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In vitro Bioassay of Allelopathic Activities of Soybean, and Three Isoflavones, Using Protoplast Co-culture Method with Digital Image Analysis

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Abstract

Inhibitory allelopathic activities of dried leaves of potted plants and protoplasts of *Glycine max* (soybean) cv. Okuhara-wase were studied using two *in vitro* bioassay methods. Strong inhibition (90%) of growth of recipient lettuce root was obtained with 50 mg soybean leaves using the lettuce seedling growth test (sandwich method), and moderate inhibition (65%) of lettuce protoplast division was obtained with 100×10^3 mL^{-1} protoplasts of etiolated soybean seedlings using the protoplast co-culture method with digital image analysis (DIA-PP method). Three isoflavones, genistein, genistin (genistein-7-*O*-glucoside), and daidzin (daidzein-7-*O*-glucoside) were investigated as putative allelochemicals using the DIA-PP method. The three isoflavones at 100 μM showed early crystal accumulation in lettuce protoplasts and inhibited lettuce protoplast division by 100%. Genistein showed the strongest inhibition. The results were compared and discussed with the previous reports on the invader leguminous plant, Kudzu, and two isoflavones, daidzein and Kudzu-specific puerarin (daidzein-6-C-glucoside), and other allelopathic plants and their putative allelochemicals.

Keywords: allelopathy, bioassay, image analysis, isoflavone, lettuce, protoplast culture, soybean

1. Introduction

Allelopathy is a strategy by which the plant produces specific allelochemicals to inhibit the growth of neighboring plants, although in the broad sense allelopathy includes stimulation effects and effects of not only plants but also of animals and microorganisms (Fujii, 2000). Several *in vitro* bioassay methods of allelopathy have been developed, *e.g.*, seedling growth test (the sandwich method for dried leaves (Fujii *et al*., 2003; 2004) and the plant box method (Fujii *et al*., 2007) for intact roots of test plants) using lettuce as a recipient. The protoplast co-culture method (Sasamoto *et al*., 2013) with digital image analysis (DIA-PP method, Sasamoto *et al*., 2017a,b) is a newly developed method to elucidate the underlying cellular mechanism(s) of allelopathy.

Glycine max (L.) Merr. (soybean) is a leguminous agricultural crop known as a source of proteins and isoflavones with different chemical structures valuable for human health (Sumardi *et al.*, 2017). Different isoflavone types, *i.e.*, glycosides of two aglycons, genistein and daidzein, have been reported in different organs tissues of soybean at different growth stages, and rhizosphere soil (Okutani *et al*., 2019; Oshima *et al*., 2016; Sugiyama *et al*., 2017) using different cultivars.

Analysis using the DIA-PP method (Kobayashi *et al*., 2021) revealed that the cotyledon protoplasts of the invader leguminous plant, *Pueraria montana* (*P. lobata*, Kudzu), at 10^4 mL^{-1} inhibited lettuce protoplast division by 100%. Two isoflavones, daidzein and puerarin (6-C-glucoside of daidzein), were evaluated as putative allelochemicals in Kudzu. Unexpectedly, puerarin at a high concentration was not inhibitory. However, daidzein at 100 μM was strongly inhibitory and was suggested to be responsible for the strong allelopathic activity of Kudzu protoplasts. Daidzein formed specific crystals in the co-cultured lettuce protoplasts (Kobayashi *et al*., 2021).

Soybean showed lower inhibitory allelopathic activity than Kudzu when examined by the plant box method

(Fujii 2000; Kobayashi et al, 2021). Kudzu (10 mg of leaves) showed moderate inhibitory activity by the sandwich method using lettuce as a recipient (Itani et al., 1998).

On the other hand, the allelopathic activities differed with the cultivar of rice, and higher activity was shown in wild species by the plant box method (Fujii 2000). Analysis by the sandwich method, revealed higher activity in young leaves of potted plants (Mori *et al*., 2015) than in leaves of field-grown mature plant of leguminous *Mucuna gigantea* (Fujii & Hiradate, 2003). Differences in the allelopathic activities with the *in vitro* bioassay methods, *i.e.,* the sandwich method and protoplast co-culture method, have been reported using several plants and putative allelochemicals, *e.g*., four bamboo species (Ogita & Sasamoto, 2017), poplar, birch and abscisic acid (Sasamoto & Yokota, 2021), and *Spiraea thunbergii* and *trans*-cinnamic acid (Suzuki *et al*., 2021).

In the present study, young leaves of the potted plant of soybean cv. Okuhara-wase, which is a very early maturing cultivar and sensitive to water-logging like other soybean cultivars, were used in the sandwich method. The etiolated seedlings of the same cultivar were also used in the DIA-PP method. As putative allelochemicals, three isoflavones, genistein and genistin (7-*O*-glucoside of genistein), and daidzin (7-*O*-glucoside of daidzein) were examined at three growth stages of recipient lettuce protoplasts, *i.e.*, cell wall formation, cell division, and yellow pigment accumulation. Early crystal formation of isoflavones in the co-cultured lettuce protoplasts was also studied. The results were compared with previous findings obtained by the DIA-PP method using Kudzu protoplasts, and two isoflavones, daidzein and Kudzu-specific puerarin (Kobayashi *et al*., 2021), and with reports on other allelopathic plants and their putative allelochemicals.

2. Method

2.1 Sandwich Method Using Soybean Leaves

Leaves of two-month-old potted plants of *Glycine max* cv. Okuhara-wase (soybean) were dried at 60˚C for 16hrs. The sandwich method was performed as previously reported (Fujii *et al*., 2003, 2004). Briefly, 10 and 50 mg of dried leaves were sandwiched between two layers of 5 ml of 0.5% agar (powder, gelling temp. 30˚C - 31˚C, Nacalaitesque Co. Ltd. Kyoto, Japan) in six multi-well plates (Nunc). Length of hypocotyls and roots of germinated seeds of *Lactuca sativa* (lettuce) cv. Great Lakes 366 was measured after three days of incubation at 20˚C in the dark. The control treatment consisted of seeds germinated in the absence of dried leaves. Data were recorded as % growth of the control and averaged with standard deviation (SD).

