

# Evaluation of Allelochemicals, Abscisic Acid and Coumarin, in Leaf-Origin Suspension Cultured Cells of *Prunus yedoensis* Using Protoplast Co-Culture Bioassay Method

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## Abstract

Dried leaves of *Prunus yedoensis* and *P. lannesiana* (50 mg) showed strong inhibitory allelopathic activities, e.g., more than 97% growth inhibition of lettuce seedling using the sandwich method. Similarly, among suspension cultures induced from leaves and peduncles of two *Prunus* species, we found the strongest inhibitory allelopathic activities of protoplasts of leaf-origin suspension cells of *P. yedoensis*, when the protoplast co-culture method for bioassay of allelopathy was applied with lettuce as a recipient plant. Effects of two putative allelochemicals, abscisic acid and coumarin, on both protoplast cultures of lettuce and *P. yedoensis* were investigated. Coumarin inhibited the growth of lettuce protoplasts from low concentrations, while abscisic acid stimulated. Abscisic acid inhibited the protoplast growth of *P. yedoensis* from low concentrations, while coumarin did not, but inhibited only at a high concentration (1 mM). Contents of abscisic acid in protoplasts were measured using small scale purification and Enzyme Linked Immuno Sorbent Assay, and contents of coumarin in leaf-origin suspension cells of *P. yedoensis* were measured using Gas Chromatography-Mass Spectrometry. Coumarin was more likely the allelochemical causing the strong inhibitory allelopathic activities of *P. yedoensis* in the protoplast co-culture bioassay. Effectiveness of the protoplast co-culture bioassay method of allelopathy was discussed.

## Keywords

Allelopathy, Bioassay, Protoplast Culture, *Prunus* Species

## 1. Introduction

*Prunus yedoensis* Matsum. (Somei-yoshino) [1] is famous for its beautiful cherry blossom in spring. The trees have been planted in many places in Japan, and are used as an environmental indicator since their growth is affected by global warming. It is a hybrid clone with *P. lannesiana* as one of its parent.

Allelopathy is a mechanism in which the plant produces allelochemical(s) to survive in nature, by inhibiting the growth of neighboring plants in the field. Fujii *et al.* investigated allelopathic activities of many plant species by measuring the effects of dried leaf litter on the growth of lettuce seedlings using an *in vitro* bioassay method, the sandwich method [2]. Allelopathy of a few fruit *Prunus* species has been known [3] [4], though its strength is not clear. The inhibitory allelopathic activity in methanol extracts of *Prunus* species was reported in 168 plant species investigated using lettuce as a recipient plant [5], though the specific species of *Prunus* is unclear.

Recently, an *in vitro* bioassay method of allelopathy, the protoplast co-culture method, was developed for herbaceous leguminous plants [6] and for woody or tree plant species using suspension cultured cells and calluses [7] [8] [9] [10]. Effects of the putative allelochemicals and related metabolites were examined using the same lettuce protoplast culture system [9] [11] [12].

In this report, we first examined the allelopathic activities of young leaves of *P. yedoensis* and *P. lannesiana* using the sandwich method. Then, after induction of suspension cultures from leaves and peduncles of two *Prunus* species, the protoplast co-culture method was applied with lettuce as a recipient plant.

Coumarin in the leaves of *P. lannesiana* is known and used for its anti-bacterial activity and specific flavor. Seasonal changes of coumarin contents were investigated in the leaves of *P. yedoensis* [13]. Coumarin is also known as a strong allelochemical in the leaves of a leguminous tree, *Gliricidia sepium* [14]. However, its role as an allelochemical in *Prunus* species has not been well investigated.

Inhibitory activities of abscisic acid (ABA), a plant hormone, on the growth of plant protoplasts and its contents were investigated [15] [16] [17] and had been discussed as an allelochemical in *Fagus* and sugar beet plants [3]. However, the role of ABA on protoplast cultures as an allelochemical in *Prunus* species has not been studied.

Here we report, the effects of both ABA and coumarin on both protoplast cultures of lettuce and *P. yedoensis*. Finally, the content of coumarin in suspension cells of *P. yedoensis* originating from leaves was investigated. The ABA content in their protoplasts was also investigated and compared with those of suspension cells originating from peduncle and petiole [16]. In addition, the differences of two putative allelochemicals were discussed as the cause of strong allelopathic activities of leaves of *P. yedoensis*.

