Protoplast Co-culture Bioassay for Allelopathy in Leguminous Plants, *Leucaena leucocephala* and *Mucuna gigantea*, Containing Allelochemical Amino Acids, Mimosine and L-DOPA

Daisuke Mori[1], Shinjiro Ogita[2], Kouhei Fujise[1], Aya Inoue[1] & Hamako Sasamoto[3,4]

[1] Graduate School of Environment and Information Sciences, Yokohama National University, Yokohama, Japan

[2] Biotechnology Research Center and Department of Biotechnology, Toyama Prefectural Unversity, Toyama, Japan

[3] Faculty of Environment and Information Sciences, Yokohama National University, Yokohama, Japan

[4] Research Institute for Integrated Science, Kanagawa University, Hiratsuka, Kanagawa, Japan

Correspondence: Hamako Sasamoto, Yokohama National University, Yokohama, 240-8501, Japan. E-mail: sasamoto@ynu.ac.jp

Abstract

Protoplasts isolated from calluses of two woody leguminous plants, *Leucaena leucocephala* and *Mucuna gigantea,* which contain high levels of the allelochemical amino acids, mimosine and L-DOPA, respectively, were co-cultured to examine their allelopathic activities. The young leaves were first confirmed to have strong inhibitory activity by the sandwich method. Callus cultures were induced and sub-cultured from seeds of *L. leucocephala* and from leaves of *M. gigantea* in Murashige and Skoog's (MS) basal media containing high concentrations of auxin and cytokinins. Protoplasts of both calluses were isolated using the separate well method under strong enzymatic conditions, in high osmotic, 0.8 M mannitol solution. Protoplasts of both species had strong inhibitory effects on the recipient protoplasts of cotyledons of *Lactuca sativa* (lettuce) co-cultured using 96 multi-well culture plates in the MS basal medium, containing 1 μM 2,4-dichlorophenoxyacetic acid, 0.1 μM benzyladenine and 0.8 M mannitol solution. Mimosine was less inhibitory than L-DOPA on the growth of lettuce protoplasts at the stage of colony formation. Amino acid profiles of calluses and protoplasts of the above two species and *Mucuna pruriens* showed very low contents of mimosine and L-DOPA, respectively. The cause of the high inhibitory allelopathic activities found in *Leucaena* and *Mucuna* protoplasts on the growth of lettuce protoplasts was discussed.

Keywords: allelopathy, amino acids, bioassay methods, callus cultures, *Lactuca sativa*, Leguminosae, protoplast cultures

1. Introduction

The tree leguminous plant, *Leucaena leucocephala* which grows in tropical and subtropical areas, is a highly invasive species. The strong inhibitory allelopathic activity of leaf litter was studied in vitro by the 'sandwich method' using dried leaves (Fujii & Hiradate, 2003; Fujii et al., 2003, 2004). Plant tissues contain a large amount of mimosine, which is a toxic non-proteinaceous amino acid (Chou & Kuo, 1986), and contain another pyridine alkaloid, trigonelline (Ogita et al., 2014). Recently, mimosine was found to have an inhibitory effect on influenza viral neuraminidase (Upadhyay et al., 2012).

Another woody leguminous plant, *Mucuna gigantea* that grows in the same (sub) tropical areas, was found to show moderate inhibitory allelopathic activity by the sandwich method (Fujii & Hiradate, 2003). This species is known to contain high amount of an amino acid, L-DOPA, similarly to another herbaceous *M. pruriens*. A variety of the latter species (Hassho-mame) grows in the temperate area. L-DOPA which is biosynthesized from tyrosine, is a strong allelochemical, and is also used for treatment of Parkinson's disease.

To study the mechanism(s) of allelopathy at the cellular level, we developed a new sensitive bioassay method 'protoplast co-culture method' using mixed culture of protoplasts of lettuce and of etiolated leaves of Hassho-mame (Sasamoto et al., 2013). We used the 'protoplast co-culture method' for the suspension cultures of

mangrove species to clarify the inverse relationship between allelopathic activity and salt tolerance. The allelopathic activities were compared to those measured using the sandwich method. (Hasegawa et al., 2014). The effects of metabolites of nicotinic acid on the growth of lettuce protoplasts were investigated to study the non-inhibitory function of trigonelline (Sasamoto & Ashihara, 2014).

In the present study, the activity in young leaves of *L. leucocephala* and *M. gigantea* was examined by the sandwich method. After callus induction and sub-culture, protoplasts of both species were isolated. Inhibitory effects of *L. leucocephala* and *M. gigantea* were investigated in mixed cultures of isolated protoplasts from calluses and protoplasts obtained from lettuce cotyledons at different protoplast densities. In order to elucidate the cause of the strong allelopathic activities of *Leucaena* and *Mucuna* protoplasts, the effect of mimosine in medium on the growth of lettuce protoplasts was investigated and compared with that of L-DOPA (Sasamoto et al., 2013).

