American Journal of Plant Sciences, 2013, 4, 1366-1374
http://dx.doi.org/10.4236/ajps.2013.47167 Published Online July 2013 (http://www.scirp.org/journal/ajps)

Relation between Amino Acids Profiles and Recalcitrancy of Cell Growth or Salt Tolerance in Tissue and Protoplast Cultures of Three Mangrove Species, *Avicennia alba*, *Bruguiera sexangula*, and *Sonneratia alba*

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Received April 22nd, 2013; revised May 23rd, 2013; accepted June 15th, 2013

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ABSTRACT

Amino acids profiles were investigated in tissues, cultured cells, *i.e.* callus or suspension cells, and their protoplasts of three mangrove species, *Avicennia alba*, *Bruguiera sexangula*, and *Sonneratia alba*. Original tissues of cultured cells of three mangrove species were cotyledons and hypocotyls, leaves, and cotyledons, respectively. In protoplasts isolated from cultured cells, glutamine and alanine were the major amino acids. Different contents of glycine, proline and serine were observed among protoplasts of three mangrove species. Large differences in the major amino acids were found among cultured cells and their protoplasts while no difference was found between callus and suspension cells independent of additional salt in culture medium. Protoplasts of original tissues, young leaves and cotyledons, contained alanine and glutamine and/or asparagine. In suspension cells of *B. sexangula*, total contents of amino acids were low while their protoplasts showed similar value as of other samples. Protoplasts of leaf and cotyledons of *A. alba* and cotyledons of *A. lanata*, *A. marina* and *S. alba* were also investigated. The total contents of amino acids and their profiles might be related to the recalcitrance for the growth and salt tolerance or halophilic nature of cells and basal media used for the maintenance of cell cultures or protoplast cultures of the mangrove species. This is the first report on callus induction from hypocotyls of *A. alba*.

Keywords: Amino Acid; Avicenniaceae; Callus Culture; Mangrove Plants; Protoplast Culture; Rhizophoraceae; Sonneratiaceae; Suspension Culture

1. Introduction

Mangrove plants, including more than 100 species of different families can be found in brackish water in tropical and subtropical regions [1,2]. Their halophilic or salt tolerance characteristics and adaptation to different osmotic conditions can serve as useful experimental systems to study mechanisms of salt tolerance. Information obtained will have potential application in improving plants' ability to tolerate higher salt levels in the future [3]. Tissue culture is a powerful tool to investigate the mechanisms of salt tolerance at the cellular and molecular levels. However, most mangrove plants remain recalcitrant for cell culture manipulations except for several species. In Sonneratiaceae, callus cultures have been obtained using pistils as explants [4] and suspension cells have successfully been obtained from cotyledons [5] of *Sonneratia alba*. In *S. caseolaris*, suspension cells were successfully generated from seedlings [6]. These cultures were maintained in the Murashige & Skoog's (MS) basal medium [7], which is commonly used in tissue cultures of not only herbaceous plants but also in woody plants. Suspension culture of *Bruguiera sexangula* in Rhizophoraceae has been developed from leaf derived callus culture and sub-cultured more than 10 years in an amino acid (AA) basal medium [8]. For callus induction, the AA medium was preferred to MS [9]. In Avicenniaceae, suspension culture of *Avicennia alba* was induced from

cotyledons and sub-cultured in a modified amino acid (mAA) medium [10]. In (m)AA medium, total nitrogen content of MS was reduced to *ca.* 1/3 and NO_3^- or NH_4^+ were replaced by amino acids [6,7]. Using cultured cells of three mangrove species, *A. alba*, *B. sexangula* and *S. alba*, the effects of sea salts on their growth were investigated and tolerance or halophilic nature were found [10,11].

Protoplast culture of *B. sexangula* was the first species successfully generated for a mangrove species and the protoplast salt tolerance property was compared to a herbaceous plant and a non-mangrove woody plant [12]. The basal medium for protoplast culture of *B. sexangula* was MS, which was different from the AA media for the suspension cells. Recently, some success in protoplasts cultures of *A. alba* suspension cells and *S. alba* cotyledons were developed using the mAA and MS basal medium, respectively, and salt tolerant or halophilic nature of protoplasts were found [13,14].

