

## Evaluation of Nutrients for the Protoplast Culture of Genus *Nicotiana*

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담배 野生種의 原形質體 培養에 미치는 無機營養素의 效果

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### ABSTRACT

Leaf mesophyll protoplast cultures from six *Nicotiana* species, *N. debneyi*, *N. rustica*, *N. amplexicaulis*, *N. glauca*, *N. glutinosa*, and *N. sylvestris* were carried out. When we reduced the  $\text{NH}_4\text{NO}_3$  and  $\text{Fe} \cdot \text{EDTA}$  concentration to 1/3 (7 mM) and 1/10 ( $10 \mu\text{M}$ ) from the Murashige and Skoog medium respectively, cell division of the protoplasts was efficiently induced in four *Nicotiana* species, *N. debneyi*, *N. rustica*, *N. amplexicaulis* and *N. glauca*. However, other two species, *N. glutinosa* and *N. sylvestris* were failed in inducing cell division at the same culture condition. The protoclone calluses derived from four *Nicotiana* species were consequently regenerated on a MS basal medium supplemented with the appropriate auxin and cytokinin.

### INTRODUCTION

Plant protoplast has been extensively used in the cell culture system for the plant improvement through the mutant isolation, somatic hybridization, and/or genetic engineering. Especially, genus *Nicotiana* has been considered as a model system for the development of genetic manipulation procedure. Hithto many successful attempts to induce cell division and plant regeneration from the protoplasts of cultivated tobacco (Nagata and Takebe, 1971; Caboche, 1980; Kim and Kim, 1986) and wild tobacco (Scowcroft and Larkin, 1980; Muller *et al.*, 1983; Douglas *et al.*, 1983; Installe *et al.*, 1985) have been achieved. Although methods for isolating protoplasts and regenerating plants have been established for genus *Nicotiana*, a generalized manipulation procedure leading from protoplasts to plants has not yet been undertaken. We have recently reported a reproducible methodology for the protoplast culture

in five cultivars of *N. tabacum*(Kim and Kim, 1986). In this paper, we describe attempts to improve the developmental potential of isolated protoplasts in general from the selected species of genus *Nicotiana*.

## MATERIALS AND METHODS

**Plant materials and chemicals.** Six *Nicotiana* species, *N. debneyi*, *N. rustica*, *N. glauca*, *N. glutinosa*, *N. sylvestris*, and *N. amplexicaulis* were used in this study. For the protoplast isolation, cellulase(Onozuka R-10) and macerozyme (Onozuka R-10) were purchased from Yakult Biochem. Co. The other chemicals used in this study were obtained from Sigma Chemical. Co.

**Protoplast Isolation.** Fully expanded leaves from 8-10 week-old plants were collected for the protoplast isolation. The leaves were surface sterilized by immersion in 70% ethanol solution for 30 s and 1% sodium hypochlorite solution for 10 min. After rinsing in sterile distilled water, lower epidermis of sterilized leaves was peeled off using fine forceps. Approximate 1 g of leaf tissue was placed in a 50 ml Erlenmyer flask containing 10 ml of the filter-sterilized enzyme solution (Table 1). The tissue-enzyme mixture was agitated at 30°C on a water bath shaker at 50 rev/min. Enzymatic digestion was continued for 3 to 6 h. Following incubation the mixture was filtered through the 50  $\mu$ m nylon mesh. For the subsequent culture the protoplasts were collected by centrifugation (100 g, 3 min) and washed three times using the liquid media described by Kim and Kim(1986).

