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Effect of High Sorbitol Concentration on Protoplast Isolation from Cotyledons of Mangroves, *Avicennia marina* and *A. lanata*

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Abstract

High and wide range of concentration of sorbitol, 0.6M to 1.9M, was effective as osmoticum in combination with 2% each of Cellulase RS and Driselase, on the isolation of protoplasts from cotyledons of *Avicennia marina* and *A. lanata*. Protoplast yield varied from 10^6 to 10^7 cells per seed. Sorbitol at 1.3-1.4M was consistently effective of good yield.

1. Introduction

Avicennia marina, a mangrove forest tree, grow on the coast of Iriomote island in Japan, and *A. lanata*, classified in the same genus, grow in tropical regions of the world [1]. They can grow in sea water. Such high salt tolerance is a good trait to introduce when breeding of forest tree species. Somatic cell fusion and genetic transformation through protoplast culture are considered to be promising tools to decrease the long time required for breeding of such species [2]. However, no report on mangrove species has been published. In this report, we describe the develop ment of a protoplast isolation technique which is prerequisite for both somatic cell fusion, and for the elucidation of the mechanisms of salt tolerance at the cell level which form the basis for genetic engineering of mangroves.

2. Materials and Methods

2.1 Materials

The seeds of *Avicennia marina* were harvested along the coast of Iriomote island, Japan. The seeds of *A. lanata* were harvested at Can Gio in Vietnam. They were dehusked by imbibing in water and were stored at room temperature, with or without further imbibition in water for 4 days to 2 months before the isolation of protoplasts.

2.2 Isolation of protoplasts

For determination of the optimal enzyme combination for isolation of protoplasts from cotyledons of *A. marina*, 24 combinations were tested, using 2% each of six enzymes in 1.3M sorbitol solution ; Cellulase RS (Yakult Honsha Co. Ltd.), Cellulase R-10 (Yakult Honsha Co. Ltd.), Hemicellulase (H-2125, Sigma Chem. Co.), Driselase (Kyowa Hakko Kogyo Co. Ltd.), Macerozyme R-10 (Yakult Honsha Co. Ltd.), and Pectolyase Y-23 (Seishin Corp.). For determination of the optimal concentration of sorbitol solutions, a range from 0.4M to 1.9M was tested with the enzyme in combination with Cellulase RS and Driselase for both *A. marina* and *A. lanata*. The cotyledons were cut in each sorbitol solution. Sliced segments (1-2mm thick) were placed into the 0.4 m*l* of enzyme solution in a well of a 24-well culture plate, and incubated at room temperature (*ca.* 25°C). The yield of isolated protoplast was determined under an inverted microscope. After 24 hrs of incubation, segments were loosened by a pair of tweezers, and the solution was transferred into a 1.5 m*l* tube. Each residual segment was washed with a 0.4 m*l* solution of each concentration of sorbitol. Combined isolated protoplast solution was centrifuged at 100g for 3 min. The precipitate was suspended in 0.1 m*l* of each sorbitol solution. The number of protoplasts was counted with a hemocytometer. Their viability was determined using final 0.01% Fluorescein diacetate (FDA) solution [3] in each concentration of sorbitol.

3. Results and Discussion

As shown in **Table 1**, Cellulase RS was found to be more effective than Cellulase R-10 for the isolation of

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Table 1.
Effect of six cell wall-degrading enzymes on the isolation of protoplasts from cotyledons of *Avicennia marina* from seeds stored at room temperature.

combination of enzymes					yield*	combination of enzymes					yield
1.	R10				+	13.	RS				2+
2.	R10	H			+	14.	RS	H			2+
3.	R10		D		4+	15.	RS		D		7+
4.	R10	H	D		+	16.	RS	H	D		5+
5.	R10			M	±	17.	RS			M	2+
6.	R10	H		M	±	18.	RS	H		M	2+
7.	R10		D	M	2+	19.	RS		D	M	4+
8.	R10	H	D	M	±	20.	RS	H	D	M	3+
9.	R10			P	−	21.	RS			P	−
10.	R10	H		P	−	22.	RS	H		P	−
11.	R10		D	P	−	23.	RS		D	P	−
12.	R10	H	D	P	−	24.	RS	H	D	P	−

yield*: −: no, ±: very low, + to 7+: low to high grade of protoplasts isolation, observed under an inverted microscope after 24 hrs incubation.

2% each of enzymes was used in 1.3M sorbitol solution.

R10: Cellulase R-10, RS: Cellulase RS, H: Hemicellulase, D: Driselase, M: Macerozyme R-10, P: Pectolyase Y-23.

Table 2.
Effect of sorbitol concentration on the yield of cotyledon protoplasts of *Avicennia marina* and *A. lanata* from imbibed seeds.

Sorbitol concentration (M)	yield($\times 10^5$cells/seed) A	B	C
	A. marina		*A. lanata*
0.4	14.2±11.8	0.6± 0.6	
0.6	41.8± 0.7	3.2± 1.7	8.0± 0.9
0.8	53.8± 3.4	6.4± 0.6	21.0± 0.8
1.0	113.8±11.5	18.1±14.8	44.6± 9.7
1.3	51.8± 6.2	25.5±11.8	43.7±16.8
1.4	53.8± 3.4	72.1±15.4	67.4± 4.4
1.6	49.4± 5.8	108.2± 8.4	80.9± 6.5
1.9	38.4± 6.7	43.9±11.0	65.1±37.0

A : Cotyledons from a seed of *A. marina* imbibed in water for 4 days.
B : Cotyledons from a seed of *A. marina* imbibed in water for 2 weeks.
C : Cotyledons from a seed of *A. lanata* imbibed in water for 2 months.
Combination of 2% each of Cellulase RS and Driselase was used.
Data were shown as the mean value of two wells twice measured.