Amino acids can have an important role to play in the development and establishment of a culture system. Ogita *et al.* [15] reported the successful maintenance of a recalcitrant conifer callus culture through measurement of endogenous levels of amino acids and application of a specific amino acid, glutamine, in the basal medium. In this report, in order to determine whether amino acids can play a role in the establishment of mangrove cell cultures, the endogenous amino acid compositions of callus, suspension cells, and protoplasts of *A. alba*, *B. sexangula* and *S. alba* were investigated. The results were compared to the amino acid profiles of protoplasts of the original tissues. In addition, the amino acid contents of leaf and cotyledons of *A. lanata and A. marina* were also determined as these species are still very recalcitrant for callus proliferation.

2. Materials and Methods

2.1. Plant Materials

Crypto-viviparous seeds of *Avicennia alba* and *A. lanata* were collected in Thailand, and seeds of *A. marina* and fruits of *Sonneratia alba* were collected from Iriomote-Island, Okinawa Japan. Seeds of *S. alba* were aseptically germinated and the cotyledons were used as explants for the generation of callus and suspension cultures [5]. For *Avicennia* species, germinated seedlings were stored in tap water [13] and were used for tissue cultures or protoplasts isolation after sterilization. Young leaf of *A. alba* was obtained from a seedling of 10 cm height which grew in tap water in a flask for 5 months.

2.2. Tissue Cultures: Callus Induction

Maintaining callus cultures of *A. alba* were induced from seedling cotyledons and hypocotyls. The seedlings were maintained in sterile tap water (changing occasionally) for 5 months after seed sterilization with 2% NaClO solution for 40 min. Basal medium was the modified amino acid medium (mAA), in which the glycine content was decreased to 1/10 of AA medium [9], containing 1 μM each of 2,4-dichlorophenoxyacetic acid (2,4-D) and thidiazuron (TDZ) and 3% sucrose [10]. The medium pH was adjusted to 6.3 with NaOH before autoclaving at 120˚C, 20 min. They were sub-cultured at 1 - 2 months intervals.

Suspension culture of *Bruguiera sexangula* [8-9] was sub-cultured at 2 - 3 weeks intervals in liquid AA medium containing 0.02 μM of 2,4-D and 2 μM of *N*-(2-chloro-4-pyridyl)-*N*'-phenylurea (CPPU, Sigma) and 3% sucrose. Fresh medium (after autoclaving) and used medium after 20 days of culture were filter-sterilized (0.22 μm) and kept under sterile condition before amino acid analysis.

S. alba suspension culture and callus cultures were originated from cotyledons [5,11]. They were sub-cultured during 3 to 5 years at 2 weeks intervals (suspension cells) and one month intervals (callus) with MS basal medium containing 3% sucrose, 0.1 μM of 2,4-D with and without 50 mM of NaCl.

All cultures were incubated in the dark at 30˚C. Suspension culture was maintained in 20 - 40 mL medium in a 100 ml flask on a rotary shaker at 100 rpm speed. Callus culture was maintained on medium containing 0.8% agar (tissue culture grade, Wako chemical co. Ltd) using 6 or 9 cm φ petri dishes.

Fresh weights of tissue or callus, or wet weights of suspension cells (10 - 20 mg) were measured before storing at −80˚C.

2.3. Protoplast Isolation

Protoplasts were isolated from cotyledons of *A. alba*, *A. lanata* and *A. marina* by 1% or 2% each of Cellulase RS (Yakult) and Driselase 20 (Kyowa Hakko Kogyo) in 1.3 M sorbitol solution as previously reported [13,16]. Leaf protoplasts of *A. alba* were isolated in 1.2 M sorbitol solution. Protoplasts of calluses originated from cotyledons and hypocotyls of *A. alba* were isolated using 1% each of enzymes in 0.6 M mannitol solution. After passing through a 95 μm sized nylon mesh, protoplasts were purified by floatation in a density gradient centrifugation using 0.6 M mannitol solution on 0.6 M sucrose solution. They were washed with 0.6 M mannitol solution three times by centrifugation at 300 *g* for 5 min.

Protoplasts of *B. sexangula* were isolated from suspension cells cultured for 12 - 19 days by 1% Cellulase RS and 0.25% Pectolyase Y-23 (Seishin) in 0.6 M mannitol solution [12].