2.2 Protoplast Co-culture Method

2.2.1 Plant Materials

Soybean seeds of cv. Okuhara-wase were sterilized with 70% EtOH solution for 10 sec and 0.5% NaClO solution for 5 min and washed with autoclaved water three times. Early swollen seeds were not used. Etiolated seedlings were aseptically grown with 1/4 sized paper towel, wetted with 10 mL autoclaved water, in a plant box covered with aluminum foil, for 7 to 14 days at 25˚C in the dark. One mL of autoclaved water was supplied every 2 days of culture to avoid water logging.

Lettuce seedlings were prepared as described previously (Sasamoto *et al*., 2013). Briefly, lettuce seeds 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 0.8% agar medium in a 15 ml tube in the light condition (60 μE s^{-1}) at 24 ℃ for 6-9 days.

2.2.2 Protoplast Isolation and Purification

Optimal combination of cell wall degrading enzymes and osmotic condition for soybean protoplast isolation were determined from preliminary experiments, using 24 combinations of six kinds of enzymes, *i.e.,* Cellulase R10, Cellulase RS, Hemicellulase, Driselase 20, Macerozyme R10, Pectolyase Y-23, in 0.4 M to 0.8 M mannitol solution in a 24-well plastic culture plate (Sasamoto *et al*., 2019). A part of the surface of etiolated seedlings was peeled off and treated with the selected enzyme combination, 1% each of Cellulase RS, Driselase 20, and Macerozyme R10 in 0.8 M mannitol solution, at 27 ℃ for 18 hrs under a static condition in the dark.

Protoplasts of lettuce cotyledons were isolated with 1% each of Cellulase RS and Macerozyme R10 (Sasamoto *et al*., 2013) in 0.8 M mannitol solution in a 100 mL flask. After filtration with a mesh (pore size of 95 μm for soybean or 63 μm for lettuce), the protoplasts were washed three times with 0.8 M mannitol solution by centrifugation at 100*g* for 5 min.

2.2.3 Culture of Soybean Protoplasts

Effects of different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D), 0, 0.1, 1, 10, 100 μM, and benzyladenine (BA), 0, 0.01, 0.1, 1, 10 μM, were investigated using 50 μL of liquid Murashige and Skoog's (MS,

Murashige & Skoog, 1962) basal medium containing 3% sucrose and 0.8 M mannitol in each well of a 96-well culture plate (Falcon No. 3072 or 3075). NH_4NO_3-free MS (MMS) basal medium with 0.6 M mannitol was also used, which made final 0.67 M mannitol condition. Five μL of soybean protoplasts in 0.8 M mannitol solution were put into the 50 μL medium. Initial protoplast densities of culture were adjusted to be 10-100 $\times 10^3$ mL^{-1}.

100 μL autoclaved pure water was added in between the wells and sealed with two layers of Parafilm^R. The protoplasts were cultured in the dark at 28 ℃ in a humid incubator (CO_2-incubator without the supply of CO_2, APC-30DR, ASTEC Co. Ltd.).

Non-spherically enlarged protoplasts, divided protoplasts and colonies were observed, and the numbers were counted under an inverted microscope (Olympus CK40) periodically. Protoplast growth was also described as the percentage of initial plated numbers of protoplasts in each well (plating efficiency).

2.2.4 Co-culture of Soybean and Lettuce Protoplasts

Fifty μL of liquid MS basal medium containing 1 μM 2,4-D and 0.1 μM BA, 3% sucrose and 0.8 M mannitol were put in each well of a 96-well culture plate. Five μL each of suspension of soybean and lettuce protoplasts at 10 times the final protoplast density in 0.8 M mannitol solution was added to each well. Final protoplast density was 5-200 $\times 10^3$ mL^{-1}. After 7-8 days of co-culture, the non-spherically enlarged and divided protoplasts of lettuce were counted under an inverted microscope. After 39 days of co-culture, the number of colonies was counted. Percentage of control values without soybean protoplasts was calculated, and the average percentage of control at different densities of lettuce protoplasts (10-200 $\times 10^3$ mL^{-1}) with standard error (SE) was obtained at different densities of soybean protoplasts. Yellow pigment accumulation of lettuce protoplasts was analyzed using digital image analysis described in *2.4*.

2.3 Protoplast Culture of Lettuce with Isoflavones

Isoflavones, daidzin, genistin and genistein were dissolved to make 10 mg mL^{-1} and diluted in filter-sterilized DMSO (Milipore PTFE membrane). One μL of each solution was put into 50 μL of the same MS basal medium containing 1 μM 2,4-D, 0.1 μM BA, and 3% sucrose as described in 2.2.4 except for 0.4 M mannitol. The final concentration of DMSO was 2% as previously described (Inoue *et al*., 2015). Number of non-spherically enlarged and divided protoplasts of lettuce was counted after 4 and 8 days of culture. Percentages of control values without isoflavone were calculated, and then the percentages at different densities of lettuce protoplasts (5-80 $\times 10^3$ mL^{-1}) were averaged with SEs. Yellow pigment accumulation in lettuce protoplasts after 28 days of co-culture was analyzed as in *2.4*.

