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Isolation and Culture of Mesophyll Protoplasts from *in vitro* Cultured *Populus alba* × *P. glandulosa*[1]

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현사시(*Populus alba* × *P. glandulosa*) 器內培養葉肉組織에서의 原形質體의 分離 및 培養[1]

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ABSTRACT

This study was carried out to investigate the optimum conditions for isolation and culture of mesophyll protoplasts from *Populus alba* x *P. glandulosa*. The results obtained from the experiments are as follows; 1) The suitable concentration of BAP for shoot multiplication was 0.4 mg/l. 2) High yield and viability of isolated protoplasts were obtained by our high enzyme-short time incubation method. 3) Optimum enzyme concentrations for mesophyll protoplast isolation were Cellulase 2%, Macerozyme 0.8%, Hemicellulase 1.2%, Driselase 2%, and Pectolyase Y-23 0.05%. 4) 0.6M mannitol in enzyme solution was the most effective for protoplast isolation and viability. 5) The most adequate pH level of enzyme solution was pH 5.6. 6) The effect of DTT and MES buffer was significant. 7) For protoplast purification, 0.6M sucrose was the most proper concentration. 8) The adding effect of Dextran T40 in floating solution was important. 9) The mesophyll protoplasts isolated through our high enzyme-short time incubation method revealed successful response to culture condition over 3 weeks of culture.

Key words: mesophyll protoplast; protoplast culture; protoplast isolation; Populus alba x P. glandulosa

ABBREVIATIONS: SDW, sterile distilled water; DTT, dithiothreitol; MES, 2(N-Morpholino) ethane sulfornic acid; BSA, bovine serum albumin; BAP, 6-benzylaminopurine; NAA, naphthalene acetic acid; MS, Murashige and Skoog.

要　　約

현사시(*Populus alla*×*P. glandulosa*)의 器內培養 葉肉組織으로부터 原形質體의 分離 및 培養을 시험하였다. 無菌의 葉肉組織을 育成하기 위한 shoot의 대량증식에는 MS기본배지에 BAP 0.4 mg/l를 첨가하였을 때 가장 증식효과가 높았으며, 原形質體의 分離를 위한 효소농도는 Cellulase 2%, Macerozyme 0.8%,

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Hemicellulase 1.2%, Driselase 2%, Pectolyase Y-23 0.05%가 가장 효과적이었다. 효소용액의 삼투압은 mannitol 0.6M이 적합하였으며, pH는 5.6이 적절하였다. 原形質體의 안정성을 높이기 위하여 DTT와 MES buffer를 첨가하여 좋은 결과를 얻었다. 分離된 原形質體의 정제를 위해서는 浮遊溶液의 sucrose 농도를 0.6M로 하였을 때가 가장 적합하였으며 dextran의 첨가효과도 높았다. 본 연구의 특징은 다소 높은 농도의 효소용액을 처리하여 단시간에 原形質體를 分離하는 것으로서 이 방법에 의해 裸出된 原形質體의 培養에 있어서 반응여부를 조사한 결과 배양 3주 후에까지도 좋은 반응을 보였다.

INTRODUCTION

In recent years much interest and speculation has been concentrated on the potential of plant tissue culture techniques including protoplast methods for genetic manipulation and improvement of plant species (Durzan, 1980, 1984; Kirby, 1982; Farnum *et al.* 1983; Bornman 1984).

The successful use of protoplasts depends on the development of a complete protoplast isolation and culture system resulting in whole plant regeneration. With appropriate choice of conditions of isolation, large number of viable protoplasts can be produced and it is the first step in order to succeed in following culture (Burger and Hackett, 1982; David *et al.* 1982).

The definition of important factors affecting protoplast yield and viability of *Populus alba* x *P. glandulosa* is the focus on this paper. In case of woody plants, the dramatic parameters on protoplast isolation can be reckoned as follows; 1) physiological conditions of source materials, 2) osmoticum concentrations, 3) enzyme concentrations and/or times of exposure to the enzyme, 4) pH of enzyme solution, 5) prevention of phenol-related toxicity (Vasil and Vasil, 1980; David *et al.* 1982; Evans and Bravo, 1984).