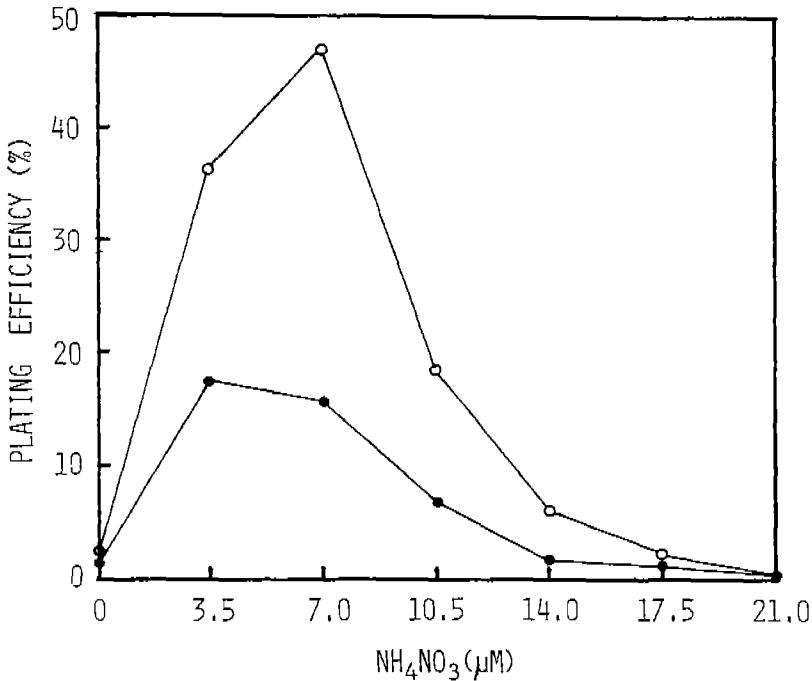
**Protoplast culture.** The freshly isolated protoplasts were resuspended in liquid medium at a cell density of  $1 \times 10^5$ /ml as measured with a hemacytometer. The suspension was mixed gently with an equal volume of melted agar medium(0.8% agar in the medium), and 1 ml aliquots were poured into glass petri dish of 5.5 cm in diameter. The final cell density in the plates was  $5 \times 10^4$ /ml. The petri dishes were sealed with parafilm and placed in the dark for 24 h at 28°C and then transferred to dim light (500 lux) for 48 h. Finally the cultures were placed at 2000 lux with 16-hour photoperiod. After four-week culture, colonies were individually transferred to the new medium which reduced the mannitol concentration to 0.2 M. Plating efficiency was defined as the percentage of protoplasts which formed the visible colonies after 3-week culture. For the plant regeneration, protoclonal calluses were transferred to the shoot induction medium and cultured under the same culture condition as above.

## RESULTS AND DISCUSSION

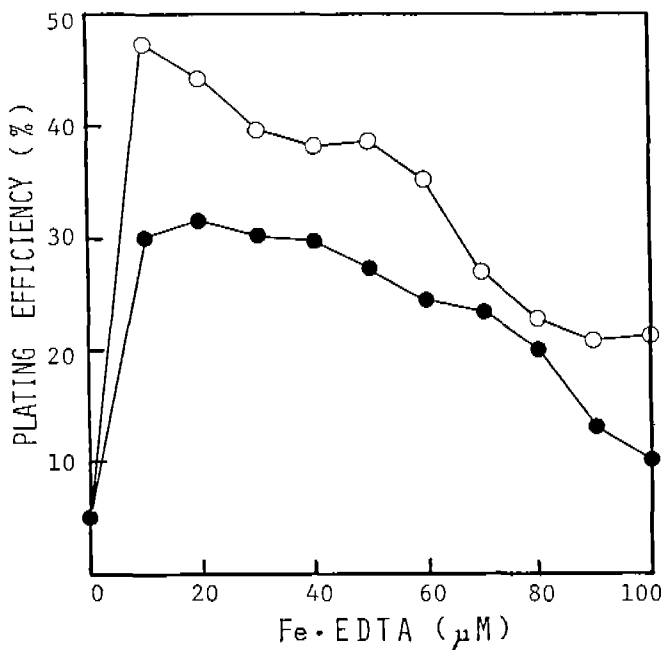
The protoplast cultures of wild species of *Nicotiana* have been extensively reported and demonstrated to require complex and diverse culture condition (Scowcroft and Larkin, 1980; Nagrutiu and Mousseau, 1980; Gill *et al.*, 1981; Muller *et al.*, 1983; Douglas *et al.*, 1983; Installe *et al.*, 1985). Therefore, we carried out protoplast culture to optimize the culture condition from six wild species of *Nicotiana*. Our preliminary results using the available culture condition showed the difficulty to induce cell division from the protoplast of wild *Nicotiana* species. Since

it was previously reported that the protoplasts from five cultivars of *Nicotiana tabacum* were successfully induced to cell division and regenerated on the modified Murashige and Skoog basal medium (1962) by reducing the  $\text{NH}_4\text{NO}_3$  and Fe · EDTA concentration (Kim and Kim, 1986), we further applied our previous results to culturing the protoplasts of *N. debneyi*, *N. rustica*, *N. amplexicaulis*, *N. glauca*, *N. glutinosa*, and *N. sylvestris*.