2.4 Digital Image Analysis of 96 Well Culture Plate

Image analysis of yellow pigment accumulation of lettuce protoplasts was performed after 3 weeks to 8 months of co-culture as described previously (Sasamoto *et al*., 2017a,b). Digital image of the 96-well culture plate was obtained using a scanner (CanoScan D2400U or Epson GTX-970). Image analysis by software Image J (Rasband, 1997-2016) 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 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 soybean protoplast or at each concentration of isoflavones was subtracted as a control. The % yellow value of control without isoflavones or soybean protoplasts was calculated at each lettuce protoplast density. Finally, the percentages were averaged with SEs at different protoplast densities of lettuce (10-100 $\times 10^3$ mL^{-1}).

3. Results & Discussion

3.1 Effect of Soybean Leaves Examined by Sandwich Method

Leaves of soybean cv. Okuhara-wase had an average length of 5.9 cm, average fresh weight of 0.18 g, and water content of 74%.

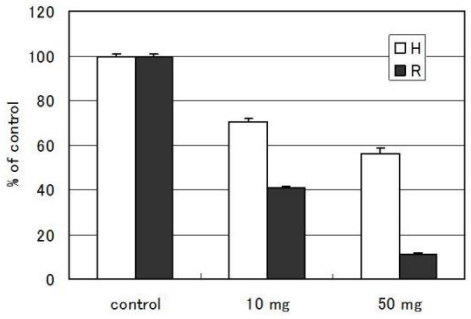

Figure 1. Effect of dried leaves of soybean cv. Okuhara-wase on the growth of hypocotyl (H) and root (R) of lettuce seedlings examined by the sandwich method

As shown in Figure 1, 50 mg of dried leaves of soybean cv. Okuhara-wase strongly inhibited (90%) the growth of recipient lettuce roots. Growth of lettuce hypocotyl was also inhibited but not as much as root growth. This resembled the strong inhibitory activity of young leaves of an invader tree, *Leucaena leucocephala,* and young leaves of the potted plant of woody leguminous plant, *Mucuna gigantea* (80-90% inhibition by 50 mg leaves, Mori *et al*., 2015), while field-grown material of the latter was only moderately inhibitory (Fujii & Hiradate, 2003). We used young leaves of two-month-old potted plants of soybean cv. Okuhara-wase, grown in the same way as the leguminous mangrove tree, *Derris indica* (*Pongamia pinnata*). *D. indica* showed strong inhibition by both the sandwich method and protoplast co-culture method, and an isoflavonoid, rotenone was identified to be the allelochemical (Inoue *et al*., 2015).

Growth of the recipient lettuce was inhibited moderately (60%) by 10 mg dried soybean leaves (Figure 1). Similar moderate inhibition (52%) by 10 mg of Kudzu leaves was reported using lettuce as a recipient plant (Itani *et al*., 1998).

By the plant box method using the intact root, the soybean root (cultivar unknown) showed weak inhibition (16%) (Fujii, 2000). It was much lower compared with the strong 80% inhibition of an invader Kudzu (Fujii *et al*., 2007; Kobayashi *et al*., 2021).

The differences in the allelopathic activity in the seedling growth tests of soybean might be due to the difference in the amount of allelochemicals in different tissues at different growth stages.

3.2 Protoplast Isolation and Culture of Soybean cv. Okuhara-wase

Straight elongated etiolated seedlings of soybean, cv. Okuhara-wase, were grown by the water supplying method as described in 2.2.1 overcoming low tolerance to water logging.

Enzyme combination, Cellulase RS, Driselase 20, and Macerozyme R10 in 0.8 M mannitol solution was selected from 24 combinations of 6 cell wall degrading enzymes. Part of the surface needed to be peeled off as in *Vicia villosa* protoplast isolation (Sasamoto *et al*., 2019).

Figure 2 shows the results of protoplast culture of etiolated seedlings of soybean in MS basal medium containing 0.8 M mannitol. Diameter of spherical isolated protoplasts of soybean varied (30-50 μm). Soybean protoplasts grow slowly compared with lettuce protoplasts cultured in the same medium condition (Sasamoto *et al*., 2013). For example, Figure 2a shows the spherical protoplasts remaining after 11 days of culture. Non-spherical enlargement (Figure 2b) and colony development (Figure 2c) occurred in MS basal medium containing both 2,4-D and BA. Figure 2b shows prominent granules in non-spherically enlarged peanut shaped soybean protoplasts. Colonies of soybean protoplasts were not yellow, unlike the lettuce protoplasts.

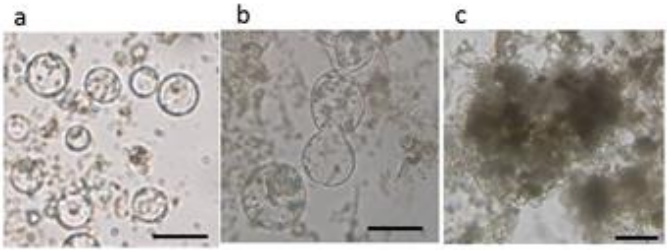

Figure 2. Photographs of cultured protoplasts of soybean cv. Okuhara-wase, in MS basal medium

a, cultured for 11 days in the medium with 1 μM 2,4-D and 0.1 μM BA. b, cultured for 19 days with 10 μM BA. c, colonies cultured for 46 days with 1 μM 2,4-D and 1 μM BA. Medium contained 3% sucrose and 0.8 M mannitol. Initial protoplast density was 20 $\times 10^3$ mL^{-1}. Bar=50 μm in a and b, and 100 μm in c.

As shown in Figure 3, both 2,4-D and BA were needed for efficient colony development from soybean protoplasts. At 1 μM 2,4-D, wide range of BA (0.1-10 μM) was effective. 2,4-D at 100 μM totally inhibited the growth (data not shown). 2,4-D at 1 μM gave the best colony development from soybean protoplasts. In Kudzu protoplasts culture, higher 10 μM 2,4-D gave better and rapid reaction within one week (Kobayashi *et al*., 2021). Soybean protoplast division and colony development occurred but was delayed when compared with those of lettuce protoplast at 1 μM 2,4-D and 0.1 μM BA in MS basal medium, which was used for the protoplast co-culture method for bioassay of allelopathy (*3.3*).

