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PROTOPLASMA

Regeneration of Plants From Mesophyll Protoplasts of *Nicotiana plumbaginifolia* Viv.

Brief Report

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Summary

Protoplasts of *Nicotiana plumbaginifolia* were isolated by a one step enzymatic method. They were cultured in Ohyama and Nitsch's medium supplemented with 2,4-D (1.0 mg/l), benzylaminopurine (1.0 mg/l) and 14% sucrose. Cell divisions were initiated after 5 days and within 3 weeks colonies were discernible without the microscope. After transfer to Murashige and Skoog's medium containing IAA and kinetin the colonies differentiated into plantlets.

1. Introduction

Being discrete and totipotent, protoplasts represent an excellent system to achieve somatic hybridization in sexually incompatible plants. They are also ideal for raising mutants in the eukaryotes, and to bring about genetic and biochemical modifications of the cells by the introduction of foreign cell organelles and nucleic acids. Considering the potentials of this technique, almost every part of the plant has been used for the isolation and culture of protoplasts. Despite considerable research in this area, however, successful culture and regeneration of plants from protoplasts, has been limited to a few plants such as *Nicotiana tabacum, Petunia hybrida, Daucus carota, Asparagus officinalis, Atropa belladonna, Brassica napus, Citrus sinensis, Datura innoxia* and *Ranunculus sceleratus* (for references see BAJAJ 1977). Among several species of *Nicotiana, N. tabacum* has been investigated extensively. Of late work has also begun on *N. sylvestris* (NAGY and MALIGA 1976) and *N. otophora* (BANKS and EVANS 1976). In this communication we report the successful regeneration and formation of plantlets from the mesophyll protoplasts of *N. plumbaginifolia* one amongst the three common species in India.

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2. Material and Methods

Leaves from 5-week-old seedlings of *Nicotiana plumbaginifolia* raised *in vitro* on MS basal medium (MURASHIGE and SKOOG 1962) were used for isolation of protoplasts. The isolation was done by the one-step method of POWER and COCKING (1970). About 0.5 g of the leaf material was cut into one cm sq. pieces and incubated in 10 ml of filter-sterilized enzyme mixture. The incubation mixture contained 2% cellulase (Onozuka R-10, Yakult Biochemicals Ltd., Nishinomiya, Japan), 0.6% macerozyme (Yakult Biochemicals Ltd., Nishinomiya, Japan) and 0.5 M mannitol. The tissue was left overnight in the incubation mixture at 25 °C for digestion without any agitation. The protoplasts formed were passed through a 43 micron steel mesh to remove the cellular debris and finally cleaned by floating on 25% sucrose solution (GREGORY and COCKING 1965). They were subsequently sedimented by centrifugation at 350 rpm for 5 minutes. The clean preparation was resuspended in the liquid medium of OHYAMA and NITSCH (1972) containing 14% sucrose and supplemented with benzylaminopurine and 2,4-D each at a concentration of 1.0 mg/l. To this suspension an equal volume of the same nutrient medium with 1.2% agar kept molten at 45 °C was added (NAGATA and TAKEBE 1971). The final density of protoplasts was maintained between 5×10^4 and 10^5 per ml. The protoplasts were cultured in 5×10 mm Corning petriplates each containing 1.5–2.0 ml of the suspension. The petriplates were sealed with parafilm or cellotape and incubated at 25 ± 1 °C, initially in dark for 48 hours (ENZMANN-BECKER 1973) and later under an alternating light and dark cycle of 16/8 hours. The light intensity was maintained at 2,500 lux.

Cell wall formation was tested by Calcofluor (NAGATA and TAKEBE 1970). For organogenesis the colonies were transferred to MS basal medium supplemented with indoleacetic acid and kinetin (THOMAS and DAVEY 1975).

3. Results and Discussion

Protoplasts were isolated from the leaves of plants raised *in vitro*. This was done to avoid the injurious effects of sterilizing agents on the leaf tissue as well as on isolated protoplasts, and also in order to obtain uniform protoplasts. The freshly isolated protoplasts were spherical, full of large green chloroplasts, indicating an active metabolic state. Nevertheless, in spite of their good appearance, the protoplasts were highly susceptible to the process of centrifugation, as losses up to 80 per cent occurred during the washing procedure. Apparently this is a common difficulty. MARETZKI and NICKELL (1973) also observed bursting of sugarcane protoplasts during the process of centrifugation and they harvested protoplasts by the process of filtration. Cell wall for-

