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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

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Abstract

Allelopathic activities of a salt-tolerant and low-temperature tolerant mangrove tree, *Kandelia obovata*, which grows in brackish water regions of sub-tropical areas, and two fast-growing trees, *Betula platyphylla* and *Populus alba*, which grow in the temperate area, were examined by two *in vitro* bioassay methods, the sandwich method using dried leaves and the protoplast co-culture method using leaf protoplasts. Lettuce root growth examined by the sandwich method, was inhibited 50% by 50 mg dried mature leaves of *K. obovata*. In the protoplast co-culture method, inhibition rates of cell division of lettuce protoplasts were 31% and 69% by leaf protoplasts of *K. obovata* at densities of 1×10^4 mL^{-1} and 5×10^4 mL^{-1}, respectively. These results were compared with the inverse relationship between allelopathic activities and salt tolerance of mangrove plants of different families. *B. platyphylla* showed 37% inhibition by the sandwich method using dried young leaves, but only 10% inhibition at 5×10^4 mL^{-1} by the protoplast co-culture method using leaf protoplasts of *B. platyphylla*. Dried young leaves of *P. alba* showed 66% inhibition, but the leaf protoplasts at the density of 5×10^4 mL^{-1} showed highly stimulatory activity. Abscisic acid, of which contents in leaf protoplasts of three tree species varies from high to low in relation to salt tolerance and recalcitrance of tissue culture, was discussed as a putative allelochemical.

Keywords: abscisic acid, allelopathy, birch, leaf protoplast culture, poplar, salt-tolerant mangrove tree

1. Introduction

1.1 In Vitro Bioassay Method of Allelopathy: the Sandwich Method

Allelopathy is one of the strategies of plants, which cannot move, to survive by emitting allelochemicals to inhibit the growth of neighboring plants sharing the same habitat. In the broad definition of allelopathy, stimulatory effects among plants and microorganisms are also included (Fujii, 2000). As an *in vitro* bioassay method, the sandwich method (Fujii *et al*., 2003), which measures the effects of 10 and 50 mg dried leaves, was used to test many plants including trees using lettuce as a recipient plant (Fujii, 2000; Bergum *et al*., 2019). Lettuce was the most sensitive among the recipient plant species examined including rice (Itani *et al*., 1998).

1.2 In vitro Bioassay Method of Allelopathy: the Protoplast Co-culture Method

The protoplast co-culture method was first developed to measure the effects of herbaceous leguminous plants (*Mucuna pruriens*) on the recipient lettuce or rice protoplasts. It has been applied to study the mechanism(s) of allelopathy at the cellular level and to simulate the possible future environmental risks in the field (Sasamoto *et al*., 2013). In the assay of herbaceous test plants, protoplasts were isolated from several tissues *e.g*., leaf (*M. pruriens*, Sasamoto *et al*., 2013; *Arabidopsis thaliana*, Sasamoto *et al*., 2017a,b); epicotyl and root (*Vicia villosa*, Sasamoto *et al*., 2019); cotyledons (*Pueraria montana*, invasive Kudzu, Kobayashi *et al*., 2021).

The number of plant species tested using the protoplast co-culture method is increasing, including woody and tree species, *e.g*., *Mucuna gigantea*, bamboo species, *Prunus yedoensis*, *Coffea canephora*, and an invasive leguminous tree, *Leucaena leucocephala* (Mori *et al*., 2015; Ogita and Sasamoto, 2017; Fujise *et al*., 2018; Ogita

et al., 2020). Tissue cultured cells (calluses and suspension cultured cells) have been used for protoplast isolation of these tree test plants. Co-culture medium was 50 μL liquid MS (Murashige and Skoog, 1962) basal medium containing 1 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.1 μM benzyladenine (BA), 3% sucrose and different concentrations of osmoticum.

1.3 Salt Tolerance of Mangrove Plants at the Cellular Level

Mangrove plants, mainly tree species of different families, are distributed in brackish waters in tropical and subtropical areas (Tomlinson, 1986; Spalding *et al*., 2010). The highly salt-tolerant or halophilic nature of seaward-side grown species of Sonneratiaceae (*Sonneratia alba*) and Avicenniaceae (*Avicennia alba*) have been studied using tissue-cultured cells and their protoplasts (Kawana and Sasamoto, 2008; Hayashi *et al*., 2009; Hasegawa *et al*., 2013; Sasamoto *et al*., 2020). In addition to stimulation by NaCl, different responses to other sea salt ions (K^+, Mg^{2+}, Ca^{2+}) added to MS basal medium were examined. Comparison of the responses of rice cells and lettuce protoplasts revealed specific Ca^{2+}-related cellular mechanisms of halophilism (Hayatsu *et al*., 2014, 2017).