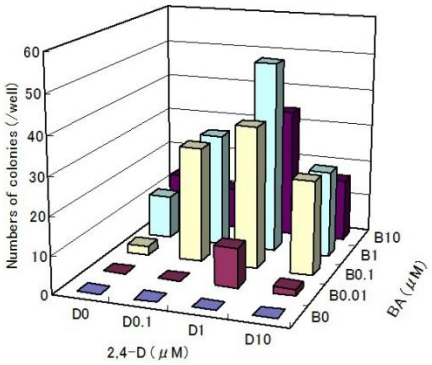

Figure 3. Effects of 2,4-D and BA on colony formation of soybean protoplasts

Average number of colonies in each well after 39-48 days of culture is shown. Initial protoplast density was 50 $\times$ 10^3 mL^{-1}. Basal medium was MS containing 3% sucrose and 0.8 M mannitol.

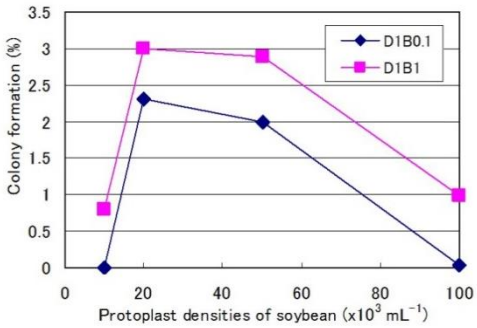

Figure.4. Effects of density of soybean protoplasts on the plating efficiency (% of colony formation) after 46 days of culture. Medium was MS basal medium with 1 μM of 2,4-D and 0.1 or 1 μM of BA, 3% sucrose and 0.8 M mannitol

Figure 4 shows the effects of initial protoplast densities of soybean on the colony development. Colony development was most vigorous at the initial protoplast densities of 20, 50 $\times 10^3$ mL^{-1}.

On the other hand, in MMS basal medium, the number of colonies formed was less than half of that in MS basal

medium (at 50 × 10^3 mL^{-1}, 1 μM 2,4-D and 0.1 or 1 μM BA, data not shown). In enlarged protoplasts, granules, which were prominent in MS basal medium (Figure 2b), were not observed in MMS basal medium (Figure 5a). Instead, some specific cell structures were observed in MMS basal medium (black spots in Figure 5b1 and 5b2).

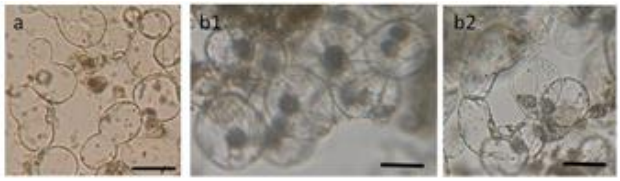

Figure 5. Photographs of cultured protoplasts of soybean, cv. Okuhara-wase in MMS basal medium

a, cultured for 19 days with 0.1 μM of 2,4-D. b1, b2: cultured for 33 days with 1 μM of 2,4-D and 10 μM of BA. Initial protoplast density was 20 × 10^3 mL^{-1} (a), and 50 × 10^3 mL^{-1} (b). Bar=50 μm. Medium contained 3% sucrose and 0.67 M mannitol.

3.3 Effects of Co-cultured Soybean Protoplasts on Lettuce Protoplasts (DIA-PP Method)

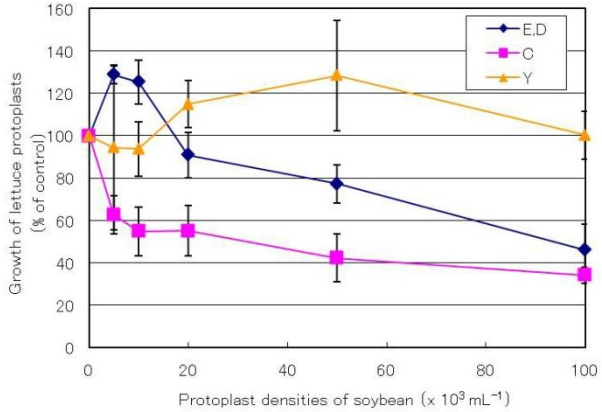

Figure 6. Effect of co-culture of soybean and lettuce examined by DIA-PP method

Non-spherically enlarged (E) and divided (D) lettuce protoplasts at day 7-8, colony formation at day 39 (C), and yellow pigment accumulation (Y) after 8 months. Medium 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 was averaged at 10-200 × 10^3 mL^{-1} of lettuce protoplasts with SE.

As shown in Figure 6, colony formation of lettuce protoplasts (c, averaged at lettuce 10-200 × 10^3 mL^{-1}) was inhibited up to 65% by soybean protoplasts at 100 × 10^3 mL^{-1}. Therefore, soybean protoplasts obtained from etiolated seedlings showed moderate inhibitory allelopathic activity. This was 10 times weaker activity than that of Kudzu protoplasts on cell division of lettuce protoplasts (100% inhibition at 10 × 10^3 mL^{-1}, Kudzu). Kudzu had the second strongest activity among the tested plants using the DIA-PP method (Kobayashi *et al*., 2021).

In Figure 6, cell wall formation and early protoplast division of lettuce after 7-8 days of co-culture was slightly stimulated by co-culture of soybean protoplasts at low densities and gradually inhibited at higher densities. Kudzu was less inhibitory at the cell wall formation stage than at the cell division (colony formation) stage, though the stimulation at a low protoplast density seen in soybean was not observed in Kudzu (Kobayashi *et al*., 2021). Strong (more than 200%) stimulation of cell wall formation by non-embryogenic coffee callus at a low protoplast density has been reported (Ogita *et al*., 2020).

