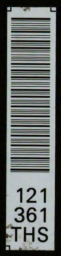

This is to certify that the

thesis entitled

ISOLATION, CULTURE AND REGENERATION
OF LEAF MESOPHYLL PROTOPLASTS
OF SELECTED ORNAMENTAL NICOTIANA SPECIES

presented by

JOAN ELIZABETH PASSIATORE

has been accepted towards fulfillment
of the requirements for

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K. C. Sink
Major professor

Date October 29, 1980

ISOLATION, CULTURE AND REGENERATION OF LEAF MESOPHYLL PROTOPLASTS OF SELECTED ORNAMENTAL *NICOTIANA* SPECIES

By

Joan Elizabeth Passiatore

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ABSTRACT

ISOLATION, CULTURE AND REGENERATION OF LEAF MESOPHYLL PROTOPLASTS OF SELECTED ORNAMENTAL *NICOTIANA* SPECIES

By

Joan Elizabeth Passiatore

In vitro procedures are described for the regeneration to whole plants of *Nicotiana alata*, *N. sanderae* and *N. forgetiana* from leaf mesophyll protoplasts. Axenic shoot tip cultures were established on semi-solid modified MS basal medium. A one-step enzymatic isolation procedure released protoplasts which were plated in media of various salts and growth regulator concentrations to test for growth to callus. Cultures were placed under 4 light regimes (dark, Gro-Lux fluorescent, cool white fluorescent, dark for 3 days and transfer to Gro-Lux) at plating densities of 2.5×10^4, 5.0×10^4 and 1.0×10^5 protoplasts/ml and kept at 25±2°C. Macroscopic calluses were transferred to regeneration media, where shoots formed. Plantlets were rooted on MS basal medium lacking growth regulators or with 0.1 mg/liter NAA. Regenerates were acclimated and transferred to greenhouse conditions where they flowered. The results indicated that genetic potential for growth in certain media may be transferred from one or both parents (*N. alata*, *N. forgetiana*) to *N. sanderae*, the hybrid, where it is expressed. Pollen viability and chromosome number determinations were made. A high degree of variation in chromosome number was found within root tip cells of individual regenerated plants.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

"omnis cellula e cellula"
- Virchow, 1858

The utilization of plant protoplasts to study cell wall biosynthesis, osmotic stress, virus infection, organelle isolation, intromission of foreign genetic materials, somatic hybridization and other biological phenomena began in the early 1960's (86). The concept of protoplast isolation, however, is not a recent one. Klercker, in 1892 (85), released protoplasts of Stratioides aloides by mechanical dissection of plasmolysed cells. Cocking (13) first isolated quantities of protoplasts from tomato root tips by dissolution of the cell walls with snail (Helix pomatia) enzyme. Since then, combinations of various commercial enzymes, primarily pectinases and cellulases, for protoplast release have been refined (20,41). Hemicellulases (26,41,62,70) and dextran sulfate have also aided in the liberation of protoplasts from various plant tissues (54,63). It is generally recognized that age and physiological condition of the plant material are two of the key factors responsible for success or failure in achieving a high yield of viable protoplasts which will subsequently undergo division at high frequencies. A systematic determination of enzyme combinations and concentrations, pH and osmotic pressure of the solution,

rate of agitation, incubation period and light and temperature regimes must be examined for each species studied. These parameters also govern the quality and quantity of protoplasts obtained (80,82).

Leaf mesophyll cells offer an ideal cell source of protoplasts for biological investigations since large, homogeneous populations of identical genetic composition are readily and inexpensively obtained (10). Mesophyll protoplasts can be plated at high efficiencies and are very uniform with respect to chromosome number at the time of isolation. In contrast, protoplasts derived from callus cells often have gross chromosomal aberrations (4,57).

To date, species of the Solanaceae are most amenable to protoplast isolation and subsequent culture. Nagata and Takebe (56) first reported cell division in cultured tobacco protoplasts; henceforth, Nicotiana protoplast systems have been extensively studied and refined (1,5,32,54,57,58,74,84), and regeneration to whole plants has been accomplished in many species. Petunia (24,35,76), Salpiglossis (8), Lycopersicon (89), Browallia (65), Solanum (75), Datura (68,69) and Atropa (33,46) are other Solanaceous crops which have been cultured to plants from isolated protoplasts.

Aside from the favored use of greenhouse-grown seedlings for isolating tobacco mesophyll protoplasts, a number of other cell sources have been found satisfactory. Protoplasts have been isolated from axenic shoot tips or nodal cultures (4,17,22,31,32,72) as well as from callus and cell suspension

cultures (8,47,62,65,82). The advantages of sterile shoot cultures are based on the physiological uniformity of the leaves as a consequence of culture in a controlled environment and growth medium, of the rejuvenation at each transfer, and by absence of contaminating organisms and, thus, the need to disinfect material by exposure to toxic agents (4).

Several techniques are used for the culture of isolated protoplasts. Most commonly, protoplasts are diluted to an optimal plating density in several standard or modified liquid media. Plating protoplasts in a low concentration of agar nutrient medium (soft agar) has also proven successful (6,79,88). An overlay of liquid medium containing the protoplasts on agar-solidified medium is reported to promote division and subsequent colony formation of several species (31,57,79). Microdroplets, where single or few protoplasts are plated in very small volumes of liquid medium, allow the observation of cell colony formation from single cells and , thus, allow the establishment of clonal plants from single protoplasts (32). Different nutrient media have been successfully employed for protoplast culture. Specific requirements for vitamins, salt concentration and organic addenda are observed among species; however, an absolute requirement of an exogenous auxin and cytokinin for inducing and sustaining cell division exists (49,56,83,88). Few protoplasts are found to divide in the absence of nitrogen. Cell wall and cleavage divisions occur when NH_4^+, high urea concentrations, and NO_3^- + glutamine are included in the culture medium as the nitrogen source (50).