Protoplasts have been isolated from tissues of many forest tree species (Redenbaugh *et al.* 1981; Ahuja, 1982, 1984a,b; David *et al.* 1982; Vardi and Spiegel-Roy, 1982; Dorin *et al.* 1983; Patel *et al.* 1984). In particular, there are several reports of protoplast isolation and/or culture from *Populus* species (Saito, 1976, 1980; Douglas, 1982; Ahuja, 1983; Verma and Wann, 1983; Chun, 1985). However, no such cases have been reported a whole plant regeneration. These are, one may think as compared with non-forest tree species, partly because of awkwardness or inadequacy in protoplast isolation conditions and partly because of the difficulty in culture and regenerating plant from tree protoplasts.

Our overall goal is directed toward the production of whole plants from cells whose potentialities have been improved by means of genetic manipulation or somatic hybridization. This report presents our initial progress toward this goal and the further culture tests are currently underway.

MATERIALS AND METHODS

1. Plant materials

The dormant branches (1-2 years old) bearing apical and axillary buds were collected from February to March from upper parts of 15 to 20-year-old *Populus alba* x *P. glandulosa*. The branches were sprouted by water cutting in culture room.

2. Sterilization of materials

The bud parts (1-2cm length) cut from sprouted shoots were rinsed with 70% ethanol for 1-2 min. Following 3 times wash in SDW they were treated for 15min in 5% sodium hypochlorite containing two drops of Tween-20 per 100ml as surfactant, then the sterilants were removed with SDW, after which the buds immersed in SDW for 1 to 2 hrs before planting on medium.

3. Bud culture

The sterilized bud parts were inoculated under aseptic condition on MS medium (Murashige and Skoog, 1962) containing 0.2 mg/l BAP and 0.1 mg/l NAA for sprouting shoots. When shoots were

initiated, they were transfered to MS medium supplemented various BAP concentrations for testing shoot multiplication. The range of BAP concentrations was ten degrees from 0.00 to 5.00mg/1. Multiplicated shoots were subcultured on half strength of MS medium without growth regulator for leaf expanding (Park and Son, 1986). Cultures were maintained at 25 ± 2°C, 70% relative humidity, and 16 hrs photoperiod (2,000-3,000 lux fluorescent light).

4. Protoplast isolation

Leaves *in vitro* cultured were sliced transversly into 1 mm strips in order to expose maximum area for enzyme penetration and action. 1g mesophyll tissues/20ml enzyme solution (ES) were incubated in dark at 30°C. The incubation was conducted for 20 min on a reciprocal shaker (120 strokes per min), after which the enzyme solution was replaced with identical fresh solution, and then the incubation was performed 3 times one after another in 30 min time unit. When the each incubation was finished, undigested debris were removed by filtering through 56 μm nylon sieve. The filtrate containing intact protoplasts was centrifuged at 100g for five minutes, the supernatant was discarded and the pellet was washed with washing solution (Table 1).

For purification, the protoplasts were resuspended in floating solution (Table 1). The final protoplast suspension was brought to a known volume and aliquots were taken to measure protoplast yield and viability.

Table 1. Compositions of washing and floating solution.

Reagents	Washing solution		Floating solution	
$CaCl_2 \cdot 2H_2O$	148	mf	24	mg
KH_2PO_4	17	mg	–	
$MgSO_4 \cdot 7H_2O$	25	mg	–	
$Ca(NO_3)_2 \cdot 4H_2O$	3	mg	–	
Dextran (T40)	–		9.6	g
B S A	–		120	mg
Sucrose	–		24.65	g
Mannitol	10.93	g	–	
H_2O	100	ml	120	ml
pH	5.6		5.6	

5. Protoplast yield and viability determination

Protoplast counts were made using a hemocytometer through an Olympus CK inverted microscope. Circular and apparently unruptured protoplasts with well-dispersed chloroplasts were counted for yield determination and their numbers given per unit of 1g fresh weight of leaves. Protoplast viability was estimated by exclusion staining with 0.2% Evans blue (Kanai and Edwards, 1973). The percentage of viability was calculated as the number of protoplasts stained with blue against total number of intact protoplasts.