Figure 6 shows the results obtained by co-culture with soybean protoplasts for a longer period examined by the DIA-PP method. As shown in Figure 2a (11 day), soybean protoplasts started cell division after a longer period of incubation. Prominent granules in the enlarged soybean protoplasts (Figure 2b) were not seen in co-culture with lettuce protoplasts. Usually, the lettuce protoplasts and the test plant protoplasts can be easily distinguished under an inverted microscope at the early cell division stage. However, in the co-culture with soybean protoplasts, elongated soybean and lettuce protoplasts were difficult to distinguish. By contrast, in the co-culture of lettuce with Kudzu protoplasts, the non-spherically enlarged Kudzu protoplasts could be distinguished by the

prominent specific dark yellow color of Kudzu observed only when co-cultured with lettuce. Accordingly, the Kudzu-specific enlargement at the early stage of co-culture could be deducted (Kobayashi *et al*., 2021).

At the lowest lettuce protoplast density tested (5×10^3 mL^{-1}), soybean-specific colony formation was distinguished under an inverted microscope (data not shown in Figure 6). Therefore, co-culture with lettuce protoplasts at higher densities ($10\text{-}200 \times 10^3$ mL^{-1}), clearly inhibited the colony formation of soybean protoplasts.

Yellow pigment accumulation of lettuce protoplasts was not inhibited during long period (8 months) co-cultured soybean (Y, Figure 6). Though, at the lowest soybean protoplast density tested (5×10^3 mL^{-1}), large colony formation of lettuce was occasionally observed. Similarly, yellow pigment accumulation was not inhibited after a long protoplast culture period using protoplasts of a mangrove tree, *Kandelia obovata* (Sasamoto *et al*,, in preparation), in which the inhibitory pattern at early growth stages (Sasamoto & Yokota, 2021) was similar to that observed in soybean (Figure 6). In contrast, Kudzu showed some inhibitory allelopathic activity at the yellow pigment accumulation stage (Kobayashi *et al*., 2021).

Differences in the stimulation and inhibition patterns among three growth stages of lettuce were observed among the plant species and putative allelochemicals studied, which seems to reflect the cellular action site of each allelochemical and its content.

3.4 Effects of Isoflavones on Lettuce Protoplasts (DIA-PP Method)

For co-culture of lettuce protoplasts with soybean protoplasts, 0.8 M mannitol, which is optimal for soybean was used (*3.3*). However, as isoflavones were dissolved in DMSO, 2% of which slightly inhibits the lettuce protoplast growth, 0.4 M mannitol concentration was used for lettuce protoplast growth (Inoue *et al*., 2015).

3.4.1 Daidzin

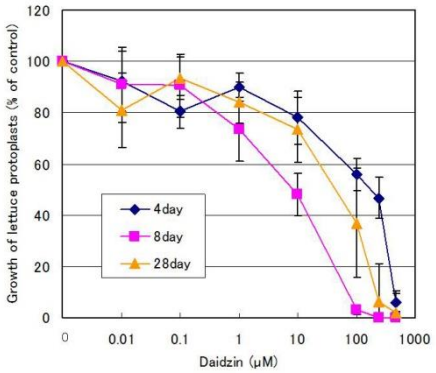

Figure 7. Effect of daidzin examined by DIA-PP method

Medium was the same as that in Figure 6 except for 0.4 M mannitol. Cell wall formation stage (day 4), cell division stage (day 8), and yellow pigment accumulation stage (day 28). Growth % of control was averaged at $5\text{-}80 \times 10^3$ mL^{-1} of lettuce protoplasts with SE.

As shown in Figure 7, lettuce protoplast division was inhibited 100% by 100 μM daidzin. Daidzin is the 7-*O*-glucoside of daidzein, which was examined previously by the DIA-PP method and showed a similar strong inhibitory pattern (Kobayashi *et al*., 2021). Inhibition of cell division by daidzin was stronger than that of daidzein at lower concentrations (1-10 μM). Inhibition of yellow pigment accumulation at high concentrations of daidzin (240-480 μM) was stronger than that of daidzein (390-780 μM) (Kobayashi *et al*., 2021).

Another isoflavone, puerarin (daidzein-6-C-glucoside), which is contained in Kudzu at a high concentration, was not inhibitory but showed slightly stimulatory activity at all three growth stages of lettuce protoplasts by the DIA-PP method (Kobayashi *et al*., 2021). Lettuce protoplasts might not degrade puerarin to daidzein, but daidzin might be incorporated and digested to daidzein.

The inhibitory activity examined by the DIA-PP method was the strongest at the cell division stage among the three growth stages of lettuce protoplasts. The pattern of inhibition was similar to that of soybean protoplasts, Kudzu (Kobayashi *et al*., 2021) and many plant protoplasts and their putative allelochemicals studied. Several putative allelochemicals, *e.g*., *trans*- and *cis*-cinnamic acid (Suzuki *et al*., 2021) and volatile compounds, tulipalin A and safranal showed the strongest inhibition at the cell wall formation stage (Mardani-Korrani *et al*., 2020).

3.4.2 Genistin

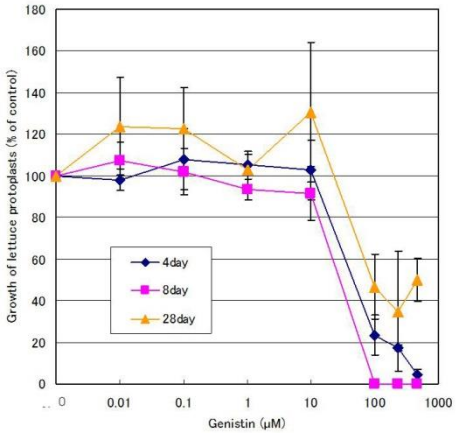

Figure 8. Effect of genistin examined by DIA-PP method

Medium was the same as that in Figure 6 except for 0.4 M mannitol. Cell wall formation stage (day 4), cell division stage (day 8), and yellow pigment accumulation stage (day 28). Growth % of control was averaged at 5-80 $\times 10^3$ mL^{-1} of lettuce protoplasts with SE.

