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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

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Abstract

Moderately strong allelopathic activities were found in four bamboo species, *Bambusa multiplex* cv. Houraichiku; *Phyllostachys bambusoides* cv. Madake; *P. nigra* cv. Hachiku; *Sasa kurilensis* cv. Chishimazasa, which are of different classification or of different ecological distributions, using the "Sandwich Method", which assays the dried leaves on growth of lettuce seedlings. Only small difference of activity was found among the four bamboo species. In addition, "Protoplast Co-culture Method" for assay of allelopathy in a 50 μL liquid medium using a 96 well culture plate, was applied to the suspension cultures of the four bamboo species. Protoplasts were isolated from two-week cultured suspension cells of four bamboo species using Cellulase RS and Pectolyase Y-23 in 0.6 M mannitol. At low protoplast densities of bamboo, *B. multiplex* and *P. bambusoides* stimulated the recipient lettuce growth, *i.e.*, non-spherically cell enlargement and cell divisions observed under an inverted microscope, while protoplasts of *P. nigra* and *S. kurilensis* were less stimulatory or inhibitory. Inhibitory effect of *S. kurilensis* was the strongest among four bamboo species. Furthermore, highly inhibitory effects of *S. kurilensis* protoplasts on yellow color accumulation of lettuce protoplasts were clearly observed by analysis of a scanned digital image of a 96-well culture plate. Differences and causes of the allelopathic activities were discussed comparing with other plant species studied using the same assay methods.

Keywords

Allelopathy, Bamboo, Bioassay, Digital Image Analysis, Protoplast Culture

1. Introduction

For application of bamboo allelopathy as natural herbicide in agriculture, allelopathic activities of 80 species of bamboo were investigated using *in vitro* bioassay method of allelopathy, "sandwich method", using lettuce as a recipient plant [1], and of several more bamboo species were investigated [2]. The "sandwich method" for dried leaf leachates of test plants using recipient lettuce seedlings growth test had been applied to large numbers of plants and very inhibitory (80% or more inhibition) plants were found [3] [4]. In bamboo species, overall not very strong inhibition (30% radicle growth of control: 70% inhibition at most), and different allelopathic activities in different cultivars were reported [1]. On the other hand, when another *in vitro* bioassay method of allelopathy, "plant box method" which measures the activities of root exudate of a living small test plant, was applied on *Sasa veitchii*, very strong activity (98% inhibition) was reported [2].

Recently, a new *in vitro* bioassay method of allelopathy, "protoplast co-culture method" was developed [5] to contribute for finding of allelochemicals and studying mechanism(s) of allelopathy at cellular level. It measures the growth of recipient lettuce protoplasts in 50 μL liquid medium using a 96-well culture plate, at both phases of cell wall formation and cell divisions. It was applied to herbaceous plants and trees growing at different environmental conditions [6]. Protoplasts of not only leaves [5], but also of calluses [7] and suspension cultured cells [6] of test plants, were used for the "protoplast co-culture method". Strong allelopathic activities were found in *Mucuna gigantea* in "protoplast co-culture method", though only moderate inhibitory activity had been reported using the "sandwich method" in this species [7]. And also quantitative effects of putative allelochemicals, e.g., L-DOPA, isoflavone, purine alkaloids, and pyridine alkaloids, were investigated in the same recipient lettuce protoplast culture system [5] [7] [8] [9] [10]. And very recently, high-throughput *in vitro* bioassay method was developed based on the yellow color accumulation of lettuce protoplasts in "protoplast co-culture method" using a 96-well culture plate [11] [12].

