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Some Factors Affecting the Isolation of Mesophyll Protoplasts from Red Clover(*Trifolium pratense* L.)

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레드 클로버의 葉肉細胞로 부터 原形質體의 裸出에 미치는 몇가지 要因의 影響

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摘 要

레드 클로버의 葉肉細胞로 부터 原形質體의 裸出에 影響을 미치는 要因들을 調査하여 다음과 같은 結果를 얻었다.

原形質體의 裸出에 使用된 酵素는 Cellulase, Macerozyme, Driselase 및 Pectolyase였으며 그 中 1%의 Cellulase ONOZUKA R-10과 0.5%의 Macerozyme R-10의 組合에서 가장 좋은 結果를 얻었다.

裸出된 原形質體의 適正 膨壓을 維持하기 爲하여 Mannitol의 濃度를 0.3~0.8M의 범위에서 6단계로 조정하였을 때, 0.7M에서 가장 높은 收率을 얻을 수 있었다. 原形質體 裸出을 爲한 酵素溶液의 pH는 5.6에서 가장 좋은 結果를 나타내었으며, 材料로 쓰인 레드 클로버 잎을 4가지 크기로 區分하여 裸出實驗한 結果, 작은 잎보다 큰 잎에서 收率이 높은 傾向이었다. 酵素處理 後 裸出된 原形質體의 정제에는 0.8M의 Sucrose가 가장 適當하였다.

I. Introduction

Protoplasts can be used for application in basic morphological and physiological studies, in plant propagation, and in the production of somatic hybrids, cybrids, and genetically transformed cells and plants. Klercker(1982) was the first to report on the mechanical protoplast isolation from *Stratiotes aloides*. Because of tedious procedure of mechanical isolation, Cocking(1960) developed an enzymatic procedure in which he used a comparatively crude cellulase preparation from the fungus Myrothecium verrucariato to isolate protoplasts from tomato roots. Takebe(1971) could get regenerated plants from protoplasts of tobacco mesophyll tissues. Melchers et al.(1978) succeeded to fuse protoplasts of *Lycopersicon esculentum* and *Solanum tuberosum* and to create the pomato, a somatic hybrid. For genetic manipulation, Ti plasmid uptake into Petunia (Davey et al., 1980a) and tobacco protoplasts (Krens et al, 1982) were investigated. Hasezawa et al. (1981) tried the fusion of *Agrobacterium* spheroplasts with protoplasts of *Vinca*. Fundamental to all these approaches is the requirement to obtain consistently high yields of viable protoplasts.

This research was conducted to elucidate the factors affecting the isolation of red clover mesophyll protoplasts in order to use them potential genetic manipulation.

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II. Materials and Methods

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Fully developed red clover leaves in the field were sliced with 1 mm intervals to allow penetration of enzyme mixtures. The composition of enzyme mixtures are shown in Table 1. Every 10 ml of enzyme mixtures is dispensed into 0.25g of sliced leaf tissue. The slurry was incubated on a reciprocal incubator with 120 strocks/min at 28°C for 8 hours. Protoplasts isolated

Table 1. Composition of four enzyme types and buffer solution for degrading mesophyll cell wall of red clover

Composition/enzyme type	I	II	III	IV
Cellulase 'Onozuka' R-10(%)	1.0	1.0	2.0	2.0
Macerozyme R-10(%)	0.5	1.0	0.5	1.0
Potassium Dextran Sulfate		1.0g		
Potassium Citrate, Monohydrate		166mg		
DTT*		30mg		
BSA**		100mg		
Calcium Chloride, Dihydrate		18mg		
Potassium Dihydrogen Phosphate		17mg		
Magnesium Sulfate		25mg		
Calcium Nitrate		3mg		
Mannitol		12.75g		
Glycine		750mg		
Distilled Water		100ml		
pH		5.6		

*DTT : Dithiothreitol
**BSA : Bovine serum albumin

Table 2. Composition of washing and floating solution

Composition	Washing solution	Floating solution
$CaCl_2 \cdot 2H_2O$	148mg	148mg
KH_2PO_4	3mg	3mg
$MgSO_4 \cdot 7H_2O$	25mg	25mg
KNO_3	10mg	10mg
Mannitol	12.75g	—
Sucrose	—	27.36g
H_2O	100ml	100ml
pH	5.6	5.6

were counted with a hemocytometer (LxW; 0.0025 mm^2, D; 0.100 mm) on the gram fresh weight basis. passed through a 56 μm nylon sieve after enzyme mixture treatment to remove undecomposed leaf debris, washed with the purified solution at 700 rpm for 3 minutes and centrifuged in a floating solution at 500 rpm for 5 minutes. The composition of purification and floating solution is shown in Table 2. Mannitol concentrations for the optimum osmoticum and treatment time were compared among 0.3, 0.4, 0.5, 0.6, 0.7 and 0.8 M with 2 hour intervals from 0 to 12 hours. Cellulase R-10 and Macerozyme R-10 were combinated to various treatments and incubated for 8 hours for cell wall digestion. The medium pH was adjusted with HCl to 5.2, 5.4, 5.6, 5.8 and 6.0 and compared among them after 8 hours incubation. Leaf sizes were also compared among 1.4 x 2.4, 1.8 x 3.4, 2.8 x 4.5 and 3.0 x 5.2 cm (width x length) under the conditions of 0.7 M mannitol, pH 5.6 and enzyme solution I after 8 hours incubation. Various sucrose concentrations were employed to separate protoplasts isolated from cellular debris after enzyme treatment and compared among 0.4, 0.5, 0.6, 0.7 and 0.8 M.