Furthermore, we investigated the amino acid profile using sub-cultured callus and the protoplasts of *L. leucocephala*, *M. gigantea*, and *M. pruriens*. Functions of two amino acids, mimosine and L-DOPA, and related amino acids as possible allelochemicals in protoplasts of *Leucaena* and *Mucuna* are also discussed.

2. Method

2.1 Plant Preparation

Seeds and young leaves of *Leucaena leucocephala* and *Mucuna gigantea* were collected on Iriomote island, Okinawa, Japan. Seedlings of *M. gigantea* were grown in a greenhouse.

Etiolated leaves of *M. pruriens* (Hassho-mame) and cotyledons of *Lactuca sativa* (lettuce) were prepared as described previously (Sasamoto et al., 2013). Briefly, seeds of *M. pruriens* were sterilized with 5% NaClO solution for 10 min were imbibed in tap water. After removing the seed coat, they were planted in autoclaved vermiculite in pots and incubated in a low light condition. 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 1 week to 1 month in a light condition (16 hr photoperiod, 60 µ*E*) at 25 ºC.

2.2 Sandwich Method

Young leaves of *L. leucocephala* and *M. gigantea* were dried at 60 ºC for 18 hrs and stored until use. The procedure of the sandwich method was as described previously (Fujii et al., 2003, 2004). Briefly, 10 and 50 mg dry weight of dried leaves were sandwiched between two layers of 5 ml of 0.5% agar (powder, gelling temp. 30-31 ºC, Nacalai tesque Co. Ltd. Kyoto, Japan) in 6 multi-well plates (Nunc). Length of hypocotyls and roots of germinated seeds of lettuce was measured after 3 days of incubation at 20 ºC in the dark. The control treatment was 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 Calluses Induction and Sub-Cultures

The seeds of *L. leucocephala* were sterilized with 1% benzalkonium chloride solution for 5 min and 70% ethanol for 10 min, then washed with autoclaved water three times. Callus was induced using the Murashige and Skoog's (MS, Murashige & Skoog, 1962) basal medium containing 3% sucrose, 30 µM of 2,4-dichlorophenoxyacetic acid (2,4-D) and 3 µM of thidiazuron, solidified with 0.3% gellangum.

Callus was induced from the young green leaves of *M. gigantea.* Leaves were sterilized with 1% NaClO solution for 10 min and washed with autoclaved water three times. Cut sections were first planted in 1 mL liquid MS basal medium containing 3% sucrose, 10 µM each of 2,4-D and benzyladenine (BA), cultured at 100 rpm speed, and proliferated callus was sub-cultured on the same medium composition solidified with 0.8% agar.

Proliferated calluses were sub-cultured in the dark at 3- to 4-week intervals at 30 ºC (*L. leucocephala*), or at 27 ºC (*M. gigantea*) and were used for protoplast isolation or stored in a freezer at -80 ºC.

Callus of *M. pruriens* was obtained from protoplast culture of etiolated leaves using MS medium with 1 µM 2,4-D and 5 µM BA as described previously (Sasamoto et al., 2013), and sub-cultured on the same medium composition solidified with 0.8% agar under the light condition as lettuce seedlings.

2.4 Protoplast Isolation

Callus of *L. leucocephala* after 2-3 weeks of culture was treated for 4-6 hrs with 0.8 M mannitol solution containing 2% each of Cellulase RS (Yakult Pharmaceutical Ind. Co. Ltd., Tokyo), Driselase 20 (Kyowa Hakko Kogyo), 1% Funcelase (Yakult) and 0.5% Pectolyase Y-23 (Kyowa Chemical Products Co. Ltd. Osaka, Japan) in wells of a 24-well culture plate under a sterile condition (the separate well method) (Sasamoto et al., 2013).

Callus of *M gigantea* cultured for 1 week was treated in separate wells for 2-3 hrs with 0.8 M mannitol solution containing 1% each of Cellulase R10, Driselase 20, and 0.5% Pectolyase Y-23.

Protoplasts of etiolated leaves of *M. pruriens* were isolated using the separate well method as previously described (Sasamoto et al., 2013). Leaf sections were treated for 18 hrs with 0.4 M mannitol solution containing 1% each of Cellulase RS, Hemicellulase (H-2125, Sigma) and Macerozyme R-10 (Yakult).

Protoplasts were isolated from lettuce cotyledons in a flask using 1% each of Cellulase RS and Macerozyme R-10 (Yakult) in 0.8 M mannitol solution with a 20 hr-treatment.