As shown in Figure 8, lettuce protoplast division was inhibited 100% by 100 μM genistin. The 7-*O*-glucoside of genistein, genistin inhibited cell division of lettuce protoplasts strongly as did daidzin (Figure 7) and daidzein (Kobayashi *et al*., 2021). Inhibition at the cell wall formation stage was stronger than that of daidzin. Inhibition of yellow pigment accumulation at a high concentration was weaker than that of daidzin, but similar to that of daidzein (Kobayashi et al., 2021). No inhibition was observed at up to 10 μM.

3.4.3 Genistein

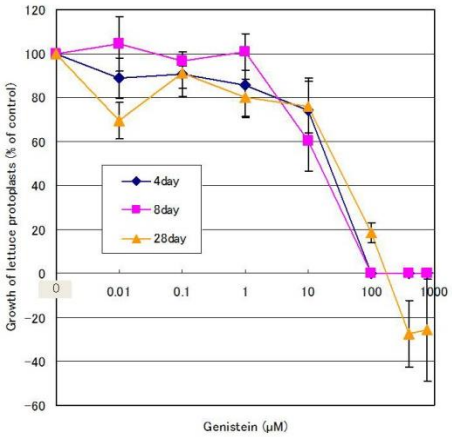

Figure 9. Effect of genistein examined by DIA-PP method

Medium was the same as that in Figure 6 except for 0.4 M mannitol. Cell wall formation stage (day 4), cell division stage (day 8), and yellow pigment accumulation stage (day 28). Growth % of control was averaged at 5-80 $\times 10^3$ mL^{-1} of lettuce protoplasts with SE.

As shown in Figure 9, 100 μM genistein strongly inhibited both cell wall formation and cell division. Genistein was the strongest inhibitor among tested isoflavones. Yellow pigment accumulation was inhibited ca.130% at high concentrations (390-780 μM).

Such strong more than 100% inhibition at high concentrations on yellow pigment accumulation was also reported with DIA-PP method of yellow carotenoids, crocin and neoxanthin. It was discussed as inhibition of yellow carotenoid(s) synthesis, and degradation of exogenously supplied yellow carotenoids by co-cultured lettuce protoplasts (Sasamoto *et al*., 2021). However, strong inhibition by genistein might be explained by the

broken protoplasts described later (3.4.4).

Daidzein (Kobayashi *et al*., 2021) and daidzin (Figure 7) showed stronger inhibition at the cell division stage than at the cell wall formation stage, genistin (Figure 8) showed slight stronger inhibition, but the activity of genistein (Figure 9) did not show any difference among the stages.

The four isoflavones, daidzein (Kobayashi *et al*., 2021), daidzin, genistein, genistin, which inhibited lettuce protoplast division 100% at 100 μM belong to the group of chemicals with strong allelopathic activity studied using the protoplast co-culture method. Similar inhibitory activity has been reported, *e.g.*, canavanine in *Vicia villosa* (Sasamoto *et al*., 2019)*;* L-DOPA in *Mucuna pruriens* (Sasamoto *et al*., 2013); isoflavonoid, rotenone in *Derris indica* (Inoue *et al*., 2015); cinnamic acid and tulipalin A (Mardani-Korrani *et a*l., 2020) in *Spiraea thunbergii* (Suzuki *et al*., 2021).

3.4.4 Crystal Formation in Lettuce Protoplasts by Isoflavones

Figures 10 and 11 show the photographs of lettuce protoplasts cultured for one day and 8 days in the medium used for co-culture with soybean protoplasts, except for 0.4 M mannitol.

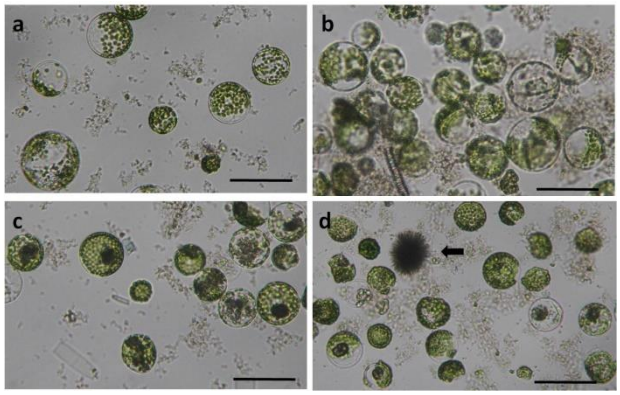

Figure 10. Photographs of lettuce protoplasts after 1 day of culture without treatment (a) or with 100 μM of daidzin (b), genistin (c) or genistein (d). Lettuce protoplast density was 10×10^3 mL^{-1}. Medium was MS basal medium containing 1 μM 2.4-D, 0.1 μM BA, 3% sucrose and 0.4 M mannitol. Bar=50 μm. Arrow head shows black spot on the outside of cells in (d)

