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Short Communication

In vitro bioassay of allelopathy of *Arabidopsis thaliana* by sandwich method and protoplast co-culture method with digital image analysis

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Abstract We examined the allelopathic activities of *Arabidopsis thaliana*, ecotype Columbia by two in vitro methods. The effect of dried leaves on the growth of recipient lettuce seedlings was examined by the sandwich method. The allelopathic activity on protoplast growth was examined by co-culture with recipient lettuce leaf protoplasts in 50 μl liquid medium using a 96-well culture plate. Non-spherically enlarged or dividing protoplasts of lettuce were counted under an inverted microscope. Inhibition of yellow accumulation during lettuce protoplast growth was quantitated by image analysis of scanned digital images of 96-well culture plates. The results were described as the percentages of control without *A. thaliana*. The results were compared with those obtained using several plants which had strong allelopathic activities on recipient lettuce using the same methods. The growth of lettuce protoplasts was inhibited at both 4 and 8 days of culture with protoplasts of *A. thaliana*. The results suggested the usefulness of the protoplast co-culture method for future studies on allelopathy.

Key words: allelopathy, *Arabidopsis*, bioassay methods, protoplast culture.

Arabidopsis thaliana (L.) Heynh., a weed belonging to Brassicaceae, is widely used as a model species in plant molecular genetics, with well established protocols for formation of transgenic plants. Allelopathic effects of co-cultivation with clover (Pedersen et al. 2013), allelochemicals of rice (Kato-Noguchi and Kitajima 2015), and an analog of amino acid (Lee et al. 2016), on the growth of *A. thaliana* have been reported. However, there have been few studies on the allelopathic activity of *A. thaliana* itself on other plants, except for the slight stimulatory effects of the seed exudates on cockscomb (Yokotani-Tomita et al. 1998).

Allelopathy of many plant species has been investigated in vitro using the sandwich method, which measures the effects of dried leaves (Fujii et al. 2003, 2004) or extracts (Takemura et al. 2013) on the elongation of hypocotyls and roots of recipient *Lactuca sativa* (lettuce) seedlings. Allelopathic activities of transgenic tree plants have also been studied by this method using lettuce as the recipient (Ishii et al. 2012). However, the effects of *A. thaliana* have not been reported.

Recently, the protoplast co-culture method was introduced for bioassay of allelopathy using protoplasts of etiolated leaves of a leguminous species and lettuce or rice protoplasts as the recipient. They were cultured in 96-well culture plates with 50 μl liquid medium per well, which is a method widely applicable to studies on allelopathy at the cellular level of various plants important in the search for future environmental risks (Sasamoto et al. 2013). For example, inverse relationship was found between allelopathic activities on lettuce protoplasts and salt tolerance using protoplasts from suspension cultured cells of three mangrove tree species, (Hasegawa et al. 2014). The effects of putative allelochemicals and metabolites of several plant species with high allelopathic activities, have also been quantitatively investigated using the same lettuce protoplast culture system (Inoue et al. 2015; Mori et al. 2015; Sasamoto and Ashihara 2014; Sasamoto et al. 2015a, b). We also developed a high-throughput bioassay method using digital image analysis in combination with the protoplast co-culture method (Sasamoto et al. 2017).

In the present study, we examined the allelopathic activity of *A. thaliana* ecotype Columbia using both the sandwich method and protoplast co-culture method with digital image analysis, using lettuce as a recipient plant.

Seeds of *A. thaliana* ecotype Columbia were grown

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in a plant growth chamber as described previously (Hayakawa et al. 2007). Briefly, they were grown on soil under 14 h-light, 10 h-dark photoperiod of 60 μmol $m^{-2}s^{-1}$ of photon flux density fluorescent light, at 24°C and 60% humidity. As nutrients, HYPONeX was supplied weekly. Aseptic seedlings of lettuce for protoplast isolation were prepared as described previously (Sasamoto et al. 2013). Briefly, lettuce seeds 'Great Lake' were sterilized with 1.5% NaClO solution for 15 min and washed with autoclaved water three times. They were cultured on 0.8% agar medium for 11 days under a continuous light condition at 25°C.