Much documentation of plant regeneration from isolated protoplasts of various Nicotiana species now exists. Vasil, et al., (85) list some species which have been regenerated including haploid N. alata (6), N. otophora, N. sylvestris x N. otophora, and N. tabacum x N. otophora (1), N. debneyi (32), N. sylvestris (1,7,58), haploid N. sylvestris (17,22), N. tabacum (57,79), haploid N. tabacum (61), N. rustica (31), N. plumbaginifolia (30) and N. acuminata, diploid N. alata, N. glauca, N. langsdorffii, N. longiflora, N. paniculata and N. suaveolens (5).

Successful regeneration of protoplasts into whole plants free from genetic aberrations is prerequisite for determining the consequences of transferring genetic materials, foreign particles, or use in somatic hybridization. The investigation reported herein describes regeneration systems for three diploid species of Nicotiana:

1) N. alata (2n=18) (Link and Otto)

2) N. forgetiana (2n=18) (Hort, ex. Hemsley)

and their hybrid,

3) N. sanderae (2n=18).

N. alata and N. forgetiana are believed to have evolved through aneuploidy and amphiploidy following chromosome deletion from earlier species possessing 12 chromosome pairs (78). These species hold potential as parents in future somatic hybridizations between other Nicotiana species and ornamental genera within the Solanaceae, thereby extending genetic variability as a means toward improvement of these ornamental species.

MATERIALS AND METHODS

Source of Protoplasts

Seeds of N. alata cv 'Sensation Mixed' were obtained from the Joseph Harris Co., Inc., Rochester , NY; N. sanderae and N. forgetiana were supplied by L.G. Burk, USDA-SEA Tobacco Research Laboratory, Oxford, NC. Seeds were surface sterilized by soaking in a 5% solution of diluted commercial Clorox (5.25% NaOCl) for 20-25 minutes, followed by 3 sterile distilled water rinses. They were sown on Murashige and Skoog(53) basal medium (MS) supplemented with (mg/liter): folic acid 0.001; kinetin (6-furfurylaminopurine) (K) 0.03; indole-3-acetic acid (IAA) 0.00875; sucrose 3.0%; agar 0.8%, pH 5.8 (before autoclaving) in 60x15 mm Petri dishes and wrapped with Parafilm(R) for germination. The dishes were kept at 25±2°C under 28-34 $uEm^{-2}s^{-1}$ (400-700 nm) cool white fluorescent light on a 16 hr photoperiod (without supplemental incandescent lighting). Germination occurred in 10-12 days and in 1 month the seedlings were transferred to modified MS medium in 100x80 mm Petri dishes for single stem growth and leaf expansion. Seedlings of N. alata were placed into MS + (mg/l) IAA 2.0; 6-benzylaminopurine (6-BAP) 1.0; agar 0.8% in 60x15 mm Petri dishes and kept under 15 $uEm^{-2}s^{-1}$ at 25±2°C on a 16 hr photoperiod. Three seedlings each of N. sanderae and N. forgetiana were placed into modified MS, half nitrate concentration, sucrose 2.5%, agar 1.0%, pH 5.8 in 100x80

mm Petri dishes and kept under 28-34 $uEm^{-2}s^{-1}$ (400-700 nm) Gro-Lux fluorescent lamps. Seedling leaves were of sufficient size (2.5-3.0x1.0-2.0 cm) for protoplast isolation approximately 45 days after transfer. After the leaves were excised, the shoot tips were recultured. These recultured shoot tips produced adequate size leaves for protoplast isolations after 30 days. Three such reinnoculations of the shoot tips were possible within the same vessel before the nutrients became depleted.

Preparation of Protoplasts

Table 1 lists the enzyme combinations that were tested for optimal release of viable leaf mesophyll protoplasts from the shoot tip cultures. Enzyme solutions were adjusted to pH 5.8 with 0.2N NaOH or 0.1N HCl before being sterilized (0.45um Nalgene [Sybron] disposable filters). Leaves were excised and placed in 100x15 mm plastic Petri dishes containing the test enzyme (1 gm leaf tissue per 10 ml solution). Leaves were sliced outwardly from the midrib to their margin at 1 mm increments with a scalpel blade while holding the entire leaf in the enzyme solution. Each dish was sealed with Parafilm(R) and statically incubated for a 3-5 hr period at 26-30°C. Gentle teasing with a Pasteur pipette aided the release of protoplasts from the digested leaf tissue. Protoplasts suspended in the enzyme solution were passed through a 61um sieve to remove large pieces of debris. The protoplast mixture was transferred to 16x125 mm culture tubes and gently pelleted by centrifugation

Table 1. Enzymes tested for the release of protoplasts from intact leaf tissue.

	Enzyme solution**								
	A	B	C	D	E	F	G	H	I
Components*(%)									
Driselase	2.5	2.5	2.5	2.5	2.5	2.5	2.0	2.0	2.5
Macerozyme	-	-	-	-	-	-	-	1.0	-
Pectinase	-	-	-	-	-	-	-	-	-
Cellulase	-	-	-	-	-	-	-	-	-
Potassium dextran sulfate	-	-	-	-	-	-	-	-	-
D-Mannitol	4.7	4.5	-	4.0	3.5	3.0	3.0	6.0	6.0
D-Sorbitol	-	-	4.5	-	-	-	-	-	-

**Enzyme solutions contained CPW salts A, 1.0 mg/l and B, 10.0 mg/l (24).

Table 1 (cont'd.).