## 2. Materials and Methods

### 2.1. Plant Materials

Young leaves and peduncles of *Prunus yedoensis* and *P. lannesiana* trees grown

in the Yokohama National University, were used directly for induction of suspension cultures, or leaves were used for the sandwich method after treatment without or with 0.5% NaClO aqueous solution for 12 min, and after drying at 60°C for 18 hrs. *Lactuca sativa* (lettuce) seeds (Great Lakes) were sterilized with 1.5% NaClO aqueous solution for 15 min, washed with autoclaved water three times and sown on 0.8% agar medium. Cotyledons of 6-to 12-day-old seedlings, which were grown aseptically under a light condition at 25°C, were used for protoplast isolation [6].

## 2.2. Sandwich Method

The sandwich method of dried leaves of *P. yedoensis* and *P. lannesiana* was performed as reported [2] [18]. Briefly, 10 or 50 mg dry weight of leaves were sandwiched between two layers of 5 mL of 0.5% agar (gelling temp. 30°C - 31°C, Nacalaitesque Co. Ltd. Kyoto Japan) in six multi-well dish (Nunc). Five seeds of lettuce were put on per well. After 3 days of incubation in the dark at 20°C, length of hypocotyls and roots was measured. Data were averaged of three wells and described as % growth of the control without leaves with standard deviation (SD).

## 2.3. Induction of Suspension Cultures

Leaves of *P. yedoensis* were sterilized with a neutral detergent and 1% NaClO aqueous solution for 12 min and peduncles of *P. lannesiana* and *P. yedoensis* were sterilized with 2.5% NaClO aqueous solution for 30 min, and washed with autoclaved water three times. Two 3 mm sections were put in a 1 mL liquid medium of Murashige and Skoog's (MS) [19] basal medium containing 10 µM each of naphthalene acetic acid and benzyladenine (BA) and 3% sucrose in flat-bottomed 10-mL tubes as reported previously for petiole and peduncle of *P. yedoensis* [16]. Maintenance of suspension culture was performed in 20 mL of the same medium composition in a 100 mL flask. They were incubated in the dark on a shaker at 100 rpm speed at 27°C. Sub-cultured 14-day-old suspension cells were used for protoplast isolation.

## 2.4. Protoplast Isolation and Purification

Leaf-derived suspension cells of *P. yedoensis* were filtered on 80 µm sized mesh and digested for 3 hrs by the enzyme combination of 1% Cellulase RS, 1% Driselase 20, and 0.25% Pectolyase Y-23 in 0.6 M mannitol, and peduncle-derived suspension cells of *P. yedoensis* were digested overnight by 1% each of Cellulase RS, Hemicellulase, and Macerozyme R10. Peduncle-derived suspension cells of *P. lannesiana* were digested by the enzyme combination of 1% each of Cellulase R10, Hemicellulase, Driselase 20, and Macerozyme R10 in 0.6 M mannitol. Each enzyme combination was preliminary selected from 24 combinations tested using six cell wall degrading enzymes in 0.4 - 0.6 M mannitol solution.

Isolated *Prunus* protoplasts were purified by passing through 63 µm sized

mesh and washed three times with mannitol solution by centrifugation at  $100 \times g$  for 5 min, and used for protoplast culture directly. Isolated suspension cell protoplasts of *P. yedoensis* originating from leaves were also stored at  $-70^\circ\text{C}$  after precipitation in a 1 mL glass tube for ABA measurement as reported [15] [16].

Lettuce cotyledon protoplasts were isolated for 20 to 24 hours in 0.6 M or 0.8 M mannitol solution containing 1% each of Cellulase RS and Macerozyme R10 and purified with mannitol solution by centrifugation at  $100 \times g$  for 5 min as reported [6].