For the sandwich method (Fujii et al. 2003, 2004), leaves of *A. thaliana* were dried at 60°C for 18 h and stored until use. Briefly, 10 mg and 50 mg of dried leaves were sandwiched between two layers of 5 ml of 0.75% agar (powder, gelling temp. 30–31°C, Nacalai tesque Co., Ltd. Kyoto, Japan) in 6-well plates (Nunc). Length of hypocotyls and roots of germinated seeds of lettuce on agar was measured after 3 days of incubation at 25°C in the dark. The control treatment consisted of seeds germinated in the absence of dried leaves. Data were recorded as the percentage growth of the control and averaged with standard error (SE).

For protoplast isolation, leaves of *A. thaliana* were sterilized with 0.2–0.5% NaClO solution for 5 min, washed with autoclaved water three times. Protoplasts were isolated in 0.4 M mannitol containing 1% Cellulase R10 and 0.2% Pectolyase Y23 for *A. thaliana* for 3 to 24 h at 25°C in the dark. Enzyme combination for *Arabidopsis* leaves was previously optimized by using six kinds of cell wall degrading enzymes (Azumi and Sasamoto 2017). Protoplasts of lettuce cotyledons were isolated in 1% of Cellulase RS and 1% Macerozyme R-10 in 0.4 M mannitol solution in a flask for 20–24 h (Sasamoto et al. 2013). Protoplasts were purified, after passing through a 63–80 μm sized nylon mesh, by washing 3 times with the mannitol solution followed by centrifugation at 100 *g* (900 rpm) for 5 min. All the procedures were performed in aseptic conditions at room temperature.

For protoplast co-culture of *A. thaliana* with lettuce, protoplast suspensions, 5 μl each in 0.4 M mannitol, were put into 50 μl of liquid medium in a 96-well plastic culture plate (Falcon No. 3075). 100 μl of autoclaved pure water (Milipore Direct-Q UV) was added to the space between the wells and the plate was tightly sealed with two layers of Parafilm®. The protoplasts were cultured at 28°C in a humid incubator (CO_2-incubator without the supply of CO_2, APC-30DR, ASTEC Co., Ltd.). Non-spherically enlarged and divided protoplasts were counted after 4 days of culture, and divided protoplasts and colonies composed of more than 4 cells were counted after 8 days of culture under an inverted microscope (Olympus IX71). The protoplast densities of *Arabidopsis* ranged from 4×10^3 to 10^5 ml^{-1}. *Arabidopsis*

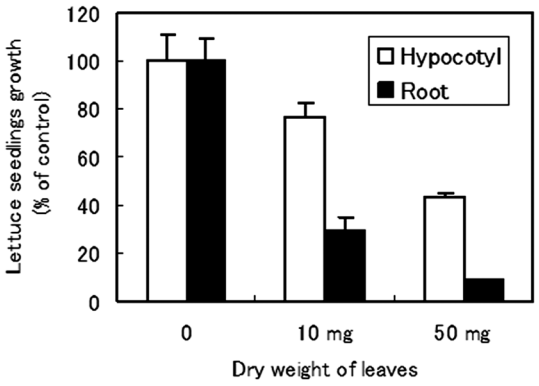

Figure 1. Sandwich method of leaves of *Arabidopsis thaliana* ecotype Columbia. Lettuce growth is the average elongation % of the control with SE of hypocotyls (white) and root (black).

protoplasts were co-cultured with lettuce protoplasts in MS (Murashige and Skoog 1962) basal medium containing 1 μM 2,4-dichlorophenoxyacetic acid and 0.1 μM benzyladenine, 3% sucrose and 0.4 M mannitol. The lettuce protoplast densities ranged from 6×10^3 to 10^5 ml^{-1}. Lettuce protoplast growth was described as the % of control without *A. thaliana* protoplasts. Data were averaged with SE at 6×10^3 to 5×10^4 ml^{-1} lettuce protoplast densities.