One of the Rhizophoraceae mangroves, *Kandelia obovata* (Sheue *et al*., 2003) previously known as *K. candel* in an *in vitro* culture study (Ogita *et al*., 2004), was transplanted to the temperate area in Japan (Masuda, 1999). It is a unique low temperature-tolerant mangrove (Kao *et al*., 2004; Suwa, 2014; Watanabe, personal communication). Studies on *K. obovata* leaf protoplasts revealed stimulation of growth by addition of high concentrations of Mg^{2+} ions, but inhibition by other cationic ions (Kaai *et al*., 2006).

1.4 Allelopathy of Mangrove Plants

An inverse relationship was found between salt tolerance and allelopathic activities among three *Sonneratia* mangrove tree species (Hasegawa *et al*., 2014), using two *in vitro* bioassay methods of allelopathy, the sandwich method using mature leaves, and the protoplast co-culture method. The trees are distributed from the seaward side (*Sonneratia alba*) to upstream side (*S. caseolaris*) and in between (*S. ovata*). High allelopathic activities were also found in the upstream-side, leguminous mangrove tree, *Derris indica*, in which an isoflavonoid, rotenone, was studied as a putative allelochemical (Inoue *et al*., 2015). Protoplasts of tissue cultured cells (suspension-cultured cells or calluses), not leaf protoplasts, of these mangroves were used in the protoplast co-culture method.

In the present study, the allelopathic activities were determined using mature leaves of the mangrove, *Kandelia obovata* by the sandwich method, and using leaf protoplasts of *K. obovata* by the protoplast co-culture method and compared with reports on other mangroves.

1.5 Allelopathy of Fast-growing Trees, Betula platyphylla and Poplus alba

In the present study, allelopathy of the young leaves of *in vitro* shoot cultures of two fast-growing broad-leaved trees, *B. platyphylla* (birch) and *P. alba* (poplar), which grow in the temperate regions, was examined by the sandwich method and compared with the allelopathy of leaf protoplasts examined by the protoplast co-culture method. These tree species had been well succeeded in regenerating plants from leaf protoplast cultures and in protoplast fusion studies of broad-leaved trees (Wakita *et al*., 2005, Sasamoto *et al*., 2006). Regenerated plants were obtained from very low numbers of electro-fused *P. alba* leaf protoplasts (Sasamoto *et al*., 2000).

The leaf protoplasts of *P. alba* showed a weak response to all added cationic ions, Na^+, K^+, Mg^{2+}, Ca^{2+}, in an Cl^- ion-depending manner under different medium pHs (Fukumoto *et al*., 2004). In leaf protoplast culture of *B. platyphylla*, tolerance to Ca^{2+} ion and acidic medium pH was reported (Seyama *et al*., 2008, though the protoplast culture methods were wrongly described).

1.6 Evaluation of Abscisic Acid as a Putative Allelochemical

Abscisic acid (ABA) is a stress-related growth retardant hormone. Exogenously supplied ABA inhibited the growth of leaf protoplasts of *Populus alba* (Sasamoto *et al*., 1995). The high ABA content of leaf protoplasts of *Betula platyphylla* was related to the recalcitrancy of their tissue culture (Sasamoto *et al*., 2002). On the contrary, the content of ABA in the cells of halophilic mangroves (*Sonneratia alba*, *Avicennia marina*, and *A. alba*) and effect of ABA on their cell growth in the culture medium varied from stimulatory to inhibitory (Kawana *et al*., 2009; Hasegawa *et al*., 2011). The leaf protoplasts of *Kandelia obovata*, showed a high ABA content and ABA added to the culture medium inhibited the growth of the protoplasts (Kaai *et al*., 2008).

In the protoplast co-culture bioassay of allelopathy, ABA in the culture medium stimulated the growth of recipient lettuce protoplasts, while the antagonistic plant hormone, gibberellic acid inhibited it (Sasamoto *et al*., 2013).

In the present study, ABA is discussed as a putative allelochemical in leaf protoplasts of *B. platyphylla, P. alba* and *K. obovata.* The present results were compared with the reported strong allelopathic activity of protoplasts of suspension cultured cells of a non-mangrove tree, *Prunus yedoensis* (Fujise *et al*., 2018). In addition, the possibility of using of the protoplast co-culture method with digital image analysis (Sasamoto *et al*., 2017a) in the search for new allelochemicals in mangrove plant cells is discussed.