### 2.5. Protoplast Co-Culture among *Prunus* and Lettuce Protoplasts

Protoplast co-culture method was performed as precisely described [6] [7] using cotyledon protoplasts of recipient lettuce. Protoplasts of different densities were put in a 50  $\mu\text{L}$  medium per well in a 96-well culture plate. Basal medium was MS containing 3% sucrose, 1  $\mu\text{M}$  of 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.1  $\mu\text{M}$  of BA and 0.6 M mannitol. The non-spherically enlarged or divided lettuce protoplasts were counted under an inverted microscope (Olympus CK40) after 4 to 12 days of co-culture in the dark at  $28^\circ\text{C}$  in a humid condition ( $\text{CO}_2$ -incubator without the supply of  $\text{CO}_2$ , APC-30DR, ASTEC Co. Ltd.). The percentage of control without *Prunus* protoplast was calculated. The % values were averaged with standard errors (SEs) at different lettuce protoplast densities ( $5 \times 10^3/\text{mL}$  -  $100 \times 10^3/\text{mL}$ ).

### 2.6. Effects of Coumarin on Lettuce Protoplast

Coumarin was dissolved in ethanol and a small volume (less than 2.5  $\mu\text{L}$ ) was put in the medium after sterilization with a filter as reported [8]. Basal medium was the same as that used for the co-culture except for 0.8 M mannitol. Non-spherically enlarged or divided lettuce protoplasts were counted under an inverted microscope after 6 days of culture. Percentage of control without coumarin was calculated at different lettuce protoplast densities ( $5 \times 10^3/\text{mL}$  -  $100 \times 10^3/\text{mL}$ ), and the average % values with SEs were obtained.

### 2.7. Effects of ABA and Coumarin on *P. yedoensis* Protoplasts

ABA was dissolved in ethanol as described for coumarin. Protoplasts of leaf-origin suspension cells of *P. yedoensis* was cultured in the same way as the co-culture. After 4 or 12 days of culture, the numbers of non-spherically enlarged and divided protoplasts were counted. The percentage of control with neither ABA nor coumarin was calculated at each *Prunus* protoplast density. The % values were averaged with SEs at different *Prunus* protoplast densities ( $5 \times 10^3/\text{mL}$  -  $25 \times 10^3/\text{mL}$ ).

### 2.8. Measurement of ABA Content in Suspension Cells Protoplasts of *P. yedoensis*

Procedures were the same as described previously [15] [16]. Briefly, protoplasts

of leaf-origin suspension cells of *P. yedoensis* were extracted with 80% methanol in a 1-mL glass tube. After evaporation to dryness, a small scale partition was performed between water (pH 2.5 with HCl) and methylene chloride. Purification by silica gel TLC was performed. The ABA fraction was eluted and assayed by Enzyme-Linked Immuno Sorbent Assay (ELISA) using the ABA-ELISA-Kit (Sigma) according to the procedure of the manufacturer, though the ABA standard solution was made separately.

### 2.9. Measurement of Coumarin Content in Suspension Cells of *P. yedoensis*

Leaf-origin suspension cells of *P. yedoensis* were filtered on 80 µm sized mesh and 30.64 g fresh weight of them were extracted by methanol and evaporated to dryness (0.997 g). Coumarin standard and methanol extracts were TMS-derivatized with *N,O*-bis (trimethylsilyl) trifluoroacetamide, and were analyzed by a gas chromatograph-mass spectrometer (TRACE DSQ GC-MS, Thermo Scientific). Coumarin in the methanol extracts were quantified with a calibration curve made by using a coumarin standard. The conditions for GC-MS analysis were as follows: ion source temp., 250°C; inlet temp., 250°C; *m/z* range, 60 - 650; column, DB-1 (15 m × 0.25 mm I.D., film thickness 0.25 µm (Agilent J&W)); carrier gas, He; flow rate, 1.5 mL/min; column temp. program, 100°C (1 min hold) → 5°C/min → 250°C (15 min hold).