In this report, we selected four bamboo species of different classification, e.g., monopodial or sympodial, and of different ecological distributions, *i.e.*, temperate, tropics, and subarctic. Suspension cultures and isolation of their protoplasts had been developed in *Phyllostachys bambsoides* Sieb. Et Zucc. (Pb, Madake) [13] [14], *Bambusa multiplex* Raeuschel (Bm, Houraichiku) [13] [15] and *Phyllostachys nigra* Munro var. *Henonis* (Pn, Hachiku) [13] [16] [17]**.** Suspension culture of *Sasa kurilensis* Makino et Shibata (Sk, Chishimazasa) were newly developed as same as others. The "protoplast co-culture method" for bioassay of

allelopathy was first applied on suspension cultured cells of the four bamboo species and compared to those of the "sandwich method" performed in this paper. Furthermore digital image analysis [11] [12] was performed on the scanned image of 96 well culture plate in protoplast co-culture method. Differences of allelopathic activities and putative allelochemicals were discussed among bamboo species and other plant species.

2. Materials and Methods

2.1. Plant Materials

Plants of four bamboo species, *i.e.*, *Phyllostachys bambusoides*, Madake (Pb); *Bambusa multiplex* Raeush, Houraichiku (Bm); *Phyllostachys nigra* Munro var. Henonis Hachiku (Pn); *Sasa kurilensis* Makino et Shibata, Chishimazasa (Sk) (**Table 1**), were cultivated using planter boxes in a greenhouse for 2 - 3 years. Fresh mature expanded leaves were collected and dried at 60˚C, for 18 hours and stored in dried condition for the sandwich method.

Suspension cultures of Bm was generated from the node and Pb and Pn were from the young shoot as described previously [13] [14] [15] [16] [17]. Suspension culture of Sk was newly induced from the node in the same way as others. They were sub-cultured with modified (680 mg/L: 4 times phosphate concentration as KH_2PO_4) Murashige and Skoog's (MS) [18] basal medium containing 3% sucrose and 10 μM of picloram. They were cultured in the dark at 27˚C on a rotary shaker at 100 rpm speed. Two-week-old dense suspension cells were used for protoplast isolation.

Lettuce seedlings were prepared as described previously [5]. Briefly, seeds of *Lactuca sativa*, cv. Great Lakes 366, were sterilized with 1.5 % NaClO solution for 15 min and washed with autoclaved water three times. They were aseptically cultured on 0.8% agar medium for 6 - 12 days in the light condition (16 hours photoperiod, 60 μ*E*) at 25˚C.

2.2. Sandwich Method

The sandwich method was performed as reported [3] [4]. Briefly, 10 and 50 mg

Table 1. Characteristics of four bamboo species used in this report.

Scientific name	Classification	a (2n)	cultivar (Common name)	Distribution	b
Phyllostachys bambusoides Sieb. Et Zucc	Bamboo Monopoidal	48	Madake	Temperate (10˚C - 30˚C) c	Pb
Phyllostachys nigra Munro var. Henonis	Bamboo Monopoidal	48	Hachiku	Temperate (10˚C - 30˚C)	Pn
Bambusa multiplex Raeush	Banboo Sympoidal	72	Houraichiku	Tropics (20˚C - 40˚C)	Bm
Sasa kurilensis Makino et Shibata	Sasa Monopoidal	48	Chishimazasa	Subarctic (<15˚C)	Sk

a. Chromosome number, b. Abbrebiations, c. an optimal temperature range for growth.

of dried bamboo 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 lettuce 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 error (SE).

2.3. Protoplast Isolation

Bamboo protoplasts were isolated by enzyme combination of 2% of Cellulase RS (Yakult) and 0.5% of Pectolyase Y-23 (Kyowa Kasei) in 0.6 M mannitol solution for 3.5 to 9 hours incubation. Protoplasts were passed through 42, 63 and 80 μm sized meshes depending on the diameters of protoplasts, and washed with mannitol solution three times by centrifugation at 100 *g* for 4 min.

Lettuce cotyledons protoplasts were isolated as described previously [5]. Briefly, they were treated in 1% each of Cellulase RS and Macerozyme R10 (Yakult) in 0.6 M mannitol solution at room temperature for 24 hrs in the dark. Protoplasts were purified by passing through a 63 μm nylon mesh and washed three times with mannitol solution by centrifugation at 100 g (800 rpm) for 5 min.

Viability of protoplasts were determined using fluorescein diacetate [19].