6. Definition of important parameters affecting yield and viability

For suitable enzyme concentration, a 2 x 2 factorial experiment was designed with cellulase at concentrations of 1, 2% and macerozyme at concentrations of 0.4, 0.8% (Table 2). And several mannitol concentrations (0.4 to 0.8M) and pH levels (5.0 to 6.8) were also tested. The adding effect of DTT and MES buffer was tested. For purification test, various sucrose concentrations (0.4 to 0.8M) were used, and the adding effect of

Table 2. Compositions of four types of enzyme solutions used to degrade cell wall.

Reagents	1	2	3	4
Cellulase 'onozuka' R-10	2.0	1.0	2.0	1.0
Macerozyme R-10	0.4	0.4	0.8	0.8
Hemicellulase 'sigma'	1.2	1.2	1.2	1.2
Driselase	2.0	2.0	2.0	2.0
Pectolyase Y-23	0.05	0.05	0.05	0.05
Potassium dextran sulfate	1.0 g			
Potassium citrate	166 mg			
DTT	30 mg			
MES buffer	3 mM			
BSA	100 mg			
Calcium chloride, dihydrate	18 mg			
Potassium dihydrogen phosphate	17 mg			
Magnesium sulfate	25 mg			
Calcium nitrate	3 mg			
Mannitol	10.93 g			
Distilled water	100 ml			
pH	5.6			

Dextran T40 (MW: 40,000) was also examined.

7. Protoplast culture

Freshly isolated protoplasts were cultured in liquid MS medium enriched with sucrose (0.1M), mannitol (0.5M), NAA (2.0mg/l) and BAP (0.5 mg/l). The culture density of protoplasts was adjusted to $5 \times 10^4 \sim 1 \times 10^5$ protoplasts/ml of culture medium. Three to five 0.2ml droplets of protoplast suspension were placed onto 60x15mm petridishes. The petridishes were then sealed with parafilm. Cultures were incubated at 25±2°C under dim light (about 200 lux). Fresh media were added at 7 days intervals and to reduce the osmotic stress and promote the growth of cell colonies. After 2 weeks of culture the osmoticum was gradually reduced by addition equal volume of fresh media with lowered osmolarity.

RESULTS AND DISCUSSION

1. Bud culture

After sterilization of buds, one-hour immersion in SDW seemed effective for prevention of autointoxication of explants by phenolic compounds existing in the plant: a condensed form and a hydrolisable one, the latter, when attacked by acid, bases, or enzymes, releases insoluble polyphenolic acids into the culture medium (Chevre, 1983). Because the amount of phenolic compounds in bud tissues is more than that in the other parts of plants, in case of culture using bud materials, soaking treatment of materials before culture establishment seems to be significant.

The effect of BAP concentration on shoot multiplication was highly significant. Within the ranges of BAP tested, the number of shoots produced per shoot transfered (multiplication) reached a maximum 5 shoots at 0.4mg/l and decreased at lower or higher concentrations (Fig. 1). Kim *et al.* (1982) and Park & Son (1986) also reported similar results in *Populus* species.

These results demonstrate that the optimum growth regulator level based on the conception of balance of endo- and exohormone is the most important for shoot multiplication.

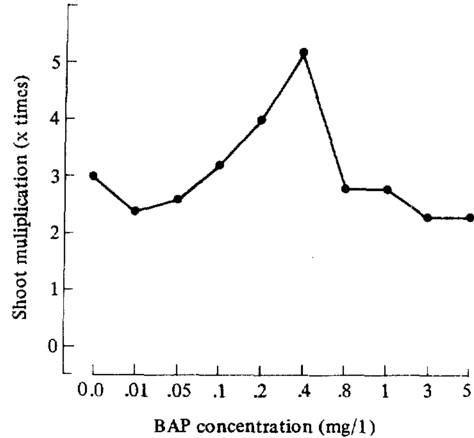

Fig. 1. Relation of shoot multiplication with BAP concentrations.