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Protoplasts of cotyledons of *S. alba* were isolated in 1% each of Cellulase RS and Macerozyme R10 (Yakult) in 0.8 M mannitol solution [17,18]. Protoplasts of suspension cells (9 - 19 days of culture) and callus (12 days of culture) were isolated in 1% each of Cellulase RS, Driselase 20 and Hemicellulase (Sigma H-2125) in 0.8 M mannitol or sorbitol solution, then purified by floatation in density gradient centrifugation on 1 M sucrose solution [18].

Protoplasts were purified with osmoticum solution by centrifugation at 100 - 300 g, 5 min after filtration through 42 - 95 μm sized nylon mesh depending on the diameters of protoplasts. Numbers of protoplasts were counted using a hemocytometer. They were stored at −80˚C in 1.5 ml micro-tubes or 1.2 ml glass tubes after centrifugation.

2.4. Amino Acid Analysis

Amino acids were extracted and analyzed from tissues directly or from isolated protoplasts as reported by Ogita *et al.* [15]. Briefly, after extraction with 80% ethanol at 60˚C 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 (pH 8.0, with 0.05 mM EDTA). Amino acids were precolumn-derivatized with 4-fluoro-7-nitrobenzo-*s*-oxa-1,3-diazole (NBD-F) [19] and analyzed using a gradient HPLC system (Gilson 305 system) at 30˚C. Column was YMC-Pack ODS-A reversed-phase column (4.6 mm × 150 mm). Flow rate was 1.0 ml/min. Fluorescence was measured at ex. 470 nm, em. 540 nm. Data were calculated from three different concentrations for each sample. And the data of two to four independently extracted samples were described as averages with standard errors in **Tables 1-3**.

3. Results and Discussion

As shown in **Table 4**, only a few mangrove species in each of the three different families had been successful in obtaining sub-culturable callus or suspension cultures and for cell divisions in protoplast cultures, using MS or

Table 1. Amino acid contents of mangrove cultured cells. Concentrations of amino acids are expressed as nmol·g^{-1} fresh weight or wet weight. The values in parenthesis show the percentage of total amino acids.

Amino acids	*A. alba* Cotyledon-callus	*A. alba* Hypocotyl-callus	*B. sexangula* suspension	*S. alba* callus	*S. alba* suspension	*S. alba* suspension NaCl 50 mM
His	423 ± 4 (3)	258 ± 28 (2)	49 ± 6 (2)	252 (1)	682 (4)	131 ± 75 (1)
Arg	906 ± 39 (6)	938 ± 104 (9)	93 ± 24 (3)	997 (6)	628 (3)	30 ± 1 (0)
Asn	4258 ± 1117 (30)	973 ± 68 (9)	17 ± 3 (1)	385 (2)	960 (5)	1372 ± 1157 (15)
Gln	1592 ± 926 (11)	592 ± 194 (6)	103 ± 18 (3)	7573 (45)	5053 (28)	3026 ± 507 (33)
Ser	479 ± 71 (3)	487 ± 105 (5)	370 ± 57 (12)	2423 (14)	2763 (15)	1031 ± 35 (11)
Asp	935 ± 80 (7)	118 ± 38 (1)	112 ± 48 (4)	285 (2)	575 (3)	136 ± 71 (1)
Gly	1126 ± 19 (8)	3046 ± 137 (29)	52 ± 10 (2)	134 (1)	256 (1)	75 ± 10 (1)
Glu	92 ± 26 (1)	220 ± 1 (2)	627 ± 87 (21)	832 (5)	774 (4)	168 ± 25 (2)
Thr	176 ± 20 (1)	109 ± 15 (1)	74 ± 26 (2)	4 (0)	19 (0)	0 (0)
Ala	943 ± 230 (7)	831 ± 21 (8)	827 ± 124 (27)	2498 (15)	2603 (14)	2397 ± 685 (26)
Pro	189 ± 38 (1)	870 ± 146 (8)	126 ± 50 (4)	262 (2)	858 (5)	241 ± 56 (3)
Met	423 ± 118 (3)	336 ± 45 (3)	96 ± 36 (3)	100 (1)	147 (1)	49 ± 3 (1)
Val	442 ± 28 (3)	264 ± 57 (3)	68 ± 12 (2)	244 (1)	685 (4)	152 ± 5 (2)
(Cys)2	23 ± 8 (0)	22 ± 9 (0)	22 ± 5 (1)	590 (3)	30 (0)	24 ± 1 (0)
Lys	521 ± 3 (4)	438 ± 75 (4)	73 ± 22 (2)	87 (1)	447 (2)	38 ± 2 (0)
Phe	292 ± 28 (2)	172 ± 32 (2)	179 ± 58 (6)	15 (0)	368 (2)	18 ± 3 (0)
Ile	477 ± 8 (3)	237 ± 34 (2)	39 ± 16 (1)	111 (1)	330 (2)	84 ± 5 (1)
Leu	481 ± 48 (3)	360 ± 94 (3)	53 ± 13 (2)	140 (1)	352 (2)	109 ± 12 (1)
Tyr	209 ± 5 (1)	122 ± 12 (1)	38 ± 9 (1)	44 (0)	482 (3)	20 ± 2 (0)
total	13,986 ± 2699 (100)	10,392 ± 617 (100)	3017 ± 398 (100)	16,978 (100)	18,011 (100)	9100 ± 55 (100)