2.4. Protoplast Co-Culture Method of Bamboo with Lettuce

Protoplasts numbers were counted using a hemocytometer. Five μL each of protoplasts in 0.6 M mannitol solution were put in the 50 μL of liquid MS medium containing 1 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.1 μM benzyladenine (BA), or 10 μM of 2,4-D as plant hormones, and 3% sucrose and 0.6 M mannitol, in a well of 96-well culture plate (Falcon 3075). Final protoplast densities of bamboo were 4×10^3/mL to 10^5/mL, and of lettuce were 6×10^3/mL to 10^5/mL. 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.). The numbers of non-spherically enlarged lettuce protoplasts and small numbers of divided cells were counted under an inverted microscope (Olympus CK40) after 4 days of culture. The numbers of divided cells including colonies composed from four or more cells were counted after 8 days of culture. Percentages of control without addition of bamboo protoplasts were calculated and averaged with SE at lettuce protoplast densities of 6 - 25×10^3/mL (6, 12, 25×10^3/mL).

2.5. Digital Image Analysis

Image analysis of yellow color accumulation of lettuce protoplasts after protoplast co-culture was performed as described previously [12]. Digital image of a 96-well culture plate was scanned using a scanner (Epson GTX-970) after 17 days and 3 months of culture. Image analysis by software Image J [20] was per-

formed as described previously. Briefly, Image, Color, Select Blue image, Draw the horizontal straight Lines at the center of wells. Analyze the line, Plot profile, List the blue plot values, Save as an excel file. In excel software, average the blue plot values in each well. Convert to yellow value by deduction of each averaged blue value from the highest blue value (control). Calculate the % yellow value of control without bamboo protoplasts at each lettuce protoplast density. And finally, percentages of control without bamboo protoplasts were averaged with SE at different cell densities of lettuce (12, 25, 50, 100 × 10^3/mL).

3. Results and Discussion

Table 1 shows the genetic classification and ecological distribution of four bamboo species used in this paper.

3.1. Sandwich Method

Figure 1 shows the sandwich method of four bamboo species. As described in the methods, growth stages of mature leaves were similar as their plants were grown in a greenhouse. Hypocotyl elongation of recipient lettuce was not inhibited except for the slight inhibition by 50 mg leaf of Pb. In contrast, root elongation was inhibited by all bamboo species. Moderate inhibition by 50 mg of dried leaves was found in Bm, Pn and Sk. Inhibition by Pb was strongest in this report (30% growth of control).

Pb and Sk and different cultivars of Pn and Bm were in the big list of sandwich method of bamboo [1]. In their report, leaf of young seedling of Pb had no inhibition, while different varieties of Pb and Pn had slightly higher inhibition (35% growth of control). Sk and different varieties of Bm were similarly moderate inhibitory.

Difference between young leaves and mature leaves was reported in *Mucuna gigantea*, whose putative allelochemical was L-DOPA, though the inhibition was higher in young than mature leaves [7]. Allelopathic activities of bamboo leaves measured using sandwich method might be different depending on growth stages and varieties. Therefore, in four bamboo species in this report, very high inhibition was not found using sandwich method.

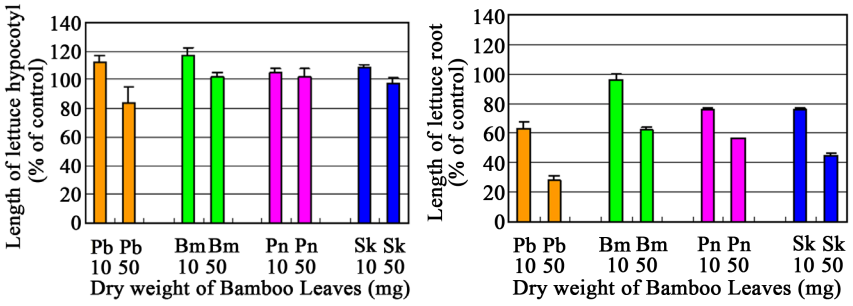

Figure 1. Sandwich method of bamboo leaves on lettuce hypocotyls (a) and roots (b). Averages with SE (N = 5). Pb, *Phyllostachys bambusoides*; Bm, *Bambusa multiplex*; Pn, *Phyllostachys nigra*; Sk, *Sasa kurilensis*.