After passing through a 42-95 μm nylon mesh, protoplasts were purified by washing three times with the mannitol solution followed by centrifugation at 100 g to 190 g (800-1000 rpm) for 5 min. All procedures were performed at room temperature.

2.5 Protoplast Cultures

Protoplast suspensions in 0.8 M mannitol solution, 5 μL each, were put into 50 μL of liquid medium in a 96-well plastic culture plate (Falcon No. 3075). *Mucuna* or *Leucaena* protoplasts were cultured with lettuce protoplasts in MS medium containing 1 μM of 2,4-D and 0.1 μM of BA, 3% sucrose and 0.8 M mannitol. The protoplast density ranged from 6×10^2 to 10^5 / mL (*L. leucocephala* or *M. gigantea*) and 5×10^3 to 10^5 / mL (lettuce). Mimosine instead of *Leucaena* protoplasts was used for culture of lettuce protoplasts where described. After adding 100 μL of autoclaved pure water (Milipore Direct-Q UV) to the space between the wells, the plate was tightly sealed with two layers of Parafilm[R]. The protoplasts were cultured at 28 ºC or at 30 ºC in a humid incubator (CO_2 -incubator without the supply of CO_2, APC-30DR, ASTEC Co. Ltd.). Numbers of enlarged- and divided- protoplasts were counted at 4 to 9 days of culture under an inverted microscope (Olympus CK40 or IX71).

Growth of lettuce protoplasts was described as the numbers of non-spherically enlarged (>50 μm diameter) and divided protoplasts or colonies composed of more than 4 cells in a well. All data were described as the percentage of the control without *Leucaena* or *Mucnuna* protoplasts or mimosine at each density of lettuce protoplasts. Averages with SE were calculated from the values at different densities of lettuce protoplast as described previously (Sasamoto & Ashihara, 2014; Hasegawa et al., 2014).

2.6 Amino Acid Analysis

Amino acids were extracted and analyzed from calluses and protoplasts as previously reported (Ogita et al., 2014; Tsuchiya et al., 2013). Briefly, after extraction with 80% ethanol at 60 ºC three times, combined fractions were evaporated to dryness using a Vacuum centrifugal evaporator (CVE-3100, EYELA, Tokyo Japan) with a glass cold trap (Uni trap UT-1000, EYELA). The residues were dissolved in 50 mM borate buffer (pH8.0, with 0.05 mM EDTA). Amino acids were precolumn-derivatized with 4 – fluoro – 7 – nitrobenzo – s – oxa – 1,3 – diazole (NBD-F) (Kotaniguchi & Kawakatsu, 1987) and analyzed using a gradient HPLC system (Gilson 305 system) at 30 ºC. Column was YMC-Pack ODS-A reversed-phase column (4.6 mm × 150 mm). The flow rate was 1.0 mL / min. Fluorescence was measured at ex. 470 nm and em. 540 nm. Mimosine was detected with the standard as for the plant tissues (Ogita et al., 2014). Data were calculated from three different concentrations for each sample.

The retention times of unidentified amino acid (u*), mimosine and L-DOPA in this system were 23.9 min, 25.5 min, and 38.1 min, respectively. The content of the unidentified amino acid was described as glycine equivalent.

3. Results and Discussion

3.1 Sandwich Method: Strong Inhibitory Activities of Young Leaves of L. leucocephala and M. gigantea

Though, some allelochemicals of secondary metabolites, might accumulate during the late growth stage of plants, higher inhibitory activity was reported in the young green leaves of soybean using the sandwich method (Sasamoto et al., 2012) compared to that of leaf litter. Strong inhibitory allelopathic activities of leaf litter of *L. leucocephala* and moderate activity of leaf fall of *M. gigantea* were obtained using the sandwich method (Fujii & Hiradate, 2003). In this report, we applied the sandwich method to the young leaves of *L. leucocephala* and *M. gigantea* species. As shown in Figure 1a (*L. leucocephala*) and 1b (*M. gigantea*), young leaves of both species had very strong inhibitory activities on root elongation of lettuce. *M. gigantea* had lower activity than *L. leucocephala* on elongation of lettuce hypocotyls. These findings were consistent with the report on *L. leucocephala* showing higher allelopathic activity and higher content of mimosine in young leaves than in mature tissues (Chou & Kuo, 1986; Xuan et al., 2006).
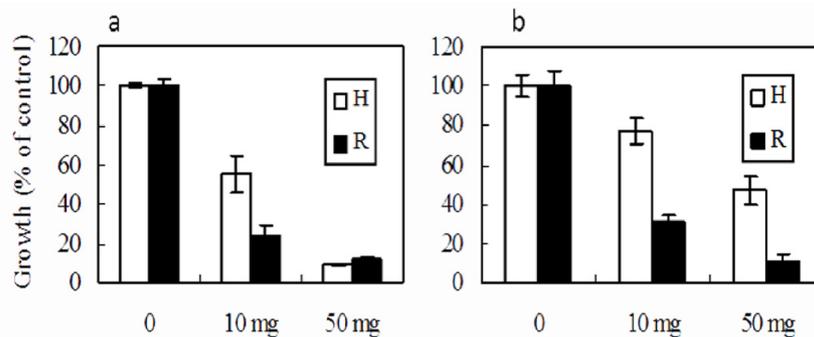
Figure 1. Bioassay of allelopathy by the sandwich method of young leaves of *L. leucocephala* (a) and *M. gigantea* (b). Data were elongation percentages of control with SE of lettuce hypocotyl (H) and root (R)