	Enzyme solution**								
	J	K	L	M	N	O	P	Q	R
Component*(%)									
Driselase	2.0	1.0	1.0	1.0	0.5	0.05	-	-	-
Macerozyme	-	1.5	1.0	-	0.5	0.02	1.5	1.0	0.5
Pectinase	-	-	-	0.5	-	-	-	-	-
Cellulase	0.5	2.5	1.0	-	-	0.1	1.0	1.0	1.0
Potassium dextran sulfate	-	-	0.5	-	-	0.5	-	-	0.5
D-Mannitol	8.0	4.0	4.0	6.0	4.5	4.7	4.0	4.0	4.5
D-Sorbitol	-	-	-	-	-	-	-	-	-

*Enzyme and Chemical Sources:

Driselase - Kyowa Hakko, Kogyo Co., Ltd., Japan
Macerozyme-R-10 - Calbiochem, USA
Cellulase-'Onozuka' R-10 - Kinki Yakult Mfg. Co., Ltd., Japan
Pectinase - Sigma Chemical Co., USA
Potassium dextran sulfate, A grade - Calbiochem, USA

(100 x g; 8 min). The supernatant was decanted and replaced with CPW salts + 15% sucrose (24) and protoplasts were separated by centrifuging (80 x g; 5 min).

The procedures utilized for N. alata protoplast isolation were slightly modified for N. sanderae and N. forgetiana. Released protoplasts of N. sanderae and N. forgetiana were pelleted at 80 x g for 7 minutes, the supernatant was decanted and the protoplasts were resuspended in CPW salts + 15% sucrose. Centrifugation at 80 x g for 7 minutes produced a band of viable protoplasts at the solution surface. Routinely, 2 washes with CPW salts were used to further remove traces of the enzyme solution. Finally, protoplasts were collected at the surface with a Pasteur pipette and suspended in 10 ml of test culture medium. A small aliquot was removed and counted on a haemocytometer. Subsequently, the protoplast solution was diluted to test densities of 2.5×10^4, 5.0×10^4 and 1.0×10^5 protoplasts/ml and dispensed as 3 ml per 60x15 mm plastic Petri dish and wrapped with Parafilm(R).

Plating of Protoplasts

Growth regulators (Table 2) were tested in combination with 5 basal salt and vitamin media (24,27,53,57,82) employed for support of protoplast division and sustained growth. Growth regulators were autoclaved in the medium (pH 5.8) at a pressure of 1.46 kg/cm^2 (121°C), for 20 minutes. Osmotic pressure was maintained with 4.5% (0.25M) mannitol. In all experiments the protoplasts were plated in liquid test media; in some they were also plated in media + agar. Plating in soft agar was done by using

Table 2. Culture media screened for the support of cell division and sustained growth of Nicotiana leaf mesophyll protoplasts.

Basal medium	Growth regulators (mg/liter)				
	K	IAA	NAA**	2,4-D**	6-BAP
MS (53)					
P1	-	-	2.0	-	0.5
PD	-	-	1.5	0.5	1.0
35	-	-	5.0	-	0.5
MS-G*					
I	-	-	3.0	-	1.0
II	-	-	2.0	-	0.5
III	-	2.0	-	-	1.0
IV	-	0.5	-	1.0	0.5
V	-	-	0.5	1.0	0.5
F5 (24)	-	-	2.0	-	1.0
NT (57)	-	-	3.0	-	1.0
UM (82)	0.1	-	0.6	-	-
B-5 (27)					
P1	-	-	2.0	-	0.5
R5	-	-	-	1.0	0.5

*MS-G = MS salts + 1.0g meso-inositol; 2.0 mg/l thiamine·HCl; 250.0 mg/l L-glutamine; 0.1 mg/l L-serine; 3.0% sucrose, pH 5.7

**NAA = alpha-napthaleneacetic acid

***2,4-D = 2,4-dichlorophenoxyaceticacid

culture medium containing 1.0% agar held at 45°C until plating. Isolated protoplasts were suspended at twice the desired final plating density in 10 ml of the same liquid culture medium and the two phases were mixed at equal volumes to obtain a final density of $5.0x10^4$ or $2.5x10^4$ protoplasts/ml. After plating, the cultures were placed under 4 test light regimes at 25±2°C:

1) dark (D)
2) cool white fluorescent light, 18-22 $uEm^{-2}s^{-1}$ (R) (without supplemental incandescent)
3) Gro-Lux fluorescent light, 28-34 $uEm^{-2}s^{-1}$ (G)
4) dark, transferred after 3 days to Gro-Lux fluorescent light, 28-34 uEm-2s-1 (D to G).

A Licor 185 (sensor LI-190) was used to measure $uEm^{-2}s^{-1}$ (400-700nm) values. Cultures were examined daily under a Nikon inverted microscope and the viability and division assessed. Reduction of osmoticum from 4.5% mannitol was accomplished by adding 1 ml of culture medium containing 2.25% or 0% mannitol to each Petri dish every 2 weeks in the case of N. alata and N. sanderae and 1 to 1½ weeks for N. forgetiana. For exceptionally high plating efficiencies, half of the protoplast solution was transferred to a separate Petri dish after 3 weeks. Experiments were duplicated at least 2 times, with a minimum of 6 Petri dishes per experiment.

Estimation of Plating Efficiency

In order to compare the efficacy of each test culture medium on the promotion of cell division and growth to callus, approximate plating efficiencies were calculated. Plating

efficiency (P.E.) was defined as the percentage of viable protoplasts which formed cell colonies after 21 days in culture. Three days after intial plating, the number of viable cells was counted. Small cell colonies were observed after 21 days; thereby enabling the percentage of viable cells forming colonies to be determined:

$$P.E. = \frac{\text{number of cell colonies}}{\text{number of colonies + number of live cells}} \times 100.$$

In some cases, protoplasts were plated in soft agar to permit a more accurate determination of the plating efficiency.