3.2. Protoplast Isolation from Suspension Culture of Bamboo

Protoplasts were well isolated by using 2% Cellulase RS and 0.5% Pectolyase Y-23 in 0.6 M mannitol, from two-week old suspension cultures of all four species tested. They were very dense culture maintained at high phosphate-containing MS basal medium. Figure 2 shows the protoplasts isolated from four bamboo species. The diameters of protoplasts of Bm were the smallest (20 - 25 μm) and were 30 - 50 μm in other three bamboo species. Suspension culture of Sk was newly developed and maintained in the same medium as suspension cultures of other species. The same strong enzymes combination, for protoplast isolation for suspension cultures of Pb, Pn and Bm, was also effective for the suspension culture of Sk. Viability of protoplasts was more than 90% by FDA staining.

Protoplasts of Pb and Bm could not be isolated from one-week-old suspension cells even after 24 hours incubation (data not shown). Two week-old culture is good growing stage for all four species.

3.3. Protoplast Co-Culture: Effect of Bamboo at Cell Wall Formation Stage of Lettuce

Figure 3 shows the effects of bamboo protoplasts on the growth of lettuce protoplasts after 4 days of co-culture. Green lettuce protoplasts were distinguished

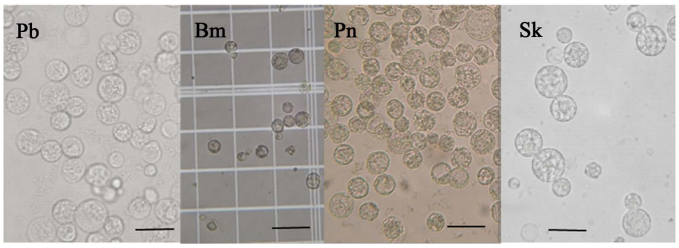

Figure 2. Isolated protoplasts of bamboo. Pb, *Phyllostachys bambusoides*; Bm, *Bambusa multiplex*; Pn, *Phyllostachys nigra*; Sk, *Sasa kurilensis.* Bar, 50 μm.

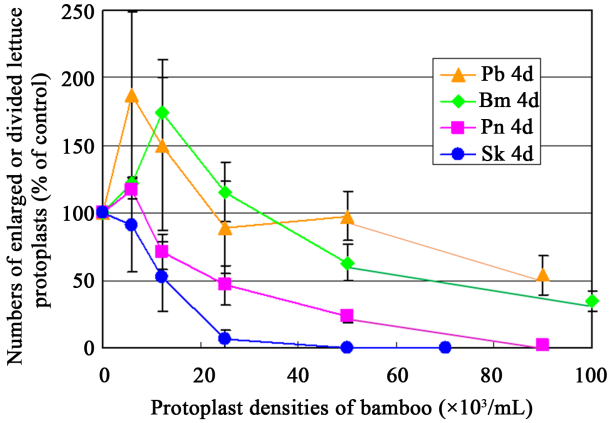

Figure 3. Effects of bamboo protoplasts on the growth of lettuce protoplasts after 4 days of culture. Data are averages with SE at lettuce protoplast densities of 6 - 25 × 10^3/mL. Pb, *Phyllostachys bambusoides*; Bm, *Bambusa multiplex*; Pn, *Phyllostachys nigra*; Sk, *Sasa kurilensis.*

from the protoplasts of bamboo suspension cells under an inverted microscope. In the control without bamboo protoplasts, 91% of lettuce protoplasts were non-spherically enlarged cells, which shows cell wall formation, and 9% were divided cells. Stimulation effects with low protoplast density (1.2×10^4/mL) of Pb and Bm were prominent. In contrast, Pn and Sk clearly inhibited the growth of lettuce at the low protoplast density and at higher densities. Almost no growth was obtained with 2.5×10^4/mL of Sk. Thus differences in inhibition on lettuce growth at cell wall formation stage were found among four bamboo species. Sk protoplasts were most inhibitory on recipient lettuce protoplasts.