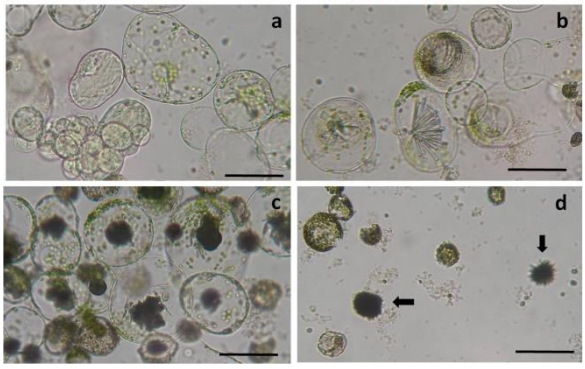

Figure 11. Photographs of lettuce protoplasts after 8 days of culture without treatment (a) or with 100 μM of daidzin (b), genistin (c), or genistein (d). Lettuce protoplast density was 40×10^3 mL^{-1} (a), 10×10^3 mL^{-1} (b, c), and 20×10^3 mL^{-1} (d). Medium was MS basal medium containing 1 μM 2.4-D, 0.1 μM BA, 3% sucrose and 0.4 M mannitol. Bar=50 μm. Arrow head shows black spot on the outside of cells in (d)

Rapid uptake of isoflavones by lettuce protoplasts occurred after 1 day of culture (Figure 10), and showed some damage on spherical enlargement of protoplast. Genistein showed the strongest activity. Crystals shown in Figure 11b (100 μM daidzin) were similar to those reported for daidzein (Kobayashi *et al*., 2021). Daidzin might be metabolized to daidzein before or after incorporation to lettuce protoplasts.

Black spot was observed in non-spherically enlarged lettuce protoplasts co-cultured with genistin (Figure 11c). Genistin at 100 μM inhibited protoplast division completely. However, alive cells were still observed depending on the lettuce protoplast density. Similar black spots were observed in enlarged soybean protoplast cultures (Figure 5b1 in the MMS basal medium).

Genistein damaged the lettuce protoplasts the most. As shown in Figure 10d, lettuce protoplasts started to shrink after 1 day of culture. Some crystals were seen in the protoplasts after 1 day (Figures 10d) and many black spots were observed on the outside of the cell after 8 day (arrow heads in Figure 11d). The strong inhibition of yellow accumulation at about one month (Figure 9) might be caused by such crystal formation and damage of cells. The strong inhibition (more than 100%) of yellow pigment accumulation might be due to the broken and shrunken lettuce protoplasts. Such phenomena might be different from those of carotenoids, which also inhibited more than 100% at 100 μM, which might be degraded by lettuce protoplasts (Sasamoto *et al*., 2021).

3.5 Evaluation of Isoflavones as Allelochemicals in Soybean

In different soybean cultivars, *e.g*., Fukuyutaka, Enrei, Shintambaguro, different contents (1-3 μmol / g dry weight) of isoflavones were obtained in sprouts, leaves and roots and in the rhizosphere soil (Okutani *et al*., 2019; Oshima *et al*., 2016; Sugiyama *et al*., 2016, 2017). Daidzin, genistin, and their malonyl-deriatives were found in both leaves and roots of soybean, while daidzein and genistein were found in roots and rhizosphere but not in leaves (Sugiyama *et al*., 2017). Such tissue differences might be a cause of the different inhibition % obtained by the bioassay methods, *e.g.*, the sandwich method using dried leaves and the plant box method using intact roots, and the DIA-PP method using protoplasts. Differences of allelopathic activities among cultivars investigated in rice using plant box method (Fujii 2000) had not been reported in soybean.

In the previous study using the DIA-PP method (Kobayashi *et al*., 2021), the invader Kudzu showed strong allelopathic activity, and daidzein was a strong inhibitor (100% inhibition at 100 μM), but the Kudzu-specific isoflavone, puerarin (daidzein-6-C-glucoside) was not inhibitory. At the predicted water content of 90%, the content of the isoflavone, daidzein (*e.g*., 0.07 mg/g dry weight) was low (30 μM) and it was considered to be one of the allelochemicals in Kudzu. However, puerarin, which was found at high concentrations (*e.g.*, 13 mg/g dry weight root), could not be the allelochemical in the Kudzu protoplast. Single chemicals found in large amounts in the plant are not necessarily the main allelochemicals.

In soybean tissues, contents of genistein, genistin, daidzein and daidzin were also calculated to be about 10 – 30 μM. In the present study using the DIA-PP method, daidzin, genistein and genistin were strongly inhibitory (100% inhibition at 100 μM). Daidzein and genistein and their 7-*O*-glucosides are likely to be allelochemicals in soybean protoplasts. Genistein was the strongest inhibitory isoflavone among the tested isoflavones.

In this study, isoflavones formed crystals in lettuce protoplasts at a very early stage of culture. Similar inhibition patterns were also observed at later growth stages of cell division. Such early observation under an inverted microscope might be usable for assessment of allelopathic activity at a very early stage and with very small number of recipient protoplasts and medium volume, and very small amount of allelochemicals.

Many cultivars of soybean are known, but most of them are not tolerant to water-logging, as described in the method of preparation of etiolated seedlings of cv. Okuhara-wase (2.2.1). In contrast, wild soybean (Tsurumame, *Glycine soja* Sieb. et Zucc.) is river side-grown and its germination and seedling growth in water is possible. Allelopathic activities of different soybean cultivars with different tolerance to water-logging might be an interesting theme.

Therefore, the DIA-PP method using lettuce as a recipient for bioassay of allelopathy will be a useful tool for elucidating the mechanism(s) of allelopathy at a cellular level.

4. Conclusion

Allelopathic activities of soybean cv. Okuhara-wase, which is a very early maturing cultivar and sensitive to water-logging, were studied by two *in vitro* bioassay methods. Growth of recipient lettuce roots was inhibited 90% by 50 mg dried leaves of soybean examined by the lettuce seedling growth test (sandwich method). The DIA-PP method revealed moderate inhibition (65%) of lettuce protoplast division with 100×10^3 mL^{-1} protoplasts of etiolated soybean seedlings. Growth at the cell wall formation stage was stimulated slightly by soybean protoplasts at a low protoplast density. No inhibition was observed at the yellow pigment accumulation stage.