Shoot Regeneration

Macroscopic calluses, approximately 2 mm in diameter, were transferred to solidified shoot regeneration media. Table 3 lists the media tested for shoot proliferation of the 3 species. All cultures were maintained at a constant 25±2°C and 15-20 $uEm^{-2}s^{-1}$ (400-700 nm) cool white fluorescent lamps on a 16 hr photoperiod. Regenerated plantlets 1 cm or taller were separated from the callus and transferred to medium for rhizogenic induction.

Rooting and Transfer to the Greenhouse

Regenerated shoots were transferred to MS medium plus 3.0% sucrose, 0.8% agar, pH 5.8 or 3.0% sucrose, 0.1 mg/l NAA and 0.35% agar to induce root formation. Rooted plantlets were transferred to VSP soil-less planting medium and covered with clear polyethylene to maintain humidity. Plantlets were kept

Table 3. Experimental media tested for shoot regeneration.

Species	Growth regulators (mg/liter)*			
	IAA	NAA	6-BAP	Zeatin
(a,s,f)	2.0	-	1.0	-
(a)	-	2.0	0.5	-
(a,s,f)	-	-	-	1.0
(a,f)	-	1.0	1.0	-
(a)	-	0.1	2.0	-

*Basal medium = MS salts + vitamins, 3.0% sucrose, 0.8% agar and pH 5.8.

a - N. alata
s - N. sanderae
f - N. forgetiana

under 74-84 $uEm^{-2}s^{-1}$ (400-700 nm) cool white fluorescent lamps on a 16 hr photoperiod and gradually exposed to ambient relative humidity. Plantlets were acclimated in 10-20 days and transferred to the greenhouse where, under standard cultural and fertilization practices, they flowered.

Pollen Viability

The percentage of viable pollen was established for the regenerated plants and compared to that of seed-grown plants of the three Nicotiana species. Pollen from flowers at anthesis was collected and placed on a 3x1 inch glass microscope slide and a drop of analine blue stain was added. After 20 minutes, examination under a Nikon Labophot microscope allowed an

accurate count of viable pollen grains. A minimum of 300 pollen grains from each regenerated plant was counted.

Chromosome Determinations

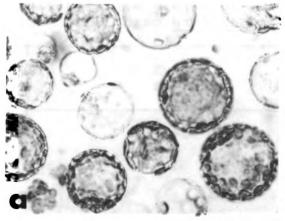
Cuttings of the 3 species were rooted in sand under intermittent mist. Root tips were collected and treated for 16 hours with 0.1% colchicine at 25°C in the dark. Subsequently, root tips were washed, fixed in 3:1 absolute alcohol to glacial acetic acid (v/v) overnight at 4°C, washed again and stored in 70% ethanol. The tips were squashed in 1.0% aceto-orcein for cytological observation (16).

RESULTS AND DISCUSSION

The isolation and culture methods developed in this study enabled the liberation of large quantities of intact mesophyll protoplasts, and their subsequent cell division and regeneration to whole plants (Figure 1). Of the enzyme combinations tested for protoplast release (Table 1), A, B, C and D all containing 2.5% Driselase + 4.0 to 4.7% mannitol or sorbitol in CPW salts solution released between 8.0×10^5 and 1.5×10^6 protoplasts per gram fresh weight of leaf tissue from N. alata. Enzyme mixture L containing 1.0% Driselase, 1.0% Macerozyme-R-10, 1.0% Cellulase 'Onozuka" R-10, 0.5% potassium dextran sulfate and 4.0% mannitol in CPW salts solution was found most effective, releasing 7.6×10^5 to 8.5×10^5 and 4.0×10^5 to 1.0×10^6 protoplasts per gram of leaf tissue from N. sanderae and N. forgetiana, respectively (Table 4). Since shoot tips were grown under sterile culture conditions and common isolation procedures were followed, the range of protoplast yields found within each species may be attributed to the variability that exists among the tissues (48). The majority of liberated protoplasts were derived from palisade and mesophyll cells and some from epidermal cells of the leaf tissue directly exposed to the enzyme solution.

The majority of protoplasts were spherical and their chloroplasts were evenly distributed at the periphery of the

Figure 1. Regeneration of leaf mesophyll protoplasts of Nicotiana alata. a. Freshly isolated protoplasts (390x). b. First cell division, 3 days after plating (1500x). c. Second division, approx. 10 days (1500x). d. Small cell colony stage after 21 days (352x). e. Callus obtained after 4-5 weeks (320x). f. Shoot regeneration from callus obtained after 2 months, actual size. g. Rooted plantlet, actual size. h. Small plants ready for transfer to greenhouse conditions after approx. 4 months, actual size.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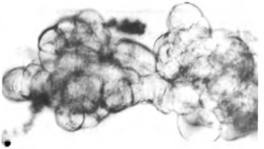
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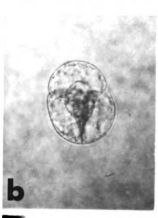
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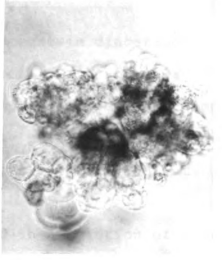
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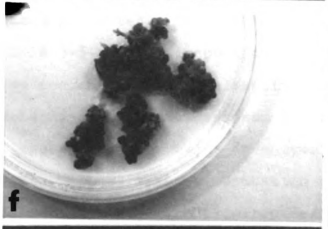
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Table 4. Relative yields of mesophyll protoplasts obtained from three Nicotiana species.