2. Method

2.1 Materials

Leaves of *Kandelia obovata* to be used in the sandwich method (*2.2*) were collected in Iriomote Island, Okinawa, Japan, washed, dried at 60^{o}C for18 hr and stored in a dry condition until use. Leaves of *K. obovata* for protoplast isolation were obtained from potted plants grown as described previously (Kaai *et al*., 2008).

Young leaves of *Betula platyphylla* and *Populus alba* were obtained from shoot cultures, which were the same strains as used for protoplast cultures and cell fusion research (Sasamoto *et al*., 2000, 2002; Wakita *et al*., 2005; Sasamoto *et al*., 2006). Medium for shoot culture of *P. alba*, was MS (Murashige and Skoog, 1962) basal medium, containing 4 μM indolebutyric acid (IBA), 3% sucrose, 1% agar, at pH 5.9 before autoclaving at 120^{o}C for 20 min. Medium for *B. platyphylla* was MS basal medium containing 2.5 μM IBA, 0.1 μM naphthalene acetic acid (NAA), 2% sucrose and 1% agar. Aseptic shoot cultures were incubated in the light condition (60 μ*E*, 16-24 hr photoperiod) at 25^{o}C.

Aseptic seedlings of *Lactuca sativa* (lettuce) were prepared as described previously (Sasamoto *et al*., 2013). Briefly, lettuce seeds 'Great Lakes 366' 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 one to three weeks in the light condition at 25^{o}C.

2.2 Sandwich Method

The sandwich method was performed as described previously (Fujii *et al*., 2003, 2004). Briefly, 10 mg or 50 mg of dried leaves were sandwiched between two layers of 5 mL of 0.5% agar (powder, gelling temp. 30-31^{o}C, Nacalai tesque Co. Ltd. Kyoto, Japan) in 6-well plates (Nunc^{TM}, ThermoFisher Scientific, Waltham, Masaachusetts, U.S.A.). Lengths of hypocotyls and roots of germinated seeds of lettuce on agar (five per one well) were measured after 3 days of incubation at 20^{o}C in the dark. The control treatment consisted of seeds germinated in the absence of dried leaves. Data were recorded as percentage growth of the control and averaged with standard deviation.

2.3 Protoplast Isolation and Purification

All procedures were as described previously. Briefly, a 7 cm-long leaf of *K. obovata* was sterilized with 1% NaClO solution for 40 min, and protoplasts were isolated using 1% each of Cellulase RS and Driselase 20 in 0.8 M mannitol solution at 30^{o}C (Kaai *et al*., 2008). Protoplasts of *B. platyphylla* were isolated using 1% each of Cellulase R10 and Driselase 20 in 0.6 M mannitol solution by overnight floating method (Wakita *et al*., 1996, 2005). Protoplasts of *P. alba* were isolated using 1% Cellulase RS, 0.25% Pectolyase Y-23 in 0.6 M mannitol solution for 2 hrs at static condition (Sasamoto *et al*., 2002, 2006). After passing through a 42-63 μm sized nylon mesh, the protoplasts were purified by washing three times with osmoticum solution followed by centrifugation at 100 *g* (800 - 900 rpm) for 3-5 min.

Protoplast isolation from cotyledons (and small leaf) of recipient lettuce was performed in a flask using 1% each of Cellulase RS and Macerozyme R-10 in the same osmoticum solution as of test plant protoplasts for 20-24 hrs (Sasamoto et al., 2013).

2.4 Protoplast Co-culture with Lettuce Protoplasts

Basically we used the method reported for herbaceous leguminous plants (Sasamoto *et al*., 2013). Test plant protoplasts with recipient lettuce protoplasts were co-cultured in liquid MS basal medium containing 1 μM of 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.1 μM of benzyladenine (BA), 3% sucrose and 0.6 M mannitol solution.

Protoplast suspensions in osmoticum solution, 5 μL each, 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^{R}. The protoplasts were cultured in the dark at 30^{o}C (*K. obovata*,), or at 28^{o}C (*B. platyphylla* and *P. alba*) in a humid incubator (CO_2-incubator without the supply of CO_2, APC-30DR, ASTEC Co. Ltd.). Numbers of non-spherically enlarged-(E) and divided-protoplasts (D), and colonies composed of more than 4 cells (C) of lettuce were counted under

an inverted microscope (Olympus CK40 or IX71) after 4 to 10 days of co-culture. The protoplast density of test plants ranged from 6-150 $\times 10^3$ mL^{-1} (*K. obovata*), and 5-100 $\times 10^3$ mL^{-1} (*B. platyphylla* and *P. alba*). The recipient lettuce protoplast density ranged from 5-100 $\times 10^3$ mL^{-1}.