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Table 2. Amino acid contents of protoplasts isolated from cultured cells. Concentrations of amino acids are expressed as nmol per 10^7 protoplasts. The values in parenthesis show the percentage of total amino acids.

Amino acids	*A. alba* Cotyledon callus PP	*A. alba* Hypocotyl callus PP	*B. sexangula* suspension PP	*S. alba* suspension PP (NaCl 50 mM)
His	211 ± 18 (3)	94 (3)	45 ± 21 (1)	1551 (5)
Arg	159 ± 6 (3)	93 (3)	260 ± 213 (4)	646 (2)
Asn	278 ± 4 (5)	65 (2)	32 ± 7 (1)	930 (3)
Gln	1012 ± 46 (17)	347 (10)	1851 ± 424 (32)	15868 (53)
Ser	335 ± 6 (6)	193 (6)	600 ± 86 (10)	1052 (4)
Asp	33 ± 10 (1)	9 (0)	43 ± 17 (1)	314 (1)
Gly	1226 ± 88 (20)	238 (7)	35 ± 12 (1)	337 (1)
Glu	51 ± 11 (1)	64 (2)	163 ± 18 (3)	246 (1)
Thr	204 ± 30 (3)	75 (2)	145 ± 54 (2)	617 (2)
Ala	668 ± 53 (11)	1103 (32)	1765 ± 139 (30)	4457 (15)
Pro	470 ± 40 (8)	361 (11)	140 ± 31 (2)	486 (2)
Met	36 ± 5 (1)	0 (0)	15 ± 15 (0)	229 (1)
Val	252 ± 10 (4)	120 (4)	172 ± 30 (3)	663 (2)
(Cys)2	45 ± 21 (1)	10 (0)	0 (0)	635 (2)
Lys	191 ± 3 (3)	73 (2)	29 ± 29 (0)	481 (2)
Phe	155 ± 10 (3)	63 (2)	66 ± 32 (1)	220 (1)
Ile	204 ± 21 (3)	123 (4)	167 ± 118 (3)	250 (1)
Leu	418 ± 6 (7)	290 (9)	250 ± 169 (4)	733 (2)
Tyr	88 ± 54 (1)	84 (2)	85 ± 41 (1)	213 (1)
total	6034 ± 92 (100)	3402 (100)	5863 ± 1267 (100)	29,927 (100)

(m)AA basal media.

In this report, in addition to the generation of callus cultures of *A. alba* from cotyledons, we were also successful in the generation of calluses from seedling hypocotyls (**Figure 1(a)**) using a mAA basal medium containing 1 μM each of 2,4-D and TDZ, the same hormonal condition for the sub-culturing of cotyledon-derived suspension cells [10]. Callus proliferation from both tissues was observed after only one month, which is much faster than the previous induction of suspension cells from cotyledons in a mAA medium containing 1 μM each of 2,4-D and benzyladenine, which took 5 months of culture. As shown in **Figure 1**, the calluses could be sub-cultured in the same composition medium as of induction and used for protoplast isolation and amino acid analysis. This is the first report on callus induction from hypocotyls of *A. alba*. Morphological characteristics of the callus cultures and their protoplasts derived from the hypocotyls (**Figure 1**) were similar to those of the cotyledon-derived callus or suspension cells and their protoplasts