III. Results and Discussion

Figure 1 shows the effect of mannitol concentrations and treatment time under the 1 % cellulase and 0.5 % macerozyme conditions. The protoplast yield was considerably higher in the treatment of 0.7 M mannitol than any other treatment. The mannitol concentrations of 0.6 and 0.8 M gave the similar result though the yield was much lower than that of 0.7 M treatment. In all time treatments, the highest yield was obtained in the 8 hours treatment except 0.3 and 0.4 M mannitol concentrations where the yield was decreased with time. The decreased yield in the low mannitol concentrations such as 0.3 or 0.4 M with time was thought be caused by bursting of cell membranes because of low osmoticum. Vasil(1976) dicussed protoplasts lost the protection of the cell wall and became highly susceptible to osmotic damage and shock as they were released into the surrounding medium during the gradual breakdown of the cell walls. He also stated, therefore, that the

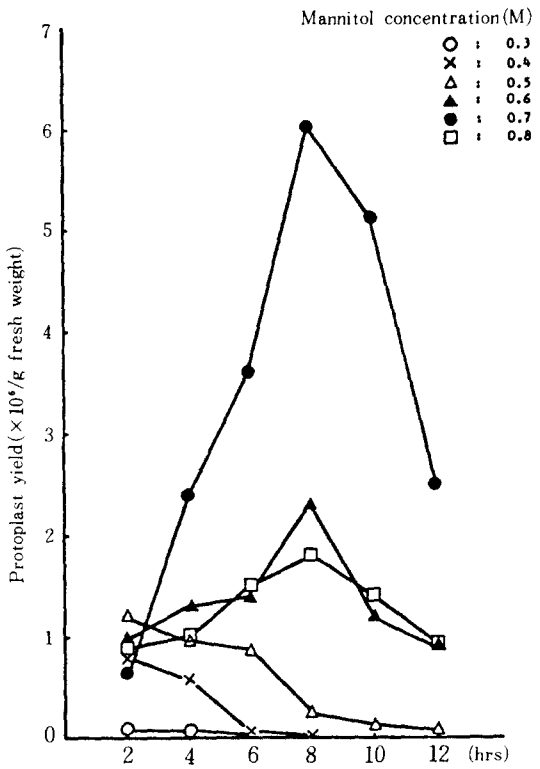

Fig. 1. Effect of several mannitol concentration on the mesophyll protoplast yield of red clover in enzyme solution I with time

isolation medium should ensure osmotic stability of the protoplasts and recommended the use of 0.4-0.8 M mannitol and/or sorbitol, sucrose or glucose. Hughes et al.(1978) reported that mannitol, sucrose, and sorbitol were all satisfactory in concentrations between about 0.3 and 0.55 M in leaves of *Hordeum Vulgare*. In this experiment with red clover, howwever, 0.7 M mannitol was most suitable.

Table 3 shows the result of various enzyme treatments with 0.7 M mannitol after 8 hours incubation. The enzyme solution I (1 % cellulase + 0.5 % macerozyme) gave the best result in which the protoplast yield was 8.16 x 10^6/g fresh weight. As the cell wall is mainly composed of cellulose, hemicellulose and pectin in herbacious plants, Cellulase R-10, Macerozyme R-10, Driselase and Pectolyase Y-23 were compared as cellulase or pectinase sources in the preliminary experiment and the combinations of Cellulase R-10

Table 3. Effect of several enzyme solutions on the mesophyll protoplast yield of red clover with 8 hour-incubation

Enzyme solution	I	II	III	IV	LSD(0.05)
Protoplast yield(×10^6)	8.16a*	4.84bc	5.96b	4.62c	1.28

*Values followed by different letters are significantly different at P=0.05, following the least significant differences test.

and Macerozyme R-10 were most effective for cell wall degradation. Pectolyase Y-23 did not show pectinase activity at all in red clover. Over 0.5 % Macerozyme and 1 % Cellulase Onozuka R-10, the protoplast yield decreased drastically because of protoplasts brust. Alan and Martin(1976) reported the use of higher concentration of enzyme resulted more protoplast yield, but it also brought decreased viable cells. Szabados and Rosa (1986) used 2 % Cellulase Onozuka R-10 and 0.2 % Pectolyase Y-23 in leaves of a tropical forage legume, *Stylosanthes guianensis*. Hock and Hartman(1983) used 2 % Cellulase Onozuka R-10 and 1 % Macerozyme

