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Studies on Factors Affecting Isolation and Fusion of Protoplasts of *Quercus* Species[1]

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참나무類의 原形質體 分離 및 融合에 影響을 끼치는 要因에 관한 硏究[1]

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ABSTRACT

Factors affecting isolation and fusion of protoplasts of three *Quercus* species were investigated and procedures for isolation, purification and fusion of protoplasts of the three species were also established. Unhardened leaves and rapidly growing callus cultures were good source of viable protoplasts. The optimum composition of enzyme mixture for rapid isolation of protoplasts from leaf mesophyll tissues and calli was Cellulase Onozuka R-10 (20g/l), Macerozyme R-10(10g/l), Pectinase(250 units/l), $CaCl_2 . 2H_2O$(14mM), $MgSO_4 \cdot 7H_2O$(1.8mM), KNO_3(1.0mM), H_3BO_3(1.0mM), KH_2PO_4(0.2mM), KI(1.0μM), 1,4-dithiothreitol (0.1mM) and mannitol (0.6M). Optimum density of protoplasts for maximum fusion was $2x10^5$/ml which was the highest protoplast density given in this study. Optimum concentration and duration of PEG 1450 treatment for inducing fusion appeared to be 29%(W/V) final PEG 1450 concentration and 5-10 minutes, respectively.

Key words: protoplast; Quercus; *Protoplast isolation; fusion.*

要 約

本 硏究는 國內 森林生態系의 優占樹種으로서 生態的 및 經濟的으로 有望한 참나무類의 育種 및 優良크론의 大量增殖을 低害하고 있는 人工交雜과 無性繁殖의 難點을 타개하기 위한 細胞培養 및 體細胞 交雜에 必要한 基礎資料를 획득하기 위하여 實施되었다. 主要試驗結果를 要約하면 다음과 같다. 1) 未硬化葉과 旺盛한 成長을 보이는 Callus가 지속적인 原形質體 供給源으로 適合하며 2) 原形質體 離出을 爲한 酵素溶液의 組成은 Cellulase Onozuka R-10(20g/ℓ), Macerozyme R-10(10g/ℓ), Pectinase(250units/ℓ), $CaCl_2$, $2H_2O$ (14mM), $MgSO_4 \cdot 7H_2O$(1.8mM), KNO_3(1.0mM), H_3BO_3(1.0mM), KH_2PO_4(0.2mM), KI(1.0μM), 1,4-dithiothreitol(0.1mM), mannitol(0.6M)을 組合한 것의 成績이 가장 좋았다. 3) 細胞融合率이 가장 높았던 原形質體의 密度는 2×10^5/ml이었다. 이는 本 試驗에 採用된 原形質體密度中 가장 높은 密度였다. 4) 細胞融合誘導를 爲한 polyethylene glycol 處理의 適正濃度와 處理時間은 各各 29%(w/v)와 5~10分으로 나타났다.

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INTRODUCTION

As dominant tree species in forest ecosystems of Korea, the genus *Quercus* has broad environmental adaptability, good pest resistance and high biomass productivity. But the development of superior clones and hybrids have been hindered by extreme difficulties of clonal propagation and controlled pollination. For those reasons, development of tissue and cell culture techniques and somatic hybridization techniques for multi-propagation of superior oak trees and development of vigourous hybrids are badly needed.

Recent advances in protoplast culture techniques have opened up new avenues for conducting basic as well as applied genetic researches in higher plants, including tree species. Although tree species are generally difficult to grow and differentiate *in vitro*, recent reports on isolation, fusion and culture of protoplasts of a few tree species are encouraging on clonal propagation and somatic hybridization of commercially important tree species.

This study was conducted to find out the optimum conditions for isolation, purification and induced fusion of protoplasts of *Quercus* species to pave the way for clonal propagation and somatic hybridization of promising *Quercus* species.