For digital image analysis of yellow color accumulation of lettuce protoplasts, each 96-well culture plate was scanned using a scanner (Epson GTX-970) after 29 days of co-culture. Image analysis was performed using Image J (NIH) and Excel software (Sasamoto et al. 2017). Briefly, in Image J, the image, color, channel, blue image was selected, a horizontal straight line was drawn through the center of the wells of 96-well culture plate. The lines were analyzed and the average values of the blue plot values of each well were obtained and saved in an excel file. These values were converted to yellow values in each well by deduction of each averaged blue value from the highest blue value (control). Percentages of control without *Arabidopsis* protoplasts at each lettuce protoplast density (6×10^3 to 10^5 ml^{-1}) were averaged with SE.

Figure 1 shows the effects of dried leaves of *A. thaliana* in the sandwich method. Dried leaves (50 mg) inhibited the growth of lettuce root by ca. 90%, but inhibited the growth of lettuce hypocotyls less than 60%. Dried leaves of a leguminous mangrove, *Derris indica*, inhibited the growth of lettuce root by 80% (Inoue et al. 2015), and the leaves of an invader plant, *Leucaena leucocephala*, inhibited the root growth by 90% in the sandwich method (Mori et al. 2015).

Figure 2 shows the protoplasts of lettuce (A) and *A. thaliana* (B) on the 1st day of culture. Both lettuce and *Arabidopsis* protoplasts were green and similar in size. The *Arabidopsis* protoplasts were greener than the lettuce protoplasts. It was difficult to distinguish two species

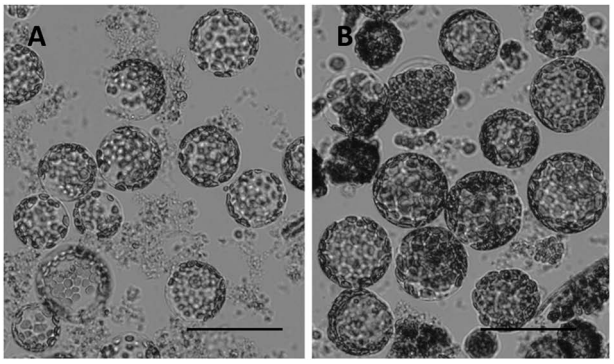

Figure 2. Protoplasts of lettuce (A) and *Arabidopsis* (B) at first day of culture in MS basal medium containing 1 μM of 2,4-D and 0.1 μM of BA, 3% sucrose and 0.4 M mannitol. Protoplast densities were 6×10^3 ml^{-1} (A) and 5×10^4 ml^{-1} (B). Bar=50 μm.

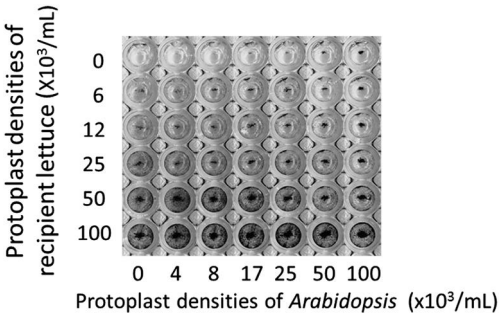

Figure 3. Blue image of 96-well culture plate showing the effects of *A. thaliana* on lettuce after 29 days of protoplast co-culture. Scanned image was analyzed using Image J.

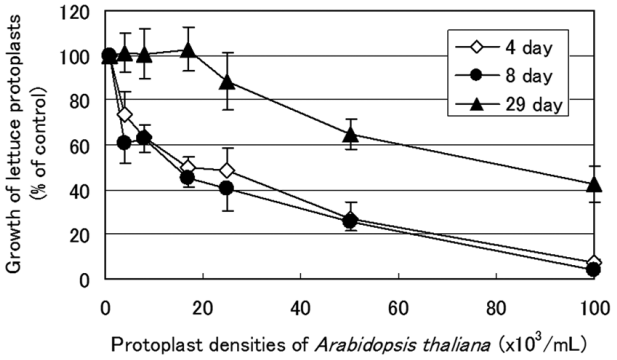

Figure 4. Effects of *A. thaliana* protoplasts on growth of lettuce protoplasts after 4 days (non-spherically enlargement and divisions), 8 days (divisions and colonies), and 29 days (yellow values) of co-culture. Medium was the same as in Figure 2. Growth of lettuce protoplasts is described as the % of control without *A. thaliana* protoplasts, and averaged with SE at different lettuce protoplast densities (6×10^3–10^5 ml^{-1}).