3.2 Hormonal Conditions for Calluses Induction and Proliferation

Calluses were induced from the hypocotyl of *L. leucocephala* seedlings on MS medium containing high concentrations of plant growth regulators; 30 μM of an auxin, 2,4-D and 3 μM of thidiazuron, which is a strong cytokinin. The same combination of plant hormones was successful for sub-culture. These concentrations of 2,4-D and thidiazuron are very high and strong even compared with those of the subculture medium for *M. gigantea,* which was 10 μM each of 2,4-D and a weak cytokinin, BA. The same hormonal condition in the sub-culture medium was reported for leaf callus cultures of other tree (*Derris indica*) and woody (*Caesalpinia crista*) leguminous plants (Inoue et al., 2010). A herbaceous leguminous plant, *e.g*., *M. pruriens*, also preferred high cytokinin condition which was 1 μM of 2,4-D and 5 μM of BA for callus proliferation (Sasamoto et al., 2013).

3.3 Strong Enzymatic Conditions for Protoplast Isolation of L. leucocephala and M. gigantea

In our previous report, for protoplast isolation from etiolated leaves of *M. pruriens,* which contains a large amount of the allelochemical, L-DOPA, the enzymatic condition; 1% each of Cellulase RS, Hemicellulase and Macerozyme in 0.4 M mannitol, was required in the separate well method using 24-well culture plate (Sasamoto et al., 2013). In this report, the same separate well method, which might reduce the formation of oxidized substances, was required for isolation of protoplasts from calluses of both *L. leucocephala* and *M. gigantea*. In addition, a higher osmotic condition (0.8 M mannitol) and strong enzymatic conditions were required as follows. For the callus of *L. leucocephala,* high concentrations of strong cell wall degrading enzymes; 2% each of Cellulase RS, Driselase 20 and 0.5% Pectolyase Y-23 and addition of 1% Funcellase, were all effective for isolation of protoplasts.

Callus of *M. gigantea* cultured for one week was used for viable protoplasts isolation. Protoplasts were isolated after 2-3 hrs incubation in 0.8 M mannitol solution containing 1% Cellulase R10, 1% Driselase 20, and 0.5% Pectolyase Y-23. Callus cultured for more than 10 days appeared brown and the efficiency of protoplast isolation was reduced. A dim red color, which might be dopaquinone, was observed in the protoplast suspension.

In the present study, lettuce protoplasts were isolated, for protoplast co-culture with *L. leucocephala* or *M. gigantea,* in 1% each of Cellulase RS and Macerozyme in 0.8 M mannitol solution instead of 0.4 M mannitol solution with *M. pruriens* (Sasamoto et al., 2013).

Figure 2 shows the isolated protoplasts of lettuce, *L. leucocephala* and *M. gigantea*. The diameters of them are around 30-50 μm. *Leucaena* protoplasts from the callus contained many starch granules which were stained with I_2-KI solution (data not shown), while lettuce protoplasts contain green chloroplasts.
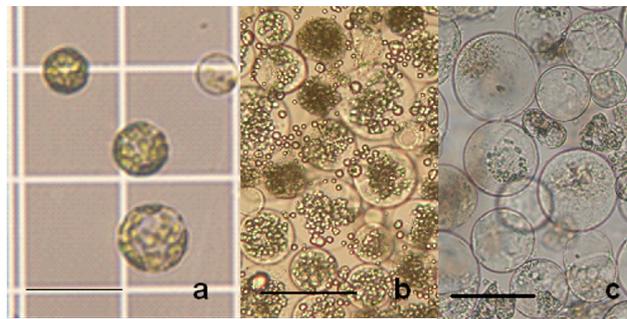
Figure 2. Isolated protoplasts of lettuce (a), *L. leucocephala* (b) and *M. gigantea* (c). Bar = 50 μm

3.4 Protoplast Co-culture Method: Strong Inhibitory Activities of L. leucocephala and M. gigantea on lettuce

As shown in Figure 3, *Leucaena* protoplasts inhibited the growth of lettuce protoplasts depending on the density. *L. leucocephala* at 2×10^4 / mL completely inhibited the growth of lettuce protoplasts. 50% inhibition was obtained with only 10^3 / mL (50 protoplasts / 50 μL medium). This is the 1/100 of the effective density of *M. pruriens* protoplasts which inhibited the growth of lettuce protoplasts (Sasamoto et al., 2013). Similarly, as shown in Figure 4, *M. gigantea* protoplasts at low protoplast density inhibited the growth of lettuce protoplasts. A protoplast density of 2×10^4 / mL completely inhibited the growth of lettuce protoplasts.