MATERIALS AND METHODS

1. Materials

The leaf materials from mature trees and 1-year-old seedlings, and rapidly growing callus cultures originated from the trees of *Quercus acutissima*, *Q. serrata* and *Q. rubra* growing on the campus of College of Agriculture, Seoul National University, Suwon, Korea were provided as the source for protoplasts. Modified Murashige and Skoog medium supplemented with 2,4-dichlorophenoxy acetic acid (0.1mg/l), indole-e-acetic acid (10mg/l) and 2-isopentenyl adenine (10mg/l) were used for callus induction in this study.

2. Methods

For all of the following procedures, the non-sterilizing systems were applied. The procedures for isolation, purification and induced fusion of *Quercus* protoplasts used in this study were as follows;

A) Isolation and Purification of Protoplasts

For efficient isolation of *Quercus* protoplasts, several 'One-Step Procedures' were quoted.[1,3,5,10,12]

(1) Cut about 1g of the leaves or calli with a sharp scalpel into small segments, and transfer the shredded materials into a 100ml side-armed Erlenmyer flask containing 25ml of the enzyme mixture (Table 1) for digesting the cell walls.

(2) Suspend the prepared materials into the enzyme mixture and submitted to a brief treatment by a weak vaccum for easy infiltration.

(3) Gently shake the flask by using a gyratory shaker at culture-room-temperature (ca. 26°C).

(4) After 5-6 hours of incubation, filter the enzyme mixture containing the protoplasts and undigested materials through a stainless-steel sieve (pore size: 74μm, Marui & Co., Ltd. Osaka, Japan., sieve No.: 200).

(5) Centrifuge the filtered suspension for 2 min at 100 rpm, and collect the pellet.

(6) resuspend the pellet with the Prefusion Solution (Table 2), and purify the viable protoplasts by the discontinuous sucrose density gradient fractionation method.

(7) Centrifuge to pellet, and wash the pellet with Prefusion Solution for 3 times by centrifugation and resupending.

(8) Adjust the protoplast density up to $2x10^5$/ml by means of haemocytometer and micro-pipette.

B) Induced Fusion of Protoplasts

For inducing the fusion of purified protoplasts, the existing methods were quoted.[4,6,7,8]

(1) Dispense droplets of 200μl of protoplast suspension in 90x15mm disposable plastic Petri dishes which were finely scratched by fine (below 200 mesh) sand-papers, 6-8 drops per dish by using a micro-pipette. After the protoplasts were settled, slowly add 140μl of PEG Solution (Table 2) at the

edge of protoplasts layer.

(2) Incubate the preparation at culture-room-temperature for 5-10 min.

(3) Gently add several drops of 200-500μl of High pH & High Ca^{++} Solution (Table 2) over a period of 15 min. Continue to add until the Petri dish is filled to two-thirds.

(4) Tilt Petri dishes and carefully remove excess liquid from settled protoplasts. Collect the protoplasts suspension in a 1.5ml Eppendorf® micro-centrifuge tubes, and wash the protoplasts adhering to the bottom of Petri dishes one more time with High pH & High Ca^{++} Solution and collect the suspension as before.

(5) Wash the protoplasts with Prefusion Solution for 3 times by centrifugation and resuspending.

(6) Inspect the result under light-microscope.

Table 1. Composition of enzyme mixture for protoplast isolation from calli and leaf mesophyll tissues of *Quercus* species

Classification	Reagent	Concentration
Enzymes	Cellulase Onozuka R-10*	20g/l
	Macerozyme R-10*	10g/l
	Pectinase**	250 units/l
CPW salts	$CaCl_2 \cdot 2H_2O$	14.0mM
	$MgSO_4 \cdot 7H_2O$	1.8mM
	KNO_3	1.0mM
	H_3BO_3	1.0mM
	KH_2PO_4	0.2mM
	KI	1.0μM
Additive	1,4-dithiothreitol**	0.1mM
Osmoticum	mannitol	0.6 M
pH		5.8

* Kinki Yakult Co., Nishinomiya, Japan.
** Sigma Chemical Co., St. Louis, U.S.A.