Species	Enzyme solution	Average protoplast yield per g fresh leaf tissue
N. alata	A-D	$8.0 \times 10^5 - 1.5 \times 10^6$
N. sanderae	L	$7.6 \times 10^5 - 8.5 \times 10^5$
N. forgetiana	L	$4.0 \times 10^5 - 1.0 \times 10^6$

plasma membrane. By staining with fluorescein diacetate (FDA) the integrity of the plasmalemma was confirmed, and thus, protoplast viability (42). Damaged protoplasts appeared broken, collapsed and had irregular or severely polarized chloroplast configurations. They also did not fluoresce with FDA but rather stained red.

A 3-5 hr leaf incubation period in a solution of high enzyme concentrations resulted in less damage to protoplasts, as determined by visual assessment, than did tissue incubated for longer durations using an enzyme mixture of lower concentrations. Thus, a short incubation period was preferred, decreasing the toxic effects often resultant after long periods of enzyme exposure (11).

The polyanionic structure of potassium dextran sulfate in mixture L exerted a protective effect on the plasmalemmae and aided in increasing the number of intact protoplasts of higher quality as revealed by FDA staining (14,63). Takebe

and coworkers (1968) suggested that Macerozyme contains some basic protein(s) toxic to tobacco cells and that dextran sulfate protects the protoplasts from damage by blocking their action. Polyanions have been found to electrostatically bind to proteins and thus inhibit the enzymatic activity of various basic proteins (3).

Plasmolysing of leaf tissue prior to enzyme treatment was found not to influence the quality or quantity of protoplasts released and was, therefore, discontinued. Tobacco and soybean mesophyll cells isolated from greenhouse-grown leaf tissue were obtained without preplasmolysis but 0.6 or 0.3 M sorbitol was included in the maceration solution (12,73). High photosynthetic rates were measured and thus, exposure to a plasmolysing agent appears not to be a general requirement for the isolation of metabolically active mesophyll cells.

A common developmental sequence was observed in protoplasts following isolation. Similar patterns have been reported in other plant species (2,22,24,28,29,39,56,61,64,71). Twenty-four hours after plating, protoplasts became slightly enlarged and chloroplasts were polarized toward one end or rearranged unevenly throughout the cytoplasm. Subsequently, cells became ovoid and chloroplasts appeared to decrease in number. Changes in shape were indicative of cell wall reformation (64) and evidence of cytoplasmic streaming and chloroplast degradation suggested the beginnings of cellular dedifferentiation. Seventy-two hours after plating, systrophy, a rosetting of chloroplasts around the nucleus occurred, cells

became reniform and first divisions occurred.

Precise determination of plating efficiency was complicated by strong aggregation of non-dissociable protoplast clumps in the culture medium; thus, plating efficiencies could only be approximated (38,64,74). Clumping was possibly due to the presence of calcium ions from $CaCl_2 \cdot 2H_2O$ which neutralizes the net negative surface charge on protoplasts by obliterating the electrostatic repulsion. Thus, aggregation occurred through attractive and constant van der Waals forces (55). The least aggregation was observed in B5 medium (150 mg/l $CaCl_2 \cdot 2H_2O$) and the greatest amount was observed in MS, MS-G, UM (440 mg/l) and F5 (850 mg/l) media.

Protoplast survival ranged from 47-78% 3 days after plating, depending on the culture medium employed. Visual observations indicated a gradual decline in viability until the time plating efficiencies were determined. Budding and release of cytoplasm occurred in many cells followed by cytolysis. Starch accumulation was evident in aging leaf protoplasts which failed to divide. Dividing cells rapidly formed small colonies (20-30 cells) in approximately 21 days. Colonies subsequently produced calluses. A small percentage of colonies suffered osmotic shock upon mannitol reduction, became necrotic and died. Shepard and Totten (74) suggested that an unreasonably high uptake of organic and inorganic

substances resulting from the reduced osmoticum produced a toxic effect on potato colonies. Ribonuclease levels within the cells are increased when cells suffer osmotic shock and thus, RNA degradation is thought to occur under conditions of lower mannitol concentrations (43); hence, injuring colonies. A priori, plating efficiencies of the 3 species at various densities (Table 5) indicated a slight increase in percent plating efficiency from $5.0x10^4$ to $1.0x10^5$ protoplasts/ml for N. sanderae and N. forgetiana. Plating efficiency of N. alata, however, decreased at the higher density. The diffusion of beneficial chemical substances throughout the medium, released by the protoplasts and presumed to be necessary for division and growth, possibly became deficient when cell densities were low (32,57). At $2.5x10^4$ protoplasts/ml, salts concentration may be sufficiently high such that toxic effects to the protoplasts occur (74). Plating protoplasts at a high density ($1.0x10^5$) had a positive effect on growth. Initial divisions occurred at higher frequencies, approximately 15% greater, but division rate decreased once cells reached a critical density. Beneficial metabolites are rapidly produced and diffuse throughout the medium promoting cell division, but cell density soon becomes the limiting factor. In three weeks, protoplast cultures were divided in half and fresh medium with a reduced osmoticum added in order to maintain the growth rate and minimize the loss of small colonies. Gleba (32) reported that preculturing protoplasts for 1-3 days in high density

Table 5. Plating efficiencies (%) of protoplasts of 3 species of Nicotiana plated at 2.5×10^4, 5.0×10^4 and 1.0×10^5/ml, 28-34 $uEm^{-2}s^{-1}$ Gro-Lux light.