The combination of plant growth regulators in the medium, 1 μM of 2,4-D and 0.1 μM of BA, was optimum for growth of lettuce protoplasts (Sasamoto et al., 2013), but protoplasts of *Leucaena* or *M. gigantea* did not divide under the same condition (data not shown). In contrast to *M. pruriens*, which stimulated the growth of lettuce protoplasts at a low protoplast density (Sasamoto et al., 2013), no stimulating effect of *M. gigantea* was observed at a low protoplast density.
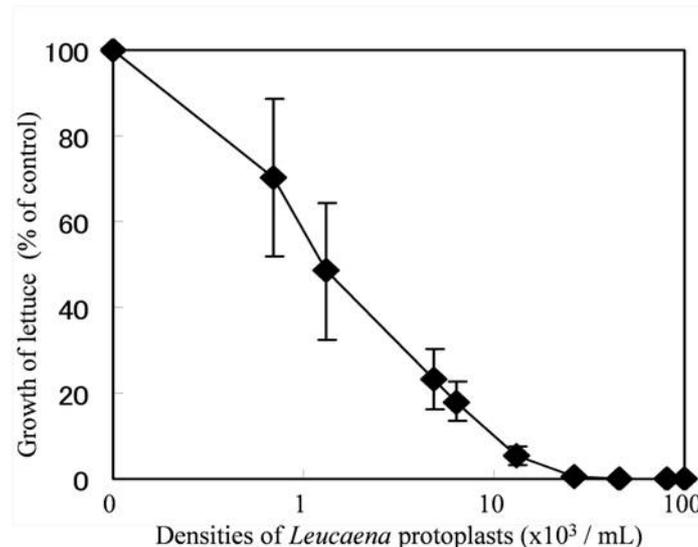
Figure 3. Effects of *L. leucocephala* protoplasts on the growth of lettuce protoplasts co-cultured for 4 days in the MS basal medium containing 1 μM 2,4-D and 0.1 μM BA, 3% sucrose and 0.8 M mannitol. Numbers of enlarged or divided protoplasts were counted and described as the % of control without *Leucaena* protoplasts. Data are averages with SE at 5-200 $\times 10^3$/mL lettuce protoplast density
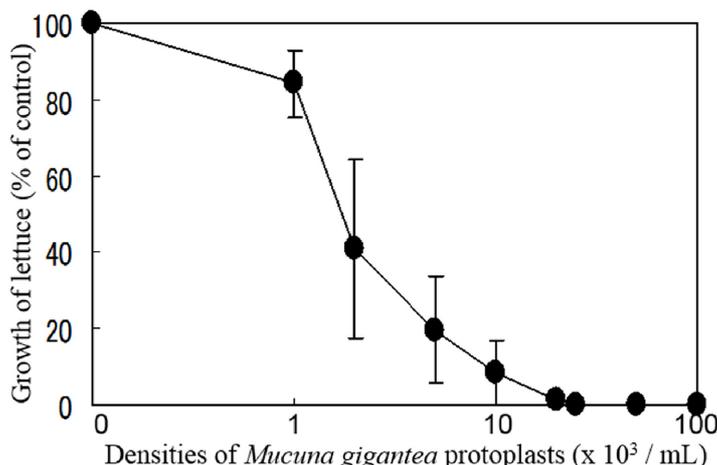
Figure 4. Effects of *M. gigantea* protoplasts on protoplast cultures of lettuce after 4-6 days of culture. Medium was the same as in Figure 3. Numbers of enlarged or divided protoplasts were counted and described as the % of control without *Mucuna* protoplasts. Data are averages with SE at 5-100 $\times$ 10^3/mL lettuce protoplast density

Percentage inhibition of growth to the control without *L. leucocephala* or *Mucuna* protoplasts, was averaged at different protoplast densities of lettuce. This method was useful for comparison of effects of three different metabolites (Sasamoto & Ashihara, 2014) and among different mangrove species (Hasegawa et al., 2014).