Lettuce protoplast growth was described as the % of control without test plant protoplasts. Data were averaged with standard errors at 5 $\times 10^3$ – 10^5 mL^{-1} lettuce protoplast densities.

3. Results and Discussion

3.1 Sandwich Method

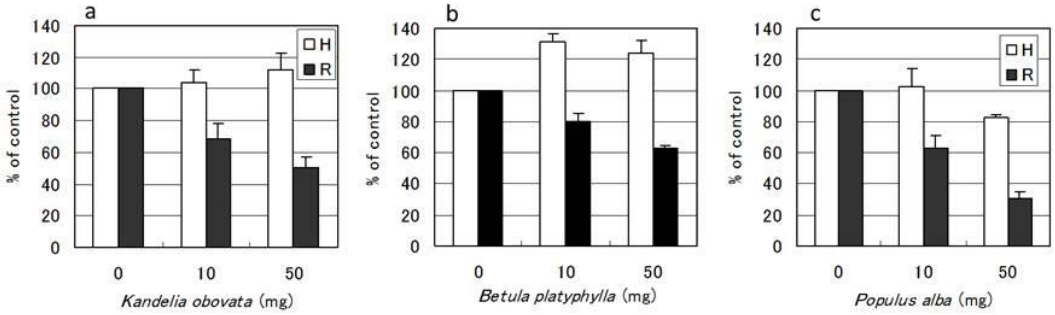

Figure 1. Effects of leaves of *Kandelia obovata* (a), *Betula platyphylla* (b) and *Populus alba* (c) on the growth of hypocotyls (white, H) and of roots (black, R) of lettuce seedlings measured by the sandwich method

As shown in Figure 1a, 50 mg of mature leaves of the mangrove, *Kandelia obovata* inhibited the root elongation of lettuce seedlings 50%. This was similar to the 53% inhibition obtained with potted plants of *K. obovata* (Hayashi *et al*., 2008). This value was similar to that obtained for the group 2 mangroves, and higher than that obtained for group 1 (the seaward-side grown, highly salt tolerant, low allelopathic activity, less than 45% inhibition), but lower than that obtained for group 3 (the upstream grown, less salt tolerant, highly allelopathic, more than 80% inhibition) (Sasamoto and Hasegawa, 2014, Sasamoto *et al*., 2014). Group 1 included *Sonneratia alba* (Hasegawa *et al*., 2014), and group 3 included *Derris indica* (Inoue *et al*., 2015) and *S. caseolaris* (Hasegawa *et al*., 2014). In the group 3 mangrove species, the growth of both hypocotyls and roots of lettuce seedling were strongly inhibited. In contrast, *K. obovata* (Figure 1a) did not inhibit the growth of the hypocotyls of lettuce.

A non-mangrove, invader tree, *Leucaena leucocephala*, which grows in sub-tropical areas, strongly inhibited the growth of lettuce root and hypocotyl (88% and 91% by 50 mg) examined by the sandwich method. Lettuce root growth was inhibited 76% by even 10 mg (Mori *et al*., 2015). Such inhibition was similar or stronger than that of the group 3 mangrove species.

Two fast-growing non-mangrove, tree species, *Betula platyphylla* and *Populus alba*, grow in temperate regions. As shown in Figure 1b, 50 mg of young leaves of *B. platyphylla* moderately inhibited lettuce root growth (37%), but stimulated the growth of hypocotyls. Mature leaves of *B. platyphylla* were reported to inhibit the growth of lettuce roots only 3% (Fujii, 2000). These trees inhibited growth less than the group 1 mangrove trees. As shown in Figure 1c, 50 mg of young leaves of *Populus alba* inhibited the growth of lettuce roots (69%), but had no effect on hypocotyls. Such moderate inhibition (56%) was reported for mature leaves of another poplar species, *Populus nigra* (Fujii, 2000). These values are similar to those obtained for the group 2 mangroves.

3.2 Protoplast Co-culture Method

3.2.1 Leaf Protoplasts of *Kandelia obovata*

Figure 2 shows the results of the protoplast co-culture of the leaf protoplasts of the mangrove species, *K. obovata*. After 10 days of co-culture, colony formation of lettuce protoplasts was inhibited depending on leaf protoplast densities of *K. obovata*. Calculated values of inhibition at 1 $\times 10^4$ mL^{-1} and at 5 $\times 10^4$ mL^{-1} of *K. obovata* were 31% and 69%, respectively.