Species	Medium	Density		
		2.5×10^4	5.0×10^4	1.0×10^5
N. alata				
	MS P_1	0	32	31
	NT	0	37	34
N. sanderae				
	MS P_1	0	32	36
	NT	0	30	32
N. forgetiana				
	MS P_1	0	13	17
	NT	0	33	34

suspensions of 1.0×10^4 - 1.0×10^5 protoplasts/ml significantly improved the plating efficiency of tobacco protoplasts. Preculturing protoplasts in a "conditioned" medium could supplant the need for nutrient-enriched medium. A density of 5.0×10^4 protoplasts/ml permitted a constant growth rate throughout the early stages of development and could be considered the standard recommendation for these 3 species.

A high propensity toward budding and cell lysis with concomitant extrusion of cytoplasm occurred with protoplasts plated in nutrient agar medium under all light regimes. Takebe, et al., (79) found that most mesophyll protoplasts

deteriorated within several days when plated in agar. Frequency of first division decreased compared to those plated in liquid medium (61). Caboche (9) suggested that the agar source be considered as an important factor in the successful growth of protoplasts. Unpurified agar, the type used in this study, may adsorb certain toxic components of the medium and thereby be unsuited for this use, which may account for the inhibited growth response. Protoplasts survived for several days during which they followed a similar developmental pattern as described for those in liquid medium. Success was obtained by plating in agar in only one experiment. N. sanderae plated in G-II medium + 0.5% agar rapidly entered first division under D and G light conditions. The most marked growth occurred after dark cultures were placed under G conditions; within 15 days after plating, visible colonies had formed. Meyer and Abel (49,50) found development of colonies to be more frequent if the medium was solidified with agar. Cells grown in agar medium tended to radiate laterally from the callus mass; whereas cells in liquid culture were more compact. The slower diffusion of beneficial compounds among the protoplasts plated in agar may explain the overall failure to obtain protoplast growth in soft agar. Agar concentrations employed may act as a physical constraint on the capability of cells to divide and inhibit sufficient oxygenation which may also influence their ability to grow. Agar purity is another factor which should be considered when culturing in semi-solid media.

Dark-grown protoplast cultures were precocious in first division in all cases; however, light-grown cultures soon at-

tained equal division rates. Cell wall regeneration of Convolvulus protoplasts was reported to occur more rapidly under dark conditions (36). The fact that cell wall regeneration is a prerequisite for cell cleavage divisions (49,71) may explain the more rapid induction of division in the dark. Cultures kept in the dark retained their bright green color approximately 3-5 days longer than those exposed to light due to accelerated chlorophyll degradation.

Cultures exposed to Gro-Lux irradience were found to divide 1-2 days earlier than cultures exposed to the other light sources. Rapid growth ensued after first division and plating efficiencies were slightly increased over most D and R conditions when cultures were kept under Gro-Lux fluorescent light (Table 6). Beneficial responses have been reported for tobacco protoplast cultures using Gro-Lux light at a constant exposure of 1000 lux (83). Cool white fluorescent light at 18-22 $uEm^{-2}s^{-1}$ (R) generally gave poorer responses in terms of division rate and frequencies, over D and G light regimes, a priori (Table 6). However, R was found promotive for N. forgetiana protoplasts cultured in NT medium a slightly higher plating efficiency was observed (Table 6).

Transferring cultures from the dark to Gro-Lux light conditions (G) after 3 days was tried with N. sanderae based on previous findings (6,46,48,58). Exposure to dim illumination for 48 hr after isolation has been shown to increase plating efficiency of Nicotiana tabacum cv 'Samsun' (18). A priori, the D to G transfer regime increased plating efficiency above R conditions; however, it did not increase plating efficiency above

Table 6. Plating efficiencies (%) of 3 species of Nicotiana in selected media under 4 light regimes; density 5.0 x 10^4 protoplasts/ml.

Species	Medium	Light regime			
		Dark (D)	Gro-Lux (G) 28-34 uEm^{-2}s^{-1} (400-700 nm)*	Cool white (R) 18-22 uEm^{-2}s^{-1} (400-700 nm)*	Dark to Gro-Lux (D to G)
N. alata	NT	34	37	30	-
	MS P_1	22	32	28	-
	MS G-I	14	15	15	-
N. sanderae	NT	48	30	16	-
	MS P_1	27	32	24	25
	MS G-II	33	32	17	21
	MS G-II, 0.5% agar	34	4	0	0
	MS P_1, 0.5% agar	0	0	0	0
N. forgetiana	NT	32	33	35	-
	MS P_1	12	13	12	-
	MS P_1, 0.5% agar	0	0	0	0
	MS P_1, 0.025% agar	0	0	0	0

*as explained in Lighting for Plant Growth. Bickford, E.D. and S. Dunn. 1972. Kent State University Press, p. 46-61.

either D or G conditions.

Of the 13 salts and growth regulator combinations tested for support of cell division and growth (Table 2), only 4 were successful (Table 7). All 3 Nicotiana species divided and produced callus in NT and MS P_1 media. MS G-I only supported the growth to callus of N. alata; MS G-II promoted division and growth of N. sanderae.

MS G-I medium contained the same growth regulator levels as in NT medium and the same salts as MS P_1, differing from MS P_1 by including 1.0g meso-inositol, 2.0 mg/l thiamine·HCl, 250.0 mg/l L-glutamine and 0.1 mg/l L-serine and lacking NH_4NO_3. N. sanderae and N. forgetiana remained viable for 7-10 days in MS G-I, but did not divide.