3.5 Effects of Mimosine on Lettuce Protoplasts

The high mimosine content of young leaves of *L. leucocephala* has been reported to be the cause of their high allelopathic activity (Xuan et al., 2006). We examined the effect of mimosine on protoplast cultures of lettuce. As shown in Figure 5, at day 4, enlargement of lettuce protoplasts was partly inhibited by 300 μM and 1 mM mimosine supplied in the medium. At 9 days of culture, colony formation was almost completely inhibited at 300 μM of mimosine, which corresponds to the inhibition by 2 $\times$ 10^4 / mL of the *Leucaena* protoplasts in co-culture (Figure 3). The mimosine content in protoplasts of *L. leucocephala* to inhibit the growth of lettuce protoplasts was calculated to be 150 μmoles / 10^7 protoplasts.
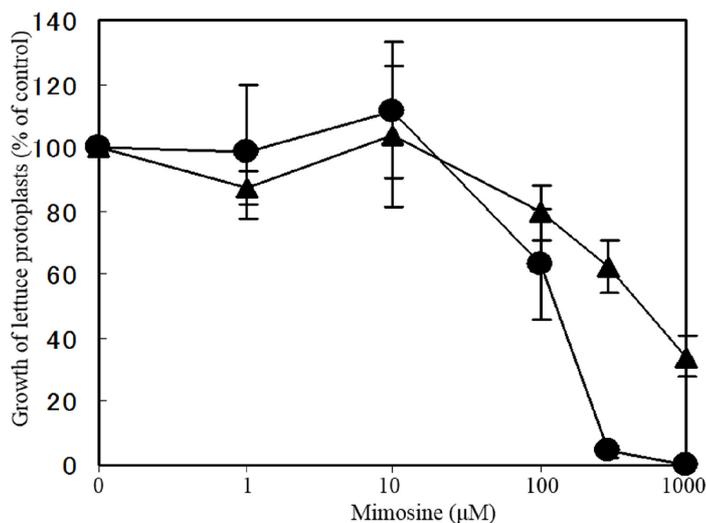
Figure 5. Effects of minosine on growth of lettuce protoplasts. Numbers of enlarged and divided protoplasts, and colonies after 4 days of culture (triangle) and after 9 days of culture (circle), were counted and described as the % of control. Medium was the same as in Figure 3. Data are averages with SE at 5 $\times$ 10^3- 10^5 / mL lettuce protoplast density

3.6 Amino Acid Profiles of Calluses and Protoplasts

The amino acid composition of protoplasts was compared with that of calluses. Amino acid profiles of *Leucaena* callus and protoplasts are shown in Table 1. The callus contained glutamic acid (11 μmoles / g fresh weight) and glutamine (8.9 μmoles / g fresh weight) as major amino acids, and a large amount of unidentified amino acid near the peak of alanine. Mimosine content was low 0.1 μmoles / g fresh weight. Protoplasts contained an extremely high content (76.5%) of glutamine, but a low content of mimosine, 0.11 μmoles / 10^7 protoplasts, which was similar to their contents in callus. Both callus and protoplasts contained mimosine only 0.2% of total amino acids, but they contained different contents of the major amino acids. Such a difference in contents of amino acids between callus and their protoplasts had been reported in several tree mangrove species, which required strong enzymatic conditions for protoplast isolation (Tsuchiya et al., 2013). For isolation of *Leucaena* protoplasts, very strong enzymatic conditions including Fancellase were needed.

Table 1. Amino acid contents of callus and protoplasts of *Leucaena leucocephala*

	Callus		Protoplasts	
	nmoles/g fresh weight		nmoles/10^7 protoplasts	
His	919	(1.8)	325	(0.6)
Arg	500	(1.0)	410	(0.8)
Asn	1977	(4.0)	289	(0.5)
Gln	8861	(17.8)	40554	(76.5)
Ser	4028	(8.1)	684	(1.3)
Asp	617	(1.2)	4	(0.0)
Gly	1068	(2.1)	68	(0.1)
Glu	10956	(22.0)	4076	(7.7)
Thr	2037	(4.1)	165	(0.3)
Ala	1540	(3.1)	969	(1.8)
u*	10588	(21.2)	2947	(5.6)
Pro	2034	(4.1)	236	(0.4)
L-Mimosine	101	(0.2)	110	(0.2)
Met	138	(0.3)	207	(0.4)
Val	1234	(2.5)	383	(0.7)
(Cys)2	14	(0.0)	42	(0.1)
Lys	179	(0.4)	274	(0.5)
Phe	1642	(3.3)	549	(1.0)
Ile	213	(0.4)	126	(0.2)
Leu	1012	(2.0)	539	(1.0)
Tyr	240	(0.5)	74	(0.1)
total	49898		53029	

u*: unidentified amino acid (nmoles glycine eq.).

Values in parentheses are Percentages.

