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Isolation and Culture of Protoplasts from Embryonic Cotyledons of *Pinus densiflora* S. et Z.[1]

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소나무 子葉을 利用한 原形質體 裸出 및 培養[1]

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ABSTRACT

Protoplasts were isolated from cotyledons of germinating *Pinus densiflora* S. et Z. seeds. The seeds were germinated for nine days, and excised embryonic cotyledons about 3-4mm long were incubated with Cellulase Onozuka R-10 and Macerozyme. After 8 hours of incubation, large number of viable protoplasts were isolated. Isolated protoplasts were cultured in a medium containing basal salts of B_5 medium, vitamines, amino acids, organic acid, sugars, and growth hormones. The first evidence of protoplast budding was observed after twelve hours in culture, and it suggested that high potential of the embryonic cotyledons for rapid cell division affected the early budding, rather than effect of culture medium was shown in twelve hours. The three- to four-cell stage was reached after three to four days of culture. Most cell divisions were achieved by additional buddings rather than equal binary cell division. No further cell division was observed beyond the four-cell stage. Protoplasts isolated from fully expanded cotyledons (germinated for 17 to 24 days) seldom initiated or failed to initiate cell division.

Key words: protoplast culture; Pinus densiflora; *protoplast isolation.*

要　　約

針葉樹類의 原形質體 培養의 可能性을 試驗하기 위하여 소나무 種子의 子葉에서 原形質體를 裸出하여 培養을 試圖하였다. 9日동안 發芽시킨 採種園産 소나무 種子에서 길이가 3～4mm 가량 되는 子葉을 잘라내서 Cellulase Onozuka R-10과 Macerozyme을 添加한 酵素溶液에서 8時間 處理하여, 活性이 높은 原形質體를 大量으로 얻었다. 裸出된 原形質體를 B_5 培地의 無機鹽, 비타민, 아미노산, 有機酸, 糖類, 植物홀몬이 들어있는 培地에 培養하였을 때, 12時間 經過 後 첫 budding이 觀察되었으며, 3～4日 後 3～4個의 細胞로 分裂하였다. 細胞分裂은 赤道板을 가운데 둔 二等分式 分裂이 아니고, 大部分 酵母菌과 같이 budding에 의한 分裂이었다. 細胞分裂은 4個의 細胞狀態에서 中斷되어 더 이상 進展되지 않았다. 소나무 種子를 17日 혹은 24日間 發芽시켜서 完全히 자란 子葉을 使用했을 때에는 原形質體 裸出은 쉽게 이루어졌으나, 培養時에 細胞分裂이 이루어지지 않았다.

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INTRODUCTION

Recent advancements in the plant tissue culture techniques have opened up a new avenue to tree breeding (McKeand and Weir, 1984). Farnum *et al.* (1983) estimated that forest productivity in certain plantations could be increased by 70 to 300 percent through silvicultural and genetic manipulation including tissue culture techniques. Particularly protoplast research is expected to offer new options for combining genotypes, even though protoplast fusion between forest trees has not been much successful (Ahuja, 1984a).

With coniferous species, isolation and culture of protoplasts have been reported in a few cases (Kirby, 1980; David *et al.*, 1984; Hakman and von Arnold, 1983; Teasdale and Rugini, 1983). All the papers reported cessation of growth after a few times of cell division or callus formation. Most of them used a range of complex media containing organic acids, vitamines, and amino acids in addition to basal salts and plant hormones (Patel *et al.*, 1984). The objective of this paper was to develop a procedure for isolation and culture of *Pinus densiflora* S. et Z. protoplasts from germinating seeds.

MATERIALS AND METHODS

1. Plant Material

Pinus densiflora seeds collected in the fall of 1984 from a seed orchard at the Institute of Forest Genetics in Suwon were used in this experiment. Intact seeds were soaked in 1% H_2O_2 solution to stimulate germination and to facilitate seed coat removal. After 9 days seed coats were removed, and seeds were surface sterilized in 0.2% $HgCl_2$ solution for two minutes with continuous shaking, and washed thoroughly ten times with sterile distilled water. Using a knife under aseptic condition, embryos were taken out from the surrounding female gametophyte. Embryonic cotyledons about 3-4mm long and upper parts of hypocotyls were excised from the embryos and further cut into several pieces.