These inhibition rates were in between those of the group 1 mangrove, *Sonneratia alba* (24%, 75%), and the group 3 mangroves, *S. caseolaris* (50%, 92%) and *D. indica* (58%, 99%) of previous reports (Hasegawa *et al*., 2014; Sasamoto *et al*., 2014; Inoue *et al*., 2015). These were obtained using protoplasts of suspension cultured cells or callus.

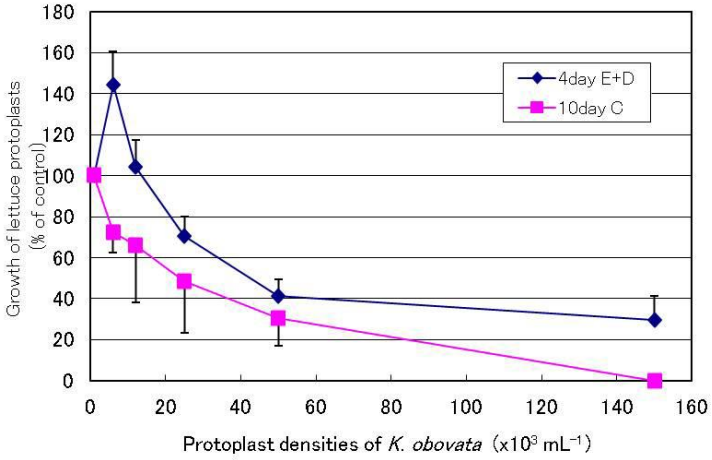

Figure 2. Activity of leaf protoplasts of mangrove species, *Kandelia obovata*, on lettuce protoplast growth at early cell division stage (day 4, diamond) and colony formation stage (day 10, square) measured by the protoplast co-culture method. The medium was MS basal medium containing 1 μM 2,4-D, 0.1 μM BA, 3% sucrose, and 0.64 M mannitol

The protoplast co-culture method has been improved by averaging the % of growth of control without test plants at different lettuce protoplast densities (5-100 $\times 10^3$ mL^{-1}) as described in *2.4*. (Sasamoto and Ashihara, 2014; Sasamoto *et al*., 2015). There was a tendency of variation in the inhibition % with the lettuce protoplast density.

After 4 days of co-culture, at the early cell division stage of lettuce, including the numbers of non-spherically enlarged lettuce protoplasts (E, cell wall formed), 44% stimulation of lettuce growth was observed at 6 $\times 10^3$ mL^{-1} of *K. obovata* (Figure 2). Such patterns of stimulation at low protoplast densities and inhibition at high protoplast densities of test plant are similar to protoplasts of suspension cultured cells of three bamboo species (Ogita and Sasamoto, 2017) and protoplasts of calluses of coffee (Ogita *et al*., 2020). Inhibition values of the non-mangrove invader tree, *Leucaena leucocephala* were 99% and 100% at 1 $\times 10^4$ mL^{-1} and at 5 $\times 10^4$ mL^{-1}, respectively, after 4 days of co-culture (Mori *et al*., 2015).

3.2.2 Leaf Protoplasts of *Betula platyphylla*

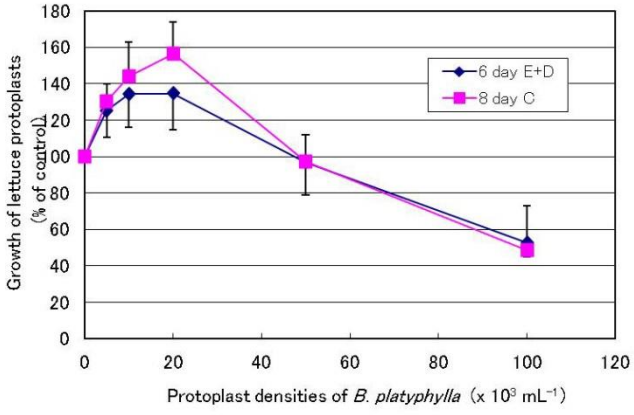

Figure 3. Activity of leaf protoplasts of fast-growing tree, *Betula platyphylla* on lettuce protoplast growth at early cell division stage (day 6, diamond) and colony formation stage (day 8, square) measured by the protoplast co-culture method. The medium was MS basal medium containing 1 μM 2,4-D, 0.1 μM BA, 3% sucrose, and 0.6 M mannitol

The allelopathy of the non-mangrove tree species, *B. platyphylla* was examined using the protoplast co-culture

method. As shown in Figure 3, after 6 days of co-culture, at the early cell division stage of lettuce protoplasts, 35% stimulation was observed at 1×10^4 mL^{-1}, and 47% inhibition at 1×10^5 mL^{-1}. Such pattern of stimulation at low protoplast densities and inhibition at high protoplast densities of test plant protoplasts were also observed in the mangrove, *K. obovata* (Figure 2).