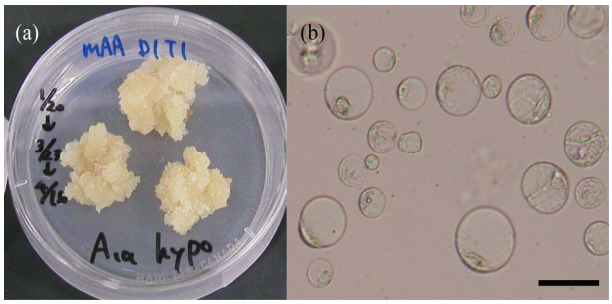

Figure 1. Hypocotyl derived callus (a) of *Avicennia alba* and their protoplasts (b). (a) sub-cultured callus in solid mAA basal medium containing 1 μM each of 2,4-D and TDZ in a 6 cm petri dish. (b) isolated protoplasts in 0.6 M mannitol solution. bar = 100 μm.

[13]. The method of storing seeds of *A. alba* in tap water at aseptic condition was based on the report on the cotyledon protoplast culture of crypto-viviparous seeds of *A. marina* [13]. Storing the seeds in pure water resulted in early browning of seeds of both *Avicennia* species.

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Table 3. Amino acid contents of protoplasts isolated from leaves and cotyledons of mangrove plants. Concentrations of amino acids are expressed as nmol per 10^7 protoplasts. The values in parenthesis show the percentage of total amino acids.

Amino acids	*A. alba* leaf PP	*A. alba* cotyledons PP	*A. lanata* cotyledons PP	*A. marina* cotyledons PP	*S. alba* cotyledons PP
His	17 (0)	1 (0)	41 ± 2 (1)	186 (2)	795 (4)
Arg	62 (2)	212 (9)	71 ± 3 (1)	184 (2)	1229 (6)
Asn	25 (1)	896 (39)	1282 ± 157 (18)	3537 (33)	1237 (6)
Gln	2246 (56)	54 (2)	1783 ± 250 (26)	592 (6)	10215 (52)
Ser	74 (2)	41 (2)	253 ± 19 (4)	244 (2)	217 (1)
Asp	24 (1)	27 (1)	260 ± 68 (4)	281 (3)	83 (0)
Gly	19 (0)	0 (0)	64 ± 9 (1)	283 (3)	21 (0)
Glu	58 (1)	68 (3)	166 ± 15 (2)	200 (2)	253 (1)
Thr	142 (4)	67 (3)	195 ± 5 (3)	235 (2)	263 (1)
Ala	630 (16)	342 (15)	1264 ± 98 (18)	2489 (23)	1934 (10)
Pro	138 (3)	85 (4)	99 ± 5 (1)	199 (2)	439 (2)
Met	0 (0)	0 (0)	0 (0)	43 (0)	113 (1)
Val	119 (3)	68 (3)	497 ± 19 (7)	502 (5)	401 (2)
(Cys)2	17 (0)	0 (0)	0 (0)	34 (0)	95 (0)
Lys	0 (0)	0 (0)	0 (0)	152 (1)	213 (1)
Phe	48 (1)	62 (3)	231 ± 18 (3)	261 (2)	672 (3)
Ile	87 (2)	123 (5)	280 ± 25 (4)	382 (4)	279 (1)
Leu	271 (7)	168 (7)	357 ± 19 (5)	764 (7)	747 (4)
Tyr	55 (1)	71 (3)	130 ± 110 (2)	182 (2)	419 (2)
total	4030 (100)	2284 (100)	6972 ± 87 (100)	10,750 (100)	19,625 (100)

Table 4. List of mangrove species which were successful for sub-culturable callus cultures or suspension cultures, and for cell divisions in protoplast cultures.