## 3. Results and Discussion

### 3.1. Sandwich Method

Effects of dried leaves of *Prunus* species on the growth of lettuce seedlings were tested using the sandwich method. As shown in **Table 1**, highly inhibitory effects of 50 mg of leaves of both *P. yedoensis* and *P. lannesiana*, which was less than 3% growth of control (more than 97% inhibition), were obtained on both hypocotyl and root growth of lettuce. Ten mg of leaves were similarly very inhibitory. Sandwich method using 10 and 50 mg dry weight of leaves were first developed using leaf leachates [2] [18], which was based on the amount of yearly leachates of broad-leaved deciduous trees is about 3 tons/ha, corresponding to 30 mg dry weight/10 cm<sup>2</sup>/10 mL [3]. Compared with the previous data of many plant species using the sandwich method [2] [18], *Prunus* in this report is in the group of very strong allelopathic activities, though young leaves of *Prunus* were used in this report. Stronger activity was also obtained in the younger leaves of *Mucuna gigantea* [9]. With the sandwich method, bacterial or fungal contamination may occur during the 3 days of incubation. However, the results obtained with leaves of *P. lannesiana* sterilized with NaClO aqueous solution before drying the leaves was not different from those obtained without the treatment (**Table 1**). Such sterilization treatment can be used to exclude the possible effect of fungal or bacterial contamination in the sandwich method.

Compared with the inhibitory effects of leaves of several plant species investigated using the sandwich method (*Derris indica* [8]; *Mucuna gigantea*, *Leucaena*

**Table 1.** Allelopathic effects of dried leaves of *Prunus* species on the growth of lettuce seedlings in the sandwich method.

Weight of <i>Prunus</i> Leaves	10 mg		50 mg		
	Lettuce	Hypocotyl	Root	Hypocotyl	Root
<i>P. yedoensis</i>		6.4 ± 5.5	6.0 ± 4.2	0	0
<i>P. lannesiana</i>		13.8 ± 1.8	4.8 ± 2.1	1.0 ± 1.8	0
<i>P. lannesiana</i> <sup>a</sup>		8.5 ± 1.8	3.6 ± 3.6	1.0 ± 1.8	1.1 ± 2.0

<sup>a</sup>Leaves were sterilized before drying. Data were described as % of control without *Prunus* leaves, and averaged with SDs.

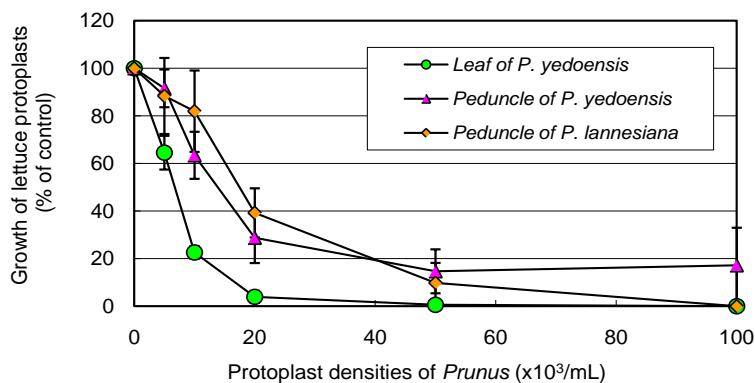
*leucocephala* [9], Bamboo species [10]), more than 90% inhibition by 50 mg leaves is very strong. Especially, no growth of lettuce root obtained with leaves of *P. yedoensis* was the strongest of all. Leaves of an invader plant, *Leucaena leucocephala*, inhibited the root growth by 90% in the sandwich method [9].

### 3.2. *Prunus* Suspension Culture and their Protoplast Isolation and Culture

In this report, suspension cultures of leaves and peduncles of *Prunus* species were induced and sub-cultured well using naphthaleneacetic acid and a cytokinin, BA, instead of *N*-(2-chloro-4-pyridyl)-*N*<sup>1</sup>-phenylurea used in the previous report on petiole and peduncle of *P. yedoensis* [16]. Selected enzyme combinations containing Macerozyme R10 for peduncles-derived suspension cells in the present study, could not be employed in the previous report, in which the 0.5% Pectolyase Y23 was used [16]. Therefore, selection method of 24-combinations of cell wall degrading enzymes in different osmotic conditions was applicable in all materials. In this paper, all protoplast cultures were performed at 28°C. This temperature was the same as that for bamboo species and co-culture with lettuce [10]. The temperature employed for the protoplast cultures of several mangrove tree species and for the co-culture with lettuce was 30°C [7]. The same range of temperatures and osmotic conditions (0.4 - 0.8 M mannitol or sorbitol) were also selected for both protoplast co-culture and test of allelochemicals and related metabolites. In lower osmotic conditions, the lettuce protoplasts grew vigorously in short days [8] [9] [11].