Table 2. Composition of solutions for inducing the fusion of protoplasts of *Quercus* species

Reagent	Concentration (mg/100ml of distilled water)		
	Prefusion Sol.	PEG Sol	High pH & High Ca^{++} Sol.*
PEG 1450**		50,000	
sorbitol	9,000		
mannitol	1,000	500	500
glucose	1,000	500	9,000
glycine			375
$CaH_2(PO_4)_2 H_2O$	50		
$CaCl_2 \cdot 2H_2O$		150	735
KH_2PO_4		10	

* add components just before use
** Sigma Chemical Co., St. Louis, U.S.A.

RESULTS AND DISCUSSION

1. Isolation and Purification of Protoplasts

Unhardened leaves from mature trees regularly provided the best mesophyll protoplasts during this study. Well-established and rapidly growing callus cultures also provided plenty of chlorophyllous and non-chlorophyllous protoplasts (Plate 1). The optimum composition of enzyme mixture for rapid isolation of protoplasts from calli and leaf mesophyll tissues of *Quercus* species found in this study was illustrated in Table 1.

Mean yield of protoplasts based on the origin of materials was best from unhardened leaves followed by mature leaves, white calli and green calli, and based on the species were in order of *Q. rubra*, *Q. serrata* and *Q. acutissima* in case of unhardened leaf mesophyll tissues. Various sizes of leaf mesophyll protoplasts were derived from unhardened leaves (Plate 1-A). Most of isolated protoplasts ranged approximately from 40μm to 70μm. Unexpectedly, protoplasts from calli had bigger diameters than those from leaf mesophyll tissues.

In this study, the discontinuous sucrose density gradient fractionation method was effective for the purification of isolated protoplasts. Debris including epidermis, broken protoplasts, chloroplasts and fibers were separated at the bottom of centrifuge tube, and most of the viable protoplasts were separated at upper portion of 19%(W/V) zone of sucrose density. Purity of protoplasts achieved by up to 95% when the discontinuous sucrose density gradient fractionation methods was applied.

2. Induced Fusion of Protoplasts

Generally, the osmolarity of the protoplast culture medium do not excess 500mOs. while the osmotic potential of PEG at a concentration of 29% (W/V) is in excess of 2,000mOs. Thus, when proto-