mangrove species	cultures	origin of cells	origin of protoplasts	basal medium	references in journals
Sonneratia alba	callus	pistil		MS	Akatsu *et al*. 1996
Sonneratia alba	callus, suspension cells	cotyledons		MS	Kawana *et al*. 2007; Kawana & Sasamoto 2008
Sonneratia alba	protoplast	-	cotyledons	MS	Kawana *et al*. 2009; Hasegawa *et al*. 2013
Sonneratia caseolaris	suspension cells	seedling		MS	Yamamoto *et al*. 2009
Bruguiera sexangula	callus, suspension cells	leaf		AA	Mimura *et al*. 1997; Kura-Hotta *et al*. 2001
Bruguiera sexangula	protoplast	-	suspension cells	MS	Fukumoto *et al*. 2004
Avicennia alba	suspension cells	cotyledons		mAA	Hayashi *et al*. 2009
Avicennia alba	protoplast	-	suspension cells	mAA	Hasegawa *et al*. 2011, 2013
Avicennia alba	callus	cotyledons		mAA	present report
Avicennia alba	callus	hypocotyls		mAA	present report
Avicennia marina	protoplast	-	cotyledons	mAA	Hasegawa *et al*. 2011

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Table 1 shows amino acids contents of callus or suspension cells of *A. alba*, *B. sexangula* and *S. alba* expressed on a gram wet weight or fresh weight basis. Major amino acids in cotyledons-callus of *A. alba* were asparagine, glutamine and glycine, while glycine, asparagine, arginine, alanine and proline in hypocotyl-callus. In *S. alba*, there was no difference in amino acid profile between suspension cells and callus cultured in the media without 50 mM of NaCl; glutamine, alanine, serine were the major amino acids detected. When comparing the amino acid profiles between the suspension cells of *B. sexangula* and *S. alba*, major differences were observed in glutamine and glutamic acid levels. In *S. alba* suspension cells cultured in the medium containing 50 mM of NaCl, asparagine was also the major amino acid.

We also investigated the amino acid composition in the fresh and used medium of suspension culture of *B. sexangula*, which were cultured in AA basal medium. Though glutamine content in the fresh AA medium was already low (281 μmoles/L, 1/20 of the expected amount) after autoclaving at 120˚C, 20 min, arginine, aspartic acid, glycine contents were at expected values (909, 1556, 1176 μmoles/L, respectively). After 20 day of culture, a very low total content (74 μmoles/L, 2% of the fresh AA medium) was found in the used medium. Proline, serine and arginine (20, 17, 11 μmoles/L, respectively) were major amino acids in the used medium and were different in suspension cells shown in **Table 1**. Amino acids in the AA medium were quickly taken up and metabolized by *Bruguiera* cells.

Though mAA basal medium contains arginine, aspartic acid and glutamine at high concentrations, very different amino acid profiles were noted in cotyledon- and hypocotyl-calluses of *A. alba* (**Table 1**). This indicates that both calluses of *A. alba* must have metabolized the medium amino acids during the course of culture.

Total amino acids contents of suspension cells of *B. sexangula* were lower than those of *A. alba* or *S. alba* (**Table 1**). Such low amino acid content of *B. sexangula* was repeatedly observed independent of culture period (2 to 3 weeks) of suspension cells. As the protoplasts are active to divide in culture, which were isolated from suspension cells of *B. sexangula* cultured for two-three weeks [12], metabolic activity of *B. sexangula*, *e.g.* protein synthesis, might remain active during the culture period. Low total amino acid content might be related to high activity of cell growth. *B. sexangula* suspension cells show salt tolerance; however, rapid growth can be obtained without additional salts while *S. alba* suspension cell cultures are halophilic [11]. In *S. alba*, the total amino acid content on the basis of g fresh (wet) weight of NaCl-containing medium-grown suspension cells was half of the callus or suspension cells grown without additional NaCl. This phenomenon might reflect the halophilic nature found in *S. alba* suspension cells. In *Avicennia*, the reported values of total amino acid content in the mature plant organs, leaves or roots, of *A. marina* are very high (68, 329 μmoles/g fresh weight) [20], compared to the values (10 - 14 μmoles/g fresh weight) of the cultured cells of *A. alba* in **Table 1**.