MS G-II contains the same salts and vitamins as MS G-I and the growth regulators of MS P_1. MS G-II permitted cell division of N. sanderae; N. alata and N. forgetiana protoplasts remained healthy in MS G-II but failed to divide. The cells declined in vigor and eventually died following strong aggregation which began 2 weeks after plating. Thus, it appears that the ability to tolerate lower ammonium levels and the amino acids, glutamine and serine, in MS G-I medium was contributed to the hybrid, N. sanderae, by N. alata. The capability of the hybrid, N. sanderae, to grow in MS G-II medium whereas neither of its parents did, suggests that complementation occurred in the hybrid which permitted division and growth under those conditions. Izhar and Power (37) proposed that several genes are responsible for and exert control over differential responses

Table 7. Culture media promoting division and growth of protoplasts of 3 Nicotiana species at $5.0x10^4$ protoplasts/ml.

Medium	Species (% P.E.)		
	N. alata	N. sanderae	N. forgetiana
MS P_1	32	32	13
MS G-I	15	-	-
NT	37	30	33
MS G-II	-	32	-

to growth factors; that is, the progressive stages of protoplast development in vitro could be under control of separate genes. Genetic complementation of the parental species in the hybrid could activate all these required genes and allow a positive growth response. Izhar and Power (37) found that in all cases, F_1 hybrids were superior to the parental lines of Petunia with regard to the range of growth regulator combinations that permitted growth. The hybrid species, N. sanderae, was responsive in the greatest number of test media and also exhibited the highest plating efficiency. The fact that neither N. alata nor N. forgetiana protoplasts divided in G-II medium, yet they retained the potential to complement when the hybrid grew on G-II, implies that a separate genetic control for each stage of protoplast development exists. Possibly, by overcoming the metabolic block for first division, genetic potential for continued division is expressed.

Table 8. Media inducing shoot regeneration from calluses of 3 species of Nicotiana.

Medium	Species		
	N. alata	N. sanderae	N. forgetiana
A. MS + 1.0 mg/l zeatin	+++	+++	+++
B. MS + 2.0 mg/l IAA; 1.0 mg/l 6-BAP	+++	+++	+
C. MS + 2.0 mg/l NAA; 0.5 mg/l 6-BAP	++		
D. MS + 1.0 mg/l NAA; 1.0 mg/l 6-BAP	++		+
E. MS + 0.1 mg/l NAA; 2.0 mg/l 6-BAP	+		

+++ prolific shoot regeneration, 5-10+ shoots/callus
++ sparce shooting, 3-5 shoots/callus
+ very sparce shooting, 1-2 shoots/callus

Regeneration media (Table 8) were selected on the basis of results obtained from earlier experiments employing callus derived from leaf sections of the 3 Nicotiana species. Shoot regeneration of protoplast-derived callus occurred for the 3 species on media A and B (Table 8). A priori, calluses derived from protoplasts were more vigorous and regenerated shoots sooner than those originating from leaf sections. Prolific shoots from callus were observed for all species except

N. forgetiana (medium B). N. alata calluses produced sparce shoots on media C and D while infrequent regeneration occurred on medium E for N. alata and medium B and D for N. forgetiana calluses. No variations were noted regarding the propensity of shoot regeneration as related to callus originating from different culture media. Anlagen appeared 3-4 weeks after calluses were transferred to regeneration media; shoot formation was observed 1-2 weeks later. Shoots formed slightly faster from callus of N. sanderae (3-4 weeks), perhaps demonstrating increased genetic potential for growth on the test media due to heterosis.

Observations from several laboratories indicated that exogenous auxin and cytokinin sources are required for in vitro morphogenetic responses of Nicotiana callus tissues (44,45, 52,59). Exogenous auxin was not required for shoot induction of the 3 Nicotiana species studied herein. Calluses readily produced shoots on medium containing only 1.0 mg/l zeatin. Auxin generally exerts its effect in proportion to the concentration of other growth regulators present in the medium (77); therefore, it seems that endogenous auxin levels were adequate to induce prolific bud differentiation on zeatin medium. It has been reported (23) that strains of Nicotiana have been selected which require neither an auxin nor a cytokinin, and suggested that variations in the chromosome complement resulting from in vitro culturing were responsible for the increased biosynthetic ability of the callus tissue. Thus, the production of higher levels of growth substances within the cells caused a

reduced need for exogenous hormones for organogenesis. A similar occurrence may have allowed autonomous production of auxin in these 3 species.

Zeatin, which has the highest biological activity of any of the natural cytokinins, is the preferred cytokinin for inducing organogenetic activity at meristemoid regions; 6-BAP, in comparison, gives a less striking response (34). Levels of 6-BAP in combination with IAA or NAA were less effective in promoting shoot regeneration. Decreasing the levels of both 6-BAP and NAA proportionately reduced the frequency of shoots and did not induce a substantial amount of shoots in the case of N. sanderae and N. forgetiana. A priori, IAA seemed to be more effective than NAA as an auxin in shoot formation in N. alata and N. sanderae, but produced only infrequent shoots from N. forgetiana callus. IAA is perhaps more potent than NAA due to its natural occurrence in vivo. Secondary products derived from IAA breakdown are apparently the active substances in tissue cultures, since photo-oxidation of IAA readily occurs (21,25,87).

Rooting (100%) occurred in 6-10 days on the regenerated shoots placed in media containing MS salts and vitamins + 3.0% sucrose, 0.8% agar or 3.0% sucrose, 0.1 mg/l NAA and 0.35% agar. Sufficient levels of naturally occurring auxin may have been present in the regenerated shoots as exemplified by the high rate and efficiency of rhizogenesis. High levels of endogenous auxin have also been found in tomato explants (40).

Within 5 months of intial protoplast isolation, regenerated plants at anthesis were assessed for pollen viability and

seed set. Seeds were produced on N. alata through sib-pollinations among the regenerated plants. Regenerated plants displayed a slight decrease in percent viable pollen when compared with seed-grown plants (Table 9). Pollen counts for N. forgetiana were not available since plants did not reach anthesis at the time the study was concluded.