Figs 1 and 2 show that  $\text{NH}_4\text{NO}_3$  and Fe · EDTA concentrations of MS medium were also highly toxic for the protoplast culture of above *Nicotiana* species as well as those of *Nicotiana tabacum* (Kim and Kim, 1986). However, when the  $\text{NH}_4\text{NO}_3$  and Fe · EDTA concentrations were reduced to 1/3 (7 mM) and 1/10 (10  $\mu\text{M}$ ) from the MS medium respectively, cell division of the protoplasts could be efficiently induced in four *Nicotiana* species, *N. debneyi*, *N. rustica*, *N. amplexicaulis* and *N. glauca*. After 3 weeks of culture, the high plating efficiencies of cell division were obtained (Table 2). However, another two species, *N. glutinosa* and *N. sylvestris* were failed in inducing the protoplast division at the same culture conditions. Our present results highly agree that high concentrations of  $\text{NH}_4\text{NO}_3$  and Fe · EDTA in plant protoplast culture could disturb the cellular metabolism and consequently inhibit cell division (Gamborg and Shyluk, 1970).



**Fig. 1.** Effect of  $\text{NH}_4\text{NO}_3$  on the protoplast culture of *N. debneyi*. Plating efficiency was determined after 3 week of culture. MS medium supplemented with 5  $\mu\text{M}$  IAA, 0.5  $\mu\text{M}$  2,4-D and 5  $\mu\text{M}$  BAP was used. Fe · EDTA concentrations on MS medium were 10  $\mu\text{M}$  (o) and 100  $\mu\text{M}$  (●).



**Fig. 2.** Effect of Fe · EDTA on the protoplast culture of *N. debneyi*.  $\text{NH}_4\text{NO}_3$  concentration on MS medium were  $3.5 \mu\text{M}$  (○) and  $7.0 \mu\text{M}$  (●). The other conditions were described in Fig. 1.

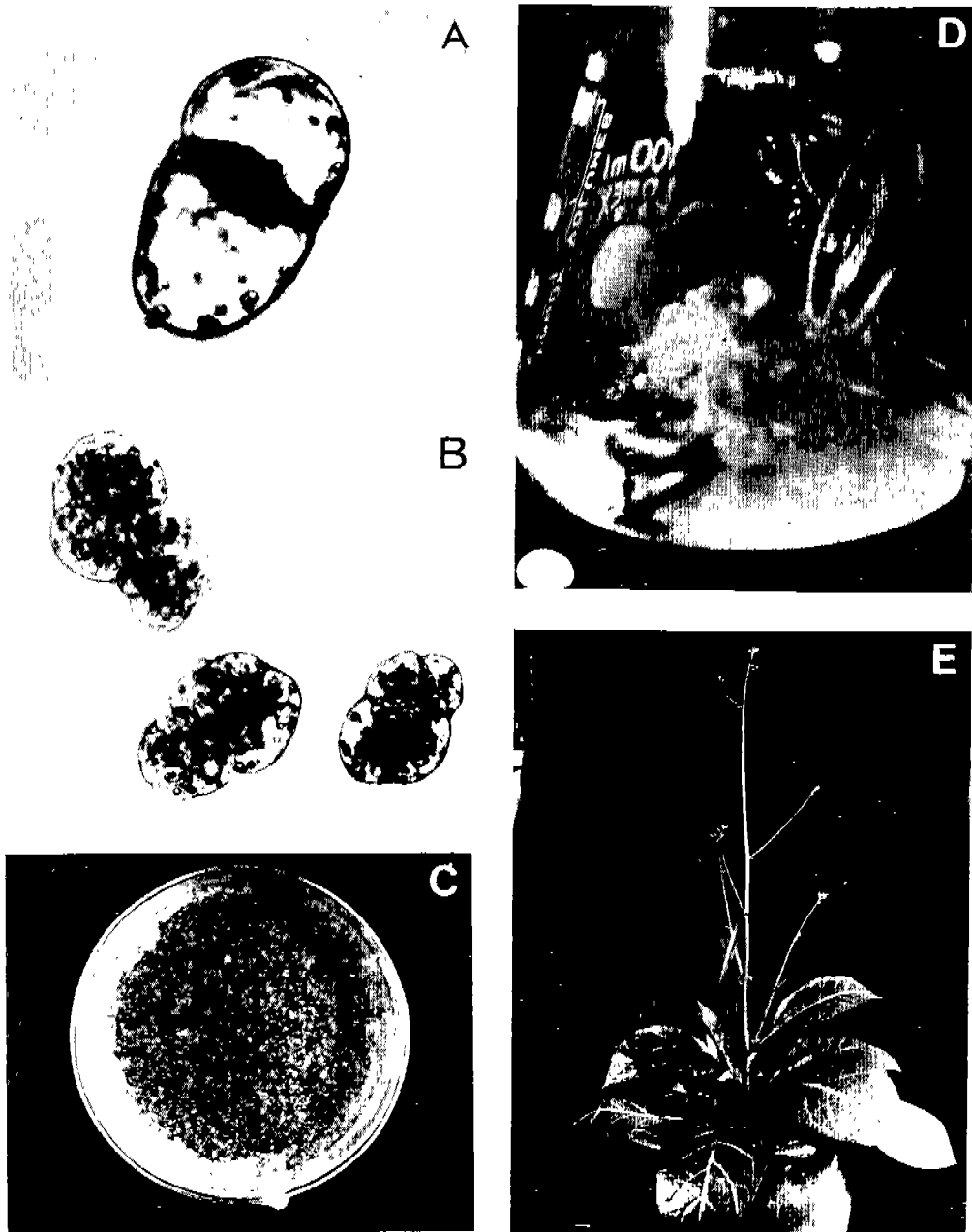
**Table 1.** Protoplast isolation from genus *Nicotiana*