Table 2 shows amino acids contents in 10^7 protoplasts isolated from cultured cells of *A. alba*, *B. sexangula*, and *S. alba*. In *A. alba* callus-protoplasts, amino acid profiles were different between calluses induced from cotyledons and hypocotyls. Major amino acids were glycine, glutamine, alanine and proline in the former protoplasts while they were alanine, proline, glutamine and leucine in the latter. In protoplasts isolated from *S. alba* suspension cells cultured with 50 mM of NaCl, major amino acids were glutamine and alanine. In *B. sexangula* protoplasts, major amino acids were glutamine, alanine and serine.

Total amino acid contents in protoplasts from *A. alba* callus and *B. sexangula* suspension cells were similar. However, the total amino acid content in protoplasts from *S. alba* suspension cells grown in NaCl-containing medium was very high. Such a high total amino acid content might reflect the recalcitrancy of protoplast culture of *S. alba* suspension cells [18,21].

From comparison between **Tables 1** and **2**, amino acid composition differs much between cultured cells and their protoplasts. In protoplasts and original cotyledon-callus of *A. alba*, glutamine content was similarly high; however, difference was observed in the contents of asparagines, alanine and proline. In protoplasts and original hypocotyl-callus of *A. alba*, alanine and proline contents were similarly high, however, difference was observed in the contents of arginine, asparagine, glutamine and glycine. In protoplasts and original suspension cells of *B. sexangula*, high alanine and high serine contents were similar each other. However, high glutamic acid content in suspension cells was different from high glutamine content in protoplasts. In *S. alba*, high contents of glutamine and alanine were similar to both protoplasts and suspension cells; however, high serine was only observed in suspension cells. These differences can be explained by the distribution of amino acids in cell wall of each cultured cells or by the metabolic changes of amino acids which might have occurred during protoplast isolation. Long incubation times, several hrs to 3 days, in strong enzymatic conditions are needed for isolation of protoplasts in mangrove species [13,17]. Therefore, such a difference in amino acids composition should affect culture conditions for them.

As reviewed in **Table 4**, some success in colony formation was already obtained in protoplast cultures of *S. alba* cotyledons [18] and *A. alba* suspension cells, and

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their halophilic or salt tolerant nature were found [14]. However, sub-culturable callus proliferation from protoplast culture was accomplished only with *B. sexangula* suspension cells [12]. Protoplast isolation and cultures of *S. alba* suspension cells were more difficult than that of cotyledons [21]. MS basal medium was used for all tissue cultured cells of *S. alba*, *i.e.* suspension cells and callus [5] and protoplasts cultures [14,18,21]. *A. alba* cotyledons-derived suspension cells preferred mAA basal medium to MS, similarly, in protoplast culture of them, colony formation was better in mAA basal medium than in MS [22]. Recently, at the optimal hormonal condition, 1 μM each of 2,4-D and TDZ, cell enlargement and some cell divisions were obtained in protoplast cultures of hypocotyls-callus of *A. alba* in the same mAA basal medium as the medium for sub-culture of original callus [23]. In contrast, in the protoplast culture of *B. sexangula*, MS basal medium was preferred to (m)AA basal medium [12,22], though AA was preferred to MS in callus culture [9]. Amino acid profile of the *B. sexangula* protoplasts showed the high glutamine content and the low glutamic acid content (**Table 2**) compared to those of suspension cells (**Table 1**).

Table 3 shows amino acids profiles of protoplasts of young leaf and cotyledons of *Avicennia* species, *A. alba*, *A. lanata and A. marina*, and cotyledons of *S. alba*. Higher total contents of amino acids were found in recalcitrant *Avicennia* species, *A. lanata* and *A. marina*, than *A. alba*, though the highest value was obtained in cotyledons of *S. alba*. In cotyledons of all three *Avicennia* species, asparagine and/or glutamine were the major amino acids, while glutamine was in cotyledons of *S. alba* and young leaf of *A. alba.* Exclusively high asparagine content (86%) in mature plant organs (leaf and root) of *A. marina* was reported [20]. In contrast, some value of alanine was obtained in protoplasts of all young tissues of *Avicennia* and *Sonneratia* species, and also in protoplasts of the suspension cells and callus of these species (**Table 2**). Such a difference in amino acids profiles might reflect the recalcitrance of mature organs for induction of callus and suspension cell cultures compared to young tissues, e.g. cotyledons and hypocotyls of a seedling. Endogenous levels of specific amino acids, e.g. glutamine, are important for the maintenance of recalcitrant conifer callus cultures [15]. However, only high glutamine content is not sufficient for induction of callus in recalcitrant mangrove tissues.

