu plant is lower than puerarin, strongly inhibited the growth of lettuce at 100 μM at three growth stages of lettuce protoplasts.

These findings suggest that one of the causes of strong inhibitory activity of

Kudzu protoplasts might be an isoflavone, daidzein, whose content varies in different tissues and growth stages of Kudzu and by extraction methods.

Further test was suggested of other isoflavones of different chemical structure, aglycons and glycosides found in Kudzu. Protoplast co-culture method with digital image analysis will contribute to know underlying mechanism(s) of allelopathy at a cellular level.

Conflicts of Interest

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

References

[1] Gulizia, J.P. and Downs, K.M. (2019) A Review of Kudzu's Use and Characteristics as Potential Feedstock. *MDPI Agriculture*, **9**, 220. https://doi.org/10.3390/agriculture9100220

[2] Liu, H.L. and Li, L. (2002) Cell Cultures of *Pueraria lobata* (Willd.): Growth and Production of Isoflavones and Puerarin. *South African Journal of Botany*, **68**, 542-544. https://doi.org/10.1016/S0254-6299(15)30382-3

[3] Kirakosyan, A., Kaufman, P.B., Warber, S., Bolling, S. and Duke, J.A. (2003) Quantification of Major Isoflavonoids and L-canavanine in Several Organs of Kudzu Vine (*Pueraria montana*) and in Starch Samples Derived from Kudzu Roots. *Plant Science*, **164**, 883-888. https://doi.org/10.1016/S0168-9452(03)00077-3

[4] Chen, T.R., Shih, S.C., Ping, H.P. and Wei, Q.K. (2012) Antioxidant Activity and Isoflavonoid Components in Different Sections of *Pueraria lobata* Root. *Journal of Food and Drug Analysis*, **20**, 681-685.

[5] Shimizu, H., Tsuduki, M. and Matsuzawa, K. (2007) Isoflavonoids in the Stalk of *Pueraria lobata* Ohwi. *Report of Nara Prefectural Institute of Industrial Technology*, **33**, 46-48.

[6] Kimura, K., Yamabe, T., Inoue, T. and Kitada, Y. (2011) Investigations of the Amount of Isoflavones Contained in Kuzu Starches and Kuzu Leaves. *Japanese Journal of Food Chemistry and Safety*, **18**, 48-52.

[7] Takeya, K. and Itokawa, H. (1982) Isoflavonoids and the Other Constituents in Callus Tissues of *Pueraria lobata*. *Chemical and Pharmaceutical Bulletin*, **30**, 1496-1499. https://doi.org/10.1248/cpb.30.1496

[8] Hakamatsuka, T., Shinkai, K., Noguchi, H., Ebizuka, Y. and Sankawa, U. (1992) Isoflavone Dimers from Yeast Extract-Treated Cell Suspension Cultures of *Pueraria lobata*. *Zeitshrift fur Naturforschung*, **47c**, 177-182. https://doi.org/10.1515/znc-1992-3-402

[9] Bergum, K., Shammi, M., Hasan, N., Asaduzzaman, M., Appiah, K.S. and Fujii, Y. (2019) Potential Allelopathic Candidates for Land Use and Possible Sustainable Weed Management in South Asian Ecosystem. *Sustainability*, **11**, 2649. https://doi.org/10.3390/su11092649

[10] Itani, T., Hirai, K., Fujii, Y., Kohdai, H. and Tamaki, M. (1998) Screening for Allelopathic Activity among Weeds and Medicinal Plants Using the "Sandwich Method". *Journal of Weed Science and Technology*, **43**, 258-266. https://doi.org/10.3719/weed.43.258

[11] Fujii, Y., Parvez, S.S., Parvez, M.M., Ohmae, Y. and Iida, O. (2003) Screening of 239

Medicinal Plant Species for Allelopathic Activity Using the Sandwich Method. *Weed Biology and Management*, **3**, 233-241. https://doi.org/10.1046/j.1444-6162.2003.00111.x

[12] Fujii, Y., Shibuya, T., Nakatani, K., Itani, T., Hiradate, S. and Parvez, M.M. (2004) Assessment Method for Allelopathic Effect from Leaf Litter Leachates. *Weed Biology and Management*, **4**, 19-23. https://doi.org/10.1111/j.1445-6664.2003.00113.x

[13] Fujii, Y. and Hiradate, S. (2003) Research Achievement of Allelopathy Laboratory in NIAES, from 1983 to 2001. Part 2. National Institute for Agro-Environmental Sciences (NIAES), Ibaraki, 579-1180.

[14] Fujii, Y., Pariasca, D., Shibuya, T., Yasuda, T., Kahn, B. and Waller, G.R. (2007) Plant Box Method: A Specific Bioassay to Evaluate Allelopathy through Root Exudates. In: Fujii, Y. and Hiradate, S., Eds., *Allelopathy New Concepts and Methodology*, 2nd Edition, Science Publishers, Inc., Enfield, 39-56.