At the colony formation stage of lettuce (8 days of co-culture), colony formation was stimulated 51% at the density of 1×10^4 mL^{-1}, and was inhibited 54% at the density of 1×10^5 mL^{-1}. This is different from the results obtained with *K. obovata*, which showed only inhibition.

3.2.3 Leaf Protoplasts of *Populus alba*

Protoplasts of non-mangrove tree species, *P. alba* were co-cultured. As shown in Figure 4, strong stimulation (100-200%) of lettuce protoplast growth was observed at the colony formation stage of lettuce. Similar but less stimulation (up to 46%) was also observed at the early cell division stage of lettuce after 6 days of co-culture. Similar 40% stimulation at the early cell wall formation stage of lettuce, at up to 1×10^5 mL^{-1}, was reported for the protoplasts of yellow callus of a halophilic mangrove *Avicennia alba*. However, inhibition was reported at the cell division stage at all protoplast densities tested (Sasamoto *et al*., 2020). Recently, protoplasts of embryogenic coffee callus cells were reported to show 150% stimulation at the early cell wall formation stage, and moderate inhibition at the cell division stage (Ogita *et al*., 2020). Compared with the responses of the protoplasts of these two trees, the leaf protoplasts of *P. alba* showed very strong stimulation at the colony formation stage.

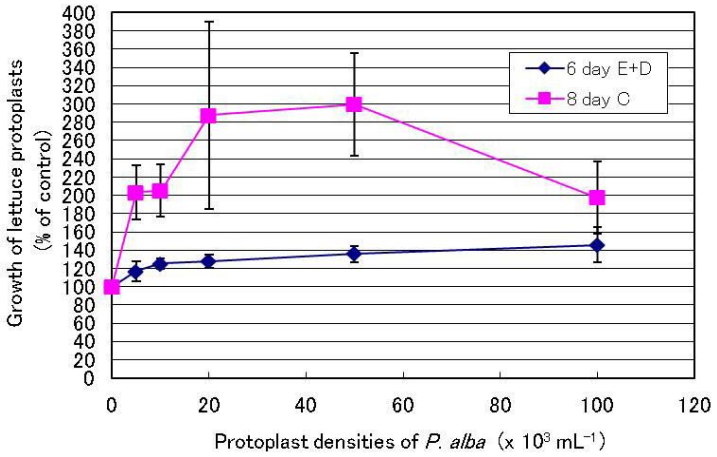

Figure 4. Activity of leaf protoplasts of fast-growing tree, *Populus alba*, on lettuce protoplast growth at early cell division stage (day 6, diamond) and colony formation stage (day 8, square) measured by the protoplast co-culture method. The medium was MS basal medium containing 1 μM 2,4-D, 0.1 μM BA, 3% sucrose, and 0.6 M mannitol

3.3 Sandwich Method and Protoplast Co-culture Method

3.3.1 *Kandelia obovata*

Both mature leaves (Figure 1a) and leaf protoplasts (Figure 2) of the mangrove, *K. obovata* showed moderate inhibitory allelopathic activities by the sandwich method and protoplast co-culture method, respectively, similar to the group 2 mangroves, between group 1 (the seaward-side grown, highly salt tolerant), and group 3 (the upstream grown, less salt tolerant) (Sasamoto *et al*., 2014).

3.3.2 Fast Growing Trees or Woody Species

Betula platyphylla showed moderate inhibition on lettuce root growth by the sandwich method (Figure 1b). However, inhibition by leaf protoplast of *B. platyphylla* was observed only at a high protoplast density, 1×10^5 mL^{-1}, by the protoplast co-culture method; and stimulation was observed at low protoplast densities (Figure 3).

Although *Populus alba* showed moderate inhibition of lettuce root growth by the sandwich method (Figure 1c), strong stimulation was observed by the protoplast co-culture method (Figure 4).

Different inhibition patterns between protoplast co-culture method and the sandwich method were also seen in four bamboo species (*Bambusa multiplex*, *Phyllostachys bambusoides*, *Phyllostachys nigra*, *Sasa kurilensis*; Ogita and Sasamoto 2017). All four bamboo species showed moderate inhibition by the sandwich method.