### 3.3. Protoplast Co-Culture Method: Effects of *Prunus* Protoplasts on the Growth of Lettuce

**Figure 1** shows the results obtained by the protoplast co-culture method using protoplasts of *Prunus* and lettuce. The growth of lettuce protoplasts was inhibited depending on the protoplast density of all three *Prunus* suspension cells. Protoplasts from peduncle-derived suspension cells of *P. yedoensis* and *P. lannesiana* were also inhibitory but less inhibitory than protoplasts of *P. yedoensis* suspension cells derived from leaves. Addition of  $2 \times 10^4$ /mL of protoplasts of leaf-origin *P. yedoensis* almost totally inhibited the lettuce protoplast growth,



**Figure 1.** Effects of protoplasts of suspension cells of *Prunus yedoensis* originating from leaves and peduncles, and *P. lannesiana* originating from peduncles on the growth of lettuce protoplasts. Numbers of divided protoplasts of lettuce (composed from 3 cells or more) were counted after 7 days of co-culture. Data were described as % of control without *Prunus* protoplasts and averaged with SE at lettuce protoplast densities of  $5 \times 10^3/\text{mL}$  -  $50 \times 10^3/\text{mL}$ . Medium was MS basal medium containing  $1 \mu\text{M}$  of 2,4-D and  $0.1 \mu\text{M}$  of BA, 3% sucrose, and 0.6 M mannitol.

which was the strongest of the three kinds of cells. This value is similar to that of *Sasa kurilensis*, which showed the strongest inhibition among the four bamboo species [10], and is between the *Derris indica* [8] and the invader plant, *Leucaena leucocephala* protoplasts, which totally inhibited the lettuce protoplast growth at  $10^4/\text{mL}$  [9]. Protoplasts of peduncles–origin *P. yedoensis* and *P. lannesiana* were less inhibitory than leaf-origin *P. yedoensis*. However, the nearly total inhibition at  $10^5/\text{mL}$  indicated very strong activity.

Recently, the allelopathic activity measured by the sandwich and protoplast co-culture methods has been reported to differ in bamboo species. Some stimulation at low protoplast densities was observed in suspension cell protoplasts of two bamboo species, which showed moderately strong allelopathic activities in the sandwich method [10].

However, no stimulation was observed even at low protoplast densities in three *Prunus* cells (Figure 1). Young growing leaves and vigorously growing suspension cells of leaves were used in our study. The same strong inhibitory activities by leaves of *P. yedoensis* were observed with both sandwich and protoplast co-culture methods.

*Prunus* protoplasts can grow slowly in the medium employed for co-culture, i.e., MS basal medium containing 2,4-D and BA, and colony developed at lower concentrations than  $10 \mu\text{M}$  of plant hormones after one month of culture [20]. Such colony development was also observed in the co-culture with bamboo protoplasts [10]. However, in early culture, only the growth of lettuce protoplasts can be well distinguished under an inverted microscope.

### 3.4. Effects of Putative Allelochemicals on the Growth of Lettuce Protoplasts

Coumarin was studied as a strong allelochemical of *Gliricidia sepium* with let-

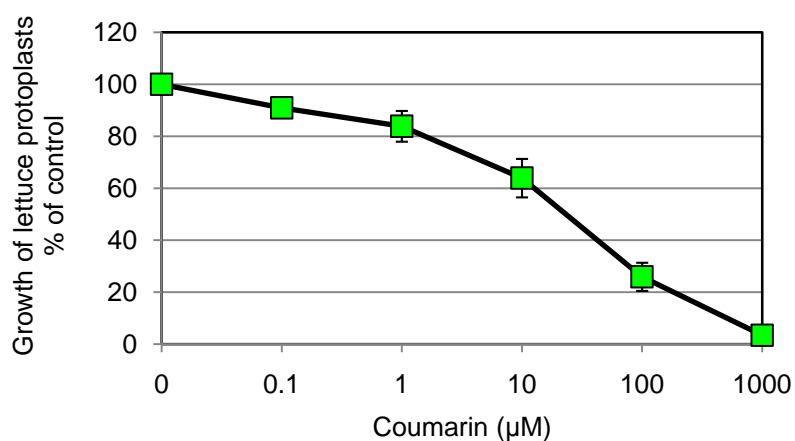


tuce as a recipient plant [14]. Recently, allelopathic effects of coumarin on adult plants of *Arabidopsis thaliana* was investigated [21]. However, its role as an allelochemical in *Prunus* species has not been well investigated. Though allelopathic activity of coumarin has not been discussed in *P. yedoensis*, content of coumarin in young leaves of *P. yedoensis* was reported to be high [13].