[15] Fujii, Y. (1999) Significance of Allelopathy as a Factor of Biodiversity and Its Application to Agriculture. In: *Techno Innovation*, Japan Association for Techno-Innovation in Agriculture, Forestry and Fisheries, Tokyo, 9(2), 27-32.

[16] Sasamoto, H., Murashige-Baba, T., Inoue, A., Sato, T., Hayashi, S. and Hasegawa, A. (2013) Development of a New Method for Bioassay of Allelopathy Using Protoplasts of a Leguminous Plant *Mucuna pruriens* with a High Content of the Allelochemical L-DOPA. *Journal of Plant Studies*, **2**, 71-80. https://doi.org/10.5539/jps.v2n2p71

[17] Sasamoto, H., Mardani, K.H., Sasamoto, Y., Wasano, N., Murashige-Baba, T., Sato, T., Hasegawa, A. and Fujii, Y. (2019) Evaluation of Canavanine as an Allelochemical in Etiolated Seedlings of *Vicia villosa* Roth: Protoplast Co-Culture Method with Digital Image Analysis. *In Vitro Cellular and Developmental Biology-Plant*, **55**, 296-304. https://doi.org/10.1007/s11627-019-09985-3

[18] Mori, D., Ogita, S., Fujise, K., Inoue, A. and Sasamoto, H. (2015) Protoplast Co-Culture Bioassay for Allelopathy in Leguminous Plants, *Leucaena leucocephala* and *Mucuna gigantea*, Containing Allelochemical Amino Acids, Mimosine and L-DOPA. *Journal of Plant Studies*, **4**, 1-11. https://doi.org/10.5539/jps.v4n1p1

[19] Inoue, A., Mori, D., Minagawa, R., Fujii, Y. and Sasamoto, H. (2015) Allelopathy in a Legu Minous Mangrove Plant, *Derris indica*: Protoplast Co-Culture Bioassay and Rotenone Effect. *Natural Product Communications*, **10**, 747-750. https://doi.org/10.1177/1934578X1501000512

[20] Fujise, K., Yokota, S. and Sasamoto, H. (2018) Evaluation of Allelochemicals, Abscisic Acid and Coumarin, in Leaf-Origin Suspension Cultured Cells of *Prunus yedoensis* Using Protoplast Co-Culture Bioassay Method. *American Journal of Plant Sciences*, **9**, 172-184. https://doi.org/10.4236/ajps.2018.92015

[21] Hasegawa, A., Oyanagi, T., Minagawa, R., Fujii, Y. and Sasamoto, H. (2014) An Inverse Relationship between Allelopathic Activity and Salt Tolerance in Suspension Cultures of Three Mangrove Species, *Sonneratia alba*, *S. caseolaris* and *S. ovata*: Development of a Bioassay Method for Allelopathy, the Protoplast Co-Culture Method. *Journal of Plant Research*, **127**, 755-761. https://doi.org/10.1007/s10265-014-0651-1

[22] Sasamoto, H., Azumi, Y. and Suzuki, S. (2017) Development of a High-Throughput Bioassay Method of Allelopathy: Protoplast Co-Culture and Digital Image Analysis. *Science Journal of Kanagawa University*, **28**, 63-70.

[23] Sasamoto, H., Azumi, Y., Shimizu, M., Hachinohe, Y. and Suzuki, S. (2017) *In Vitro* Bioassay of Allelopathy of *Arabidopsis thaliana* by Sandwich Method and Protoplast

Co-Culture Method with Digital Image Analysis. *Plant Biotechnology*, **34**, 199-202. https://doi.org/10.5511/plantbiotechnology.17.1204a

[24] Ogita, S. and Sasamoto, H. (2017) *In Vitro* Bioassay of Allelopathy in Four Bamboo Species; *Bambusa multiplex*, *Phyllostachys bambusoides*, *P. nigra*, *Sasa kurilensis*, Using Sandwich Method and Protoplast Co-Culture Method with Digital Image Analysis. *American Journal of Plant Sciences*, **8**, 1699-1710. https://doi.org/10.4236/ajps.2017.87117

[25] Sasamoto, H., Iwashina, T., Suzuki, S., Azumi, Y. and Fujii, Y. (2018) 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. *Journal of Plant Studies*, **7**, 1-10. https://doi.org/10.5539/jps.v7n2p1

[26] Suzuki, S., Wasano, N., Kimura, M., Yasuda, R., Nakagawa, W., Sasamoto, Y., Fujii, Y. and Sasamoto, H. (2018) Evaluation of Putative Allelochemicals, Cinnamic Acid and an Anthocya Nin, in *Spiraea thunbergii* and *S. cantoniensis* Using *in Vitro* Bioassay Method of Allelopathy, the Protoplast Co-Culture with Digital Image Analysis. *Proceedings of the* 4*th International Conference of Asian Allelopathy Society*, Tokyo, 33, 72.