Table 9. Mean percent pollen viability as determined by analine blue staining of pollen from regenerated and seed-grown Nicotiana species.

Species	Regenerates	Seed-grown
N. alata	75*	89
N. sanderae	67**	73
N. forgetiana	not available	64

*probability of a larger X^2 value 0.10 between regenerates and seed-grown plants.
**probability of a larger X^2 value 0.05 between regenerates and seed-grown plants.

Chromosomal variants are a common occurrence in plants regenerated from protoplast-derived callus cultures (1,6,59,67, 76). Overall, regenerated plants appeared morphologically identical to seed-grown plants. Upon careful examination, however, split corollas, flower size variations or slight flecking in the petals was apparent through parts of the population, indicating that genetic changes may have occurred. Variations in chromosome number (from the normal 2n=18) from 7-27 were observed in 6 plants counted of N. alata and in 3 plants of N. sanderae. Number variations occurred within a single root tip as well as between root tip cells of different plants tested. The high levels of

aneuploidy and euploidy often found in tobacco cell cultures (6, 66,67) can be explained by unequal or asynchronous divisions, multinucleate protoplasts formed through spontaneous fusion or chimeral associations of abnormal cells that had aggregated and produced callus of mixed ploidy. Reports of regeneration in aneuploid, polyploid, hypotriploid and hypotetraploid cells have been made (67), thus, restoration of whole plants through diplontic selection as suggested by Nishiyama and Taira (59) is not necessarily favored. These marked differences in chromosome number can account for the changes in morphology, decreased fertility and capability of autotrophic production of growth regulators for differentiation normally supplied exogenously.

Multinucleate cells have been found in cell cultures resulting from spontaneous fusion events during the isolation process or through endomitosis (51,80). Under low osmotic levels during isolation, a higher percentage of multinucleate protoplasts have been found (19). Plasmolysing the leaf tissue prior to the enzyme treatment has been shown to reduce the occurrence of multinucleate protoplasts by severing plasmodemata, thereby preventing the fusion of adjacent cells (24). Since no preplasmolysing treatment was used, a greater number of multinucleate cells may have been anticipated. Ogura (60) reported a wide range of chromosome numbers from regenerates of Nicotiana tabacum tissue cultures; such plants were regarded as cytologically chimeric or mixoploid in the root tips. D' Amato (15) reported evidence of polyploidy, aneuploidy and chromosome number mosaicism (chimerism) in cultured cells and regenerated plants of tobacco and other species, indicating that these are relatively frequent phenomena.

SUMMARY

The study conducted herein established the *in vitro* protocol for three species of *Nicotiana*: *N. alata*, *N. sanderae* and *N. forgetiana* from leaf mesophyll protoplasts to whole plants. Several points emerge from the results obtained:

1. Optimal protoplast release from leaf tissue of *N. alata* occurred using an enzyme mixture containing 2.5% Driselase and 4.0-4.7% mannitol in CPW salts solution. *N. sanderae* and *N. forgetiana* leaf tissue responded best to a solution of 1.0% Driselase, 1.0% Macerozyme R-10, 1.0% Cellulase 'Onozuka' R-10, 0.5% potassium dextran sulfate and 4.0% mannitol in CPW salts solution for release of protoplasts.

2. Generally, a plating density of 5.0×10^4 protoplasts/ml permitted a constant growth rate throughout the early stages of protoplast development for all 3 species.

3. Gro-Lux light at 28-34 $uEm^{-2}s^{-1}$ (400-700 nm) was, overall, the best of test light regimes in promoting early first division of the 3 species.

4. It appears that a complementation of accumulated genes in N. sanderae, the hybrid, from the parental species may be responsible for its positive growth response in media in which the parents were non-responsive. Protoplasts of N. sanderae developed into callus in the greatest number of test media as well as exhibiting the highest plating efficiency of the 3 species.

5. Variations in chromosome number within root tip cells of N. alata and N. sanderae suggest chromosome instabilities of the developing tissues, in vitro, perhaps attributable to spontaneous fusion of the protoplasts, unequal mitoses or chimeral associations of abnormal cells, or a combination of these events.

RECOMMENDATIONS

Further investigations are warranted in light of the successful results obtained from the preceeding study. A more thorough understanding of the cytogenetics of cultured cells derived from protoplasts is necessary to explain the variations in chromosome number found in the regenerated plants. To reduce the possibility of multinucleated protoplasts formed from spontaneous fusion during the isolation procedure, a more complete study of the effects of pre-plasmolysis should be conducted. In addition, experiments testing agars of different purity may improve methods of plating in soft agar to increase plating efficiencies. Improvement in the semi-solid agar culture method may eliminate or lessen the degree of chimeral association of the cultured cells forming callus.

Selection systems for the identification of callus derived from somatic cell hybrids are necessary before attempts at parasexual hybridizations are made. Albino seedling of N. sanderae were found during the course of this study and shoot tip cultures were established and maintained. Cells of the albino mutant may serve as potential parents in protoplast fusion studies utilizing albino complementation as a selection system.

The somatic hybridization between N. alata and

N. forgetiana to produce N. sanderae can be attempted in order to characterize morphological or biochemical differences, if any, in the somatic hybrid versus the sexually-produced hybrid. Careful attention to the cytogenetics may reveal gross chromosomal aberrations which may account for any differences observed.

Since regenerating systems have now been established for N. alata, N. sanderae and N. forgetiana, somatic hybridization with other Solanaceous species, especially those possessing a diploid chromosome number of 18 (e.g., Petunia parviflora, N. bonariensis, N. langsdorffii) should be attempted for the purpose of combining desirable characteristics and, consequently, creating new germplasm.

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