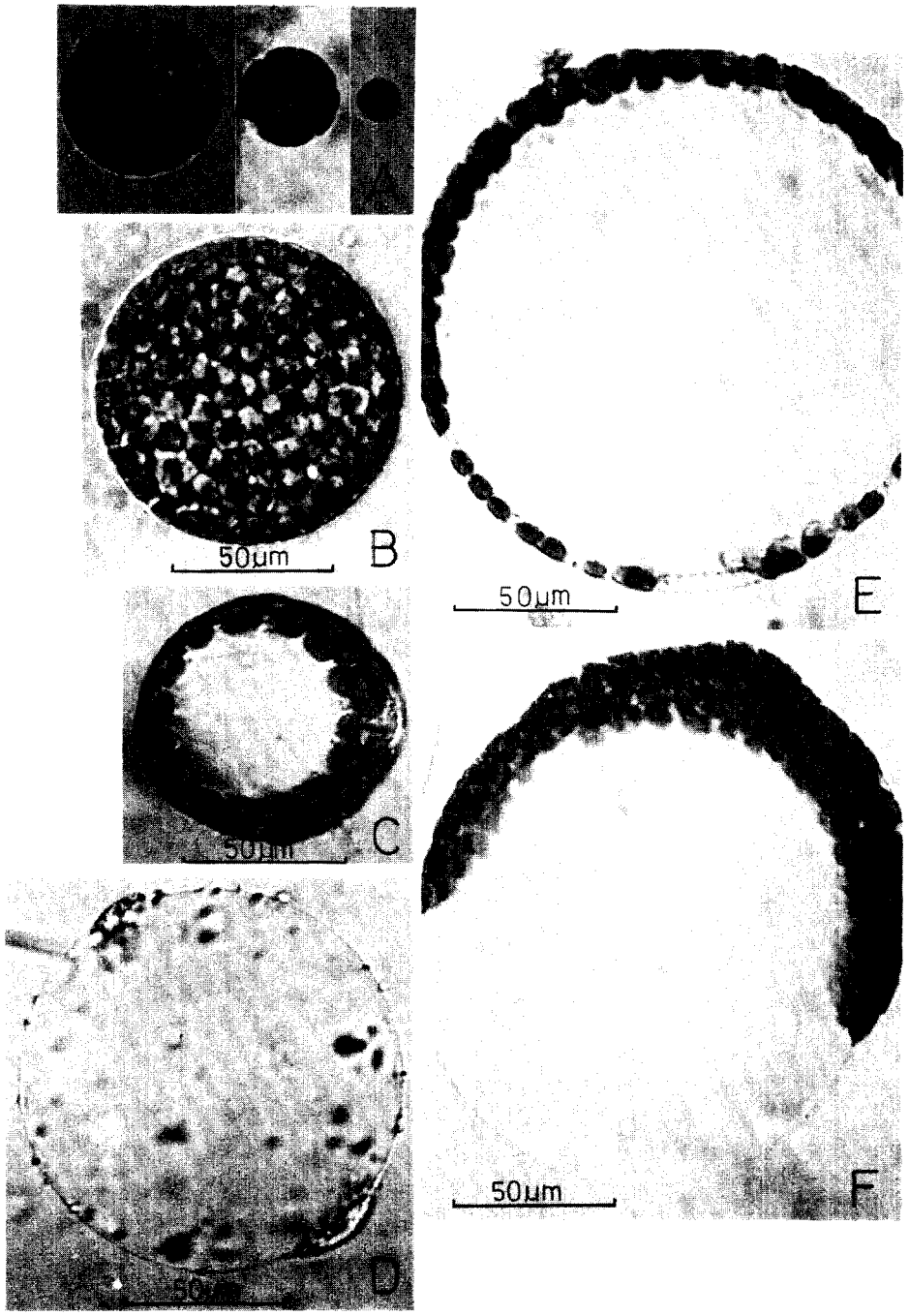

Plate 1. Various types of isolated protoplasts with vacuole
(A) Various size of protoplasts from a leaf of *Q. acutissima.*
(B) Isolated mesophyll protoplast of *Q. serrata.*
(C) Chlorophyllous protoplast from callus culture of *Q. rubra.*
(D) Non-chlorophyllous protoplasts from callus culture of *Q. acutissima.*
(E) Mega-protoplast contains a large vacuole from a leaf of *Q. rubra.*
(F) Vacuole emerged from broken mega-protoplast of *Q. rubra.*