[27] Sasamoto, H., Hayatsu, M. and Suzuki, S. (2020) High Allelopathic Activity of Carotenoid-Accumulating Callus of a Halophilic Mangrove Plant, *Avicennia alba*: Protoplast Co-Culture Method with Digital Image Analysis. *Journal of Plant Studies*, **9**, 1-12. https://doi.org/10.5539/jps.v9n1p1

[28] Sasamoto, H., Fujii, Y. and Ashihara, H. (2015) Effect of Purine Alkaloids on the Proliferation of Lettuce Cells Derived from Protoplasts. *Natural Product Communications*, **10**, 751-754. https://doi.org/10.1177/1934578X1501000513

[29] Ogita, S. and Sasamoto, H. (2018) *In Vitro* Bioassay of Allelopathy in Caffeine-Containing Coffee Cells Using Protoplast Co-Culture Method with Digital Image Analysis. *Proceedings of the* 4*th International Conference of Asian Allelopathy Society*, Tokyo, 13, 52.

[30] Ogita, S., Asrori, M.I. and Sasamoto, H. (2020) Establishment of Pluripotent Cell Cultures to Explore Allelopathic Activity of Coffee Cells by Protoplast Co-Culture Bioassay Method. *Plants* (*MDPI*), **9**, 1170. https://doi.org/10.3390/plants9091170

[31] Mardani-Korrani, H., Sasamoto, H., Suzuki, S., Sasamoto, Y. and Fujii, Y. (2020) Application of the Protoplast Co-Culture Method for Evaluation of Allelopathic Activities of Volatile Compounds Safranal and Tulipalin A. *Results in Chemistry*, **2**, 1-5. https://doi.org/10.1016/j.rechem.2020.100030

[32] Murashige, T. and Skoog, F. (1962) A Revised Medium for Rapid Growth and Bioassays with Tobacco Tissue Cultures. *Physiologia Plantarum*, **15**, 473-497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

[33] Rasband, W.S. (1997-2016) Image J. U.S. National Institute of Health, Bethesda, Maryland, USA. https://imagej.nih.gov/ij

[34] Sasamoto, H., Suzuki, S., Mardani-Korrani, H., Sasamoto, Y. and Fujii, Y. (2021) Allelopathic Activities of Three Carotenoids, Neoxanthin, Crocin and β-Carotene, Assayed Using Protoplast Co-Culture Method with Digital Image Analysis. *Plant Biotechnology*, **38**. https://doi.org/10.5511/plantbiotechnology.20.1211a

[35] Sasamoto, H. and Ashihara, H. (2014) Effect of Nicotinic Acid, Nicotinamide and Trigonelline on the Proliferation of Lettuce Cells Derived from Protoplasts. *Phytochemistry Letters*, **7**, 38-41. https://doi.org/10.1016/j.phytol.2013.09.008

[36] Tamura, S., Chang, C.F. and Suzuki, A. (1969) Chemical Studies on "Clover Sick-

ness". Part I. Isolation and Structural Elucidation of Two New Isoflavonoids in Red Clover. *Agricultural and Biological Chem*istry, **33**, 391-397. https://doi.org/10.1080/00021369.1969.10859321

[37] Chang, C.F., Suzuki, A., Kumai, S. and Tamura, S. (1969) Chemical Studies on "Clover Sickness". Part II. Biological Functions of Isoflavonoids and Their Related Compounds. *Agricultural and Biological Chem*istry, **33**, 398-408. https://doi.org/10.1080/00021369.1969.10859325

[38] Rashid, M.H., Asaeda, T. and Uddin, M.N. (2010) The Allelopathic Potential of Kudzu (*Pueraria pueraria*). *Weed Science*, **58**, 47-55. https://doi.org/10.1614/WS-09-106.1

[39] Oshima, A., Mine, W., Nakada, M. and Yanase, E. (2016) Analysis of Isoflavones and Coumestrol in Soybean Sprouts. *Bioscience*, *Biotechnology*, *and Biochemistry*, **80**, 2077-2079. https://doi.org/10.1080/09168451.2016.1196577

[40] Sugiyama, A., Yamazaki, Y., Hamamoto, S., Takase, H. and Yazaki, K. (2017) Synthesis and Secretion of Isoflavones by Field-Grown Soybean. *Plant and Cell Physiology*, **58**, 1594-1600. https://doi.org/10.1093/pcp/pcx084

[41] Okutani, F., Hamamoto, S., Aoki, Y., Nakayasu, M., Nihei, N., Nishimura, T., Yazaki, K. and Sugiyama, A. (2019) Rhizosphere Modeling Reveals Spatiotemporal Distribution of Daidzein Shaping Soybean Rhizosphere Bacterial Community. *Plant*, *Cell and Environment*, **43**, 1036-1046. https://doi.org/10.1111/pce.13708

[42] Kato-Noguchi, H. (2003) Allelopathic Substances in *Pueraria thunbergiana. Phytochemistry*, **63**, 577-580. https://doi.org/10.1016/S0031-9422(03)00195-X

[43] Kobayashi, K., Sasamoto, H. and Fujii, Y. (2020) Search and Evaluation of Substances; to Control the Twining Weeds. *Proceedings of the* 59*th Weed Science Society of Japan* (*WSSJ*) *Annual Meeting*, Nagano, 11-12 April 2020, 27, 97.