Species	Mannitol (M)	Cellulase (%)	Macerozyme (%)	Incubation time(h)	yield (No./ml)
<i>N. debneyi</i>	0.5	1.5	0.5	5.0	$9.5 \times 10^6$
<i>N. rustica</i>	0.5	0.8	0.3	3.5	$1.0 \times 10^7$
<i>N. amplexicaulis</i>	0.4	0.8	0.3	3.0	$9.0 \times 10^6$
<i>N. glauca</i>	0.5	1.0	0.3	4.0	$9.0 \times 10^6$

**Table 2.** Plating efficiency of protoplast culture from genus *Nicotiana*

species	First cell division (day)	Plating efficiency (%)
<i>N. debneyi</i>	5	40
<i>N. rustica</i>	7	20
<i>N. amplexicaulis</i>	6	50
<i>N. glauca</i>	7	40

Modified MS medium supplemented with  $5 \mu\text{M}$  IAA,  $0.5 \mu\text{M}$  2,4-D and  $5 \mu\text{M}$  BAP was used at an initial culture stage. Plating efficiency was counted after 3 weeks of culture.



**Fig. 3.** Representative protoplast culture of *N. debneyi* leaf mesophyll tissue A, two cell stage of protoplast after 5 day in culture on modified MS medium ; B, protoplast-derived colonies after 2 weeks; C, protoplast-derived colonies after 3 weeks of culture period ; D, Shoots from the protoclone calluses after 4 weeks on the regeneration medium; E, regenerated plant.

Fig. 3 shows the sequential culture of the representative protoplast of *N. debneyi*. Cell division of the protoplasts in the semi-solid agar medium could be seen at beginning on the 5–7 day of culture (Fig. 3A). In this culture period the modification of inorganic salts and hormone combination on MS medium was critical for the continuous cell division of protoplasts. Generally, two or three phytohormone combinations such as IAA+2,4-D or 2,4-D+NAA as auxin and BAP+kinetin as cytokinin were effective (Kim and Kim, 1986). The first cell division took place in *N. debneyi* within 5 days and *N. amplexicaulis* within 6 days after culture. In *N. glauca* and *N. rustica* the first cell division occurred after a week (Table 2). It was necessary to culture in darkness for 24 h at an initial culture stage for the induction of cell division.