in the same well early in culture. However, *Arabidopsis* protoplasts did not divide well in the same medium condition, growth of lettuce protoplasts (Sasamoto and Ashihara 2014; Sasamoto et al. 2015a, 2015b, 2017), was distinguished from *Arabidopsis* protoplasts under an inverted microscope. Enlarged and divided lettuce protoplasts had yellow color. The yellow color accumulation can be quantified by digital image analysis of scanned image of 96 well culture plate (Sasamoto et al. 2017). Figure 3 shows the blue image obtained from the scanned image using Image J after 29 days of protoplast co-culture.

Figure 4 shows the growth of lettuce, i.e., numbers of non-spherically enlarged protoplasts and divided cells including colonies at early stages of culture, and the accumulation of yellow color after 29 days of co-culture, as the average % of control. Non-spherically enlarged protoplasts are at the cell wall formation stage. The percentage of non-spherically enlarged protoplasts at 4 days of culture was 78%, and that of colonies at 8 days of culture was 46%, in the control without *A. thaliana*. Addition of 10^5 ml^{-1} of *A. thaliana* protoplasts inhibited both cell enlargement and division of recipient lettuce protoplasts at both 4 and 8 days of culture. The inhibitory pattern was similar to that of *D. indica* protoplasts and an isoflavonoid, rotenone, on lettuce protoplast growth (Inoue et al. 2015). However, the protoplast density, 3×10^4 ml^{-1} of *D. indica* totally inhibited lettuce growth. It had less inhibitory activity compared to the invader plant *L. leucocephala* protoplasts, which almost totally inhibited the lettuce protoplast growth at 10^4 ml^{-1} (Mori et al. 2015).

In contrast, the pattern of inhibition of yellow color accumulation at 29 days of culture was different from the above two-stage pattern of cell wall formation and cell divisions. There was no inhibition up to 1.7×10^4 ml^{-1} of *A. thaliana*. Protoplasts at a density of more than 2×10^4 ml^{-1} was inhibitory and only 60% inhibition was observed at 10^5 ml^{-1} of *A. thaliana*. There was no significant difference in the inhibitory pattern analyzed from scanned images between this study (29 days) and a previous study (43 days) (Sasamoto et al. 2017).

This is the first report of strong inhibitory allelopathic activities of leaves of *Arabidopsis* ecotype Columbia obtained using both the sandwich method and the protoplast co-culture method. Similar work is under way on another ecotype of *A. thaliana*, Landsberg.

The cause of the stimulatory effect of root exudates of *Arabidopsis* on cockscomb (Yokotani-Tomita et al. 1998), was considered to be at lower concentrations of allelochemicals to show inhibitory activities (Tomita-Yokotani et al. 2003). Such stimulation at low protoplast concentrations of test plants on the growth of recipient lettuce protoplasts were reported in *Mucuna pruriens* (Sasamoto et al. 2013), and bamboo species (Ogita and Sasamoto 2017), while inhibitory activities were found at high protoplast concentrations. However, differences of test tissues (root) and recipient plant species need to be considered. Strong inhibitory allelopathic activity of *M. pruriens* was reported using another bioassay method of allelopathy, plant box method, which measure the activities of exudates of intact roots (Fujii et al. 2007).

Similarly, the inhibitory allelopathic activities of etiolated leaf of *M. pruriens* have been examined by protoplast co-culture bioassay using both cotyledons and roots of lettuce (Sasamoto et al. 2013). Therefore, by comparing the effects of test plant protoplasts and their metabolites on the growth of lettuce protoplasts at three different stages, i.e., cell wall formation, cell division and yellow color accumulation, it may be possible to find putative allelochemicals and to clarify the mechanism(s) of allelopathy at the cellular level (Sasamoto et al. 2015a, 2015b). Furthermore, the allelopathic activities of transgenic plants of *A. thaliana* containing putative allelochemicals on recipient lettuce remain to be investigated.

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