protoplasts from cotyledons of *Avicennia marina* stored at room temperature. The most efficient protoplast isolation was achieved after 24 hrs incubation, using the combination No.15, 2% each of Cellulase RS and Driselase (**Table 1**). In this condition, 1.3×10^6 protoplasts/seed with very good viability (100%) were recovered after centrifugation. Hemicellulase, Macerozyme R-10 and Pectolyase Y-23 were not effective or rather inhibitory on the isolation of protoplasts after 24 hrs incubation. When Pectolyase Y-23 was used, many released cells were not spherical. In other forest tree species, *e.g. Populus* [4], *Betula* [5, 6], *Chamaecyparis* [2], treatment with a 1 % solution of the enzymes and a short incubation time (2 hrs) were effective on digesting cell walls, when the same 24 combinations of enzymes were tested. The need for high concentration of enzymes, 2% or 4% (data not shown), and long incubation times might be characteristic of protoplast isolation from *A. marina*.

Only very high sorbitol concentrations, 1.2M and 1.3 M were effective for the isolation of protoplasts and the results were reproducible, when seeds were stored at room temperature before isolation.

On the other hand, as shown in **Table 2**, when the seeds of *A. marina* and *A. lanata*, were soaked in water before protoplast isolation treatment, a high and wide range of sorbitol concentrations, 0.8M to

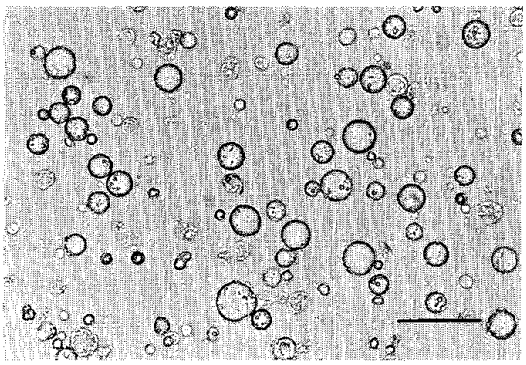

Fig. 1 Isolated protoplasts of *A. marina* with 2% each of Cellulase RS and Driselase in 1.6M sorbitol solution. Photographed on the hemocytometer. Bar=100μm.

1.9M, was effective. Under an inverted microscope, protoplasts were observed only after 1 hr of incubation at 0.6M and 0.8M , however, almost no protoplasts were observed at concentrations higher than 1.2M sorbitol. During 24 hrs of incubaion, more protoplasts were released, however, before loosening with a pair of tweezers, most protoplasts remained in the cut tissue segments at high sorbitol concentrations. Similarly, protoplasts of *Betula* remained in the leaves before loosening, although their leaves were floated on the enzyme solution and uncut [5, 6]. Such a difference might suggest a low diffusion rate of enzymes from the cut surface of segments of *A. marina* and *A. lanata*. The sorbitol concentration which gave the best yield (1.1×10^7cells/seed) varied from 1.0M to 1.6M in different experiments (**Table 2**). However, 1.3M or 1.4M sorbitol concentrations were effective in both species *A. marina* and *A. lanata* on the high yield of $5\text{-}7\times10^6$cells/seed and the results were reproducible.

As shown in **Fig. 1**, two different sizes of protoplasts were obtained from cotyledons of imbibed seeds of *Avicennia marina*, when isolated under 2% each of Cellulase RS and Driselase. One is smaller than 20μm in diameter, and the other is larger than 25μm and up to around 50μm. The former might originate from epidermal tissue, and the latter from inside tissue layers. No difference was found among different sorbitol concentrations, in the percentage distribution of the numbers of different size of protoplasts. The former was 25% and the latter was 75%. Average viability of spherical protoplasts measured by FDA was 84%, and no difference was found among different sorbitol concentrations.

As shown in **Fig. 2**, protoplasts of two different sizes were also observed in *A. lanata* imbibed for 2 months. In this case, viability was very low at 0.6M (20%). The average viability was 75% at the higher sorbitol concentrations.

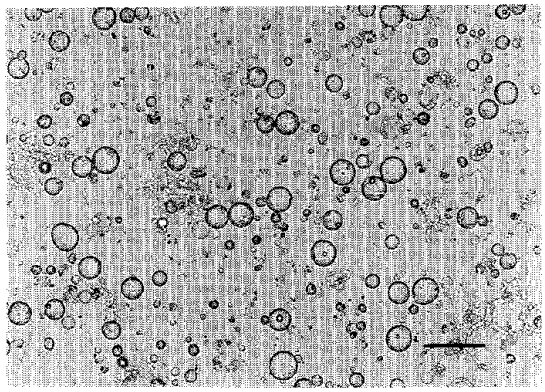

Fig. 2 Isolated protoplasts of *A. lanata* with 2% each of Cellulase RS and Driselase in 1.9M sorbitol solution. Bar=100μm.

In this report, viable and spherical protoplasts were isolated from salt tolerant seeds of mangroves, *A. marina* and *A. lanata*. Interestingly, the same high sorbitol concentrations were effective for the isolation of protoplasts as with highly salt-tolerant herbaceous plants, *Atriplex* (1.2-1.5M)[7] and *Zostera* (0.7-1.5M) [8]. Isolated protoplasts will serve as good material for further investigation of regulatory mechanism(s) of osmotic conditions in mangrove cells and the distribution of contributing solutes [7, 9]. Such work will construct the basis for genetic engineering. As for development of cell fusion techniques with other forest trees [10], the very high optimal sorbitol concentration of *A. marina* and *A. lanata* might be an inhibitory factor for fusion with protoplasts of other species, however, it is encouraging that many viable protoplasts were obtained at lower sorbitol concentrations, 0.6 to 1.0M.

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