However, *Sasa kurilensis* showed strong inhibition by the protoplast co-culture method, and the other three bamboo species showed stimulation at lower protoplast densities. Such a discrepancy might be explained by the differences of endogenous levels of putative allelochemical(s) among protoplasts of young and mature leaves of test plants. Another explanation is the use of dead leaf leachates for the sandwich method and living cells for the protoplast co-culture method (Ogita and Sasamoto, 2017). In *Sasa* species, another bioassay method of allelopathy, the plant box method, which measures the effect of exudates from intact roots (Fujii, 2000, Fujii *et al*., 2007), was similarly inhibitory as in the protoplast co-culture method. Though, small seedlings are needed for the plant box method, and it is difficult to obtain them in tree species all year round.

3.4 Abscisic Acid (ABA) as a Putative Allelochemical in Protoplasts

3.4.1 ABA in *Betula platyphylla* and *Populus alba*

ABA is a plant hormone related to stress tolerance and known as a growth retardant. Exogenously supplied ABA inhibited the growth of leaf protoplasts of *P. alba* (Sasamoto *et al*., 1995). Protoplast culture of *B. platyphylla* was recalcitrant to antagonize endogenous ABA, when the strong cytokinins, thidiazuron or *N*-(2-chloro-4-pyridyl)-*N'*-phenylurea (4-PU or CPPU) were not used (Wakita *et al*., 1996). Very high content of ABA in leaf protoplasts of *B. platyphylla* (16.7 pmoles / 6 $\times 10^6$ protoplasts) and low content (0.81 pmoles / 6 $\times 10^6$ protoplasts) in leaf protoplasts of *P. alba* were found using micro scale extraction, partition, and ELISA test (Sasamoto *et al*., 2002). As the diameter of these protoplasts was 20 μm, the calculated ABA concentration in each protoplast of *Populus alba* was 0.03 μM, and 0.67 μM in *B. platyphylla* protoplast (Table 1).

Table 1. Calculated concentrations of ABA in leaf protoplasts

Plant species	ABA (μM)	ABA (pmoles/6 $\times 10^6$ protoplasts)	References
Populus alba	0.03	0.81	Sasamoto *et al*. 2002
Betula platyphylla	0.67	16.7	Sasamoto *et al*. 2002
Kandelia obovata	0.36	8.92	Kaai *et al*. 2008

*Diameter of protoplasts was 20 μm.

However, co-cultured poplar leaf protoplasts stimulated the growth of lettuce (Figure 4). The growth of recipient lettuce protoplasts was stimulated by exogenously supplied ABA at 0.1 μM up to 10 μM; the highest stimulation at 1 μM (Sasamoto *et al*., 2013). Growth of lettuce protoplasts was strongly stimulated by co-culture with *P. alba* leaf protoplasts (Figure 4). Co-culture with *B. platyphylla* was stimulatory at low densities and inhibitory at a high density (Figure 3). This might be explained by the low and high ABA content of protoplasts of these two trees. Therefore, ABA in leaf protoplasts of *P. alba* and *B. platyphylla* might act as an allelochemical on lettuce protoplast growth in the protoplast co-culture method.

On the other hand, ABA has been reported to have a moderate inhibitory effect on seedling growth of recipient lettuce (Hiradate *et al*., 2005). However, the effect of ABA alone could not explain the stronger inhibition of *P. alba* (Figure 1c) than that of *B. platyphylla* (Figure 1b) on the lettuce root growth examined by the sandwich method. Such discrepancy might be partly explained by the differences in endogenous levels of ABA and antagonistic plant hormones among protoplasts and seedlings of lettuce. In *P. alba*, exogenous ABA inhibited the growth of leaf protoplasts. However, exogenous ABA stimulated the protoplast growth of suspension cultured cells of *P. alba*, to antagonize the high endogenous content of gibberellins (Sasamoto *et al*., 1995).

Very strong inhibitory allelopathic activity was reported in a flower tree, *Prunus yedoensis* using both the protoplast co-culture method and the sandwich method (Fujise *et al*., 2018). Though exogenous 1 μM ABA strongly inhibited the growth of *Prunus* protoplasts themselves, low calculated content of ABA (0.09 μM) in isolated protoplasts of suspension cells of *Prunus yedoensis* (Yokota *et al*., 2005) was not sufficient to be considered as a cause of strong inhibitory allelopathic activity of *Prunus* protoplasts on lettuce protoplasts, and coumarin was suggested as an allelochemical in *Prunus* protoplasts.

3.4.2 ABA in *Kandelia obovata*

The inhibitory effect of ABA in tissue culture has also been reported in the mangrove, *K. obovata* leaf protoplasts, along with high endogenous ABA content in leaf protoplasts (8.92 pmoles / 6 $\times 10^6$ protoplasts) (Kaai *et al*., 2008). As the diameter of *K. obovata* leaf protoplast was 20 μm, the calculated ABA concentration in each protoplast of *K. obovata* was 0.36 μM (Table 1). This value was several times higher than that of *P. alba* leaf protoplasts (Sasamoto *et al*., 1995), but several times lower than that of *B. platyphylla* leaf protoplasts (Sasamoto *et al*., 2002).