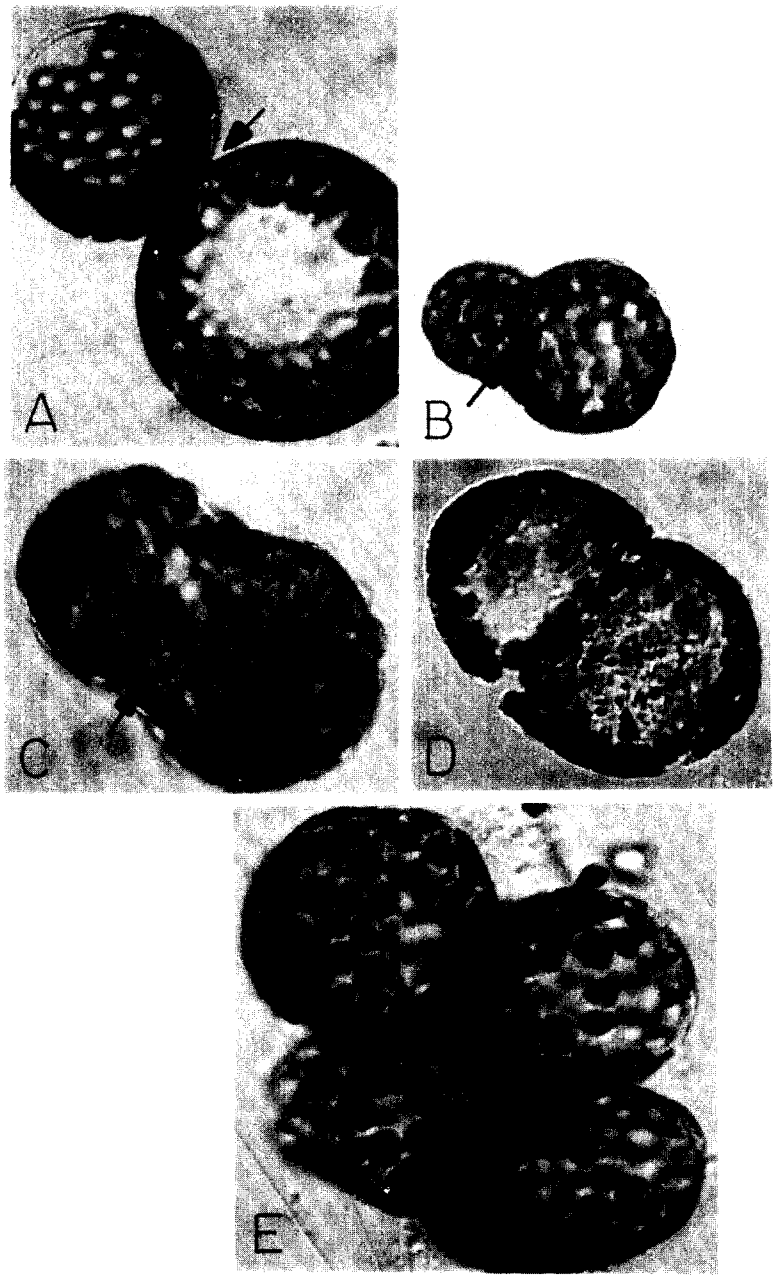

Plate 2. Various stages during the PEG-mediated fusion event of protoplasts from mesophyll tissues and callus cultures

(A) Initiation of fusion event. Note a flattening of protoplasts in the area of membrane contact (arrow).

(B) Agglutination of *Quercus acutissima* mesophyll protoplasts in pair. Note the vesicle-like formation (arrow) in the contact area.

(C) Enlarging a membrane contact area to form a spontaneous fused protoplasts. Note the vesicle-like formation (arrow).

(D) Vacuolar fusion between two contacted protoplasts was not initiated. Note the accumulated vacuous deposits are crowded to one side of fused protoplasts.

(E) Aggregation of Q. serrata mesophyll protoplasts in quadruplet.