2. Isolation of Protoplasts

The pieces of cotyledons were soaked in 10ml of a protoplast isolation mixture in Petri dishes (Table 1). The mixture contained 2% Cellulase Onozuka R-10, 1% Macerozyme, 11% mannitol and CPW salts. Preliminary study with poplars and pines in our laboratory showed that hemicellulase was not essential for the isolation of protoplasts. The isolation mixture was previously filter sterilized using a Sartorius filter unit equipped with 0.2 μm filters. The Petri dishes were incubated for 8 hours in dark at 27-28°C on a shaker at 40 rpm. At the end of the incubation period, isolation mixture with digested plant materials was filtered through an about 90 μm wire sieve. The filtrate was centrifuged at about X 100g for five minutes. The supernatant was discarded and protoplast pellet was resuspended with washing solution (Table 1). The washing processes of centrifugation and resuspension were repeated three times to remove excess emzymes in the protoplast isolation mixture. At the final washing, protoplast culture medium was added and washed one more time.

Table 1. Composition of protoplast isolation mixture for embryonic cotyledons of *Pinus densiflora*. The CPW salts are the same as Frearson *et al.* (1973). Washing solution consists of all the reagents below except enzymes.

Type	Reagent	Concentration
Enzymes:	Cellulase (Onozuka R-10)	20 g/l
	Macerozyme	10 g/l
CPW Salts	KH_2PO_4	27.2 mg/l
	KNO_3	101 mg/l
	$CaCl_2 \cdot 2H_2O$	1480 mg/l
	$MgSO_4 \cdot 7H_2O$	246 mg/l
	KI	0.16 mg/l
	$CuSO_4 \cdot 2H_2O$	0.025 mg/l
Osmoticum	Mannitol	110 g/l
	pH adjusted to 5.8	

3. Culture of Protoplast

The protoplasts were cultured in filter sterilized

B_5 medium (Gamborg *et al.*, 1968) supplemented with amino acids, vitamines, organic acids, sugars, and growth regulators as shown in Table 2. Supplement of vitamines and organic acids are modification from Kao (1982) and that of amino acids and sugars are modification from Patel *et al.* (1984). Final density of protoplasts was $10^4 - 10^5$ protoplasts per ml of culture medium. Protoplasts were cultured in 5cm Petri dishes at 25±1°C under cool white light of about 1,000 lux.

RESULTS AND DISCUSSION

Large number of viable protoplasts with active cytoplasmic streaming were obtained from the embryonic cotyledons which had been germinated for nine days in 1% H_2O_2 solution (Plate 1). They looked perfect round nad contained many chloroplasts.

The first evidence of protoplast budding was observed in some protoplasts after twelve hours in culture (Plate 2). Even though cell wall regeneration of protoplasts was not confirmed in this experiment, some round protoplasts became oval shaped and initiated budding, suggesting cell wall might have regenerated before these events. The new bud protruding from mother protoplast in Plate 2 had thinner membrane than the mother protoplast. The thinner membrane of the new bud probably lacked cell wall, while mother protoplast with thicker outer layer probably had regenerated cell wall. Initiation of budding in 12 hours appeared to be fast compared with other reports. For example, Ahuja (1984b) reported first initiation of budding after 5 to 7 days in beech protoplasts, and David and David (1979) after six days in *Pinus pinaster* protoplasts. First cell division was reported to initiate after 10 days in *Pinus taeda* protoplasts (Teasdale and Rugini, 1983). The first budding observed after twelve hours in culture in the present study suggested that high potential of the embryonic cotyledons for rapid cell division affected the exceptionally early budding, rather than the effect of culture medium was shown in twelve hours.

The first three to four-cell stage was observed after three to four days of culture as shown in Plates 3 and 4. The three to four-cell stage was achieved by second or third budding as observed by Ahuja (1984b), while equal binary cell division with equatorial cell plate as shown by David and David (1979) was seldom observed in our study with *Pinus densiflora*. It was uncertain whether different culture media used by different investigators affected the types of cell division in protoplasts.

No further cell division was observed in the present experiment. On the fifth day of culture we reduced the concentration of sugars to about 8% by addition of new medium lacking sucrose from the Table 2. No response was noticed after the dilution, indicating some unknown factors other than osmotic condition might have been involved. Patel *et al.* (1984) suggested accumulation of

Table 2. Composition of protoplast culture medium for *Pinus densiflora*. Macro- and micro-elements are same as B_5 medium (Gamborg *et al.*, 1968), vitamines and organic acid are modification of Kao (1982), and amino acids and sugars modification of Patel *et al.* (1984).