The leaf protoplasts of *K. obovata* showed inhibitory allelopathic activities at 1/10 density of *B. platyphylla*, by the protoplast co-culture method (Figure 2). Accordingly, the high ABA content calculated in isolated protoplasts of *K. obovata*, could not be the single and direct cause of the moderate inhibitory allelopathic activity. Different allelochemical(s), which mimic the stimulatory effect of ABA, might exist in *K. obovata* protoplasts.

3.5 Finding Allelochemicals in Mangrove Plant Cells Using Protoplast Co-culture Method

The contents of ABA in the protoplasts of halophilic mangroves (*Sonneratia alba, Avicennia alba, A. marina*) varied from high to low, and the growth of protoplasts has been reported to be inhibited or stimulated by ABA in the culture medium (Kawana *et al*., 2009; Hasegawa *et al*., 2011). Though, an inverse relationship was found between allelopathic activity and salt tolerance of *Sonneratia* mangrove plants (Hasegawa *et al*., 2014), inhibitory allelopathic activities were found in all mangroves tested using both the sandwich method and the protoplast co-culture method (Figure 1a, Figure 2; Mori *et al*., 2012; Sasamoto *et al*., 2014; Inoue *et al*., 2015). Some allelochemical(s) with inhibitory activities, which mimic the stimulatory activity of ABA, might be found even in the seaward-side grown mangrove species using the protoplast co-culture method.

The content of a putative allelochemical, ABA, in protoplasts was analyzed using the small-scale extraction and fractionation (purification) method in combination with the sensitive assay method (ELISA test; Sasamoto *et al*., 2002). In the protoplast co-culture method for the assay of allelopathic activities, the amount of chemical(s) in a 50 μL medium, and the protoplast numbers needed are very low (500 protoplasts at 1×10^4 mL^{-1}, and 2500 at 5×10^4 mL^{-1}). In addition to the chemicals, already found in tissues of test plants, or in tissue cultured cells, finding of new allelochemicals might be possible by using a combination of such small-scale methods. A single chemical of large amount might not be the only cause of strong inhibitory allelopathic activity of plants (Kobayashi *et al*., 2021).

Recently, digital image analysis has been applied to the protoplast co-culture method (DIA-PP method) for leaf protoplasts of a herbaceous plant, *Arabidopsis thaliana* using lettuce as a recipient (Sasamoto *et al*., 2017a,b). In addition to the effects on cell wall formation and cell division stages of protoplasts of the recipient plants, effects on the yellow pigment accumulation stage, which is specific to recipient lettuce protoplasts after 3 weeks to 2 months of co-culture, was quantitatively analyzed by digital image analysis. Using the DIA-PP method, a carotenoid, neoxanthin, was found as an allelochemical of yellow callus of a group 1 mangrove, *Avicennia alba*. Strong inhibition at the cell division stage, and moderate inhibition at the yellow pigment (including a carotenoid) accumulation stage were observed (Sasamoto *et al*., 2020; Sasamoto *et al*., 2021). The yellow callus was not yellow in the original cultured cells after induction in the dark (Hasegawa, 2014; Tsuchiya *et al*., 2013), of which protoplasts were highly salt tolerant (Hasegawa *et al*., 2013). In addition, using the DIA-PP method, an anthocyanin, cyanidin 3,5-di-*O*-glucoside, was found as an allelochemical of red callus of a less salt-tolerant, group 2 mangrove, *S. ovata* (Hasegawa *et al*., 2014; Sasamoto *et al*., 2014; Sasamoto *et al*., 2018). Moderate inhibition of cell division and no inhibition at the yellow pigment accumulation stage were observed in co-cultured protoplasts of *S. ovata* (Sasamoto *et al*., 2018). Similarly, no inhibition at the yellow pigment accumulation stage was observed in the DIA-PP method of *Kandelia obovata* (Sasamoto et al., in preparation).

Protoplast co-culture method and DIA-PP method will contribute to the understanding of the underlying mechanism(s) of allelopathy at a cellular level, and they can be applied under different culture conditions, *e.g*., high and low temperatures, and different salts conditions, in order to simulate the possible environmental risks of genetically modified plants and invasive plants (Fujise *et al*., 2012; Sasamoto *et al*., 2013; Suzuki *et al*., 2018).

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