The callus and protoplasts of *L. leucocephala* had amino acid profiles totally different from those of *Leucaena* plant tissues, *e.g.* leaves, flowers and seeds (Ogita et al., 2014), which were used to examine the relation between major amino acids, mimosine and asparagine. The leaves had a mimosine content of 20-40 μmoles / g fresh weight, which was 400-800 times that in the callus. Callus often have a lower content of metabolites than the original plant tissues (Sasamoto et al., 2013). As the volume of a spherical protoplast is 14 pL (30 μm diameter) and 65 pL (50 μm diameter), the concentration of mimosine in *Leucaena* protoplasts was calculated to be 0.78

mM to 0.17 mM. These mimosine concentrations were sufficient for inhibition of growth in lettuce protoplast culture (Figure 5).

Amino acid profiles of *M. gigantea* callus and protoplasts are shown in Table 2. Callus contained alanine (1.5 μmoles / g fresh weight), serine (0.6 μmoles / g fresh weight) and glutamine (0.6 μmoles / g fresh weight) as major amino acids. In contrast, alanine (15.8%) and an unidentified amino acid (23.6%) were the major amino acids in the protoplasts. L-DOPA was not detected in this species, though a high content of tyrosine, the precursor of L-DOPA biosynthesis, was detected in both the callus (7.6%) and protoplasts (5.8%). The content of the allelochemical L-DOPA was also lower in callus cultures than in the original plant tissues (Sasamoto et al., 2013).

Table 2. Amino acid contents of callus and protoplasts of *Mucuna gigantea*

	Callus		Protoplasts	
	nmoles/g fresh weight		nmoles/10^7 protoplasts	
His	33	(0.9)	356	(2.2)
Arg	34	(1.0)	597	(3.6)
Asn	29	(0.8)	320	(1.9)
Gln	555	(16.1)	838	(5.1)
Ser	580	(16.8)	1258	(7.6)
Asp	3	(0.1)	70	(0.4)
Gly	12	(0.4)	345	(2.1)
Glu	69	(2.0)	148	(0.9)
Thr	41	(1.2)	516	(3.1)
Ala	1455	(42.1)	2612	(15.8)
u*	41	(1.2)	3896	(23.6)
Pro	63	(1.8)	780	(4.7)
Met	33	(0.9)	189	(1.1)
Val	90	(2.6)	974	(5.9)
(Cys)2	13	(0.4)	-	
Lys	33	(1.0)	741	(4.5)
Phe	36	(1.0)	503	(3.1)
Ile	24	(0.7)	544	(3.3)
Leu	45	(1.3)	848	(5.1)
L-DOPA	-		-	
Tyr	263	(7.6)	957	(5.8)
total	3453		16493	

u*: unidentified amino acid (nmoles glycine eq.)

-: not detected. Values in parentheses are percentages.

Table 3 shows the amino acid profile of *M. pruriens* callus and protoplasts. Protoplasts isolated from etiolated shoots had a high content of asparagine and alanine. Callus had a high content of alanine (18.5 μmoles / g fresh weight), serine (4.3 μmoles / g fresh weight) and glutamine (3.8 μmoles / g fresh weight) as major amino acids. High alanine and serine contents were similar to that of *M. gigantea* callus (Table 2). L-DOPA content was high in protoplasts (3.5%), and low in the callus (0.1%), while the tyrosine content was lower in protoplasts. The concentration of L-DOPA in *M. pruriens* protoplasts with a diameter of 20 μm (4.2 pL) was calculated to be 5.2 mM. In contrast to *M. gigantea* protoplasts and callus, *M. pruriens* protoplasts had a high L-DOPA content and low tyrosine content.

The protoplasts of *M. gigantea* and callus of *L. leucocephala* had a high content of an unidentified amino acid, which was not detected in *M. pruriens*. These results suggest no common correlation between the contents and strong allelopathic activities of protoplasts of these three species.

Table 3. Amino acid contents of callus and protoplasts of etiolated leaves of *Mucuna pruriens*

	Callus		Protoplasts	
	nmoles/g fresh weight		nmoles/10^7 protoplasts	
His	1081	(3.0)	169	(2.8)
Arg	435	(1.2)	276	(4.5)
Asn	1198	(3.3)	2954	(48.3)
Gln	3800	(10.5)	259	(4.2)
Ser	4323	(12.0)	253	(4.1)
Asp	639	(1.8)	42	(0.7)
Gly	735	(2.0)	74	(1.2)
Glu	1360	(3.8)	54	(0.9)
Thr	12	(0.0)	143	(2.3)
Ala	18494	(51.2)	723	(11.8)
u*	-		-	
Pro	921	(2.5)	99	(1.6)
Met	167	(0.5)	31	(0.5)
Val	1091	(3.0)	216	(3.5)
(Cys)2	35	(0.1)	22	(0.4)
Lys	102	(0.3)	189	(3.1)
Phe	247	(0.7)	92	(1.5)
Ile	440	(1.2)	121	(2.0)
Leu	383	(1.1)	170	(2.8)
L-DOPA	36	(0.1)	216	(3.5)
Tyr	641	(1.8)	13	(0.2)
total	36140		6116	

u*: unidentified amino acid (nmoles glycine eq.).