Cell colony developed in the four species were microscopically visible in the agar medium after 3 weeks of culture (Figs. 3B and C). When transferred to new medium in which mannitol concentration was reduced to 0.2 M, small calluses grew rapidly and green mass was obtained. When grown to 5 mm in size, individual colony was picked out with a fine forceps and was placed on MS agar plate supplemented with 5  $\mu$ M IAA and 5  $\mu$ M kinetin. The colonies grew actively on this medium and differentiated shoot mostly without root (Fig. 3D). Shoot subsequently produced roots on root induction medium which was composed of MS medium supplemented with 5  $\mu$ M IAA and 0.032  $\mu$ M kinetin and developed into plantlet. These plantlets were grown to mature plants and seeded after flowering. The Tobacco plants regenerated from mesophyll protoplasts appeared to be normal in their morphology (Fig. 3E). Our results obtained from culturing the protoplasts from four wild *Nicotiana* species and five cultivars (Kim and Kim, 1986) in the same medium and culture conditions appeared to be easily applicable to diverse studies using *Nicotiana* protoplast cultures.

## 摘 要

담배의 야생종, *N. debneyi*, *N. rustica*, *N. amplexicaulis*, *N. glauca*, *N. glutinosa*, *N. sylvestris*의 엽육 원형질체 배양 및 식물체 재분화 실험을 수행하였다. MS배지의  $\text{NH}_4\text{NO}_3$ 와 Fe·EDTA의 농도는 이들 원형질체의 초기 배양과정에서 매우 유해하였기 때문에 그농도를 각각 1/3 (7mM)과 1/10(10  $\mu$ M)로 감량하여 사용하였으며, 이 결과 원형질체는 높은 생존율을 유지하면서 세포분열이 유도될 수 있었다. 이와같은 조건에서 *N. debneyi*, *N. rustica*, *N. amplexicaulis*, *N. glauca*의 원형질체 배양을 성공시켰으며 식물체 재분화를 유도할 수 있었다.

## REFERENCES

- Caboche, M. 1980. Nutritional requirements of protoplast-derived, haploid tobacco cells grown at low cell densities in liquid medium. *Planta* **149**: 7–18.
- Douglas, G.C., L.R. Wetter, W.A. Keller and G. Setterfield. 1983. Production of sexual hybrids of *Nicotiana rustica* x *N. tabacum* and *N. rustica* x *N. glutinosa* via in vitro culture of fertilized ovules. *Z. Pflanzenzuchtg.* **90**: 116–129.

- Gamborg, O.L. and J.P. Shyluk. 1970. The culture of plant cells with ammonium salts as the sole nitrogen source. *Plant Physiol.* **45**: 598–600.
- Gill, R., A. Rashid and S.C. Maheshwari. 1981. Dark requirement for cell regeneration and colony formation by mesophyll protoplasts of *Nicotiana plumbaginifolia* Viv. *Protoplasma* **106**: 351–354.
- Installc, P., I. Negrutiu and M. Jacobs. 1985. Protoplast-derived plants in *Nicotiana plumbaginifolia* Viviani: Improving the regeneration response of wild type and mutant cultures. *J. Plant Physiol.* **119**: 443–454.
- Kim, S.-G. and D.J. Kim. 1986. Protoplasst culture in five cultivars of *N. tabacum* L. by modified Murashige and Skoog medium. *Korean J. Bot.* **29**(3): 197–205.
- Muller, J.F., C. Missionier and M. Caboche. 1983. Low density growth of cells derived from *Nicotiana* and *Petunia* protoplasts: Influence of the source of protoplasts and comparison of the growth-promoting activity of various auxins. *Physiol. Plant.* **57**: 35–41.
- Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiol. Plant.* **15**: 473–497.
- Nagata, T. and I. Takebe. 1971. Plating of isolated tobacco mesophyll protoplasts on agar medium. *Planta* **99**: 12–20.
- Negrutiu, I. and J. Mousseau. 1980. Protoplast culture from in vitro grown plants of *Nicotiana sylvestris* Spegg. and Comes. *Z. Pflanzenphysiol.* Bd. **100**. S. 373–376.
- Scowcroft, W.R. and P.J. Larkin. 1980. Isolation, culture and plant regeneration from protoplasts of *Nictiana debenyi*. *Aust. J. Plant Physiol.* **7**: 635–644.

(1988.9.10 接受)