2. Protoplast isolation

In the present investigation large number of viable protoplasts were obtained by short-period enzyme treatment from *in vitro* cultured mesophyll tissues of *Populus alba* x *P. glandulosa*. Isolations were made quickly, usally within 110 min after enzyme incubation began. The freshly isolated protoplasts were spherical and evenly distributed chloroplasts, but their sizes were various (Fig. 3A and B). Ahuja (1983) grouped the protoplasts having various sizes into two categories: Normal and Mega protoplasts. The origin of them is currently not known.

The characteristic of our method is that high amount of protoplasts can be isolated in a considerably short time. The rapidity of protoplast isolation may be a result of both the high level of enzyme concentration and the supplement of several cell protective additives. In this experiment, a diversity of additives added to the enzyme solution includes $CaCl_2$ (to 'inhibit swelling' and 'improve membrane integrity'), BSA (to reduce possible damage to the plasmamembrane), DTT (to maintain reducing conditions), and potassium dextran sulphate (to get the protective effect on the plasmalemma). The specific function of these additives is unclear, but the importance of these additives has been

accentuated (Schmidt and Poole, 1980, Passiatore and Sink, 1981; Weyers *et al.* 1983).

3. The effect of enzyme concentration in relation to incubation time

The best yield was obtained with ES3, followed by ES1, ES4 and ES2. As the concentrations of enzymes increased, so did the yield of protoplasts. However, as the concentrations of enzymes increased, viability (percentage of viable protoplasts) was decreased (Fig. 4). To complete digestion of incubated mesophyll tissues, we didn't need any longer time than 110 min and this seemed mainly due to high level of enzymes. In general, to obtain sufficiently high amount of protoplast it made no difference using higher enzyme concentration for a shorter time or vice versa. Saito (1976) was successful in protoplast isolation from mesophyll cells of *Paulownia fortunei* and *Populus euramericana* within 240 min using high concentration of enzymes. A low enzyme concentration increase treatment time and a long treatment period (several hours) can be deleterous effect on protoplast yield

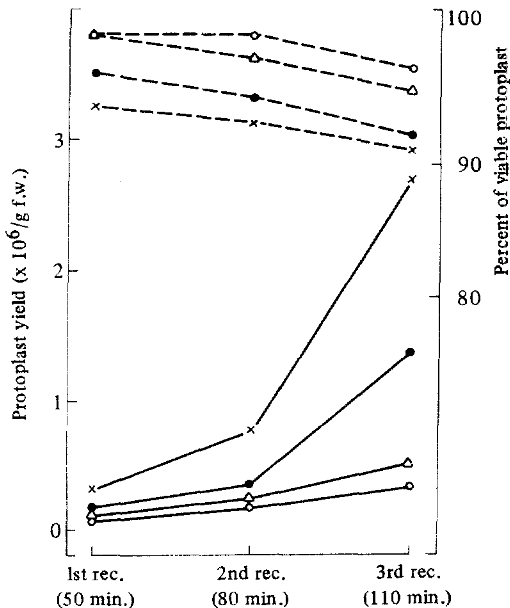

Fig. 4. The effect of several enzyme concentrations on protoplast yield (——) and viability (------) in relation to incubation time.

and viability. It is, therefore, necessary to adjust enzyme concentrations to get high yield and viability.

4. Effect of osmoticum concentrations

The data presented in Fig. 5 show that protoplast yield was greatest when 0.6M mannitol was used and dropped when lower (0.4 and 0.5M) and higher (0.7 and 0.8M) mannitol concentrations were used. These results essentially comply with those of Saito (1976) in *Populus* mesophyll cells and Burger and Hackett (1982) in *Citrus* cotyledons. Viability was shown the best result when 0.8M mannitol concentration was used, but the effect of mannitol concentrations on viability didn't reveal any wide differences. It may be resulted from the fact that incubation time is very short (50-110 min). Nevertheless, it should be pointed out that protoplast lysis after cell wall removal is protected by the use of an appropriate osmoticum.