This report is the first report that 50% or more stimulation effect was observed by protoplasts of test plants on lettuce protoplasts at cell wall formation stage. In other woody plants, which had very inhibitory allelopathic activities in both sandwich method and protoplast co-culture method, e.g., *Derris indica* [9], *Leucaena leucocephala*, *Mucuna gigantea* [7], and *Sonneratia* mangrove species [6], no stimulation was observed at cell wall formation stage.

3.4. Protoplast Co-Culture: Effect of Bamboo at Cell Division Stage of Lettuce

Effects of bamboo protoplasts on the growth of lettuce protoplasts after 8 days of culture were shown in **Figure 4**. Divided protoplasts of lettuce [6] [8] [12] were distinguished from the protoplasts of bamboo suspension cells under an inverted microscope. In the control without bamboo protoplasts, numbers of divided cells composed of 2 - 3 cells were 53% and colonies composed of 4 or more cells was 47%. Stimulation by all four bamboo protoplasts at low protoplast density (6×10^3/mL) was observed, though stimulation % was the lowest in Sk. At higher

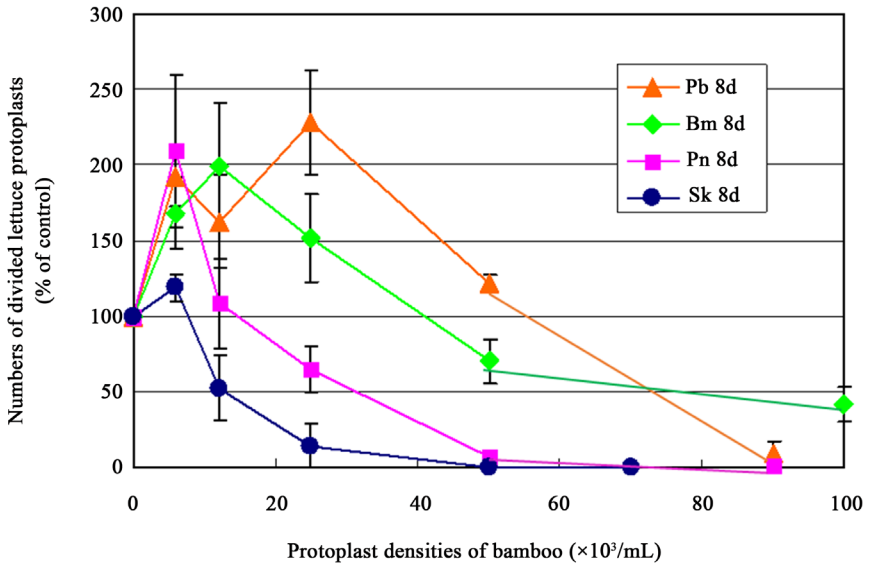

Figure 4. Effects of bamboo protoplasts on the growth of lettuce protoplasts after 8 days of culture. Data are averages with SE at lettuce protoplast densities of 6 - 25 × 10^3/mL. Pb, *Phyllostachys bambusoides*; Bm, *Bambusa multiplex*; Pn, *Phyllostachys nigra*; Sk, *Sasa kurilensis*.

protoplasts densities of bamboo up to 2.5 × 10^4/mL, Pb and Bm were stimulatory, while Pn and SK were similarly inhibitory as of 4 day pattern in Figure 3. The stimulation % of Pb and Bm at cell division stage was very high, 100% around. Similar patterns of stimulation and inhibition were observed in four bamboo species, between cell wall formation stage and cell division stage. Sk is most inhibitory compared to other three bamboo species.

No difference between cell enlargement stage and cell division stage was clearly reported in one of tree mangrove species, *Derris indica.* And the effect of a putative allelochemical, rotenone showed the same pattern of inhibition at both stages [9]. We found differences in stimulation and inhibition of metabolites of purine alkaloids, caffeine [10], and of pyridine alkaloid, trigonelline [8], between two lettuce growth stages. Investigation of such difference and similarity of the inhibitory effects of test plant protoplasts in two growth stages might contribute to clarify the mechanism of allelopathy through finding putative allelochemicals.