-: not detected. Values in parentheses are percentages.

3.7 Cause of Strong Allelopathic Activity in Protoplasts

The high inhibitory activity of young leaves of *L. leucocephala* was confirmed by the sandwich method (Figure 1a, section 3.1). The protoplasts also showed similar high inhibitory activity by protoplast co-culture method using callus cultures of *L. leucocephala* (Figure 3, section 3.4).

Leucaena plant tissues contain a large amount of pyridine alkaloids, mimosine and trigonelline (Ogita et al., 2014). However, as trigonelline did not inhibit the growth of lettuce protoplasts, it has been discussed as a detoxification product (Sasamoto & Ashihara, 2014). Non-proteinaceous amino acid, mimosine is thought to be the main allelochemical in *Leucaena* plant (Xuan et al., 2006). Here we examined the direct effect of mimosine in the medium, and found that it inhibited the growth of lettuce protoplasts (Figure 5). However, mimosine was inhibitory only at high concentrations (100-300 μM). As 50 μM of L-DOPA inhibited the growth of lettuce protoplasts completely (Sasamoto et al., 2013), 6 times higher concentration of mimosine in the medium was needed for inhibition. The value, 150 μmoles / 10^7 protoplasts, calculated (section 3.5), is much higher than the experimental result (Table 1). Considering the low content of mimosine in callus and protoplasts (1/200 – 1/400

of leaf tissues, section 3.6), the strong inhibitory activity of *Leucaena* protoplasts co-cultured with lettuce protoplasts, did not correlate directly with the low mimosine content in isolated protoplasts.

Using the sandwich method, young leaves of *M. gigantea* with high inhibitory activity showed strong inhibitory allelopathic activities (Figure 1b). Similar high inhibitory activity of protoplasts was found in the 'protoplast co-culture method', developed using callus culture of *M. gigantea* (Figure 4, section 3.4).

Plants of *M. gigantea* contain L-DOPA as plants of *M. pruriens*. L-DOPA at 50 μM totally inhibited growth of lettuce protoplasts, while no effect on rice protoplasts was obtained. Such phenomena were similar to those of field test of allelopathy (Sasamoto et al., 2013). As the same strong inhibition was obtained with *M. gigantea* by the sandwich method and protoplast method, the mechanism is assumed to be the same. L-DOPA was detected in *M. pruriens* protoplasts (Table 3), but not in *M. gigantea* protoplasts while the precursor tyrosine content was high in both protoplasts and callus (Table 2). The strong inhibitory activities of two *Mucuna* species could not be explained by the low L-DOPA contents of freshly isolated protoplasts detected by amino acid analysis. Concentrations of each putative allelochemical in each protoplast were calculated to be 0.17 to 0.78 mM mimosine in *L. leucocephala*, 5.2 mM L-DOPA in *M. pruriens*. These values are high enough to inhibit the growth of lettuce protoplasts. *M. gigantea* protoplasts were calculated to have a tyrosine content of 1.5 to 6.7 mM.

There are several possible explanations for this discrepancy. (1) *Leucaena* or *Mucuna* protoplasts quickly synthesize mimosine or L-DOPA during culture under non-optimal hormonal conditions in a high osmotic solution. (2) Secondary effects of low contents of mimosine or L-DOPA, during several hrs of treatment for isolation of protoplasts, or cell death during culture may cause a different allelopathic effect during culture. (3) The chemicals dissolved in the medium were not efficiently incorporated into lettuce protoplasts, but cell to cell interaction might accelerate the process; (4) Some specific cellular mechanism(s) can affect lettuce protoplasts through only small amount of chemicals.

Therefore, this protoplast co-culture method using *L. leucocephala* and *M. gigantea* calluses may be useful as a sensitive bioassay of allelopathy. The inhibitory activities of protoplasts on the growth of lettuce protoplasts could be quantitated using a small number (50-5000 / 50 μL). Investigation of putative allelochemicals in recipient lettuce protoplast cultures will help to elucidate the cellular mechanism underlying the allelopathic activity. Furthermore, the method can be applied on different recipient plant species under different culture conditions, e.g., high and low temperature or different nutritional conditions, in order to simulate the possible environmental risk of invasiveness of these species (Sasamoto et al., 2013).

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