Effects of coumarin was investigated on the growth of lettuce protoplasts (Figure 2). Inhibition at higher than 10  $\mu\text{M}$  was clearly observed along the concentrations of coumarin. One mM of coumarin totally inhibited the non-spherical enlargement nor divisions of lettuce protoplasts. Such inhibition is stronger than those of another allelochemical, caffeine, on lettuce protoplasts growth [12].

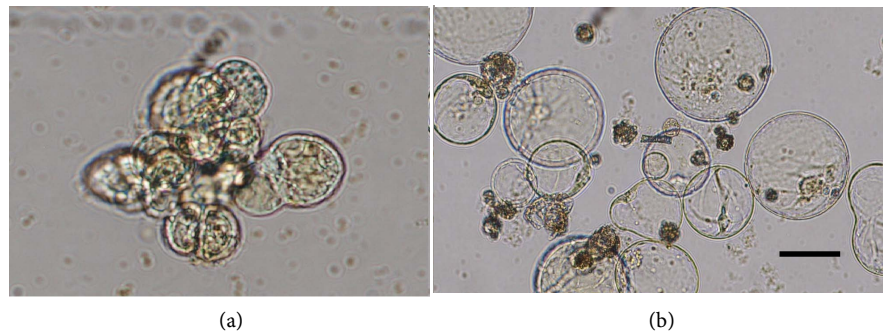
Lettuce protoplasts can develop colonies without allelochemicals after 20 days of culture (Figure 3(a)). Figure 3(b) shows the spherical protoplasts of lettuce by addition of 1 mM coumarin. Such a spherical enlargement without yellow color was also observed indicating the strong inhibitory effect of coumarin, while an alkaloid trigonelline, synthesized from it, had no inhibitory effect in the same lettuce protoplast culture system [11]. Lettuce protoplasts can develop yellow colonies (Figure 3(a)) after cell wall formation and divisions. Such yellow substance of lettuce was a carotenoid and applicable for quantitative bioassay of allelopathy with digital image analysis [10] [22].

Though the inhibitory effects of ABA on the growth of plants cells and protoplasts are well known [15] [16] [17], a different phenomenon was reported in the lettuce protoplast culture. Stimulation of growth was observed by ABA in the range of 0.1 to 10  $\mu\text{M}$ , while antagonistic plant growth regulator,  $\text{GA}_3$  inhibited the lettuce protoplast growth [6]. Such stimulation effect of ABA was also observed in the protoplast cultures of highly salt-tolerant mangrove tree species [23] [24]. However, the role of ABA on protoplast cultures as an allelochemical in *Prunus* species has not been studied.



**Figure 2.** Effects of coumarin concentrations on the growth of lettuce protoplasts. Numbers of non-spherically enlarged and divided lettuce protoplasts were counted after 6 days of culture. Medium was the same as of Figure 1 except for 0.8 M mannitol. Data were described as % of control without coumarin and averaged with SE at lettuce protoplast densities of  $5 \times 10^3/\text{mL}$  -  $50 \times 10^3/\text{mL}$ .





**Figure 3.** Effects of coumarin on protoplasts growth of lettuce after 20 days of culture. Medium was MS basal medium, 2,4-D 1  $\mu$ M, BA 0.1  $\mu$ M, 3% sucrose, and 0.6 M mannitol without (a) and with 1 mM of coumarin (b). Bar = 100  $\mu$ m.