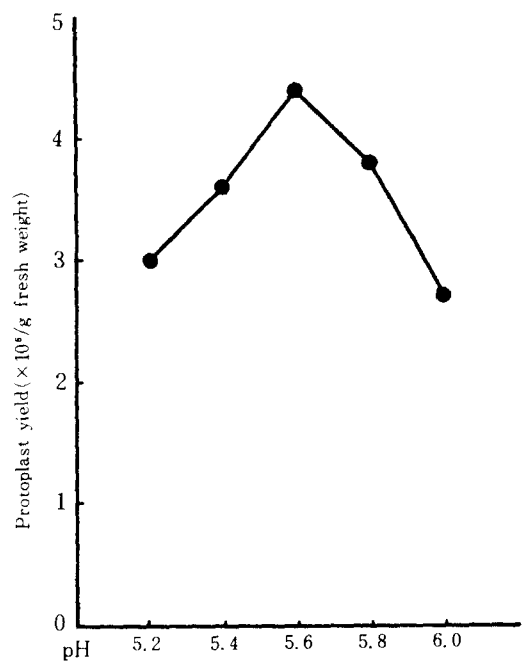

Fig. 2. Relationship between pH gradient and the mesophyll protoplast yield of red clover in enzyme solution I with 8 hour-incubation

in *Phaseolus vulgaris* from hypocotyls, which were much higher enzyme concentrations than those used in this experiment. As *Graminae* generally contains higher concentrations of fiber, more cellulase is used. Hughes et al. (1978), for example, used 4 % Cellulase R-10 and 1 % Macerozyme R10 in coleoptile segment of *Hordeum vulgare.*

The relationship between pH gradient and the mesophyll protoplast yield are shown in Figure 2. The yield was increased with pH increment from 5.2 to 5.8 and showed a drastic decrease afterwards. The yield at pH 5.8 was 4.4 x 10^6/g fresh weight. Evans and Bravo suggested the optimum pH range of 5.4-6.2 in protoplast isolation. Scott et al.(1978) reported a wide optimum pH range of 4.6-5.4 for the protoplast isolation in *Hordeum vulgare.* However, the sharp peak was observed in pH 5.6 as shown in the figure in this experiment.

Table 4. Relationship between some leaf sizes and the mesophyll protoplast yield of red clover with 8 hour-incubation

Leaf size	I	II	III	IV	LSD(0.05)
Protoplast yield($\times 10^4$)	1.60c*	1.91c	4.49b	5.69a	0.82

*Values followed by different letters are significantly different at P=0.05, following the least significant differences test.

Leaf size also affected the protoplast isolation as shown in Table 4. Lesves should be separated according to age, but they were separated by leaf sizes in this experiment because they were grown in the field and the accurate age could not be calculated. The yield was increased with the size expansion in which the msximum yield was 5.69 x 10^6 cells/g fresh weight in leaf size IV of 3.0 x 5.2 cm (width x length).

After isolation of protoplasts by cellulase-macerozyme medium, it is necessary to remove the surrounding enzymes from the surface of the isolated protoplasts and to separate them from cell debris. Isolated protoplasts by enzymes were recovered by centrifugation on the several sucrose concentrations. At 0.4 M sucrose, the yield was extremely low and only 1.3 % of total isolated protoplasts were recovered. The recovery rate, however, increased with sucrose concentration from 0.4 to 0.8 M and the maximum rate was 57 % at 0.8 M sucrose as shown in Table 5.

IV. Summary

An experiment was conducted to elucidated some factors affecting the isolation of protoplasts from red clover mesophyll tissues. Among the four enzymes of Cellulase, Onozuka R-10, Macerozyme R-10, Driselase and Pectolyase Y-23, the combination of 1 % Cellulase R-10 and 0.5 % Macerozyme R-10 gave the best result for the isolation of viable protoplasts from red clover mesophyll tissues. For the osmoticum, 6 concentrations of mannitol, 0.3-0.8 M were compared and 0.7 M brought the maximum protoplasts yield. The optimum enzyme solution pH for the protoplast isolation was 5.6 among the five treatments from 5.2-6.0. When red clover leaves were divided into four sizes, the larger leaves resulted in better yield of protoplasts. The sucrose concentration for purification of protoplasts isolated and to recover protoplasts from cell debris was 0.8 M and the recovery rate was 57 % at that concentration.

Table 5. Effect of several sucrose concentrations in floating solution on the protoplast purification

Sucrose concentration	0.4M	0.5M	0.6M	0.7M	0.8M	LSD(0.05)
Protoplast yield($\times 10^6$)	0.04 d**	0.39 c	0.64 c	1.16 b	2.000 a	0.25
R. Rec.*(%)	1.3%	11%	18%	35%	57%	

*R. Rec. : Rate of recovered protoplasts

**Values followed by different letters are significantly different at P=0.05, following the least significant differences test.

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