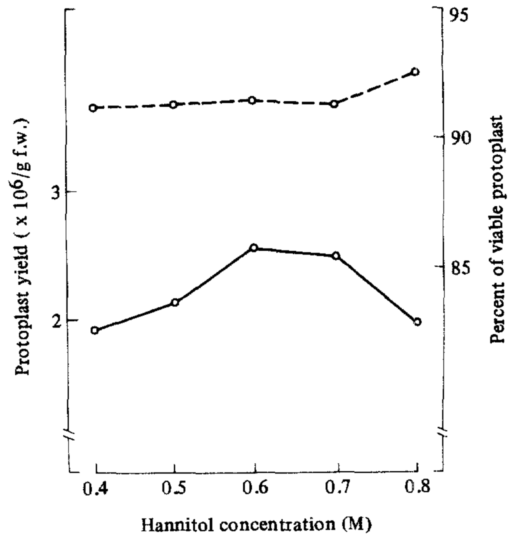

Fig. 5. The relation of protoplast yield (——) and viability (-----) to mannitol concentration in enzyme solution.

5. Effect of pH

Protoplast yield and viability were greatest at pH5.6, and decreased at lower and higher levels. These results are given in Fig. 6.

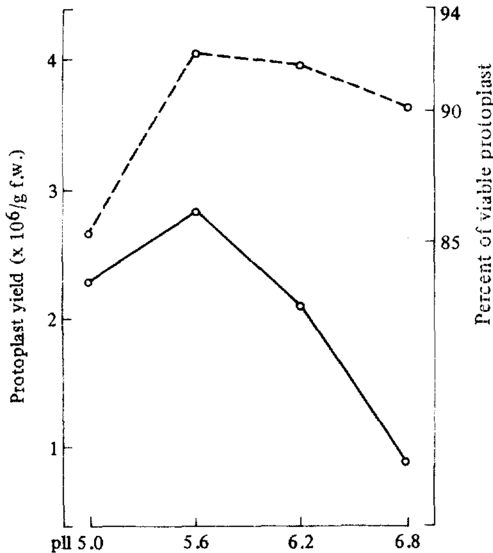

Fig. 6. The relation of protoplast yield (———) and viability (- - - -) to pH of enzyme solution.

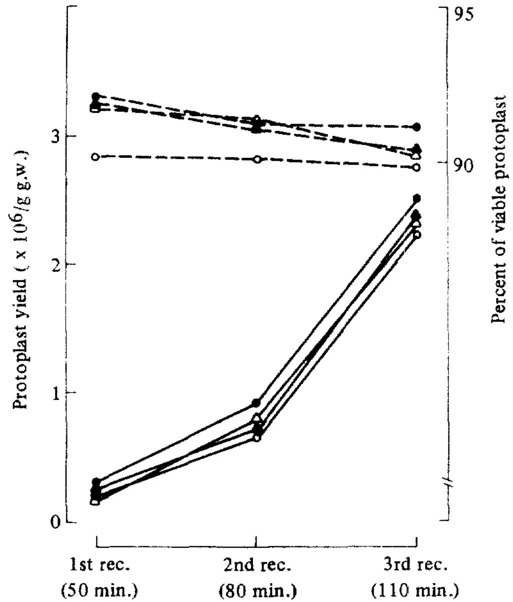

Fig. 7. The effect of DTT and MES buffer on protoplast yield (———) and viability (- - - -) in relation to incubation time.
* control : ○ * DTT alone : ■
MES buffer alone : □
DTT and MES combination : ●

6. The adding effect of DTT and MES buffer

Protoplast yield and viability were increased by supplementing both DTT as reducing reagent and MES buffer as pH stabilizer (Fig. 7). Wallin *et al.* (1977) reported that a pretreatment with sulfhydryl reagents such as DTT increased protoplast yield. Schmidt and Poole (1980) found that the addition of DTT evidently prevented the negative alteration of the cell wall structure in beet tissue. The reaction of these additives is not exactly understood. Nevertheless, it is likely that adding DTT and MES buffer to the enzyme solution during incubation is significant for protoplast yield and viability.