3.5. Protoplast Co-Culture with Digital Image Analysis: Effect of Bamboo on Yellow Color Accumulation

Figure 5 shows the effects of four bamboo protoplasts on yellow color accumulation of lettuce protoplasts in each well after 17 days of culture. Data were described as % of control without bamboo protoplasts and averaged at different protoplast densities of lettuce. Effects of 7 × 10^4/mL Sk totally inhibited the yellow color accumulation. Such quantitative analysis with image analysis was developed recently and the yellow substance was carotenoid [11] [12]. After quick

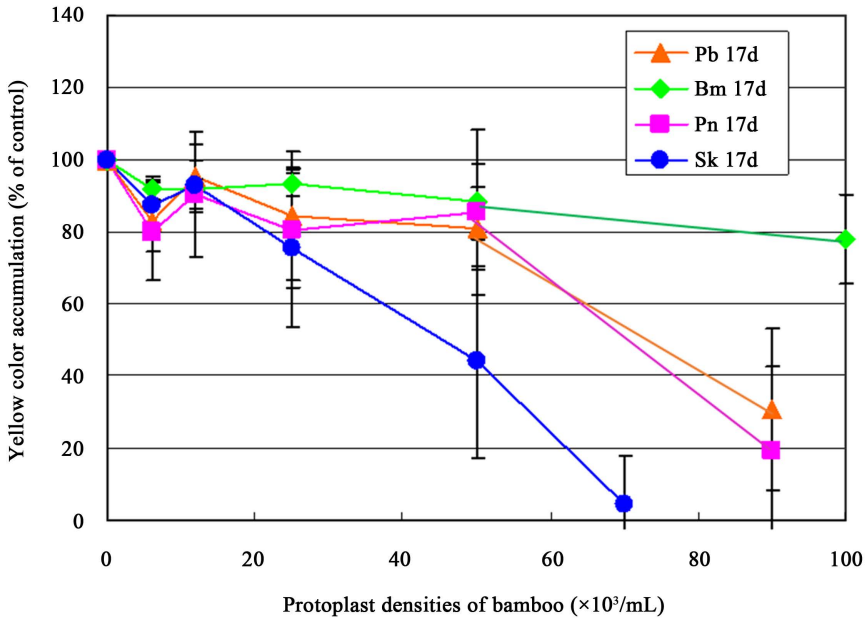

Figure 5. Effects of bamboo protoplasts on yellow color accumulation of lettuce protoplasts after 17 days of culture. Averages with SE at lettuce protoplast densities of 25 - 100 × 10^3/mL. Pb, *Phyllostachys bambusoides*; Bm, *Bambusa multiplex*; Pn, *Phyllostachys nigra*; Sk, *Sasa kurilensis.*

scanning of 96-well culture plate using an inexpensive scanner, analysis with free software Image J [20] was performed. Averaging % of control of yellow intensity in each well at different lettuce protoplast densities, is the same method as those of reacted numbers of protoplast in each well. In contrast to the numbers of protoplasts counted under an inverted microscope, no stimulation was obtained in four bamboo species.

In this report, at 17 day of culture, 6×10^3/mL data which yellow color intensity was low, were deleted and the data at lettuce protoplast densities of 1.2×10^4/mL to 10^5/mL were averaged.

Calculated yellow values at high bamboo densities gave sometimes minus value because we deducted the values of bamboo themselves without lettuce protoplasts [12]. As shown in the scanned digital images after three months of culture (**Figure 6**), bamboo protoplasts developed eye-visible colonies at high protoplast densities (7×10^4/mL to 10^5/mL) of Pn, Pb, Sk, without lettuce protoplasts. However, at the lowest lettuce density (6×10^3/mL), no such colony formation was observed, which shows that bamboo colony formation was mutually inhibited by the addition of lettuce protoplasts. Therefore, strong inhibition by Sk protoplasts were repeatedly observed on yellow color accumulation of lettuce protoplasts.