### 3.5. Effects of Putative Allelochemicals on the Growth of Protoplasts of *P. yedoensis*

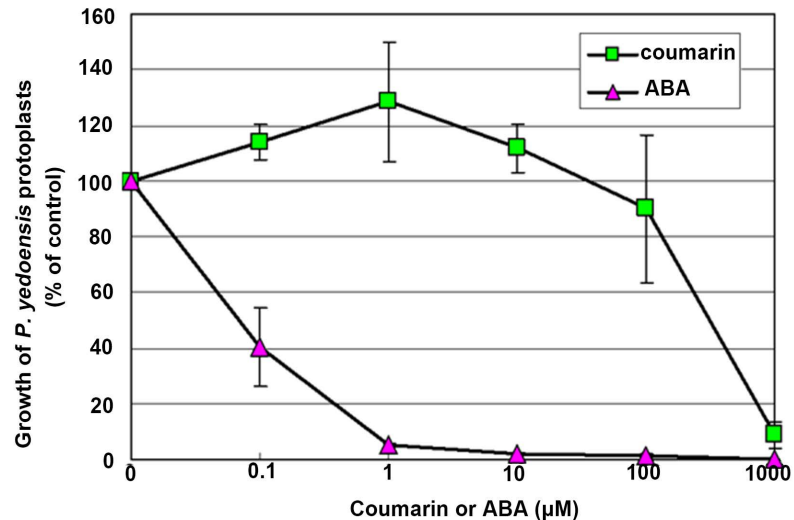
**Figure 4** shows the effects of coumarin and ABA on the growth of protoplasts isolated from suspension cells of *P. yedoensis* originating from leaves after 4 days of culture.

Coumarin was not inhibitory at up to 100  $\mu$ M, but showed strong inhibition at the highest concentration, 1 mM. While ABA showed strong inhibition at even 0.1  $\mu$ M. Compared with the strong inhibitory effects on the growth of lettuce protoplasts (**Figure 2**), coumarin had little inhibitory effect on *P. yedoensis* protoplasts. Similar differences between coumarin and ABA were obtained after 12 days of culture when numbers of colonies composed of 4 cells or more were counted (data not shown). In addition, a highly inhibitory effect of ABA on *P. yedoensis* was inverse of the stimulatory effect on lettuce protoplast reported [6].

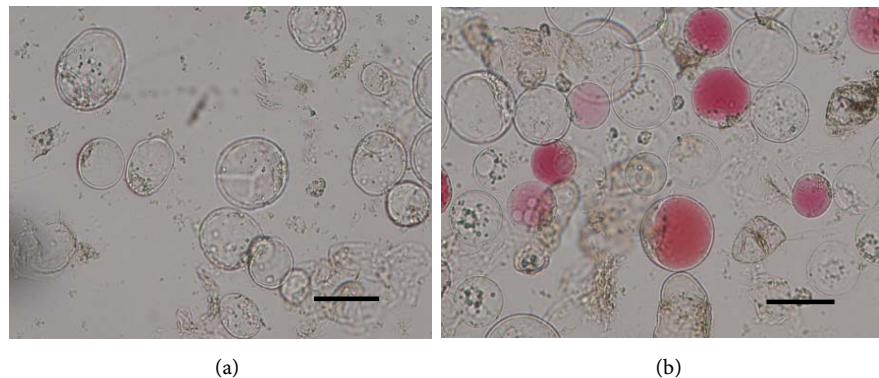
Non-spherical protoplasts observed without allelochemicals, which is the sign of cell wall formation before cell divisions (**Figure 5(a)**), were totally inhibited at 10  $\mu$ M of ABA (**Figure 4**, **Figure 5(b)**). Red spherical protoplasts of *P. yedoensis* were prominent at 10  $\mu$ M of ABA (**Figure 5(b)**).

### 3.6. ABA Content in Protoplasts of *P. yedoensis*

Exogenously supplied ABA strongly inhibited the growth of *P. yedoensis* protoplasts at low concentrations, 0.1 - 10  $\mu$ M (**Figure 4**), but stimulated the growth of lettuce protoplasts [6]. The difference in ABA content in *P. yedoensis* might cause such different effects of ABA in protoplast co-culture. ABA content of protoplasts has been reported for *P. yedoensis* suspension cells derived from peduncle and petiole, but not from leaves, using small scale extraction and purification steps and monoclonal antibody measurement method [16]. We examined the ABA content in leaf-derived suspension cells using the same protoplasts method after partition purification steps and ELISA. Though ELISA is possible with protoplast samples [15]. The content of ABA in protoplasts of leaf-origin suspension cells of *P. yedoensis* was 34.3 pmol/ $10^7$  protoplasts, 2.9 pmol/mg dry weight. These values are similar to those of petiole-derived



**Figure 4.** Effects of coumarin and ABA on the growth of protoplasts isolated from suspension cells of *Prunus yedoensis* originating from leaves. Basal medium was the same as of **Figure 1**. Numbers of enlarged and divided protoplasts after 4 days of culture were counted and described as % of control. Data were averaged with SE at densities of *P. yedoensis* ( $5 \times 10^3/\text{mL}$  -  $25 \times 10^3/\text{mL}$ ).