7. Effect of sucrose concentration on protoplast purification

By the enzymatic maceration of plant leaves with complex enzymes, intact protoplasts were released together with fragments of vascular strands, broken protoplasts, and cellular organelles (Kanai and Edwards, 1973). However, after wash with washing solution, when the mannitol prepared protoplasts were suspended onto sucrose solution, intact protoplasts could be recovered at the mannitol-sucrose interface while broken protoplasts, organells and other cellular debris sedimented on the bottom of the centrifuge tube (Hughes *et al.* 1978).

Table 3 shows that the highest percent recovery of protoplasts was obtained when 0.6M sucrose was used. The protoplasts were recovered over 97% pure at 57.7% efficiency. Higher (0.7 and 0.8M) sucrose concentrations gave similar results to 0.6M sucrose but lower (0.4 and 0.5M) sucrose concentrations had poor efficiency of recovery. On the other hand, sucrose concentrations had no significant effect on viability of protoplasts.

Table 3. Effect of several sucrose concentrations in floating solution on protoplast purification

	0.4 M	0.5 M	0.6 M	0.7 M	0.8 M
Density(x10^5)	8.8	10.3	14.1	13.5	13.2
Viability(%)	95.5	96.1	96.2	96.0	94.4
E.R.* (%)	36.1	42.1	57.7	55.2	54.1
E.D.** (%)	2.3	2.3	2.8	5.2	2.4

* E.R. : Efficiencies of recovery of protoplasts
** E.D. : Efficiencies of debris

8. The adding effect of Dextran T40 on protoplast purification

The efficiency of recovery and viability of protoplasts increased by adding Dextran T40 in floating solution. Protoplasts were recovered with 57.7% efficiency in the presence of Dextran T40 (8%), whereas the efficiency dropped to 46.4% in the absence of Dextran T40. Viability also showed the same results with the case of efficiency of recovery (Table 4). Kanai and Edwards (1973) reported that

Table 4. Effect of Dextran T40 in floating solution on protoplast purification

Dextran T40	Density ($x10^5$)	Viability (%)	E.R.* (%)	E.D.** (%)
8 %	14.1	96.2	57.7	2.8
none	11.3	93.5	46.4	2.4

* E.R. : Efficiencies of recovery of protoplasts
** E.D. : Efficiencies of debris

a Dextran-PEG system could be useful for seperating intact protoplasts from borken protoplasts and chloroplasts. Larkin (1976) developed the lymphocyte purification method which utilize a density buffer containing sodium metrizoate and Ficoll. Dextran as well as Ficoll have agglutinating activity. When used to purify protoplasts it is not clear whether the debris and walled cells are agglutinated or they are sufficiently dense to sediment through the density buffer without agglutination (Larkin, 1976). Nevertheless, our this method is probably applicable for protoplast purification.

9. Protoplast culture

In this experiment, the primary purpose of protoplast culture is to prove whether the poplar protoplasts isolated through our high enzyme-short time incubation method are capable, or not, of responding to culture conditions.

After 3 days of culture, the protoplasts enlarged in size and some of the protoplasts underwent their first division (Fig. 8A), and many showed sustained divisions (Fig. 8B). These protoplasts could grow to form cell clumps (Fig. 8C). After addition of fresh medium with lower osmolarity (from 0.6M to 0.5M) cell clumps maintained division to form micro-colonies after 3 weeks (Fig. 8D). Eventhough the cultures sustained successfully to some extant, it is not certain whether the protoplast system is successful or not. Therefore more precise investigation on culture methods is required to find out the optimum conditions for protoplast growth and whole plant regeneration from protoplasts.