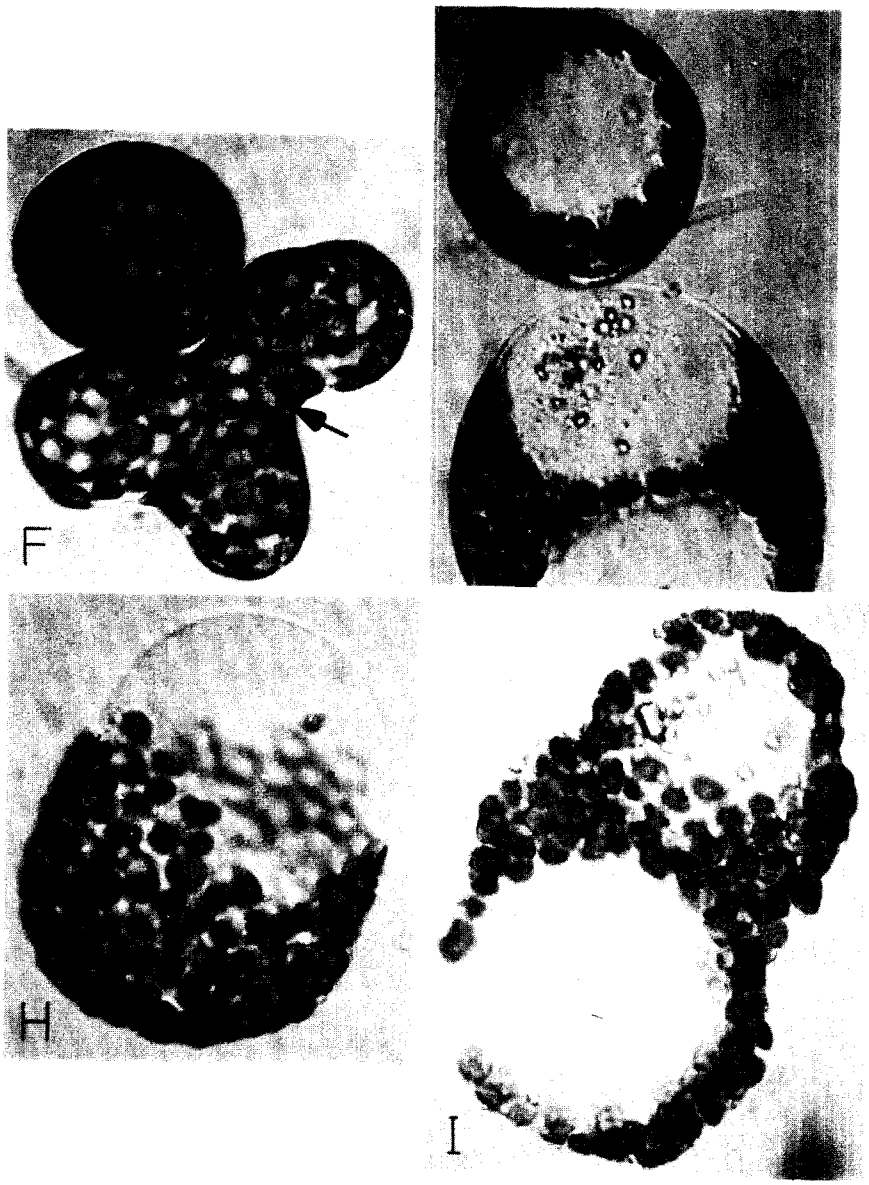

Plate 3.

(F) Agglutinated triplet protoplasts and adhered protoplast which were identical protoplasts with (E). Note the expansion of plasmodesmata (arrow).

(G) Non-fused protoplast (upper) and elliptical fused protoplasts (below) from callus cultures of *Q. rubra*.

(H) Fusion product between callus protoplast of *Q. acutissima* and mesophyll protoplast of *Q. serrata*. Diffusion of chloroplasts was already initiated.

(I) Agglutinated protoplasts were damaged by highly concentrated PEG solution during fusion event.

plasts are treated with PEG at such concentration, they get a severe osmotic shock.[6] The addition of concentrated solution of PEG and slow dilution treatment to protoplasts resulted various changes during a treatment. These include 1) aggregation of more than one protoplasts, and flattening of protoplasts in the area of membrane (Plate 2-A, E), and 2) enlarging a membrane contact area to form a vesicle-like formation[13] (Plate 2-B, C), and 3) initiation of cytoplasmic diffusion between two contacted protoplasts (Plate 2-D, Plate 3-F, H). Similar effects of PEG on protoplasts have been reported by Kao and Michayluk.[8]

A short exposure to PEG (5-10 min) was sufficient to induce adherence and fusion of protoplasts. With longer treatment, e.g., 25 min, the incidence of cell mortality increased while the incidence of fusion decreased. The incidence of fusion of protoplasts following slow dilution of PEG depended on the duration of PEG treatment, the final concentration of PEG and the density of protoplast. The maximum fusion was obtained by 10 min treatment with 29% final PEG 1450 concentration. So, majority of the following experiments in this study were conducted by treating for 10 min with 29% of final PEG 1450 concentration. Maximum fusion of protoplasts (ca. 67%) occurred at the highest protoplast density ($2x10^5$/ml) given in this study. When the density of protoplast were reduced to $5x10^4$/ml, the fused-ratio were also reduced to 52%. In the case of the protoplasts treated with more than 60% (W/V) of PEG stock solution or with longer duration, the incidence of protoplast breakage increases during fusion event (Plate 3-I) for increasing of the susceptibility to external damage immediately after induction of fusion. These results were very similar to those of Bajaj *et al.*[2]

There was no marking on one of fusion partners in this study. When two same-sized protoplasts were fused, a fusion product would be doubled in volume while its diameter would be increased only 1.4 times. Thus, fusion products of chlorophyllous protoplasts from calli of *Q. rubra* which had large vacuoles subsequently showed an elliptically shaped big protoplasts with two tonoplasts (Plate 3-G). Depending on foregoing criterion, maximal fusion of chlorophyllous protoplasts from calli of *Q. rubra* was calculated up to 67% (246 fused protoplasts/369 counted protoplasts) at 29% (W/V) final concentration of PEG treatment for 10 min.