**Figure 5.** Inhibitory effect of 10 μM of ABA on *P. yedoensis* protoplasts after 4 days of culture. Non-spherical protoplasts of *P. yedoensis* without (a), and red spherical protoplasts with 10 μM of ABA (b). Basal medium was the same as of **Figure 1**. Bars are 50 μm.

suspension cells measured previously (43.9, 5.0 respectively) and higher than peduncle-derived suspension cells [16]. As the diameter of *P. yedoensis* protoplasts is 50 μm, volume of a protoplast is 39.3 pL, the calculated concentration of ABA was 0.09 μM in a *P. yedoensis* protoplast. This value is inhibitory on *P. yedoensis* protoplasts themselves (**Figure 4**) but stimulatory on lettuce protoplasts [6]. Addition of  $2 \times 10^4/\text{mL}$  *P. yedoensis* protoplasts totally inhibited the lettuce protoplast growth (**Figure 1**). Even when all of the endogenous ABA was eluted from the *P. yedoensis* protoplasts, this value is much lower than expected for inhibition of growth as an allelochemical. ABA is not likely the allelochemical of *P. yedoensis*. However, rapid increase of ABA content during co-culture and the secondary effect of endogenous ABA in *P. yedoensis* might be considered.

### 3.7. Coumarin Content in Leaf-Origin Suspension Cells of *P. yedoensis* and Evaluation as an Allelochemical

As coumarin at low concentrations does not affect *P. yedoensis*, and it inhibits the growth of protoplasts and seedlings of the recipient lettuce, coumarin is a candidate allelochemical of *P. yedoensis*. By using GC-MS analysis, the coumarin content of 3.26 µg/g fresh weight was obtained in the suspension cells of *P. yedoensis* originating from leaves. This value corresponds to 22 µM, at which concentration did not inhibit the growth of *P. yedoensis* protoplasts (Figure 4), and inhibited strongly the growth of lettuce (Figure 2) when exogenously supplied in the media. In the protoplast co-culture among *P. yedoensis* and lettuce,  $2 \times 10^4$ /mL of *P. yedoensis* (1000 protoplasts per 50 µL medium) totally inhibited the growth of lettuce protoplasts (Figure 1). As suspension cells contain cell wall portions, the coumarin content in the cytoplasm of cells might be higher than that calculated. Coumarin is more likely the allelochemical of *P. yedoensis* protoplasts in co-culture with lettuce protoplasts.

## 4. Conclusion

In this report, strong inhibitory allelopathic activity of leaves of *P. yedoensis* was found using the sandwich method. Strong inhibitory allelopathic activity was also found using the protoplast co-culture method, during the early and vigorous growing stage in the suspension culture originating from leaves of *P. yedoensis*. Coumarin was evaluated as the allelochemical of leaf-origin suspension cells of *P. yedoensis* in co-culture with lettuce protoplasts. These results were consistent with the report that the coumarin content of *P. yedoensis* was high in the young leaves in spring, but not in autumn [13]. Allelopathic activities are thought to be related to some secondary metabolites accumulated after stopping the plant growth in leaf leachates. However, the small amount of allelochemicals formed during the growth stage must also be involved. Another bioassay method of allelopathy, the plant box method [3] [25], which measures the activities of exudates of intact roots, might be similar to the protoplast co-culture method [6] [10]. Small numbers of protoplasts are sufficient for bioassay of allelopathy using protoplast co-culture method and for the measurement of allelopathic activities of putative allelochemicals [22]. Though lettuce seems to be sensitive as a recipient plant, the protoplast co-culture method is applicable to any plant species simulating the neighboring environment before the big-scale field test [6].

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