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Legend for Figures

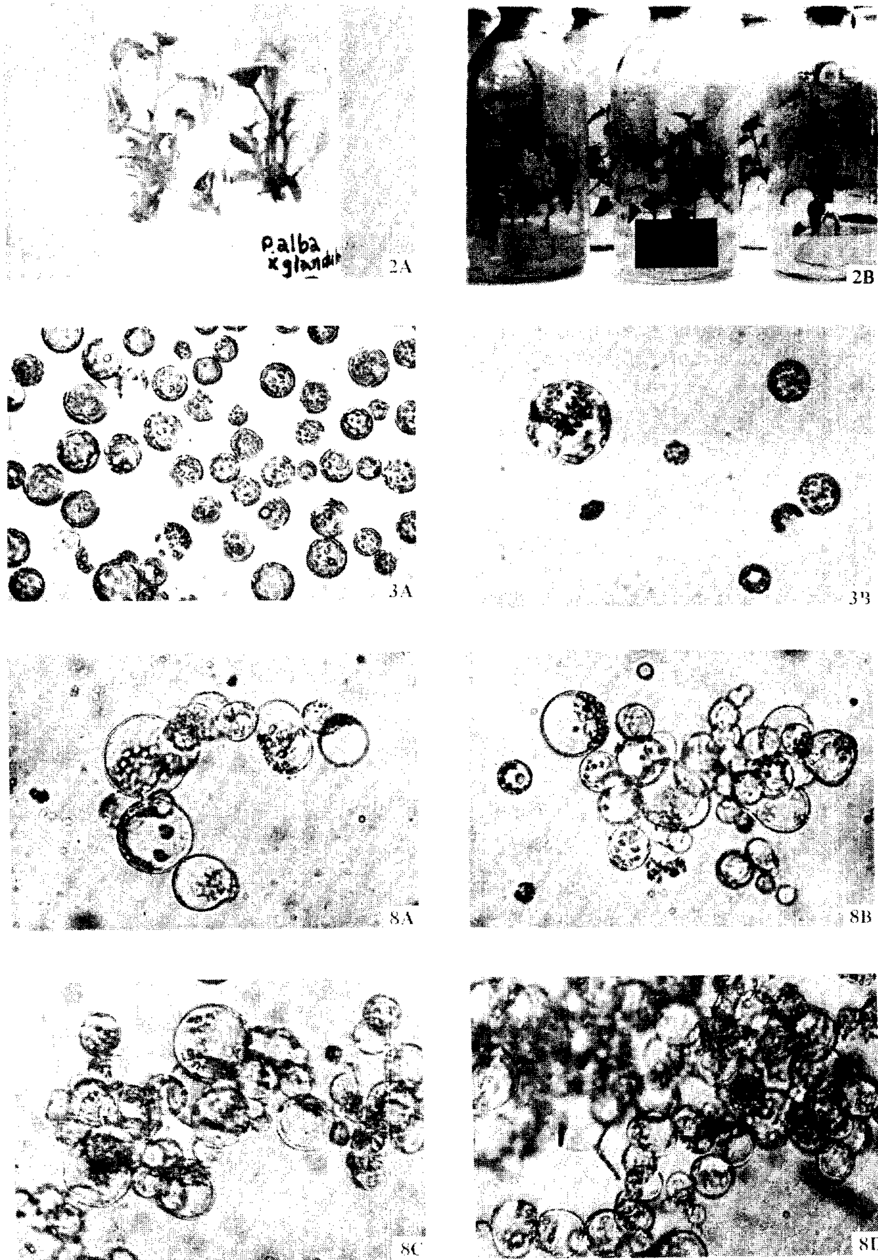

Fig. 2A: Shoot multiplication of *Populus alba* x *P. glandulosa* at MS + 0.4mg/l BAP.
2B: Leaf expanding and growth at 1/2 MS.
Fig. 3A: Freshly isolated protoplasts from mesophyll tissues of *Populus alba* x *P. glandulosa*.
3B: Normal and mega protoplasts from *Populus alba* x *P. glandulosa*.
Fig. 8A: First cell division after 3 days of culture.
8B: Sustained cell division after 1 week of culture.
8C: Cell colony formation after 2 weeks of culture.
8D: Micro callus formation after 3 weeks of culture.