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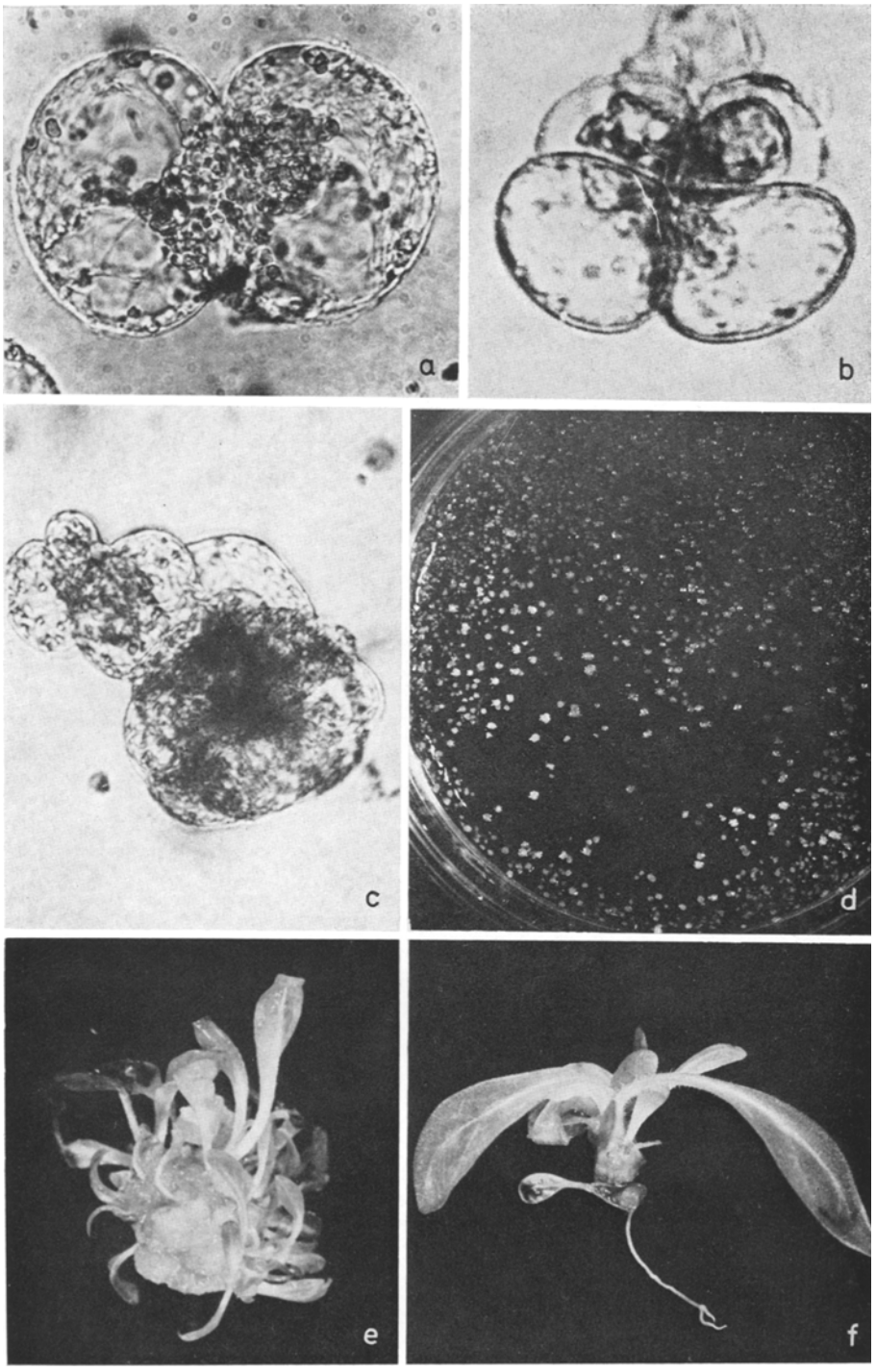
Figs. 1 *a–f*. Different stages in the culture and regeneration of plants from mesophyll protoplasts of *Nicotiana plumbaginifolia* Viv. *a* Six-day-old dividing protoplast on Ohyama and Nitsch's medium supplemented with 2,4-D (1.0 mg/l), benzylaminopurine (1.0 mg/l), 14% sucrose and 0.6% agar. *b* Four-celled stage after 10 days, on the same medium. *c* Four-week-old colony, developed from protoplast, on the same medium. *d* General view of culture showing one-month-old colonies. *e* Fourteen-week-old callus showing formation of shoots on differentiation medium (MS + IAA 4.0 mg/l + kinetin 2.56 mg/l). *f* Twentytwo-week-old plantlet differentiated from protoplast-derived callus on MS medium supplemented with IAA (1.0 mg/l) and kinetin (0.04 mg/l)



Figs. 1 *a–f*

mation, as detected by Calcofluor fluorescence, took place within 24 hours. The first noticeable changes were an increase in volume, and a rearrangement of the chloroplasts accompanied by wall formation in about 60% of the protoplasts. These cells were seen to undergo first cell division (Fig. 1 *a*) after 4 to 5 days and within 3 weeks light green colonies were visible (Fig. 1 *c*). The plating efficiency, *i.e.*, percentage of plated protoplasts producing viable colonies, was calculated after one month and was found to be 2–3%. This plating efficiency in *N. plumbaginifolia* is comparable to that of *N. sylvestris* (BANKS and EVANS 1976). It is, however, poor as compared with that of *N. tabacum* (BINDING 1975) and *N. otophora* (BANKS and EVANS 1976) where the highest reported values are 80% and 40% respectively.

The colonies were transferred to MS medium enriched with 4 mg/l of indoleacetic acid and 2.56 mg/l of kinetin for the differentiation of shoots (Fig. 1 *e*) which followed in 2 weeks. These gave rise to roots (Fig. 1 *f*) on MS medium supplemented with 1 mg/l of indoleacetic acid and 0.04 mg/l of kinetin.

Presently, experiments are underway to enhance the plating efficiency.

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