3.6. Differences and Causes of Allelopathyin Bamboo Species

In the sandwich method, not very strong inhibitory allelopathic activities were repeatedly found in four bamboo species tested. However, in the protoplast co-culture method, with digital image analysis, Sk was most inhibitory among four bamboo species. In all three stages of recipient lettuce protoplast growth, *i.e.*, non-spherical cell enlargement, cell divisions and yellow color accumulation, Sk had the strongest inhibitory effect. At stages of non-spherical cell enlargement and cell divisions, Bm and Pb were much stimulatory at lower protoplast densities of bamboo. Pn was in between of Bm and Pb, and SK. No stimulation on yellow color accumulation was observed in four bamboo species tested.

Such a difference of activities among assay methods of allelopathy was also reported. *Sasa veitchii* (*Sasa glabra*), Kokumazasa, was very inhibitory (2% radicle growth) in another assay method, plant box method, which measure the effects of living root exudates on lettuce seedlings growth, however, only 33% growth of control was reported in sandwich method [2].

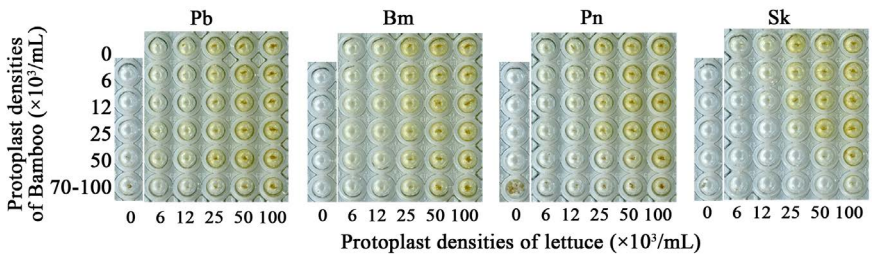

Figure 6. Digital images of effects of bamboo protoplasts on yellow color accumulation of lettuce protoplasts in a 96 well culture plate after three months of culture.

SK plants are subarctic which naturally grow in northern Hokkaido island, Japan and were planted in highway slopes, while Pb and Pn are temperate and Bm is tropics. In this report, protoplast co-culture method was performed at 28˚C. Test at lower temperature can be considered. However, though 20˚C is the best for lettuce seedlings growth in the sandwich method, lettuce protoplasts could be well cultured up to 30˚C in the co-culture method with mangrove species which grow in the tropical area [9]. Optimal temperature for growth might differ between plant and cellular levels.

Different inhibition patterns in protoplast co-culture assay and differences among different bioassay methods might be caused by different concentrations of the same allelochemical or difference of allelochemicals among four bamboo species. Secondary metabolites synthesized in the late growth stage of plant development are thought to be allelochemicals in leaf litter. Leaf leachate and aqueous extracts of a tropical bamboo, *Phyllostachys edulis* was reported to be inhibitory to the growth of recipient plants including lettuce [21]. Allelopathic activities of methanol extracts of Pb [22] had been reported. However, in this report, suspension cells of good growing stage of four bamboo species were used for protoplast co-culture assay. As the protoplast co-culture assay method is based on protoplast isolation from intact living cells, the method is similar to the plant box method which measure the exudate of intact roots.

Bamboo is well known as a source of plant hormones, e.g., gibberellins, for rapid growth of shoots. However, in the lettuce protoplast co-culture assay system, gibberellic acid was inhibitory, while antagonistic hormone, abscisic acid (ABA), stimulated the lettuce protoplast growth at 0.1 - 10 μM range [5]. ABA is known as one of allelochemicals which inhibits growth of recipient Fabaceae plants and *Beta vulgaris* [2]. Therefore, different allelochemical (s) might be considered for Sk protoplasts.

A study is underway to investigate chemical constituents in protoplasts of bamboo species by using microscopy image analysis of flavonoid and lipid [23]. Such a study will contribute to find new allelochemicals in bamboo cells which are effective in only a small amount in protoplast co-culture method.

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