The results obtained by the DIA-PP method showed that genistein, genistin, and daidzin, at 100 μM inhibited 100% lettuce protoplast division. Early crystal accumulation of each isoflavone was observed in lettuce protoplasts under an inverted microscope. Genistein showed the strongest inhibition.

The reported contents of the four isoflavones, genistein and daidzein and their 7-*O*-glucosides, in soybean tissues indicated that they were putative allelochemicals in soybean.

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References

Fujii,Y. (2000). *Allelopathy*, Nobunkyo, Tokyo.

Fujii, Y., & Hiradate, S. (Eds.). (2003). *Research achievement of allelopathy laboratory in NIAES, 1983 to 2001*. pp. 1072.

Fujii, Y., Parvez, S. S., Parvez, M. M., Ohmae, Y., & 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

Fujii, Y., Shibuya, T., Nakatani, K., Itani, T., Hiradate, S., & Parvez, M. M. (2004). Assesment 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

Fujii, Y., Pariasca, D., Shibuya, T., Yasuda, T., Kahn, B., & Waller, G. R. (2007). Plant Box Method: A specific bioassay to evaluate allelopathy through root exudates. In Y. Fujii & S. Hiradate (Eds.), *Allelopathy new concepts and methodology* (pp. 39-56). Science Publishers, Inc.: Enfield, New Hampshire, USA. https://doi.org/10.1201/9780367813673-4

Inoue, A., Mori, D., Minagawa, R., Fujii, Y., & Sasamoto, H. (2015). Allelopathy in a leguminous mangrove plant, *Derris indica*: Protoplast co-culture bioassay and rotenone effect. *Natural Product Communications, 10*(5), 747-750. https://doi.org/10.1177/1934578X1501000512

Itani, T., Hirai, K., Fujii, Y., Kohdai, H., & Tamaki, M. (1998). Screening for allelopathic activity among weeds and medicinal plants using the "Sandwich Method". *Journal of Weed Science and Technology, 43*(3), 258-266. https://doi.org/10.3719/weed.43.258

Kobayashi, K., Sasamoto, H., Sasamoto, Y., Sugiyama, A., & 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

Mardani-Korrani, H., Sasamoto, H., Suzuki, S., Sasamoto, Y., & 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

Mori, D., Ogita, S., Fujise, K., Inoue, A., & 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

Murashige, T., & Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum, 15*, 473-497. http://dx.doi.org/10.1111/j.1399-3054.1962.tb08052.x

Ogita, S., & 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

Ogita, S., Asrori, M. I., & Sasamoto, H. (2020). Establishment of pluripotent cell cultures to explore allelopathic activity of coffee cells by protoplast co-culture bioassay method. *Plants, 9*, 1170. https://doi.org/10.3390/plants9091170

Okutani, F., Hamamoto, S., Aoki, Y., Nakayasu, M., Nihei, N., Nishimura, T., Yazaki, K., & Sugiyama, A. (2019). Rhizosphere modeling reveals spatiotemporal distribution of daidzein shaping soybean rhizosphere bacterial community. *Plant, Cell and Environment*, 1-11. https://doi.org/10.1111/pce.13708

Oshima, A., Mine, W., Nakada, M., & 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

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

Sasamoto, H., Murashige-Baba, T., Inoue, A., Sato, T., Hayashi, S. & 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

Sasamoto, H., Azumi, Y., & Suzuki, S. (2017a). Development of a high-throughput bioassay method of allelopathy: Protoplast co-culture and digital image analysis. *Science Journal of Kanagawa University, 28*, 63-70.

Sasamoto, H., Azumi, Y., Shimizu, M., Hachinohe, Y., & Suzuki, S. (2017b). In vitro bioassay of allelopathy of *Arabidopsis thaliana* by sandwich method and protoplast co-culture method with digital image analysis. *Plant Biotechnology, 34*(4), 199-202. https://doi.org/10.5511/plantbiotechnology.17.1204a

Sasamoto, H., & Yokota, S. (2021). *In vitro* bioassay of allelopathic activities of a mangrove tree, *Kandelia obovata*, and fast-growing trees, *Betula platyphylla* and *Populus alba*, using protoplast co-culture method. *Journal of Plant Studies, 10*, 8-19. https://doi.org/10.5539/jps.v10n2p8

Sasamoto, H., Suzuki, S., Mardani-Korrani, H., Sasamoto, Y. & 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*, 101-107. https://doi.org/10.5511/plantbiotechnology.20.1211a

Sasamoto, H, Mardani-Korrani, H, Sasamoto, Y., Wasano, N., Murashige-Baba, T., Sato, T., Hasegawa, A., & 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 & Developmental Biology-Plant, 55*, 296-304. https://doi.org/10.1007/s11627-019-09985-3

Sugiyama, A., Yamazaki, Y., Yamashita, K., Takahashi, S., Nakayama, T., & Yazaki, K. (2016). Developmental and nutritional regulation of isoflavone secretion from soybean roots. *Bioscience, Biotechnology, and Biochemistry, 80*, 89-94. http://dx.doi.org/10.1080/09168451.2015.1062714

Sugiyama, A., Yamazaki, Y., Hamamoto, S., Takase, H., & 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

Sumardi, D., Pancoro, A., Yulis, E., Musfiroh, I., Pasetiyono, J., Karuniawan, A., & Syamsudin, T. S. (2017). Potential of local black soybean as a source of the isoflavones daidzein and genistein. *International Food Research Journal, 24*, 2140-2145. Retrieved from http://www.ifrj.upm.edu.my

Suzuki, S., Kimura, M., Yasuda, R., Wasano, N., Sasamoto, Y., Fujii, Y., & Sasamoto, H. (2021). 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. *American Journal of Plant Sciences, 12*, 1673-1690. https://doi.org/10.4236/ajps.2021.1211117