CONCLUSION

Unhardened leaves from mature trees and rapidly growing callus cultures regularly provided the best protoplasts during this study. The optimum composition of enzyme mixture for rapid isolation of protoplasts from calli and leaf mesophyll tissues was Cellulase Onozuka R-10(20g/l), Macerozyme R-10 (10g/l), Pectinase (250 units/l), $CaCl_2 \cdot 2H_2O$(14.0 mM), $MgSO_4 \cdot 7H_2O$(1.8mM), KNO_3(1.0mM), H_3BO_3(1.0mM), KH_2PO_4(0.2mM), KI(1.0μM), 1,4-dithiothreitol(0.1mM), mannitol(0.6M), and pH adjusted to 5.8.

The discontinuous sucrose density gradient fractionation method was revealed as an effective method for the purification of isolated protoplasts. Most of the viable protoplasts were separated at the upper portion of 19%(W/V) zone of sucrose density. Purity of protoplasts achieved by up to 95% when the discontinuous sucrose density gradient fractionation method was applied.

A short exposure to PEG (5-10 min) was sufficient to induce adherence and fusion of protoplasts. The maximum fusion appeared to occur by 10min. of treatment with PEG 1450, and the optimum final concentration of PEG appeared to be 29% (W/V). Maximum fusion of protoplasts (ca. 67%) occurred at the highest protoplast density ($2x10^5$/ml) given in this study.

LITERATURE CITED

1. Ahuja, M. R. 1982. Isolation, culture and fusion of protoplasts; problems and prospects. Silvae Genetica 3:66-77.
2. Bajaj, Y. P. S., J. Reinert and G. Gosch. 1975.

Differential sensitivity of protoplasts of various origin to polyethylene glycol (PEG). pp. 78. In: Abstr. 4th Intern. Symp. on Yeast and Other Protoplasts. Nottingham Univ., Nottingham.

3. Chalupa, V. 1974. Isolation and division of protoplasts of some forest species. In: 3rd Omter. Congr. Plant Tissue and Cell Culture. Leicester, Abst. No. 174.
4. Constabel, F., H. Koblitz, J. W. Kirkpatrick and S. Rambold. 1980. Fusion of cell sap vacuoles subsequent to protoplast fusion. Can. J. Bot. 58: 1032-1034.
5. Cocking, E. C. 1960. A method for the isolation of protoplasts and vacuoles. Nature (London) 187:927-929.
6. Eriksson, T., H. T. Bonnett, K. Glmelius and A. Wallin. 1974. Technical advances in protoplasts isolation, culture and fusion. Pages 301-327 *in* H. E. Street, ed. Tissue culture and plant science. Academic Press.
7. Gosch, G., Y. P. S. Bajaj and J. Reinert. 1975. Isolation, culture and fusion study on protoplasts from different species. Protoplasma 85: 327-336.
8. Kao, K. N. and M. R. Michayluk. 1974. A method for high frequency inter-generic fusion of plant protoplasts. Planta 115: 355-367.
9. Pearce, R. S. and E. C. Cocking. 1973. Behavior in culture; Isolation of protoplasts from "Paul's Scarlet Rose" suspension culture cells. Protoplasma 77: 165-180.
10. Power, J. B. and E. C. Cocking. 1970. Isolation of leaf protoplasts: Macro-malecule uptake and growth substance response. J. Exp. Bot. 21: 64-70.
11. Vasil, I. K. and V. Vasil. 1980. Isolation and culture of protoplasts. Int. Rev. Cytol. Suppl. 131:1-20.
12. Zimmermann, V. and V. Jorg. 1984. Electric field-mediated cell to cell fusion. In: Cell fusion; Gene transfer and transformation. pp. 171-187. Raven Press.