Type	Reagent	Concentration
Macro- and Micro- elements	B_5	
Additional salt	$CaCl_2 \cdot 2H_2O$	900 mg/l
Vitamines	Inositol	100 mg/l
	Thiamine HCl	10 mg/l
	Ascorbic acid	1 mg/l
	Nicotinic acid	1 mg/l
	Pyridoxine HCl	1 mg/l
	Calcium pantothenate	0.5 mg/l
	Choline chloride	0.5 mg/l
	Folic acid	0.2 mg/l
Amino acids	L-glutamine	146 mg/l
	L-asparagine	150 mg/l
	Arginine	10 mg/l
Organic acid	Citric acid	10 mg/l
Sugars	Mannitol	40 g/l
	Sucrose	30 g/l
	Glucose	25 g/l
	Ribose	500 mg/l
	Xylose	300 mg/l
Growth hormones	BAP	1.0 mg/l
	2,4-D	1.5 mg/l
	pH adjusted to 5.8	

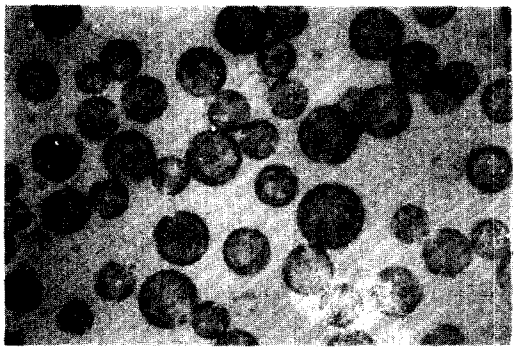

Plate 1. Protoplasts isolated from embryonic cotyledons of *Pinus densiflora* S. *et* Z. after 8 hours incubation with cell wall degrading enzymes

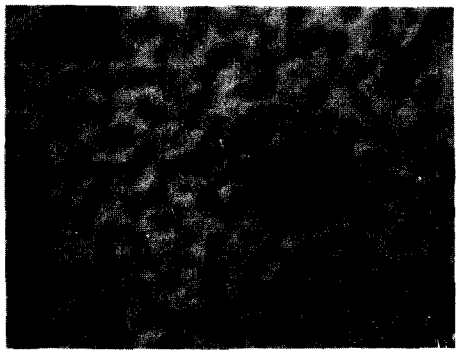

Plate 2. First budding of an isolated protoplast after 12 hours in culture

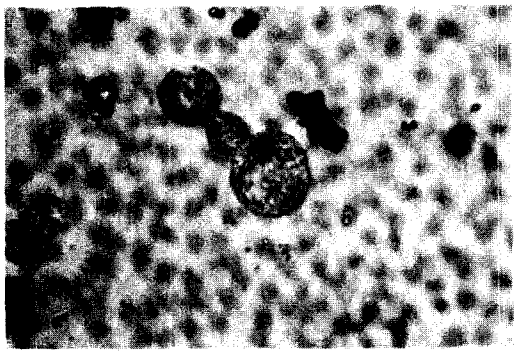

Plate 3. A three-cell stage of a dividing protoplast after three to four days in culture.

phenolics to be a main obstacle to further cell division. Further study is needed to elucidate limiting factors for rapid cell proliferation in protoplast

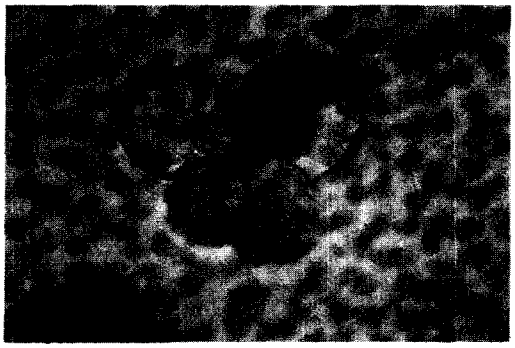

Plate 4. A four-cell stage of a dividing protoplast after three to four days in culture

culture of coniferous species.

In the present study we used embryonic cotyledons germinated for nine days and about 3 to 4 mm in length for protoplast isolation. Even though at this stage large number of embryos were required to obtain adequate density of protoplasts for culture as reported by David *et al.* (1982), protoplasts isolated from embryonic cotyledons appeared to have more rapid regenerating potential than protoplasts from cotyledons at later stage of development. In our preliminary study with *Pinus densiflora*, cotyledons excised from seedlings germinated for 17 or 24 days produced abundant viable protoplasts, but failed to initiate cell division when cultured in the same media in Table 2.

The protoplast culture medium in Table 2 was modification from Patel *et al.* (1984). Our preliminary study with different media suggested that Patel's complex medium appeared to be better than simpler media used by Kirby (1980). When Murashige and Skoog's medium supplemented with L-glutamine and plant hormones (auxins and cytokinins) was tested on the same embryonic cotyledons, no cell division was observed. It suggested that cell division of protoplasts required more complicated nutrients, such as organic acids, and amino acids in addition to vitamines and plant hormones.

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