Proline is one of amino acids which is known to relate to stress tolerance and used in tissue culture [24]. High proline content along with high content of osmoregulator (glycinebetaine) was reported in protoplasts of cotyledons of *A. marina* [25]. Low proline data of *A. marina* shown in **Table 3** might be caused by the long seed-storage period in tap water in this report. During the long storage time, the endogenous content of stress-related plant hormone, abscisic acid, also decreased and some cell enlargement and cell divisions were observed in protoplast culture [13]. Genes related to proline utilization in *A. marina* was reported [26], though no proline was detected in mature plant organs of it [20]. In this report, proline was present in *A. alba* hypocotyl-derived callus and protoplasts of cotyledon-derived callus and it did not totally inhibit growth of them.

By investigation of the differences between wall containing suspension cells and protoplasts, we can know the site of precise mechanisms for halophillic or salt tolerance and the osmotic tolerance in mangrove cells [14]. From the total amino acid data on wet weight basis shown in **Table 1**, ca. 10 - 20 mM concentration was calculated for *S. alba* suspension cells. Protoplasts are simple system; removing cell walls, under osmotic pressure, are spherical shaped and cell numbers can be counted easily, while suspension cells or callus are difficult to measure their cell numbers. Volume of 10^7 protoplasts can be calculated from their diameters, *i.e.* 42 μL (20 μm), 141 μL (30 μm) and 393 μL (50 μm). The concentrations of endogenous plant hormones in a protoplast were calculated in conifer protoplasts [27]. Similarly, as diameter of protoplasts of *S. alba* suspension cells was 30 to 50 μm [18], total amino acid concentration of them was calculated to be 150 mM at the highest when amino acids distribute uniformly in a cell. Considering some compartmentation in organelles of a protoplast, a part of osmotic adjustment might be regulated by amino acids and might contribute to the halophilic nature of *S. alba* cells. Lower values of total amino acid concentration were calculated for other materials.

4. Conclusions

Callus cultures of *A. alba* were induced from cotyledons and hypocotyls, respectively, of small seedlings in a mAA basal medium containing 1 μM each of 2,4-D and TDZ. This is the first report on callus induction from hypocotyls of *A. alba*.

Amino acids profiles were investigated in tissues, in cultured cells, *i.e.* callus or suspension cells, and in their protoplasts of three mangrove species, *Avicennia alba*, *Bruguiera sexangula*, and *Sonneratia alba*. Large differences in major amino acids were found among cultured cells and their protoplasts while no difference was found between callus and suspension cells nor between cells cultured with or without NaCl in *S. alba*. In suspension cells of *B. sexangula*, which were sub-cultured in AA basal medium, total contents of amino acids were low and glutamic acid was observed along alanine and serine. Low total amino acid content which might reflect high

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metabolic activity can be related to the success of subculturable callus culture or suspension culture of mangrove species. In protoplasts isolated from cultured cells of the three mangrove species, glutamine and alanine were the major amino acids. Different contents of glycine, proline and serine were observed among protoplasts. In *B. sexangula* protoplasts, which were successful for protoplast culture in MS basal medium, high glutamine content was observed. Amino acid profiles and contents in protoplasts of original tissues, leaves and cotyledons of *A. alba* and cotyledons of *S. alba*, were compared to those of recalcitrant mangrove species, *A. lanata* and *A. marina*. Cotyledons of all three *Avicennia* species contained asparagine and/or glutamine and alanine, while cotyledons of *S. alba* and young leaf of *A. alba* contained glutamine and alanine. They were very different from the report of mature organ of *A. marina*, which were recalcitrant for culture.

The total contents of amino acids and their profiles might be related to the recalcitrance for the growth and salt tolerance or halophilic nature of cells and basal media used for the maintenance of cell cultures or protoplast cultures of the mangrove species.

5. Acknowledgements

Authors thank to Dr. Reiko Minagawa for supplying the seeds of *Avicennia alba* and *A. lanata*, and thank to Prof. Edward C. Yeung of the University of Calgary for his valuable suggestions on this ms.

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